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Title: Next-generation RNA-Sequencing of serum small extracellular vesicles discovers potential diagnostic biomarkers for dementia with Lewy bodies

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Abstract:

Objectives: There is an urgent clinical need for identifying blood-based diagnostic biomarkers for Dementia with Lewy Bodies (DLB). Transcriptomic studies have reported unique RNA changes in post-mortem DLB brains. Small extracellular vesicles (SEV) that transport RNA between brain and peripheral circulation enable identifying molecular changes in living human brain. Hence, we aimed to identify differentially expressed RNA in serum SEVs from people with DLB.

Methods: We investigated serum SEV total RNA profiles in people with DLB (n=10) and age and gender matched comparisons (n=10) using next-generation RNA-sequencing. SEVs were separated by ultracentrifugation with density gradient and were characterized by nanoparticle analysis and western blotting. We verified identified differentially expressed genes (DEG) using high-throughput qPCR. Functional implications of identified DEG were evaluated using Ingenuity pathway analyses.

Results: We identified 846 nominally significant DEG including 30 miRNAs in DLB serum SEVs. We identified significant downregulation of pro-inflammatory genes, *IL1B*, *CXCL8* and *IKBKB*. Previously reported post-mortem DLB brain DEGs were significantly enriched (χ^2=4.99; df=1; p=0.03) among the identified DEGs, and the differential expression of 40 post-mortem DLB brain DEGs could be detected in serum SEVs of people living with DLB. Functional pathway and network analyses highlighted the importance of immunosenescence, ubiquitin proteasome system (UPS) dysfunction, DNA repair and RNA post-transcriptional modification deficits in DLB pathology.

Conclusion: Identified DEGs, especially reduced expression levels of inflammation and UPS associated RNA, may aid diagnosing DLB, and their biomarker potential warrants further investigation in larger clinical cohorts. Our findings corroborate the absence of chronic neuroinflammation in DLB.

Introduction:

Dementia with Lewy bodies (DLB) is the second most common neurodegenerative dementia (1) that causes earlier mortality (2), earlier nursing home admissions, higher costs (3) and more caregivers' burden than Alzheimer's disease (AD). DLB (4) and AD are diagnosed by their clinical diagnostic criteria. Currently, the three available indicative diagnostic biomarkers (4) for DLB are not routinely used in clinical settings (5), and there is no reliable biological fluid based biomarker for aiding DLB diagnosis. Failure to diagnose DLB accurately and treating visual hallucinations and challenging behaviors, more frequent in DLB than in AD, with any antipsychotic medication can lead to potentially fatal adverse effects including neuroleptic malignant syndrome (5). Moreover, early diagnosis of DLB is essential for formulating appropriate multidisciplinary management plans. Hence, there is an urgent clinical need for identifying reliable blood-based diagnostic biomarkers for DLB, but pertinent research remains sparse (6).

Better understanding of molecular pathology of DLB is important for identifying reliable biomarkers. Two genome-wide association studies (7,8) and at least 73 candidate gene association studies have investigated the molecular genetics of DLB so far (9). Genetic associations between DLB and variants in *APOE, GBA, SNCA* and *MAPT* have been replicated by two or more studies. Other reported genetic associations of DLB that need further replication include the variants in *BCHE-K, BCL7C, CHRFAM7A, CNTN1, GABRB3,* mtDNA, *NOS2A, PSEN1, SCARB2, TREM2, ZFPM1* and *UCHL1* (9). Gene expression studies investigating RNA levels clarify functional implications of identified genetic associations and their dysfunctional molecular networks. RNA changes indicate the effects of gene-environment interactions, and the changes in non-coding RNA levels add functional information that cannot be provided by studies investigating DNA and proteins. Three next-generation RNA sequencing (RNA-Seq) studies (10), and at least 21 quantitative gene expression studies have

investigated gene expression changes in people with DLB (11). Most of them have studied gene expression changes in post-mortem DLB brains and they have reported 4,842 statistically significant differentially expressed genes (DEG) in post-mortem DLB brains (10). We have previously reported DEGs, identified by RNA-Seq, and metabolic reprogramming in post-mortem anterior cingulate and dorsolateral prefrontal cortices of pathology-verified DLB (10,11). We identified four genome-wide statistically significant DEGs (*CTSG*, *SELE*, *GIPR* and *PSPHP1*), and have documented significant downregulation of several pro-inflammatory genes in post-mortem DLB brains (10). Although prior evidence have confirmed unique RNA expression changes in post-mortem DLB brains (11), identifying differentially expressed RNA in biological fluids such as blood or cerebrospinal fluid (CSF) of people living with DLB is necessary for discovering novel clinically applicable diagnostic biomarkers. Yet, studies investigating RNA levels in biological fluids of people with DLB are sparse (11). One study has reported significant downregulation of miR-125b in DLB CSF (12) and another two studies that investigated peripheral leukocytes (13,14) have reported significant upregulation of *SNCA*-126 isoform and significant downregulation of four mitochondrial genes, *ATP8, MT-CO2, MT-CO3* and *MT-ND2*, in DLB.

Each neurodegenerative disorder is hypothesized to have its own unique peripheral RNA signature (15). The discovery of small (30-100nm) extracellular vesicles (SEV) that can cross the blood-brain barrier and can transport RNA between brain and peripheral circulation has opened up an avenue for studying molecular changes in living human brain by investigating peripheral blood samples (16). Studies investigating extracellular vesicles (EV) have been increasing exponentially since the last decade, and the minimal information for studies of extracellular vesicles (MISEV2018) guidelines was published by the International Society of Extracellular Vesicles in 2018 (17). The MISEV2018 guidelines discourage using the previously popular term “exosomes” that assumed specific biogenesis, and they urge naming

EVs based on their physical or biochemical characteristics (17). Nearly 100 EV-based diagnostic and prognostic biomarkers have been identified for various malignancies (18). The diagnostic biomarker potential of serum or plasma SEVs for neurodegenerative disorders is increasingly recognized (19), and several potential diagnostic SEV RNA biomarkers for AD have been identified (20). Moreover, CSF derived SEVs from people with DLB can induce α-synuclein aggregation *in-vitro* (21). However, only one study has investigated CSF, serum, or plasma SEV RNA profiles of people with DLB so far (22). That exploratory study has sequenced only microRNA (miRNA) profiles of plasma SEV RNA from seven people with DLB, and it could not identify any statistically significant differentially expressed miRNA between DLB and comparisons (22). SEV long RNA including messenger RNA (mRNA) profiles in DLB have not been investigated so far. Hence, we aimed to conduct a RNA-Seq study investigating serum SEV total RNA profiles of people living with DLB.

Methods:

Serum samples:

We obtained serum samples from the biobanks of three Norwegian cohorts (23-25). The dementia study of western Norway (DemWest) is a relatively large longitudinal cohort study (23) including people with probable DLB (26). The DLB diagnosis was based on a standardized set of clinical and biomarker analyses, longitudinal follow-up, and pathological confirmation of a subset (27). Serum samples from 10 people living with probable DLB, and three gender and age (±3 years) matched comparisons without cognitive impairment or Parkinson's disease (NDC; No-dementia comparison) were obtained from this cohort. Another seven gender and age (±3 years) matched NDC serum samples were obtained from two cohorts at Akershus University Hospital dementia research centers (24,25). All three cohorts allowed 30 minutes for coagulation of their blood samples, centrifuged their samples at room

temperature and stored them at -80°C until use. There were minor variations in the duration and speed of their centrifugation procedures. Supplemental digital content (SDC-1) provides age, gender, cohort, and centrifugation details of all 20 samples. The data on the Mini-mental status examination total score (28), the Clinical Dementia Rating Scale global score, and the number of years of formal education were available only for the DemWest cohort samples, and they are presented in the SDC-1. Further details of the cohorts have been published elsewhere (23-25). These studies have obtained generic ethical approval for further studies using their serum samples.

Separation of EVs:

SEVs were separated using an ultracentrifugation and OptiPrep^{TM} (Sigma-Aldrich, UK) density gradient approach based on their buoyant density. In brief, a discontinuous iodixanol gradient was prepared by diluting a stock solution of OptiPrep^{TM} (60%w/v) with 0.25M sucrose/10 mM Tris (pH 7.5) for generating 40%, 20%, 10% and 5%w/v iodixanol solutions. The solutions were sequentially layered (3 ml/layer) in an UltraClear (Beckman Coulter, USA) centrifuge tube, and 0.5ml of serum/ sample was overlaid. The tubes were centrifuged at 100,000g at 4°C for 16 hours. Subsequently, 12 fractions of one ml were collected from the top of the gradient. Ninth to 11th fractions were diluted in phosphate buffered saline (PBS) (29). They were centrifuged further at 100,000g for one hour at 4°C in an Optima-Max centrifuge (Beckman Coulter, USA), and this was repeated once more. After discarding the supernatant, the SEV pellet was reconstituted with 200µl PBS.

Characterization of EVs:

Size distribution and concentration of separated EVs were verified using the Malvern NanoSight LM10 nanoparticle analyzer (Malvern Instruments Ltd., UK) using standard manufacturer's instructions. Separation of SEVs was confirmed by Western blotting using antibodies against Flotillin-1 and CD63, which are widely used exosomal markers (17).

RNA extraction:

Total SEV RNA including all mRNA, miRNA, and other small RNA were purified using the Invitrogen total exosome RNA isolation kit (Thermo Fisher Scientific, USA). Quality of purified RNA were assessed using the NanoDrop™ One/One microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, USA), and the mean 260/280 absorbance ratio of purified RNA was 2.05 (95%CI 1.88-2.22).

Next-generation RNA-sequencing (RNA-Seq):

Purified serum SEV RNA were sequenced at the Wellcome Centre for Human Genetics, Oxford, UK, using the Illumina HiSeq-2500 (Illumina, USA). A NEBNext Ultra-II directional cDNA library and another NEBNext small RNA cDNA library (New England Biolabs, USA) were prepared for each sample. The cDNA libraries underwent single-end sequencing (50 base pairs/read), and we obtained a minimum of 10 million reads/sample.

RNA-Seq data analyses:

Quality control of RNA-Seq reads removed the reads that included an ambiguous base, and the reads that had less than 90% of bases with less than 1% sequencing error. Remaining reads were aligned to the human genome (Homo_sapiens.GRCh38) with corresponding gene model annotation (Homo_sapiens.GRCh38.88.gtf) using the *HISAT2* (30). Gene-wise counting of aligned reads was completed using the *featureCounts* tool (31). DEGs in DLB serum SEVs were identified by a previously experimentally validated (32) *edgeR* 3.18.1 algorithm employing generalized linear models with tag-wise dispersion (33), and Benjamini-Hochberg transcriptome-wide false discovery rate (FDR) correction (5%). The *edgeR* p-values were derived by employing exact tests (no df) after fitting gene-specific quasi-negative binominal models and estimating dispersion using the quantile adjusted conditional maximum likelihood method (33).

Verification of identified DEGs:

Differential expression levels of 48 identified DEGs (SDC-2) in serum SEVs of people living with DLB were evaluated using high-throughput quantitative polymerase chain reactions (qPCR). Total SEV RNA were purified using the Invitrogen total SEV RNA isolation kit (Thermo Fisher Scientific, USA) from 0.5ml aliquots of serum samples that had been sequenced (N=20). 100ng of SEV RNA/sample were reverse transcribed using the miScript®-II RT Kit and its 5X miScript HiFlex buffer (Qiagen, UK). After 19 cycles of specific target amplification with the PreAmp master-mix (Fluidigm, USA), high-throughput qPCR was performed using the BioMark HD, GE 96.96 dynamic arrays (Fluidigm, USA), and SsoFast EvaGreen low ROX kit (Bio-Rad, USA). SDC-3 provides further details of the qPCR verification. A DEG would be verified if it met the following criteria (34): (i) Both RNA-Seq and qPCR showed same direction of differential expression, and (ii) differential expression fold change, estimated by qPCR, was either above 1.25 or below 0.80 (logarithmic fold change (L_2FC) cut-off ±0.3219).

Functional analyses of identified DEGs:

Functional implications of identified DEGs (*edgeR* $p<0.05$; no df) were analyzed by Ingenuity Pathway Analysis (IPA) using the Ingenuity knowledge base (Ingenuity, USA). The IPA is a powerful functional analysis tool that helps identifying potential biomarkers within the context of biological systems. Our IPA analysis settings included stringent filters with only experimentally observed relationships, and they helped identifying disrupted functional pathways and dysfunctional molecular networks in serum SEVs of people with DLB. The IPA p-values were calculated by estimating the ratio of the number of DEGs that map to a canonical pathway to the total number of molecules that map to that canonical pathway, and then by using Fisher's exact test (no df) for determining the probability that the association between the DEGs and the canonical pathway is explained by chance alone (35).

Results:

Serum SEV RNA profile:

The Malvern NanoSight LM10 nanoparticle analysis revealed that 94.47% (95%CI 92.13-96.81%) of the separated EVs were between 30 and 100nm in size. Mean amount of total purified SEV RNA/ sample, estimated by the NanoDrop™ One/One microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, USA), was 227.80 (95%CI 149.39-306.21) ng. All RNA-Seq data files are available in the NCBI BioProject database under accession number PRJNA530121 (https://www.ncbi.nlm.nih.gov/sra/PRJNA530121). RNA, expressed from 429 miRNA genes and 8,863 other genes, were found in the investigated serum SEVs. Only 244 (56.9%) miRNA genes and 3,314 (37.4%) other genes were expressed in both DLB and NDC serum SEVs. RNA, expressed from 28 mitochondrial genes, were found in both DLB and NDC serum SEVs. 112 (26.1%) miRNA genes and 2,173 (24.5%) other genes were expressed only in DLB serum SEVs.

Serum SEV DEGs in DLB:

We identified 846 statistically significant (*edgeR* $p<0.05$; no df) DEGs in serum SEVs of people with DLB, compared to NDC serum SEVs (SDC-4). There were 737 downregulated and 109 upregulated DEGs. They included 30 differentially expressed miRNA and one mitochondrial gene, *MT-TS2*, encoded RNA. None of the DEGs reached transcriptome-wide statistical significance (*edgeR* $q<0.05$; no df) after Benjamini-Hochberg FDR correction. However, differential expression levels of 77.1% (37/48; 95%CI 65.2-89.0%) of the nominally significant DEGs (*edgeR* $p<0.05$; no df) could be verified (34) by high-throughput qPCR, and Table-1 presents those 37 qPCR verified DEGs. RNA expression levels of pro-inflammatory genes, *IL1B*, *CXCL8* and *IKBKB* were significantly (*edgeR* $p<0.05$; no df) downregulated in DLB serum SEVs, and their differential expression were also verified by qPCR. Other statistically significant (*edgeR* $p<0.05$; no df) downregulated DEGs in DLB, verified by qPCR,

included pro-apoptotic *BID* and *TNFRSF1A*, ubiquitin proteasome system (UPS) associated *UBE3A*, *USP47*, and *PSMD4*, as well as *PSEN1* regulating β-amyloid synthesis. Moreover, qPCR-verified significantly (*edgeR* $p<0.05$; no df) upregulated DEGs in DLB serum SEVs included *PTPRF*, *MIR556,* and *SMG9* that plays a critical role in the nonsense-mediated mRNA decay (NMD). *PTPRF* encodes a protein tyrosine phosphatase that contributes to the regulation of cell growth, differentiation, mitotic cycle, insulin resistance and oncogenesis (36). *MIR556* has been reported to be associated with the proliferation and migration of various malignancies (37). SDC-5 presents the z-scores of *edgeR* normalized expression levels of the 37 qPCR verified DEGs. z-scores above two in at least one of the following three genes, *SMG9*, *PTPRF*, and *MIR556*, could classify the people with DLB with 60% sensitivity, 100% specificity, 100% positive predictive value, and 71.43% negative predictive value.

Comparing with DEGs in post-mortem DLB brains:

We cross-linked our post-mortem cortical transcriptomic data that have been reported elsewhere (10) with the DLB serum SEV RNA profiles, and found statistically significant ($\chi^2=4.99$; df=1; $p=0.03$) enrichment of post-mortem DLB brain DEGs (*edgeR* $p<0.05$; no df) among the DEGs (*edgeR* $p<0.05$; no df) identified in this study. Table-2 presents the 40 DEGs that were differentially expressed in both post-mortem DLB brains and serum SEVs of people living with DLB. Expression levels of pro-inflammatory genes, *IL1B*, *CXCL8* and *IFI44L* were significantly (*edgeR* $p<0.05$; no df) downregulated in both post-mortem DLB brains and DLB serum SEVs. In addition, *ABCA13*, involved in active transmembrane transport of lipid species, *PARVB*, involved in GTPase activation and integrin signaling, and a transcriptional activator *LBH* were significantly (*edgeR* $p<0.05$; no df) downregulated in both datasets. Moreover, *JUND* that antagonizes apoptosis and slows down cell growth as well as *AVP* encoding vasopressin, neurophysin 2 and copeptin were significantly (*edgeR* $p<0.05$; no df) upregulated in both post-mortem DLB brains and DLB serum SEVs.

Functional analyses of identified DEGs:

We investigated the functional implications of the 846 identified DEGs (*edgeR* $p<0.05$; no df) using IPA. SDC-6 presents the molecular pathways that were significantly enriched among the serum SEV DEGs in people with DLB. Huntington's disease signaling (Fisher's exact $p=3.05X10^{-5}$; no df), regulation of eIF4 and p70S6K signaling (Fisher's exact $p=4.21X10^{-5}$; no df), and glucocorticoid receptor signaling (Fisher's exact $p=5.96X10^{-5}$; no df) associated genes were significantly enriched among the identified DEGs. Pro-inflammatory pathways such as role of PKR in interferon induction and antiviral response (Fisher's exact $p=1.89X10^{-4}$; no df), TNFR1 signaling (Fisher's exact $p=6.65X10^{-4}$; no df), IL-6 signaling (Fisher's exact $p=6.88X10^{-4}$; no df), IL-8 signaling (Fisher's exact p=0.001; no df), interferon signaling (Fisher's exact p=0.004; no df) and T-cell receptor signaling (Fisher's exact p=0.01; no df) were significantly downregulated in DLB serum SEVs. Integrin signaling (Fisher's exact $p=3.44X10^{-4}$; no df) and Integrin linked kinase signaling (Fisher's exact $p=3.27X10^{-4}$; no df) pathways were significantly enriched among the identified DEGs, and the overlapping DEGs were predominantly downregulated in DLB serum SEVs. Moreover, protein ubiquitination pathway was significantly (Fisher's exact p=0.007; no df) enriched among the identified DEGs, and 15 identified significantly (*edgeR* $p<0.05$; no df) downregulated DEGs overlapped with this pathway. Furthermore, IPA upstream and causal network analyses indicated that downregulation of transcription regulators, *TP53* (Fisher's exact $p=1.38X10^{-7}$; no df), *NUPR1* (Fisher's exact $p=2.72X10^{-4}$; no df) and *WT1* (Fisher's exact $p=4.32X10^{-4}$; no df) and of *TYROBP* (Fisher's exact $p=5.65X10^{-4}$; no df) encoding a transmembrane receptor were probable upstream causes that may explain the identified DEGs in DLB serum SEVs.

The network analyses showed that the direct and indirect interactions of several identified DEGs converge on the following three downregulated DEGs that interact among themselves, (i) *IL1B* encoding Interleukin-1β, (ii) pro-apoptotic *CASP3*, and (iii) *PSEN1* that

is essential for γ-secretase complex cleaving β-amyloid from amyloid precursor protein (APP) (Figure-1A). This dysfunctional molecular network can explain how the interactions between the DEGs may impact neuronal survival, organismal injury, and apoptosis in DLB. Moreover, several identified DEGs and their interactions can influence transcriptional regulation of many downstream genes, RNA post-transcriptional modification, and DNA replication and repair (Figure-1B).

Discussion:

This is the first study that systematically investigated serum SEV total RNA profiles of people living with DLB and their overlap with post-mortem DLB cortical transcriptomic data. The study has confirmed the feasibility of measuring gene expression changes in serum SEVs of people living with DLB like measuring such changes in post-mortem DLB brains. It has identified differentially expressed mRNA and miRNA, and their dysfunctional molecular networks in serum SEVs from people with DLB. We have observed that previously reported post-mortem DLB brain DEGs (10) were significantly enriched among the DEGs identified by this study, and that the statistically significant differential expression of 40 post-mortem DLB brain DEGs (10) could be detected in serum SEVs of people living with DLB. Our findings have highlighted the importance of immunosenescence, UPS dysfunction, and the deficits in DNA repair, RNA post-transcriptional modification, and APP processing in DLB pathology.

Chronic microglial activation and neuroinflammation contribute to AD pathology (38). However, prior evidence supporting chronic neuroinflammation in DLB have been inconsistent, and several immunohistochemical, transcriptomic and proteomic studies have documented notable absence of chronic neuroinflammation in post-mortem DLB brains (9,11,39). Transcriptomic studies using gene expression microarray and RNA-Seq methodologies have reported statistically significant downregulation of several pro-

inflammatory genes including *IL1B*, *IL2*, *IL6*, *CXCL2*, *CXCL3*, *CXCL8*, *CXCL10*, and *CXCL11* in post-mortem DLB brains (10,40). Another transcriptomic and proteomic study corroborated the absence of chronic neuroinflammation in post-mortem pulvinar region of DLB brains (41). Moreover, immunohistochemical staining with IBA1 and CD68 antibodies revealed reduced microglial density and more microglial dystrophy in post-mortem DLB brains (42). In this study, we have observed statistically significant reduced expression of pro-inflammatory genes, *IL1B*, *CXCL8*, *IFI44L* and *IKBKB*, and statistically significant downregulation of several inflammatory pathways, such as TNFR1, IL-6, IL-8, interferon and T-cell receptor signaling pathways in DLB serum SEVs. Neuronal survival and synaptic plasticity require optimal microglial activation (43), and immunosenescence leading to impaired neuronal survival may contribute more to DLB pathology than chronic neuroinflammation (10,11). Hence, reduced expression levels of the identified pro-inflammatory DEGs in serum SEVs may aid diagnosing DLB early and may help distinguishing DLB from AD. Their diagnostic and prognostic biomarker potential needs to be investigated in larger clinical cohorts.

The UPS is an important intracellular degradation system for clearing pathologically misfolded proteins, and its dysfunction substantially contributes to the progression of α-synucleinopathies (44). Our findings revealed significantly reduced levels of UPS associated genes, *UBE3A*, *USP47*, and *PSMD4*, and significant downregulation of the protein ubiquitination pathway in DLB serum SEVs. An earlier study that investigated RNA profiles of substantia nigra dopaminergic neurons of people with Parkinson's disease has reported statistically significant downregulation of *USP47* and *PSMD4* (45). The UPS closely interacts with the autophagy lysosomal pathway, and its dysfunction has been demonstrated to be sufficient for inducing Lewy body-like inclusions in mice models (46). Downregulation of the protein ubiquitination pathway aggravates α-synuclein aggregation and cytoplasmic accumulation of other misfolded proteins that can set off a vicious cycle by inhibiting the

neuronal autophagy lysosomal pathway. However, systematic research investigating the diagnostic biomarker and therapeutic potential of the UPS molecules in DLB remain sparse. Significant downregulation of *UBE3A*, *USP47*, and *PSMD4* in serum SEVs in people with DLB could be detected by both RNA-Seq and qPCR, and their diagnostic biomarker potential warrants further research.

α- synuclein is a DNA binding protein and it regulates DNA damage response and DNA repair (47). DNA repair deficits and consequent increased levels of DNA double strand breaks are associated with Lewy body pathology and neurodegeneration in mice models and post-mortem human DLB brains (47). Moreover, transcriptional RNA processing such as RNA splicing contributes to α-synuclein aggregation (48). We have presented a dysfunctional molecular network involving several identified DEGs that can affect DNA repair and RNA post-transcriptional modification. Downregulation of *RPA1* that plays an important role in DNA replication, and downregulation of *CUL4A*, an ubiquitin ligase protein that regulates DNA repair, can impair neuronal survival by affecting DNA damage response in DLB. *CUL4A*, and *RNMT* that catalyzes mRNA cap methylation interact with RNA polymerase II (49) and their downregulation may lead to reduced expression of many downstream genes that are essential for neuronal survival in DLB. As SEVs deliver RNA to recipient cells and modify DNA damage response in recipient cells (50), this dysfunctional molecular network may contribute to the progression of DLB pathology.

Downregulation of *PSEN1* can increase α-synuclein aggregation independent of its γ-secretase activity, and it may explain varying degrees of comorbid AD pathology in DLB (51). Besides, significantly reduced expression levels of pro-apoptotic *CASP3* and *BID* in DLB serum SEVs are intriguing. Apoptosis and mitosis maintain tissue homeostasis, and decreased apoptosis may pathologically prolong survival of dysfunctional cells (52). The cell cycle two-hit hypothesis that involves non-dividing neurons re-entering into steady state G1 phase, and

then losing their ability to undergo apoptosis because of chronic oxidative stress has been studied extensively in AD (53). Although α- synuclein is known to alter cell cycle progression (54), the contributions of cell cycle re-entry and decreased apoptosis towards DLB pathology have not been investigated. Molecular mechanisms underlying reduced expression of *CASP3* in DLB serum SEVs and its impact on autophagy lysosomal pathway in recipient cells (55) warrant further investigation. Moreover, ATP-binding cassette family genes, *ABCA7* and *ABCA13*, were significantly downregulated in DLB serum SEVs. Rare variants in *ABCA13* have been associated with various neuropsychiatric disorders (56), and common variants in *ABCA7* have been associated with AD (57). Future studies may consider investigating their contributions towards the molecular pathology of DLB.

We acknowledge the limitations of this study including its small sample size and investigating the total serum SEV population that was not enriched for neuronal origin. Further studies should compare DLB SEV RNA profiles with those of people with AD for disentangling disease-specific effects. However, circulating SEV RNA have opened a new avenue for identifying blood-based diagnostic biomarkers for DLB, and diagnostic biomarker potential of identified DEGs warrant further evaluation in large replication cohorts. Multiplexing biomarkers improves their diagnostic accuracy and predictive values, and potential RNA biomarkers can be investigated together by developing a multiplex RNA biomarker assay (58). The diagnostic accuracy including sensitivity and specificity of such biomarker assay will need further investigation. Moreover, gene expression changes are dynamic, and they differ with disease progression. Measuring the expression levels of identified DEGs at various clinical stages of DLB may help diagnosing DLB early, identifying novel prognostic biomarkers, and advancing our understanding of molecular pathology of rapidly progressive DLB (59). Furthermore, investigating SEVs that are enriched for neuronal origin by L1CAM immunoprecipitation can enhance study power and biomarker discovery

(19). However, the caveat is that only 5-10% of circulating SEVs are considered to be of neuronal origin (19), and limited starting volumes of clinical blood samples make the investigation of neuronally enriched serum or plasma SEV RNA profiles methodologically challenging. Several methodological challenges have been identified in separation, enrichment, quantification, characterization, and functional analysis of EVs, and the MISEV2018 guidelines have made specific recommendations for improving methodologies of further research in this important area (17). Optimizing the immunoprecipitation and RNA-Seq library preparation methods for investigating very low input neuronally enriched SEV RNA will aid identifying reliable blood-based diagnostic biomarkers and novel therapeutic targets for DLB.

Figure-legends:

Figure-1: Functional network analyses of identified differentially expressed genes in serum small extracellular vesicles of people living with dementia with Lewy bodies.

A: A network of 32 identified DEGs that may impact cell death and survival, and organismal injury and abnormalities; **B**: Another network of identified DEGs that can influence gene expression, RNA post-transcriptional modifications, DNA replication, recombination, and repair; **(A-B)** Green represents downregulated genes, and red represents upregulated genes. Solid lines represent direct interactions and dotted lines represent indirect interactions.

Supplemental Digital Content (SDC):

Note: Supplementary information are available in the online version of the paper.

1. SDC-1 presents the sample characteristics (.docx file).
2. SDC-2 presents forward and reverse primer sequences that have been used for high-throughput qPCR verification of 48 identified differentially expressed genes (DEG) (first worksheet) and eight reference (second worksheet) genes (.xlsx file).
3. SDC-3 presents an overview of separation of small extracellular vesicles, RNA extraction, cDNA synthesis, specific target amplification and high-throughput qPCR verification procedures (.docx file).
4. SDC-4 presents differential expression analyses of RNA-Seq data from serum small extracellular vesicles of people living with dementia with Lewy bodies (n=10) and of age and gender matched comparisons without cognitive impairment or Parkinson's disease (n=10) (.xlsx file).
5. SDC-5 presents the Z-scores of *edgeR* normalized expression levels of the 37 qPCR verified DEGs
6. SDC-6 presents the results of Ingenuity pathway analyses that identified the canonical pathways, which were statistically significantly enriched among the identified DEG in serum small extracellular vesicles from people living with DLB (.xlsx file).

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Author contributions:

APR, AH, CB, and DA were involved in the conception and the design of this research. JL, and TF provided the serum samples. AH isolated SEV from serum samples, and APR extracted RNA from SEV. APR analyzed the RNA-Seq data and completed subsequent functional analyses. APR, YRM, and AH performed high-throughput qPCR verification and analyzed the data. APR and AH drafted the initial manuscript. All authors were involved in the critical revisions and final approval of the manuscript.

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