Potential of extracellular vesicles as biomarkers of mitochondrial dysfunction

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BACKGROUND

- ✓ Mitochondrial disorders (MD) are caused by dysfunction within the respiratory chain and can be classified as primary or secondary MD.
- ✓ Primary mitochondrial disease (PMD) are caused by known mtDNA and nDNA mutations, while secondary mitochondrial dysfunction (SMD) is due to inherited or acquired disorders affecting mitochondrial function and dynamics.
- ✓ Extracellular vesicles (EVs) are important mediators of intercellular communication and show promise as disease biomarkers, but remain unexplored in MD.
- ✓ We hypothesize that EVs can serve as biomarkers of MD.

AIM

- ✓ To test our hypothesis, we characterized plasma-EVs from a known PMD (MELAS, N=1), 2 patients with hypophosphatasia (HPP) a disorder of skeletal mineralization recently associated with mitochondrial dysfunction and matched controls.
- ✓ Compared results to EVs from a hydrogen peroxide (H_2O_2)-induced model of mitochondrial dysfunction.

METHODS

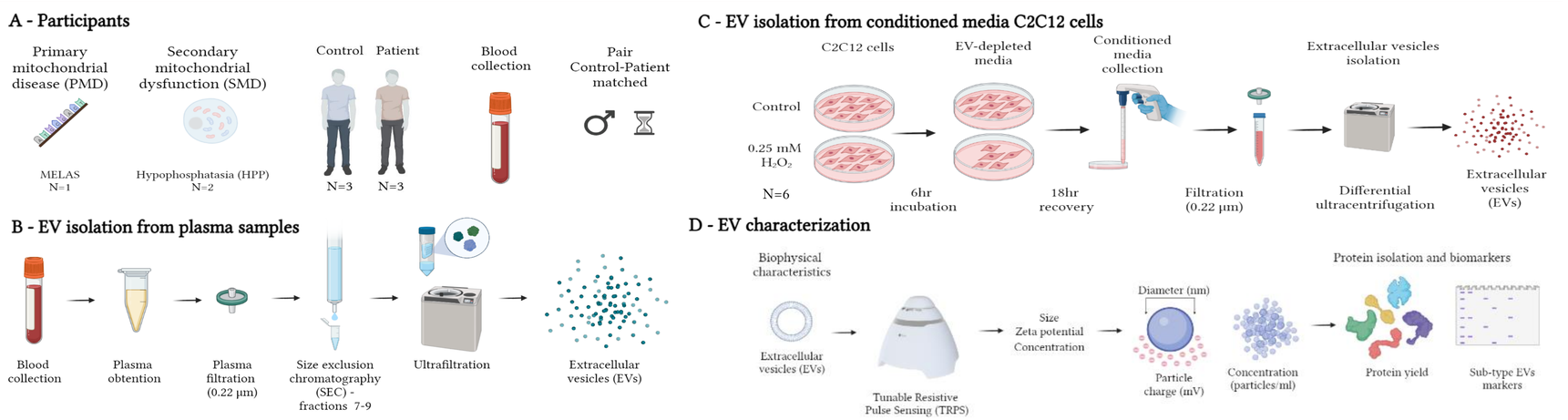

Figure 1 – Experimental design. **(A)** Ethical approval for this study was obtained from the University of Manitoba Research Ethics Board (REB# HS25169 (H2021:341) to Dr. Ayesha Saleem). Blood samples were obtained from 3 consenting adult patients with PMD (MELAS; N=1) and SMD (Hypophosphatasia; N=2). The recruited patients were matched with healthy control subjects (N=3) by age and sex. **(B)** Plasma was isolated from the blood and filtered (0.22 µm) and used for EV isolation via size exclusion chromatography (SEC, qEV columns, IZON) and concentrated by ultrafiltration (F7-9). **(C)** C2C12 cells were incubated with 0.25 mM H_2O_2 (6hr) to induce mitochondrial dysfunction in vitro. EV-depleted growth media was replaced and after 18h of recovery conditioned media (CM) from control and H2O2-treated cells was collected and filtered (0.22 µm). EVs were isolated from CM (N=6) via differential ultracentrifugation. **(D)** Biophysical characteristics (size, zeta potential and concentration) of fresh EVs were evaluated by tunable resistance pulse sensing (TRPS, IZON®). Proteins from EVs were extracted and protein yield was assessed by micro-BCA protein assay. Western blot was used to measure the expression of small-EVs (TSG101 and Flotillin-1), medium/large EVs (Cytochrome-C) and non-specific protein markers (ApoA1). The number of subjects for MELAS (N=1) and HPP (N=2) patients in this study did not permit us to perform statistical analyses on the data. The statistical analyses were performed on H_2O_2-EVs study using Unpaired Student's t-test.

RESULTS

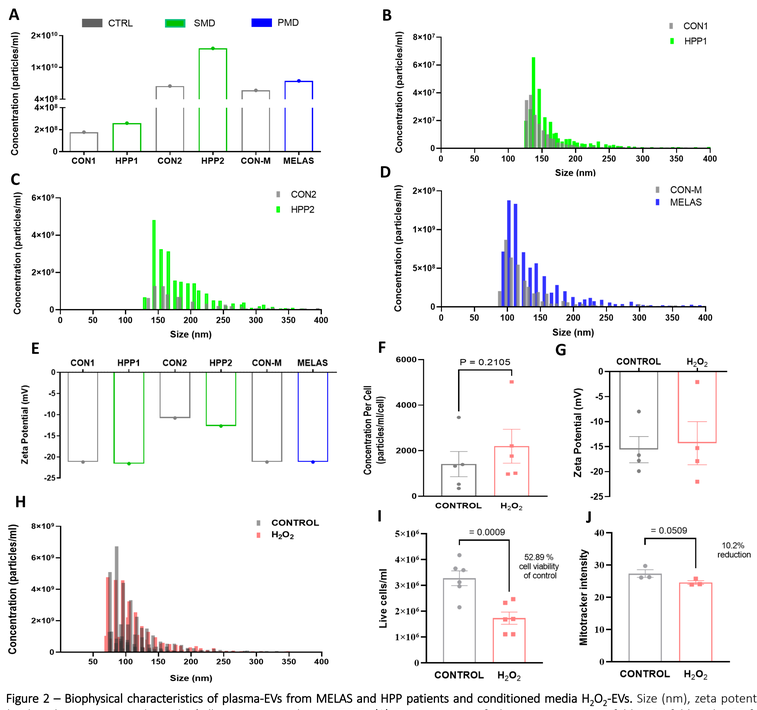

Figure 2 – Biophysical characteristics of plasma-EVs from MELAS and HPP patients and conditioned media H_2O_2-EVs. Size (nm), zeta potential (mV) and concentration (particles/ml) were assessed using TRPS. **(A)** Concentration of plasma-EVs was 1.96-fold, 1.48-fold and 2.96-fold higher in MELAS, HPP1 HPP2 *vs.* respective control, **(F)** and 1.58-fold higher in H_2O_2-EVs vs. control-EVs. *vs.* plasma/EVs **(E)** Zeta potential remained unchanged between patient-EVs and control, also for control-EVs and H_2O_2-EVs **(G)**. Size distribution histogram of plasma-EVs from **(B)** HPP1, **(C)** HPP2 and **(D)** MELAS patients and respective control matched, **(H)** and control-EVs and H_2O_2-EVs. **(I)** The viability of C2C12 cells after H_2O_2 incubation (0.250 mM, 6h) was assessed using Trypan blue staining and expressed as the number of live cells/ml. **(J)** Mitotracker staining was performed to evaluate mitochondria content in control and H_2O_2 cells. Data was grouped as PMD in blue, SMD in green and control in grey. The statistical analyses were performed on H_2O_2-EVs study using Unpaired Student's t-test (N=6).

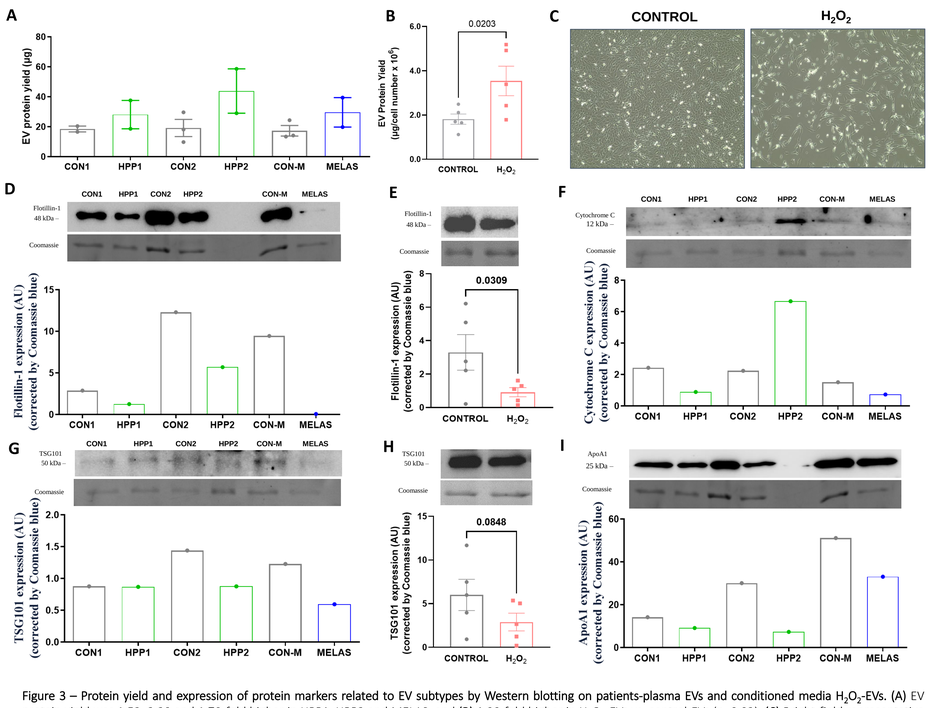

Figure 3 – Protein yield and expression of protein markers related to EV subtypes by Western blotting on patients-plasma EVs and conditioned media H_2O_2-EVs. **(A)** EV protein yield was 1.52, 2.29 and 1.70-fold higher in HPP1, HPP2 and MELAS, and **(B)** 1.90-fold higher in H_2O_2-EVs vs. control-EVs (p=0.02). **(C)** Bright-field representative images of control cells and cells treated (6hrs) with H_2O_2 after 18hrs of recovery. **(D)** Flotillin-1 expression was reduced by 69.54% in patient-EVs, and **(E)** by 35.4% in H_2O_2-EVs (p=0.03). **(F)** Cyt-C was differentially expressed in HPP-EVs and lower on HPP1 and undetectable in H2O2-EVs. **(G)** TSG101 was reduced by 39.1% in MELAS-EVs, 30.48% in HPP-EVs, and **(H)** 51.81% in H2O2-EVs (p=0.08). **(I)** ApoA1 expression was lower in patient-EVs vs. controls. Bands were corrected for loading by Coomassie, with multiple proteins run on one gel. The statistical analyses were performed on H_2O_2-EVs study using Unpaired Student's t-test (N=6).

CONCLUSIONS

- ✓ Mitochondrial dysfunction increased EV concentration and protein yield, and decreased Flotillin-1 expression in both patient-EVs and H_2O_2-EVs.
- ✓ EV subtype-related proteins were expressed differentially. EVs as biomarkers for MD require further investigation but hold viable potential.

FUTURE DIRECTIONS

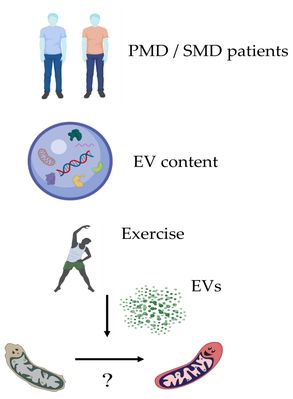

Figure 4 – Future directions of the study. **(A)** Increase the number of patients with primary and secondary mitochondrial disease (PMD/SMD). **(B)** Investigate the PMD/SMD EV content. **(C)** Investigate if EVs derived post-exercise can rescue mitochondrial dysfunction using a cell culture model.

ACKNOWLEDGMENTS

Dr. Souza was funded by Postdoctoral Fellowship from Research Manitoba. This work was done primarily with grants from CHRIM and CHF to Dr. Saleem.