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IDENTIFICATION OF SPECIFIC BIOSIGNATURES FROM PLASMA SAMPLES FOR THE DIFFERENTIAL DIAGNOSIS OF DEMENTIA WITH LEWY BODIES

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1. Project summary

1.1 Introduction

Dementia with Lewy bodies (DLB) is the second most common type of degenerative dementia after Alzheimer's disease (AD), accounting for 20-30% of dementia cases. It is a very heterogeneous and complex disease, and belongs together with Parkinson's disease (PD) to the group of Lewy body disorders, characterized by the presence of alpha-synuclein positive inclusions in the brain.

DLB shows overlapping features with AD hindering accurate diagnosis and correct management of DLB. Frequently, psychiatric symptoms are not readily identifiable and the presence of cognitive impairment or motor symptoms may lead to an AD or PD misdiagnosis. Correct diagnosis of DLB is of paramount importance, since this condition is characterized by an aggressive clinical course and high overall mortality.

Characteristic irreversible neurodegeneration begins long before clinical symptoms of DLB or AD become evident, hampering effective treatments. Therefore, the early identification of patients is an important goal in dementia research.

Peripheral blood samples include different sources of biological material for biomarker discovery. Of these, platelets are characterized by their content of several neurotransmitters, their transporters and receptors, and additionally by a complete miRNA pathway. Another source of biological material derived from peripheral blood is extracellular vesicles including exosomes. The latter are increasingly being considered a valuable source of cell-derived information. Exosomes are nanometre-sized endosome-derived vesicles secreted by many cell types and found in most body fluids. Exosomes contain specific cytoplasmic material from the cell of origin, including nucleic acids (especially small RNA species and miRNAs), proteins and metabolites, making them attractive in biomarker discovery.

1.2 Objectives

1. To collect blood samples from DLB and AD patients, and control individuals, and to isolate DNA, platelets and extracellular vesicles.

2. To carry out genetic analysis, including mutation and expression analyses, of the GBA gene as a major DLB risk factor.

3. To determine by whole transcriptome analysis specific mRNA and miRNA expression changes in platelets and extracellular vesicles from DLB, AD and controls.

4. To determine by whole proteome expression changes in platelets and extracellular vesicles from DLB, AD and controls.

5. To analyse expression levels of those mRNAs and miRNAs showing disease-specific expression changes in either the platelets or extracellular vesicles.

6. To establish a biosignature of specific mRNA/miRNA to distinguish DLB from AD and controls.

1.3 Study design

Prospective cross-sectional study of patients with DLB and AD diagnosed at two tertiary hospitals in the Barcelona metropolitan area controlled by control subjects of the same age range: extraction of DNA, platelets, extracellular vesicles, mRNA and miRNA expression analysis.

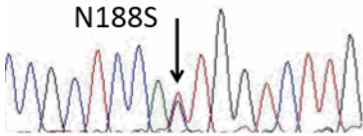
Samples and subjects to be included: Clinical diagnosis of DLB and AD patients was carried out in the Departments of Neurology at the Hospitals Germans Trias i Pujol, Badalona, and Bellvitge, L'Hospitalet. The same departments recruited age- and gender-matched control subjects. All participants signed an informed consent, previously approved by the Ethics Committee of the Hospital Germans Trias i Pujol. **Sample obtaining:** Ten mL of blood was collected in (1) ACD tubes to minimize platelet activation, and (2) in EDTA tubes (both: BD Vacutainer). **DNA and platelet extraction from blood** were carried out following standard procedures. **Extraction of extracellular vesicles from blood** was performed by enriching the supernatant after platelet obtaining, using a column-based approach. Extracellular vesicle-containing fractions were monitored by the expression of vesicle-associated markers such as CD9 and CD81 by flow cytometry. **RNA/miRNA extraction from platelets and extracellular vesicles** was carried out with the mirVana™ miRNA Isolation Kit based on the use of a glass fibre filter (GFF). The method isolated total RNA ranging in size

from kbs (large RNA fraction) down to 10-mers (small RNA fraction; <200 nt). A quality control assay was performed using a High Sensitivity ScreenTape in a Bioanalyzer. **Small RNA expression profiling of platelets and extracellular vesicles** was performed with the TruSeq Small RNA Sample Preparation Kit which is based on a direct ligation to two adapters to each RNA molecule and a subsequent RT reaction to create single stranded cDNA. The cDNA was PCR amplified and loaded on a MiSeq System (Illumina) for clustering and subsequent sequencing. **Reverse transcription and mRNA/miRNA expression analysis by qPCR:** potential mRNA biomarkers were validated by real-time PCR and potential miRNA biomarkers by the use of predesigned Pick-&-Mix panels and LNA-technology. In both cases, relative expression changes were evaluated by the deltadeltaCt method. **Data analysis of mRNA/miRNA expression:** Tertiary data analysis was performed by applying a standard workflow for this kind of data consisting of (i) performing quality control of the sequences obtained to rule out low quality sequences, (ii) aligning remaining sequences against the reference genome, (iii) counting known mRNAs and also any new splicing, exon-junctions or other available variants, (iv) quantifying differential expression between the different study groups, and (v) performing biological significance analyses of the elements found by checking enrichment in target annotations databases and afterwards against different databases (GO, KEGG, etc.) to facilitate interpretation of the results. **Establishment of the biosignature:** Regardless of the methodology used adjusted p-values less than 0.05 represented significant expression changes. The significant expression change of at least one mRNA or miRNA consistently present in all samples analysed in the present study was accepted as a marker.

Based on selected biomarkers a predictive signature was built and validated. This was done using a 5-fold cross-validation approach to select the best predictive model and the best features to be included in it. Cross-validating all the process allowed to avoid selection bias and warranted unbiased estimates of predictive accuracy that ensure reproducibility of the derived signature in new independent datasets.
Within the 3 years of project duration we were be only able to propose which miRNAs could constitute the final biomarker profile for the early and differential diagnosis of DLB. All corresponding validation studies in independent and multi-centre cohorts are ongoing.

2. Results

2.1 Glucocerebrosidase gene mutations precipitate onset of Lewy body dementia and prevail in males.



In this study, GBA mRNA sequences were analysed in a neuropathological cohort including 50 DLB, 44 PD and 34 control brain samples and in a clinical cohort of 47 DLB patients and 131 unaffected individuals. Six different GBA mutations were identified in 16 patients with relevant overrepresentation in brains with pure DLB (35.7%, p=0.001). The most common mutation was E326K, which was strongly associated with pure DLB and PD with dementia. We confirmed that E326K was not a common polymorphism in our population. Correlation analyses with clinical data revealed that GBA mutations are associated with earlier DLB onset (64.7 vs 72.4, p=0.014) and are mainly present in males (90% in mutation-carriers vs 52.5% in non-mutation-carriers, p=0.021). **Conclusions:** Our results show that *GBA* mutations confer risk for DLB development also in the Spanish population. They are associated with earlier age at onset and are more prevalent in male patients.

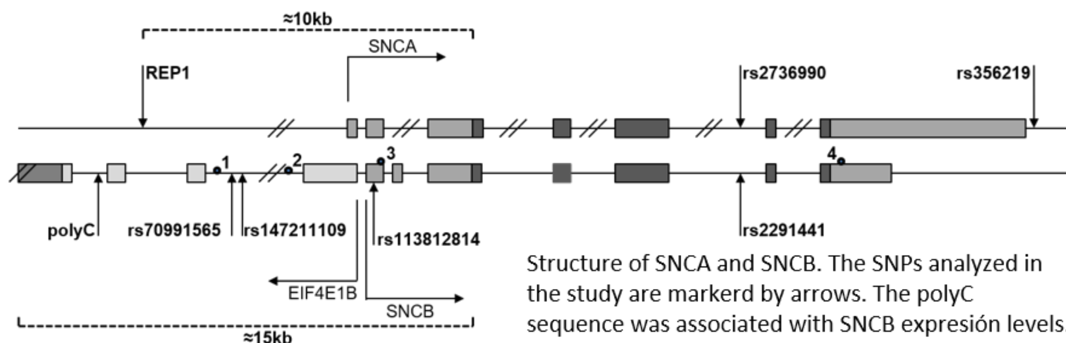
2.2 Glucocerebrosidase gene mutations are accumulated in idiopathic REM sleep behaviour disorder.

All GBA coding exons from 69 polysomnography-confirmed IRBD patients and 84 matched controls were sequenced by the Sanger method. As a result, seven missense mutations (E326K, L444P, A446T, A318K, R329C, T369M, N370S) were identified in eight (11.6%) IRBD patients and in one (1.2%) control (P=0.026).

GBA-variant	cDNA change	aa change	MAF	SNP
A318G	c.1070C>G	p.A357G	n/a	n/a
E326K	c.1093G>A	p.E365K	0.011	rs2230288
R329C	c.1102C>T	p.R368C	1.22 e-5	rs374306700
T369M	c.1223C>T	p.T408M	0.0061	rs75548401
N370S	c.1226A>G	p.N409S	0.0022	rs76763715
N392N	c.1303A>G	p.N431N	2.52 e-5	rs777049786
L444P	c.1448T>C	p.L483P	0.0013	rs421016
A446T	c.1453G>A	p.A485T	2.53 e-5	rs759859002

After a mean follow-up of 8.9±3.8 years from IRBD diagnosis, five subjects with GBA mutations developed a Lewy body disorder (3 DLB and 2 PD) and three remained disease-free. The risk of developing a Lewy body disorder was similar in IRBD subjects with GBA mutations and in those without mutations (log rank test, p=0.935). In conclusion, in IRBD, GBA mutations are 1) frequent, 2) associated with impending PD and DLB, but 3) not indicative of a short-term risk for Lewy body disease after IRBD diagnosis. IRBD patients carrying GBA mutations could be studied with disease-modifying interventions aiming to restore the GBA metabolic pathway.

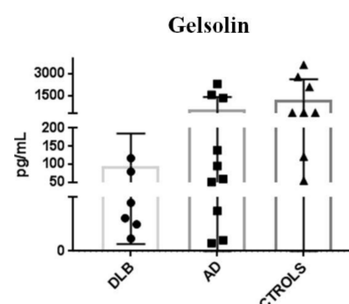
2.3 INDEL length and haplotypes in the beta-synuclein gene may be a key to differentiating dementia with Lewy bodies. In this study, we aimed to analyse the genotype distribution of potentially functional SNPs in *SNCA* and *SNCB*, to perform haplotype analysis for *SNCB*, and to identify functional insertion and deletion (INDEL) variations within the regulatory region of *SNCB* which might be responsible for the drastically diminished beta-synuclein levels reported for pure DLB. Thus, we genotyped brain samples from AD, DLB, PD and healthy controls for two *SNCA* and four *SNCB* SNPs. We also analysed INDEL variations upstream of *SNCB*, determined *SNCB* expression levels and correlated INDEL lengths with expression levels.



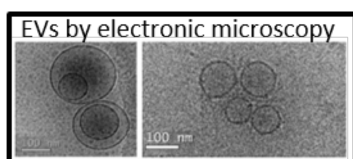
Structure of SNCA and SNCB. The SNPs analyzed in the study are markerd by arrows. The polyC sequence was associated with SNCB expresión levels.

Applying Fisher's exact, chi-square, ANOVA tests, and the deltadeltaCt method, we found disease-specific genotype distribution of *SNCA* and *SNCB* SNPs. Additionally, we identified three INDEL variations upstream of *SNCB* and showed that the INDEL allele lengths were associated with *SNCB* expression levels. INDEL alleles associated with low *SNCB* expression were accumulated in pure DLB. Finally, one major and four minor DLB specific *SNCB* haplotypes were identified with Haploview and Arlequin. In summary, our study showed that differential genotype enrichment in *SNCA* and *SNCB* contributes to the development of either PD or DLB, and that genotypes associated with low *SNCB* expression are accumulated in DLB.

2.4 Characterization of the proteomic profile of plasma-derived exosomes obtained from DLB patients. Two hundred and twenty different proteins were identified by using an in-solution digestion with LysC and Trypsin and, an in-gel-based analysis followed by Trypsin digestion. The comparison of both proteomic methods suggested that gel digestion is a more sensitive method. In addition, decreased levels of gelsolin and butyrylcholinesterase were found in DLB compared to controls. The additional ELISA validation, which also included a group of patients with AD, demonstrated that gelsolin levels are decreased in plasma exosomes of DLB but not in controls and AD. The detection of gelsolin as a putative biomarker in plasma by a conventional ELISA assay should be further explored in independent studies.

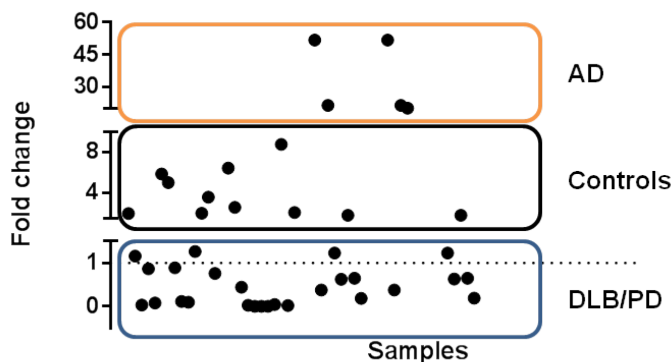


2.5 microRNA profiles from plasma-derived extracellular vesicles differ between Alzheimer's disease and dementia with Lewy bodies. In this study the microRNA (miRNA) content of plasma-derived extracellular vesicles (EVs) from patients suffering from DLB and AD and from healthy age-matched controls was isolated and characterized. Results uncovered a set of 5 miRNAs (hsa-miR-21-5p, hsa-let-7i-5p, hsa-miR-126-3p, hsa-miR-451a and hsa-miR-26a-5p) as significantly down-regulated in AD samples compared with DLB patients and controls; and a group of 4 miRNAs (hsa-miR-24-3p, hsa-miR-183-5p, hsa-miR-143-3p and hsa-miR-423-5p) which were over-expressed in AD compared to DLB and controls, though without statistical significance. ROC curve analysis revealed that hsa-miR-21-5p, hsa-let-7i-5p, hsa-miR-126-3p and hsa-miR-451a with an AUC of about 0.9 in all cases could be promising AD biomarkers. All these miRNAs might represent a possible biosignature for the differential characterization and diagnosis of DLB versus AD. Among the highest scored protein-coding genes identified by predicted target gene analysis, genes encoding for phosphorylation enzymes and proteasome-related proteins, and genes involved in neuroinflammation, cell death or neuron-development, were found. Although validation of these results in larger cohorts is required, altogether, our data suggest that miRNA content of plasma-EVs may reflect the pathogenesis of dementia-related disorders.

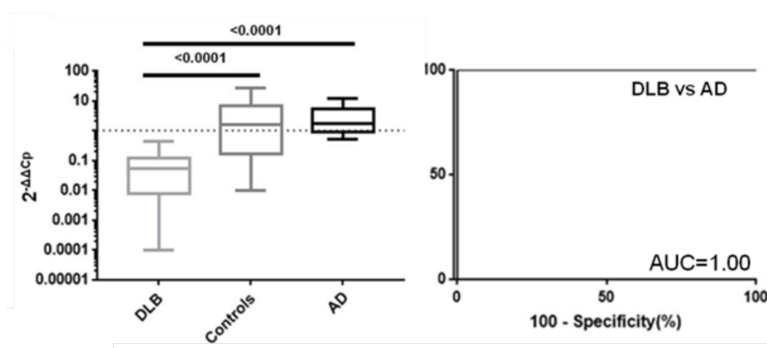


2.6 Platelet hsa-miR-150-5p levels discriminate between synucleinopathies and Alzheimer's disease. This study was carried out in two phases, a discovery phase and a validation phase. To assure valid source material, the platelet rich pellet was characterized by flow cytometry, and contamination of the obtained pellet with leucocytes could be ruled out. During the discovery phase, the whole miRNA transcriptome of platelets was analysed by NGS in 22 DLB patients and 24 controls, and 22 miRNAs were identified as possible biomarkers. After a **first validation study** of DLB (n=14) and controls (n=15), 12 miRNAs met criteria to be further investigated. Two subsequent validation studies including a final blind study were carried out:

- **Second, independent study:** DLB (n=12), Alzheimers' disease patients (n=10), controls (n=10)
- **Blind study:** DLB (n=18), AD (n=6), PD (n=5), age matched controls (n=14).



The blind study showed that the expression of the miRNA with highest discrimination properties could be divided into three levels, low, intermediate and high (**see Figure on the left**). Five out of six AD samples were located within the high-level expression group and 10 out of 14 control samples within the medium-level expression group. All DLB and PD samples were grouped within the low-level expression group. ROC-curve analyses revealed that the miRNA differentiates DLB from AD with very high specificity and sensitivity (**see Figure below**).



These data clearly suggest that the miRNA panel may be a biomarker for the differential diagnosis of DLB versus AD and could be a very useful tool to be introduced into clinical practice on one hand, and to be used for the inclusion of patients in clinical trials on the other.

3. Relevance with possible future implication

The main result of the project is of high clinical relevance, since we have identified a peripheral biomarker to distinguish between dementia with Lewy bodies (DLB) and Alzheimer's disease (AD). This biomarker has been characterized in three independent studies, the initial and two validation studies, and it is of sensitivity and specificity higher than 95%. The findings have been patented (EP18382540.5, date of application: 07/19/2018) and are expected to be validated in proof of concept studies throughout this year. Our final goal is to be able to offer a tool for the differential diagnosis of DLB to be used in clinical practice.

The biomarker identified thanks to this project is the first biomarker for the differential diagnosis of DLB, and although we had identified a biomarker for the identification of one of the DLB subgroups, the one established here is valid for the identification of all patients.

DLB is the second most frequent cause of dementia after AD. Due to the neuropathological overlap of both diseases, the clinical diagnosis of DLB is very difficult and up to 80% of patients receive an erroneous diagnosis, in most cases of AD. The treatment received by poorly diagnosed patients corresponds to that of AD and causes adverse reactions in 50% of them. In this context, the discovery of the biomarker represents an important advance to ensure a correct diagnosis of DLB, avoiding erroneous treatments, to significantly improve the quality of life of patients with DLB and also of their relatives and caregivers. Additionally, its use in clinical practice will contribute to decrease expenses of the health system.

Another very important point is that the application of our biomarker will also contribute directly to the improvement of drug development for the treatment of DLB but also of AD. Many of the recent AD trials have failed since the recruitment of homogenous patient groups is not always guaranteed. With our marker we intend to offer a tool that makes it possible to identify all patients with DLB mistakenly classified as AD and thus contribute to the successful execution of clinical trials related to AD or DLB.

4. Generated literature

4.1 Publications

1. Gámez-Valero A, Prada-Dacasa P, Santos C, Adame-Castillo C, Campdelacreu J, Reñé R, Gascón-Bayarri J, Ispierto L, Álvarez R, Ariza A, Beyer K. GBA Mutations Are Associated With Earlier Onset and Male Sex in Dementia With Lewy Bodies. Mov Disord. 2016, 31, 1066-70; doi: 10.1002/mds.26593. **IF: 6.01, Quartile: 1**

2. Gámez-Valero A, Monguió-Tortajada M, Carreras-Planella L, Franquesa Ml, Beyer K, Borràs FE. Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents. **Sci Rep. 2016**, 6, 33641; doi: 10.1038/srep33641. **IF: 5.228, Quartile: 1**

3. Gámez-Valero A, Beyer K. Alternative splicing of alpha- and beta-synuclein genes plays differential roles in synucleinopathies. **Genes 2018,** 9, 63; doi:10.3390/genes9020063.
IF: 3.600, Quartile: 1

4. Gámez-Valero A, Iranzo A, Serradell M, Vilas D, Santamaria J, Gaig C, Álvarez R, Ariza A, Tolosa E, Beyer K. Glucocerebrosidase gene variants are accumulated in idiopathic REM sleep behavior disorder. **Parkinsonism Relat Dis 2018**, 50:94-98; https://doi.org/10.1016/j.parkreldis.2018.02.034. **IF: 4.721, Quartile: 1**

5. Gámez-Valero A, Canet-Pons J, Urbizu A, Anillo A, Santos C, Ariza A, Beyer K. INDEL length and haplotypes in the b-synuclein gene: A key to differentiate dementia with Lewy bodies? **JAD 2018**, 65:207-219; doi: 10.3233/JAD-180074. **IF: 3.731, Quartile: 2**

Two additional manuscripts are under review:

6. Gámez-Valero A, Campdelacreu J, Vilas D, Ispierto L, Reñé R, Álvarez R, Armengol MP, Borràs FE, Beyer K. microRNA profiles from plasma-derived Extracellular Vesicles differ between Alzheimer's disease and Dementia with Lewy bodies. **Submitted to:** *Journal of Extracellular Vesicles*

7. Gámez-Valero A, Campdelacreu J, Reñé R, Beyer K, Borràs FE. Comprehensive proteomic profiling of plasma-derived EVs from dementia patients points to gelsolin as differential biomarker.

A third manuscript will be submitted after increasing the sample number:
8. Gámez-Valero A, Campdelacreu J, Vilas D, Ispierto L, Gascón-Bayarri J, Reñé R, Álvarez R, Armengol MP, Borràs FE, Beyer K. Can opposite platelet-derived hsa-miR-150-5p expression levels distinguish between dementia with Lewy bodies and Alzheimer disease?

4.2 Congresses

1. Gámez-Valero A, Borràs FE, Beyer K. MicroRNA signature from plasma-derived EVs for Lewy body dementia as promising non-invasive biomarker source. 7th Annual Meeting of the International Society for Extracellular vesicles; Barcelona (Spain), May **2018**: **POSTER**

2. Gámez-Valero A, Borràs FE, Beyer K. Specific platelet microRNA signature for Dementia with Lewy bodies as promising biomarker. Molecular Neurodegeneration Course Wellcome Genome Campus; Hinxton-Cambridge (UK), January **2018**: **POSTER**

3. Gámez-Valero A, Borràs FE, Beyer K. Specific platelet microRNA signature for Dementia with Lewy bodies as promising biomarker. Brain Disorders Conference; Madrid (Spain), November **2017**: **ORAL**

4. Gámez-Valero A, Carreras L, Monguió-Tortajada M, La Franquesa M, Beyer K, Borràs FE. Isolation and characterization of Extracellular Vesicles isolated by SEC, PEG and PROSPR: A Comparative Study. International Society for Extracellular Vesicles (ISEV) Congress, Rotterdam (Netherlands), May **2016**: **POSTER**

5. Gámez-Valero A, Pérez-Roca L, Canet Pons J, Campdelacreu J, Reñé R, Ispierto L, Katrin Beyer. Beta-synuclein genotypes indicate increased risk to develop Lewy body pathology in the brain. Genomics of Brain disorders Conference. Wellcome Genome Campus; Hinxton- Cambridge (UK), April **2016: POSTER**

6. Gámez-Valero A, Adame C, Beyer K. Glucocerebrosidase Gene in Lewy Body Diseases. 16th Congress Spanish Society Neuroscience, Granada (Spain), September **2015**: **POSTER.**