Extracellular vesicles from aged individuals trigger mitochondrial dysfunction in hematopoietic progenitor cells

The role of extracellular vesicles (EVs) in hematopoietic stem cell (HSC) aging remains poorly explored. We have collected evidence to suggest that EVs from older human individuals significantly alter the proteome and transcriptome of hematopoietic progenitor cells (HPC) and specifically impair mitochondrial activity. This work implies that EVs contribute to the decline of HPC functionality during aging, which we propose creates a vulnerability for age-based blood disease development.

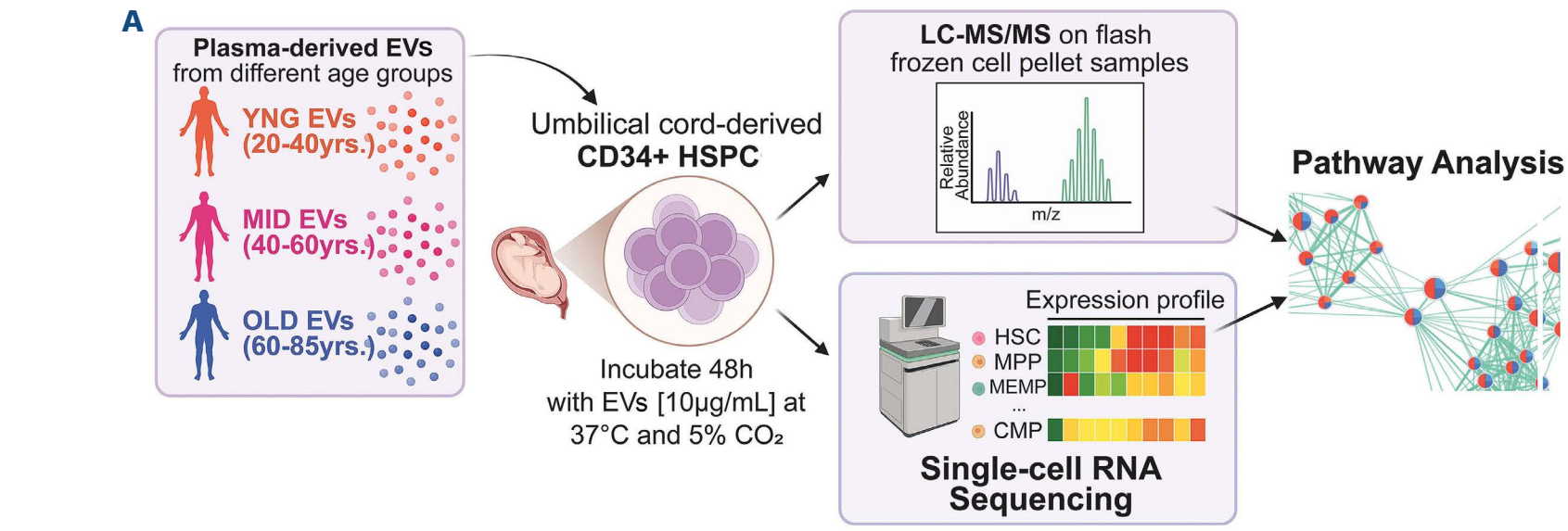
Aging alters hematopoietic stem and progenitor cell (HSPC) biology, leading to the decline of blood and immune cell function. Significant strides have been made in understanding cell autonomous factors during HSPC aging, namely genetic and epigenetic alterations, decreased quiescence, impaired autophagy and mitochondrial dysregulation.[1] With the identification of clonal hematopoiesis (CH) and its associated risk of progression to myeloid neoplasms, a concerted effort has been made toward being able to accurately predict risk and develop therapeutic approaches to avert/delay progression of CH. Recently, three publications have identified mitochondrial metabolism as a central target in HSPC with mutated DNMT3a, suggesting that during aging, CH clones commandeer the mitochondria, leading to an enhancement of oxidative phosphorylation.[2-4] Questions remain as to what are the cellular conditions that make cells permissive to CH mutation interactions.

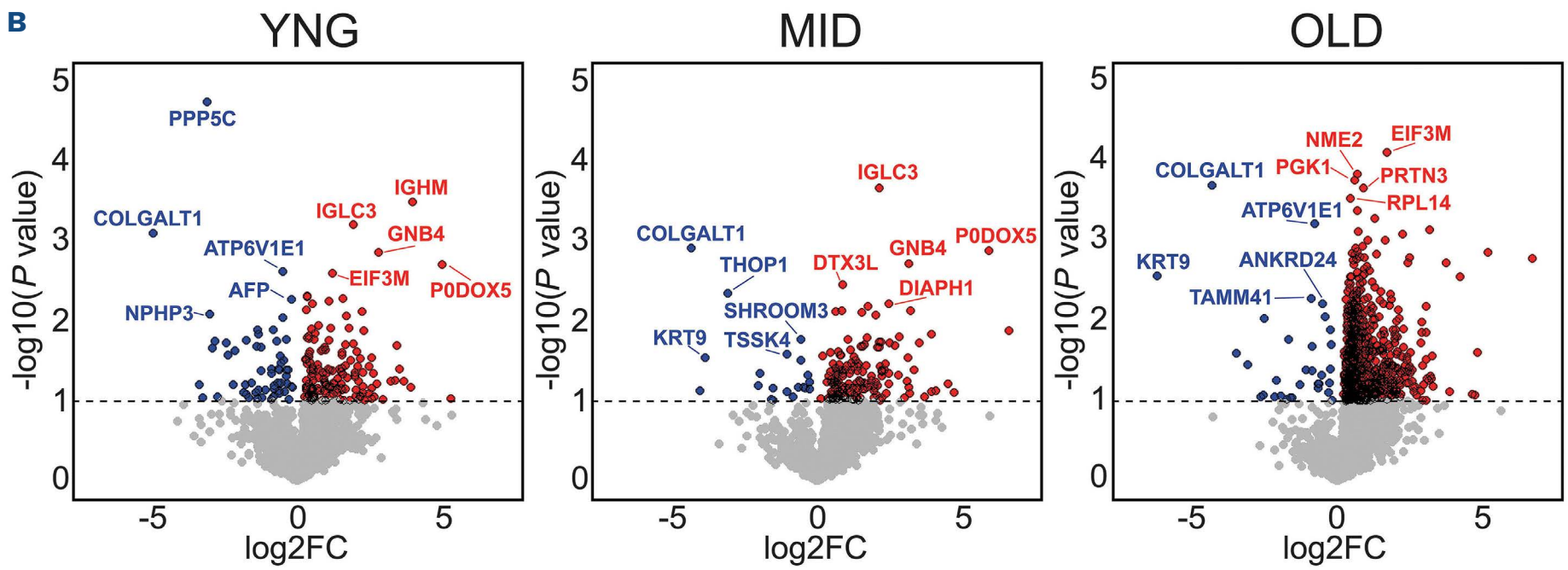
Mitochondria are the quintessential metabolic hubs that coordinate energy production via oxidative phosphorylation with a network of biosynthetic pathways critical for cell biomass production. These primary functions are further interlinked with the regulation of apoptosis and redox balance, which altogether control activation of homeostatic signaling pathways and ultimately overall cell survival.[5] Mitochondrial dysfunction is one of the aging hallmarks[6] and is commonly accepted to be essential in leukemogenesis, to support the increased metabolic needs in rapidly proliferating transformed leukemic stem cells.[1] Notably, the majority of studies investigating mitochondria, aging and cancer have primarily focused on cell intrinsic factors,[7,8] and less on the external factors present in the pre/leukemic microenvironment.

Extracellular vesicles have emerged as critical mediators of cell-to-cell communication. These nano-sized (30 nm -10 microns) phospholipid-enclosed particles are secreted by cells which mediate intercellular communication through the transfer of proteins, lipids, and nucleic acids. In blood cancers, tumor-derived EVs are well documented to remodel the bone marrow microenvironment, suppressing normal hematopoiesis and promoting leukemic expansion.[9] Additionally, EVs are being explored for their therapeutic potential as disease biomarkers due to their ability to mirror the physiological or pathological state of their cells of origin. Although few studies have explicitly examined connections between EVs and HSPC aging, some evidence suggests an age-based decline in functional mitochondria in multiple subpopulations of plasma EVs.[10]

Our previous work demonstrates that aging alters blood EVs content,[11,12] thereby having the potential to contribute to HSPC dysfunction.[13] To expressly investigate exactly how blood EVs affect HSPC in the context of aging, plasma-derived EVs were enriched from young (20-40 years old), middle-aged (40-60 years), and older (60-85 years) individuals. Human sample collection followed the principles of the Declaration of Helsinki and was approved by the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (HSREB). Approval for the collection of human umbilical cord blood was obtained prior to commencement of the study (Department Code; DBMS-093-18, TRAQ# 6024642). Informed verbal consent was obtained from all blood donors undergoing total hip arthroplasty surgery according to HSREB regulations.

Extracellular vesicle purification and characterization methods were based on guidelines from the Society of Extracellular Vesicles.[13] Post enrichment, EVs were incubated with umbilical sourced $CD34^+$ cells for 48 hours (h), with cells subsequently washed with PBS and prepared for proteomics (Figure 1A). When comparing the differential expression of bulk proteins compared to phosphate-buffered saline (PBS) control samples, volcano plots suggested a progressive increase in the number of differentially expressed proteins following exposure to EVs from individuals as they age (Figure 1B). Using proteins (N=1,916 proteins identified after pre-processing and duplicate removal) identified in the mass spectrometry data, we implemented the ActivePathways algorithm as a means to perform integrative pathway enrichment analysis for significant biological processes triggered by EVs. The resulting enrichment map of biological pathways reveals that only EVs from older subjects significantly alter the proteome of HSPC (Figure 1C). EVs from older subjects were observed to trigger pathways involved in translation, cellular response to stress/stimuli, ribosomal RNA processing, nonsense-mediated decay and regulation of mitochondrial membrane potential (Figure 1C). Few to no biological processes were triggered in the presence of EVs from either young or middle-aged subjects. Ingenuity pathway analyses





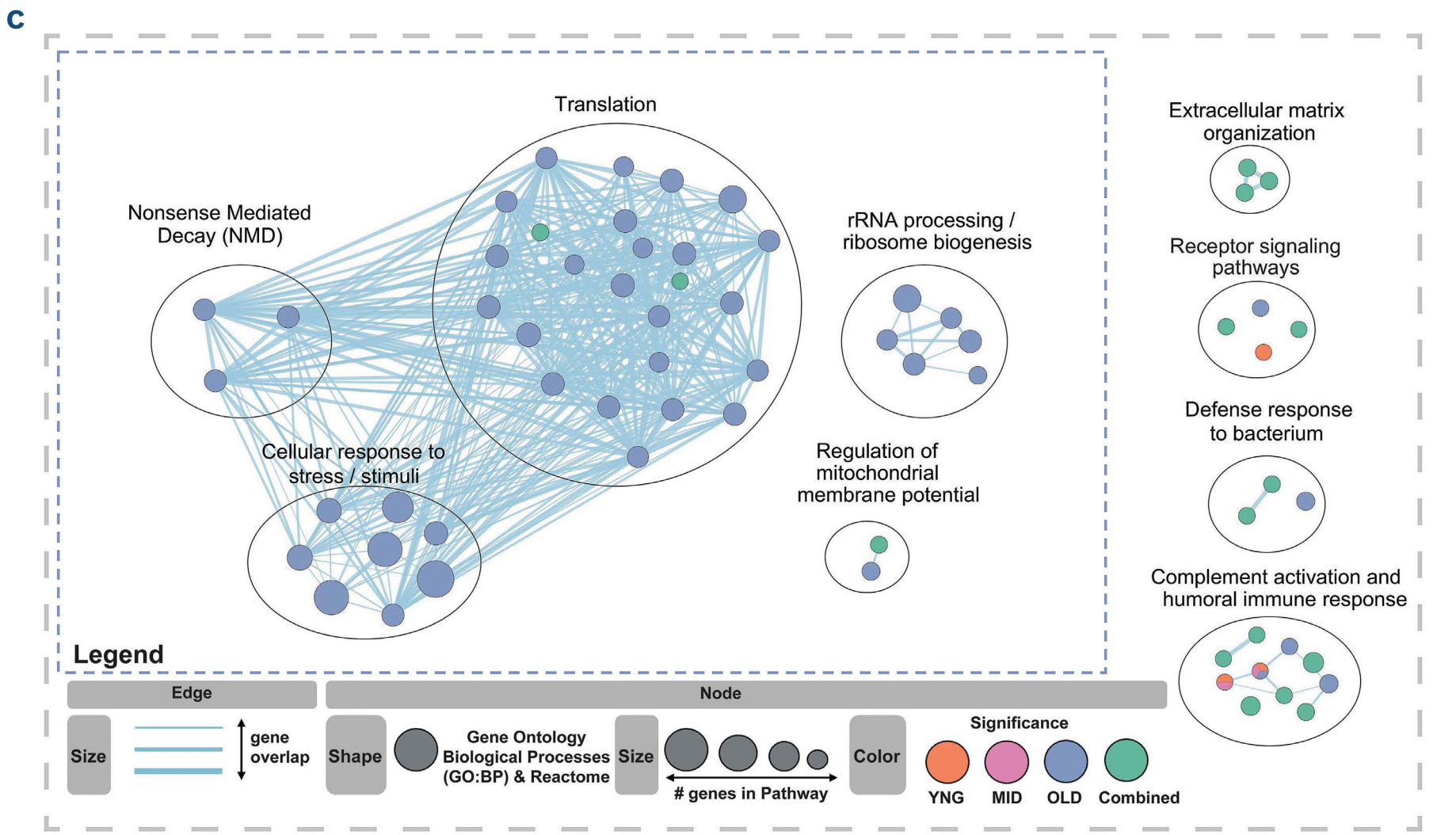


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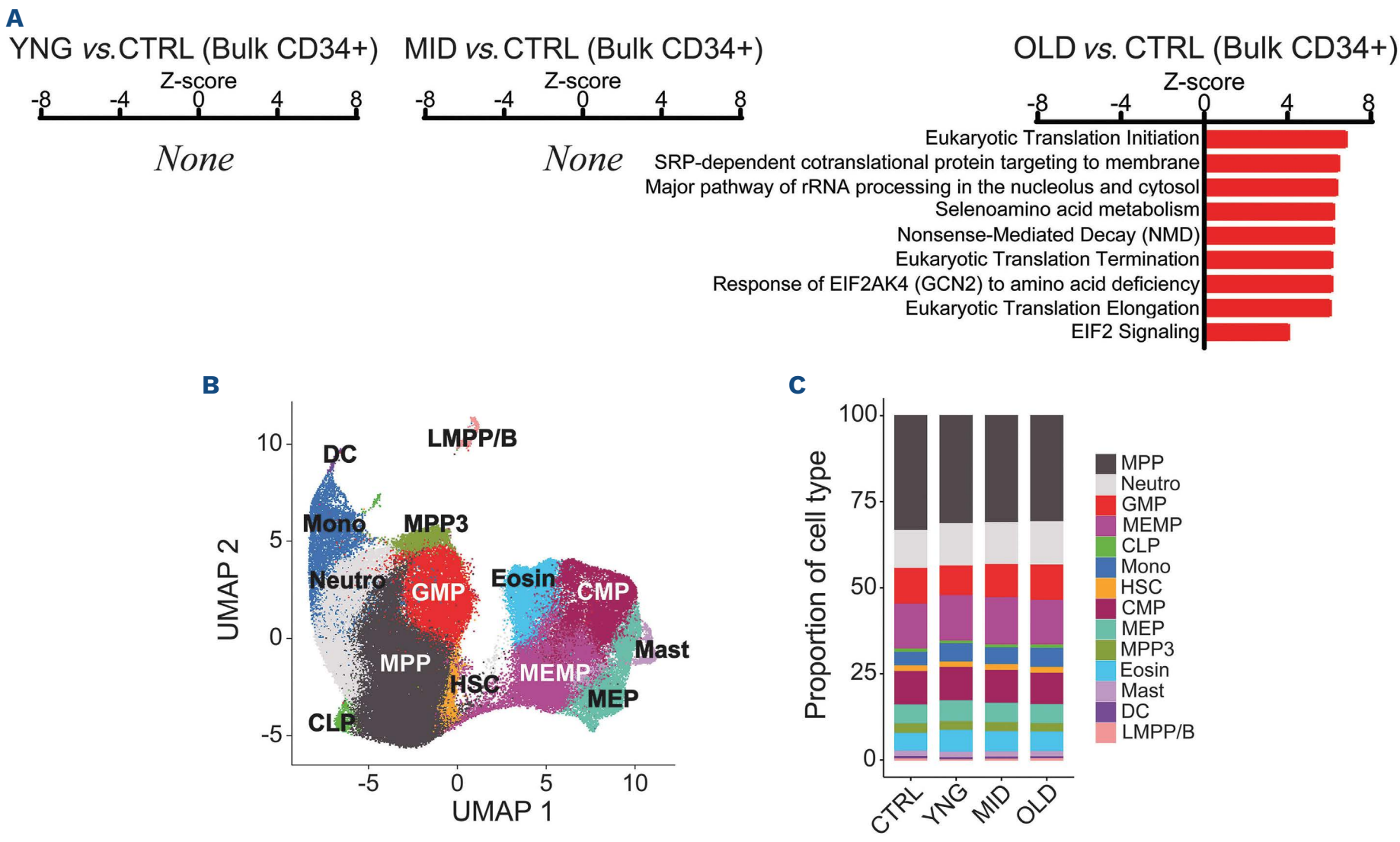
Figure 1. Extracellular vesicles from older individuals alter the expression of various biological processes in the proteome of hematopoietic stem and progenitor cells. CD34⁺ cells were incubated for 48 hours (h) with or without extracellular vesicles (EVs) from various age groups: phosphate-buffered saline (PBS) control; young (YNG) 20-40 years; middle-aged (MID) 40-60 years; OLD 60-85 years. (A) Schematic of experimental design for proteomics and single cell transcriptomics. (B) Volcano plots comparing hematopoietic stem and progenitor cell (HSPC) protein post 48-h EV incubation. (C) Enrichment map of biological processes (Gene Ontology) and molecular pathways. Reactome for each EV age group compared to control (False Discovery Rate <0.05). Circled areas indicate common biological themes. Statistical analysis was performed by unpaired t test with Welch correction.

(IPA) further supported enrichment of the select processes similar to ActivePathways, and additionally provided directionality of pathway (i.e., activation or inhibition) based on z-score (Figure 2A), supporting a predicted activation of eukaryotic translation initiation (z-score = 6.856; adjusted *P* value [adj-pval] = 1.84E-05), eukaryotic initiation factor 2 (EIF2) signaling (z-score = 4.082; adj-pval = 2.31E-05), major pathway of rRNA processing in the nucleolus and cytosol (z-score = 3.807; adj-pval = 1.56E-04), and nonsense-mediated decay (NMD) (z-score = 3.807; adj-pval = 1.56E-04) (Figure 2A, B). Thus, EVs from older individuals are altering normal physiological processes.

To investigate how EVs might regulate specific HSPC populations at a transcriptional level, we used single-cell transcriptome analyses (as outlined in Figure 1A) (N=3 biological EVs samples per group). Cell type annotation of the heterogeneous HSPC population was performed using normalized gene expression of top differentially expressed and key cell lineage marker genes, identifying 14 unique hematopoietic cell subsets. Uniform Manifold Approximation and Projection (UMAP) display the 14 distinct HSPC subtypes based on their transcriptomic profiles (Figure 2B).

Despite the proportion of the 14 hematopoietic cell types remaining unchanged post 48 h incubation with plasma-derived EVs from young, middle-aged and older individuals compared to control (Figure 2C), a disproportionate number of differentially expressed genes (DEG) were identified depending on both the cell type and the donor age of EVs (Figure 2D). Interestingly, the multipotent progenitor (MPP) population and downstream myeloid precursor cells displayed the highest number of DEG (Figure 2D). The MPP cell subset (representing 30.9-31.4% of identified cells following EVs exposure was the most transcriptionally sensitive out of the 14 HSPC populations to EVs exposure (Figure 2E). Results suggests that while EVs from different aged subjects affect a similar number of genes, the magnitude and significance of these changes is more pronounced in response to exposure to EVs derived from older individuals. We continued our analysis on HSC, MPP, myeloid-biased multipotent progenitor (MPP3), megakaryocyte-erythroid-mast cell progenitor



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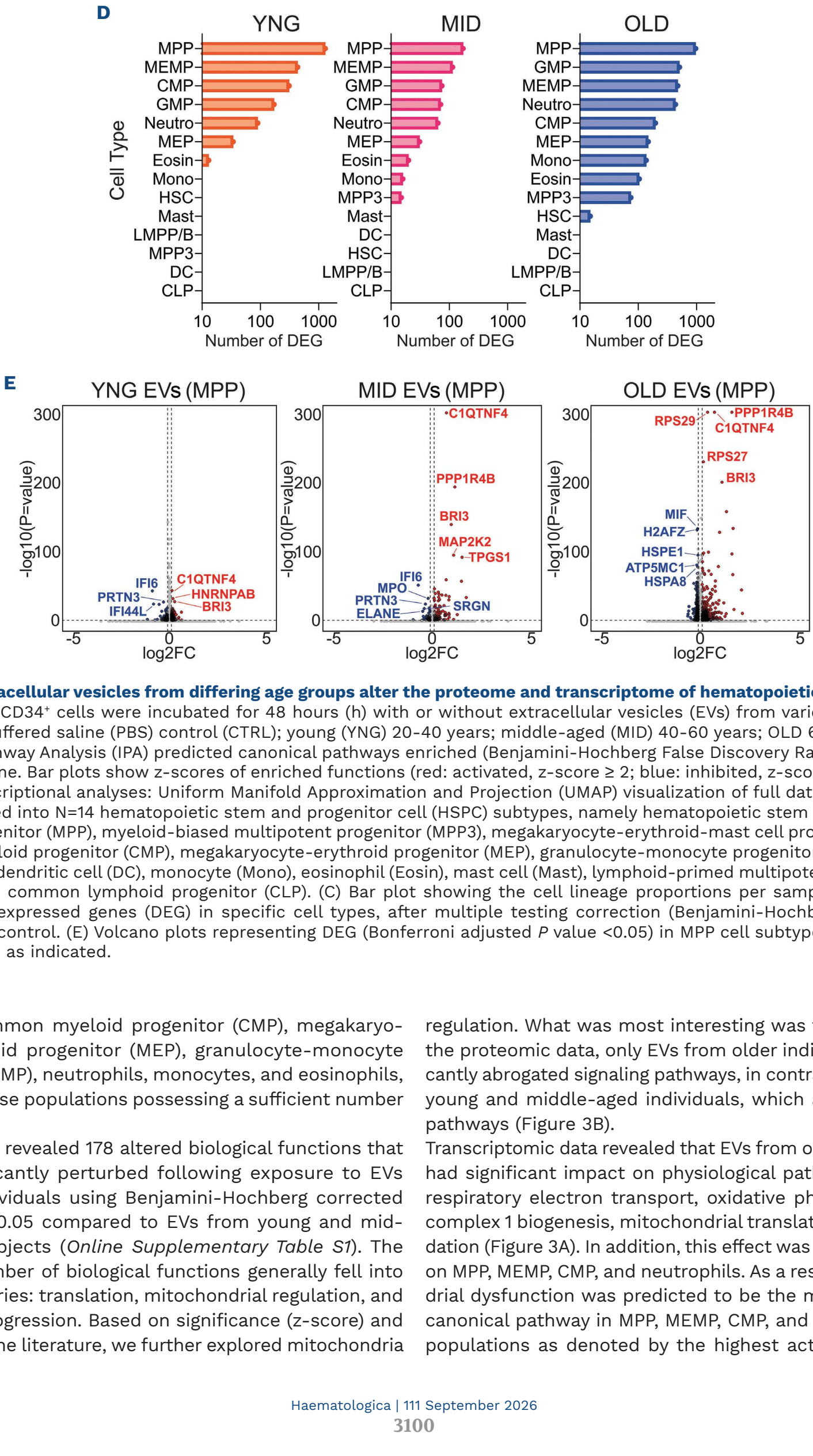

Figure 2. Extracellular vesicles from differing age groups alter the proteome and transcriptome of hematopoietic stem and progenitor cells. CD34⁺ cells were incubated for 48 hours (h) with or without extracellular vesicles (EVs) from various age groups: phosphate-buffered saline (PBS) control (CTRL); young (YNG) 20-40 years; middle-aged (MID) 40-60 years; OLD 60-85 years). (A) Ingenuity Pathway Analysis (IPA) predicted canonical pathways enriched (Benjamini-Hochberg False Discovery Rate [FDR] < 0.05) in the proteome. Bar plots show z-scores of enriched functions (red: activated, z-score ≥ 2; blue: inhibited, z-score ≤ -2). (B) Single cell transcriptional analyses: Uniform Manifold Approximation and Projection (UMAP) visualization of full dataset (N=106,267 cells) clustered into N=14 hematopoietic stem and progenitor cell (HSPC) subtypes, namely hematopoietic stem cell (HSC), multipotent progenitor (MPP), myeloid-biased multipotent progenitor (MPP3), megakaryocyte-erythroid-mast cell progenitor (MEMP), common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP), granulocyte-monocyte progenitor (GMP), neutrophil (Neutro), dendritic cell (DC), monocyte (Mono), eosinophil (Eosin), mast cell (Mast), lymphoid-primed multipotent progenitor/B cell (LMPP/B), common lymphoid progenitor (CLP). (C) Bar plot showing the cell lineage proportions per sample. (D) Bar plot differentially expressed genes (DEG) in specific cell types, after multiple testing correction (Benjamini-Hochberg FDR <0.05) compared to control. (E) Volcano plots representing DEG (Bonferroni adjusted *P* value <0.05) in MPP cell subtypes following exposure to EVs as indicated.

(MEMP), common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP), granulocyte-monocyte progenitor (GMP), neutrophils, monocytes, and eosinophils, based on those populations possessing a sufficient number of DEG.

Our analyses revealed 178 altered biological functions that were significantly perturbed following exposure to EVs of older individuals using Benjamini-Hochberg corrected *P* value of <0.05 compared to EVs from young and middle-aged subjects (*Online Supplementary Table S1*). The greatest number of biological functions generally fell into three categories: translation, mitochondrial regulation, and cell cycle progression. Based on significance (z-score) and evidence in the literature, we further explored mitochondria regulation. What was most interesting was that, similar to the proteomic data, only EVs from older individuals significantly abrogated signaling pathways, in contrast to EVs from young and middle-aged individuals, which altered few/no pathways (Figure 3B).

Transcriptomic data revealed that EVs from older individuals had significant impact on physiological pathways such as respiratory electron transport, oxidative phosphorylation, complex 1 biogenesis, mitochondrial translation and degradation (Figure 3A). In addition, this effect was most profound on MPP, MEMP, CMP, and neutrophils. As a result. mitochondrial dysfunction was predicted to be the most perturbed canonical pathway in MPP, MEMP, CMP, and neutrophil cell populations as denoted by the highest activated z-score

among all other biological processes present in each of the above-mentioned cell types.

To confirm our pathway analyses, we decided to functionally assess the mitochondrial membrane potential (ΔΨm) of

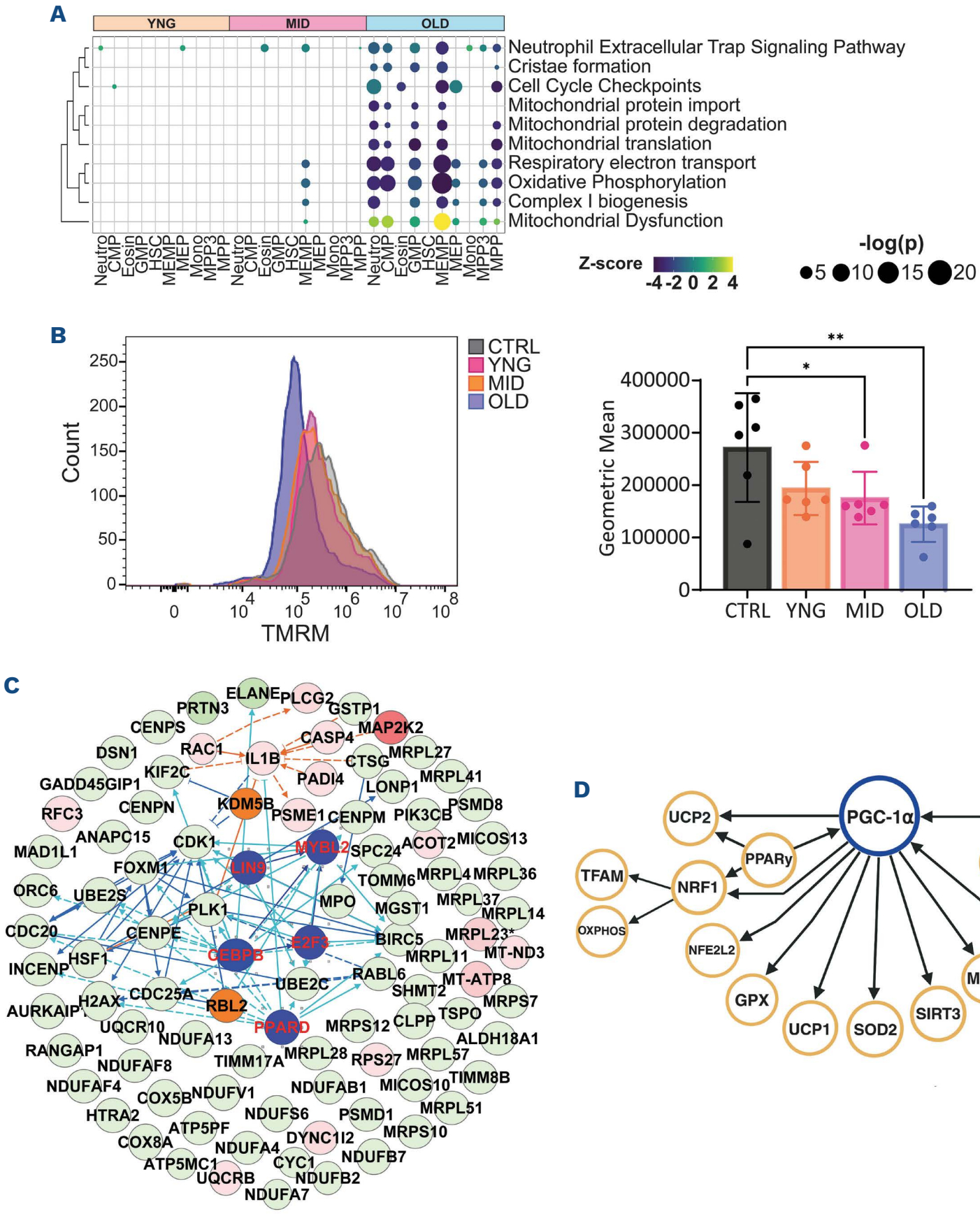

Figure 3. Extracellular vesicles from older subjects augment mitochondrial dysfunction in multipotent progenitors and downstream progenitors. (A) Mitochondrial-related pathways significantly modulated in screen. Dot plots separated by extracellular vesicle (EV) exposure displaying altered canonical pathways according to age group: young (20-40 years) = pink; middle-aged (40-60 years) = orange; old (60-85 years) = blue. Activated pathways (z-score ≥ 2) or inhibited pathways (z-score ≤ -2). All highlighted pathways significant (Benjamin-Hochberg False Discovery Rate [FDR] < 0.05). The dot size represents the negative log *P* value. (B) Tetramethylrhodamine, methyl ester (TMRM) staining of $CD34^{+}CD38^{-}$ cells post EV incubation (representative plot and averaged data from N=4 hematopoietic stem and progenitor cell [HSPC] and N=6 EV samples). Statistical Analysis: RM one-way ANOVA, with Holm-Šídák's multiple comparisons test *P≤0.05, **P≤0.01. (C) Mitochondrial genes identified in multipotent progenitor (MPP), treated with EVs from older subjects; down-regulated genes in green, up-regulated genes in red, predicted activation in orange, and predicted inhibition in blue. Please note that different font colors have no other meaning than making them easier to read. (D) The predicted inhibition of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PPARGC1A, commonly known as PGC-1α), a crucial regulator of mitochondrial regulation. Pathway analyses performed using Ingenuity Pathway Analysis (IPA) (Benjamin-Hochberg FDR <0.05). CTRL: control.

umbilical cord sourced $CD34^{+}CD38^{-}$ cells (N=4), incubated with EVs from young, middle-aged and older individuals (N=6 per age group). Following a 48-h incubation, as previously completed, Tetramethylrhodamine, methyl ester (TMRM) staining was performed and assessed using flow cytometry. Results demonstrate that blood EVs compromise mitochondrial function in an age-dependent manner, confirming our bioinformatic analyses (Figure 3B). Considering that the MPP cell type comprises a large portion of all cells analyzed and was the most primitive hematopoietic population identified, being upstream of the MEMP, CMP and neutrophils, we further probed the genes responsible for the predicted mitochondrial dysfunction. All genes captured in our transcriptomic dataset involved in mitochondrial regulation are indicated in Figure 3C. Based on genes affected, four out of five Complexes within the Electron Transport Chain were impacted by the exposure of EVs from older individuals. Taking a more global approach, we utilized the molecule activity predictor (MAP) function of IPA, to identify key regulators and identified peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PPARGC1A, commonly known as PGC-1α) as a central regulator (Figure 3D) that was predicted to be down-regulated. These data suggest EVs from older individuals de-regulate mitochondrial function at the single-cell level in both primitive and progenitor hematopoietic cells. Interestingly, another study performing reciprocal experiments[14] shows PGC-1α is not only down-regulated in older mice, but also that injected EVs from young mice stimulate PGC-1α, complementing our own observations of EVs from older humans dampening PGC-1α. In conclusion, we have collected evidence to suggest that blood EVs from older human individuals significantly alter the proteome and transcriptome of HPC when compared to EVs from younger individuals, corroborating results that blood-sourced EVs have little effect on the most primitive HSC population.[15] Importantly, EVs from older individuals initiate differential expression of genes in primitive multipotent and myeloid progenitors, and up-regulate mitochondrial dysfunction, specifically dampening PGC-1α activity, a critical modulator in energy metabolism and mitochondrial biogenesis. Our work suggests that as individuals age, EVs that are released into the bloodstream are detrimental to HPC function and specifically impair mitochondrial activity. This work implies that blood EVs contribute to the decline of HPC functionality and may preclude age-based disease development.

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Contributions

SAA is responsible for study concept, methodology, formal analyses, funding acquisition, supervised the study, wrote the original draft, and reviewed and edited the final version of the work for publication; CJW, SMH and IJGP are responsible for methodology, formal analyses, visualization, investigation, wrote the original draft, and reviewed and edited the final version of the work for publication; JK is responsible for methodology, formal analyses, visualization, investigation and supervised the study; MS is responsible for methodology, visualization and investigation; CH and MV are responsible for methodology and investigation; JFR, SM, KT, EB, SAD, JR, MH and SMC are responsible for methodology; PT is responsible for funding acquisition and supervised the study; EYWC supervised the study, and reviewed and edited the final version of the work for publication; LMP, AWC and JR supervised the study.

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Data-sharing statement

All data are available in the main text, the Online Supplementary Appendix or will be deposited in an appropriate repository.

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