IMI1 Final Project Report
Public Summary

Project Acronym: PHARMA-COG

Project Title: Prediction of cognitive properties of new drug candidates for neurodegenerative diseases in the early clinical development

Grant Agreement: 115009
Project Duration: 01/01/2010 - 31/12/2015

1. Executive summary

1.1. Project rationale and overall objectives of the project

Alzheimer's disease (AD) is a progressive brain disorder that causes a gradual and irreversible decline in memory and cognitive abilities. Until today, the pharmacological therapy of AD is still limited to symptomatic temporary improvement or stabilization of cognitive performance and the reduction of neuropsychiatric symptoms of the disease. Five drugs are currently marketed for the treatment of AD including four cholinesterase inhibitors – Tacrine, Donepezil, Galantamine, and Rivastigmine – and one glutamate antagonist (Memantine). However, owing to the extensive and multifocal nature of neurodegeneration in AD, the effects of transmitter modulators are modest. In recent years, a new therapeutic approach (disease modifying approach) has emerged. Unlike treatments that target symptoms, disease modifying therapies should interact on the natural course of the disease by interrupting early pathologic events thus preventing underlying pathophysiological processes. Although they are very promising, to date no disease modifying therapies have been clearly shown to be efficacious on clinical symptoms. In this context, the development of new drugs with symptomatic effects remain necessary together with those acting on the neurodegenerative processes.

The clinical development of drugs in AD has been confronted with challenging methodological difficulties. Taking into account the cost involved taking drug candidates to the phase III stage of development and the risk of investing time and resources fruitlessly in the evaluation of poor candidate drugs, the crucial decision remains whether to proceed from phase II to phase III (Go/Nogo). The aim of phase II studies is to select a molecule likely to be effective in phase III, but also to eliminate candidate-drugs with an inadequate effect. No consensus currently exists on the best possible design of Phase II studies in AD to inform the Go/No Go decision optimally. At the present time, clinical scales and neuropsychological-based tools, mainly "paper and pencil", are the most established and approved method of assessing outcomes in AD pharmacotherapy, in part because they are widely available and do not require technological instrumentation. However, failure of a new drug to produce an effect on clinical and neuropsychological scores cannot be easily understood without information on the effect of the drug on neurobiological and neurophysiological endpoints. Therefore, results of such failed clinical trials do not provide any useful information in promoting the understanding of the disease and pharmacological interactions of the drugs with the disease. Furthermore, several variants of AD and cases of mixed dementia disorders (Dubois et al., 2014; Lancet Neurology, 13(6):614-29) can further confound that understanding. hBecause of the difficulties in demonstrating the efficacy of a candidate-drug using only clinical and cognitive tools, development of new assessment tools including "matrices" of "fluid", neuroimaging, and neurophysiological biomarkers have become more important over the past few years.

The objectives of the PharmaCog project were to test innovative hypotheses about the value and utility of a matrix of computerized cognitive markers (i.e. CANTAB battery) and biomarkers in the multi-modal characterization of prodromal AD subjects with amnesic mild cognitive impairment (aMCI) in work package 5 (WP5), and the back-translation of this matrix to healthy young volunteers subjected to experimental conditions inducing reversible cognitive impairment ("the challenges") in WP1 as a platform for testing new compounds in the early stages of drug discovery. Furthermore, the reliability of these biomarkers in healthy young volunteers was tested in WP3 and its value (i.e.

feasibility, utility) for preclinical research was tested in WP2, WP4, and WP6. As for any innovative project, the work plan was adapted as the project developed to ensure the maximum impact of the project deliverables. Overall, the key strategic objectives of the project were reached and the deliverables and core messages are now available for all public and private stakeholders in the field of AD in line with the conditions of access reported in the PharmaCog contract agreement.

1.2. Overall deliverables of the project

The PharmaCog project has almost reached its goal to develop an innovative multidimensional matrix that combines system biology, pharmacology, neuroanatomy, neurophysiology, biochemistry, functional imaging and neuropsychology. Parallel studies were performed or analyzed in animals, healthy volunteers and selected patients, those results are described through the deliverable reports of the different workpackages, around three key overall outputs: (i) development of translational and reversible challenge models ; (ii) development and validation of pharmacodynamic markers; (iii) development of predictive markers related to early stages of disease progression necessary for stratification of patients in clinical trials. These outputs are essential for further development of symptomatic or disease-modifiers drugs that are both necessary for AD patient treatment. We have recently demonstrated a first success of the output of the project as evidenced by the fact that a start-up who are developing a novel symptomatic compound for AD are using some biomarkers and methodology of PharmaCog . This work has been funded by the French Ministry of Industry and the clinical study in healthy volunteers will be performed through the platform and network associating Lille, Marseille and Toulouse.

1.3. Summary of progress versus plan since last period

WP1

During the last period of the project, all experiments were finalized, management, monitoring, and generation of statistical analyses was completed and dissemination of some key results (see sections below) was achieved. Finally, storage of the generated data on the Internet-based X-Nat database managed by the Toulouse centre was undertaken. This action will be maintained after the end of the project to protect them and ensure established access to the PharmaCog data.

The main deviation regarding the initial work plan of WP1 has been the postponement of the hypoxia study due to its low reliability as a method to induce cognitive impairment in humans, according to the revised literature. Hence, the Steering Committee endorsed an enabling, non-pharmacological study combining cognitive, EEG, and blood markers would be conducted during the last period of the project on a limited sample (N=20) of young healthy volunteers. As stated in the Steering Committee meeting in Lille, Dec 2015, the Marseille and Lille centres will pursue and undertake the validation of this challenge study outside the official timeline of the PharmaCog project and using their own funds, and PharmaCog will be acknowledged when findings regarding this challenge will be disseminated and published.

WP3

All data of WP3-study 1 assessing the impact of Donepezil, a well-known acetylcholinesterase inhibitor, on the biomarker battery in healthy young volunteers have been stored on the X-Nat database, and the core

analysis of “fluid”, neuropsychological, neuroimaging, and EEG biomarkers has been finalized. The X-Nat WP3 database will be maintained after the end of the project to protect them and ensure established access to the PharmaCog data.

When the Donepezil study was launched, we worked in parallel on the methodological and regulatory process for Memantine (a NMDA antagonist) study i.e. since January 2014. Unfortunately, this WP3-study 2 assessing the impact of Memantine on the biomarker battery has not been implemented since the therapeutic units were only obtained in January 2016 after many administrative and regulatory processes related to the form of Memantine conditioning provided by the pharmaceutical company. This delay explains the impossibility to deliver on the comparison of the impact of Memantine and Donepezil on the PharmaCog biomarker battery. Nevertheless, we have planned to continue the studies after the end of the official PharmaCog project, since it remains important for the teams involved to obtain complete information to support the implementation of the battery in AD drug development. We plan to complete the studies and publish the results with the acknowledgement of the PharmaCog project in the first semester of 2017. The principal investigators of the Lille, Toulouse, and Marseille centres formally ensure that the results of the Memantine study of WP3 will be presented in the AAIC conference with reference to the PharmaCog project, as soon as the final results will be obtained in 2017 or 2018.

WP5

In Year 6, follow-up visits of patients with amnesic mild cognitive impairment (aMCI) enrolled in each clinical centre continued until August 2015. At that time, all the centres received close-out visits to mark the successful completion of the WP5 longitudinal study. All data of WP5 have been stored on the Internet-based Intellimaker neuGRID platform (https://neugrid4you.eu) databases, and the core analysis of “fluid”, neuropsychological, neuroimaging, and EEG biomarkers has been finalized. The neuGRID WP5 database will be maintained after the end of the project to protect them and ensure established access to the PharmaCog data.

Since September 2015, data analyses were carried out to identify biomarkers sensitive to AD progression with reference to parallel clinical and cognitive decline. Moreover, we compared all cross-sectional clinical and biomarker data in the aMCI patients divided into three groups (“positive”, “intermediate” or “negative”) based on the Gaussian statistical distribution of their baseline Aβ42 levels and Aβ42/t-tau and Aβ42/p-tau ratios in cerebrospinal fluid (CSF). The positive group was that of probable prodromal AD, the negative group was that of probable non-AD while the intermediate group was probably mixed. The focus of WP5 was to unveil differences in the clinical/neuropsychological scores and biomarkers (i.e. the so-called PharmaCog matrix of cognitive markers and biomarkers) between the groups of positive and negative aMCI at baseline (cross-modal study) and across the follow ups (longitudinal study) for a characterization of prodromal AD in aMCI subjects. *The results of the WP5 unveiled the PharmaCog matrix of cognitive markers and biomarkers for testing their back-translation value in healthy volunteers (WP1, WP3) and animal models of AD (WP2, WP4, and WP6)*.

One of the partners, Exonhit (EHT), unfortunately had to leave the Consortium. Brescia centre (IRCCS-FBF) took up the task of carrying out Transcriptomics analyses ("Exhonit" analyses). The principal investigator of the Brescia centre formally ensures that the results of the Transcriptomics study of WP5 will be presented in the AAIC conference 2017 or 2018 with reference to the PharmaCog project.

WP7

It has been acknowledged since the second year of the PharmaCog project that the final refined version of the clinical and preclinical protocols made it them unsuitable for an adequate evaluation of the pharmacokinetic-pharmacodynamics (PK/PD) relationships. This limitation is partly due to the need to reformulate the original working hypotheses in the light of the outcome of the review of the most recent literature (see the reviews authored by the Consortium in the WPs 1-6). Based on that outcome, we decided to concentrate the resource on the most relevant and promising directions. These directions implied some restrictions in the clinical (e.g., use of therapeutic doses only) and the pre-clinical (e.g., high dose levels are not well tolerated) protocol design. Due to the limited resources and the exploratory nature of the working hypotheses in the preclinical and clinical WPs, the experiments did not include an administration of psychoactive drugs and serial longitudinal recordings (>2) of biomarkers and cognitive measurements in animal and human models. As a consequence, the experimental data sets did not have the kind of information to support standard PK/PD modelling. In this restructuring context, the role of the WP7 changed. The WP7 members played the role of reviewing the protocol design and the oncoming data from the different WPs in the context of a feasibility analysis, with the objective of establishing the suitability of the experimental data for the implementation of the analyses descried in deliverables 7.2, 7.3, and 7.4.

WP8

The PharmaCog initiative was driven by a wish to investigate and better understand translational signals between platforms and modalities through qualitative techniques.

Whereas these notions were a powerful guiding force for the study as a whole, most of WP8's work concentrated on building a foundation for such an analysis by concentrating on supporting and promoting good practices in experimental design and analysis. This was done through general interactions with other IMI partners. This policy was driven by the industrial partners' experience that where statistical resource is limited, such approaches have proved to be very effective. Given the volume and breadth of the PharmaCog project, WP8 would not otherwise have had the resource to provide a global model of statistical support.

In this capacity acting alone, and in conjunction with WP7, WP8 gave input on experimental design and analysis in numerous clinical and pre-clinical experiments both in general and WP-specific IMI meetings and off-line by mail and phone conversations. Depending on the nature of the work required, this either pointed scientific colleagues to alternative more effective methods or, selectively, led to more concrete support in the form of written reports and model analyses. The overall aim was to help scientific colleagues perform effective analyses rather than directly intervening ourselves. The model was however flexible with statistical groups in partners who were also contributing experimental work themselves notably providing higher levels of support.

Specific platforms such as EEG were not universally covered by WP8. Although support was sometimes provided, many of the required analysis techniques were platform specific and well covered by other more skilled experts elsewhere in the IMI.

Beyond support for single platforms, WP8 was also instrumental in trying to work with other WP partners to develop a systematic approach to the cataloguing and sharing of experimental data. WP8 did this either by reviewing various database systems or making independent suggestions itself for the numbering and identification of datasets. This later work was presented and multiple IMI meetings and adopted as a data standard in many parts of the collaboration.

Whereas our work on data integration was seen as a key step to the multivariate cross-analysis and the investigation of signals across modalities and platforms – the MATRIX idea – ultimate progress in this direction was limited by the unavailability of data and corresponding biological signals. This was largely scientific given the difficulty in finding such translation effects but further complicated by the wide range of data that was generated. Indeed, where possible signals were found – such as EEG – they were adequately covered by the EEG platform groups themselves.

In summary, WP8 was successful in providing statistical support across a wide range of projects and activities during the course of this collaboration. Within the levels of resource available and the nature of our challenges, this was adapted during the duration of the collaboration to changing challenges and questions. Contributing not only to their analysis but also their design, WP8 thus affected many experiments and so ultimately made a positive contribution to the project as a whole.

WP10

In 2015-2016, the PharmaCog partners developed an intensive activity of dissemination under the supervision of the WP10 members in respect of the agreed communication plan. Concerning the communication for the members of the Community of clinical neuroscientists and pharmacologists, 11 papers were published in peer-reviewed international neuroscience journals. Furthermore, the most important experimental results were disseminated in 29 oral communications or posters during international congresses of Clinical Neuroscience and Pharmacology. More details can be found in the Table 3.2. For non-experts, news and updates of the PharmaCog projects were systematically reported in the project website up to date (Alzheimer Europe staff), namely www.pharmacog.org. Furthermore, specialists of Alzheimer Europe published 4 project updates in its monthly e-newsletters. In 2015, these updates regarded the following important dissemination events: 25 February, PharmaCog partners hold Steering Committee meeting; 28 June, PharmaCog researchers show results at EACPT conference; 3 October, PharmaCog researchers show progress at ECCN conference; 18 December, Final meeting of the PharmaCog Steering Committee. A special emphasis was given to the event of 2 December 2015, when Prof Régis Bordet presented the project at Alzheimer Europe's "Alzheimer Association Academy" in Brussels. This event was attended by 50+ delegates, including people with dementia, representatives from AE member organisations and pharmaceutical companies, as well as scientists from IMI and from the AETIONOMY and EPAD projects.

1.4. Significant achievements since last report

WP1 Sleep deprivation (SD) cognitive challenge

During the present period report, we have finished all residual experimental data collection parts in the recruiting centres (i.e. Marseille, Toulouse, and Lille) and concluded all data management and monitoring

processes. Data of SD study were verified according to the Good Clinical Practices. During this process, we have also concluded the principal statistical analysis of data, both as regards the impact of the cognitive challenge itself as well as to test reversibility with pharmacological agents (i.e. Donepezil and Memantine). In addition, Foggia/Rome (for EEG) and Marseille (other joint markers) centres led transversal analyses between WP1 (SD and TMS studies) and WP3. Results summarizing the main findings of the SD challenge are described in the next section.

WP1 Transcranial magnetic stimulation (TMS) challenge

As in the case of the SD challenge, during this last period the remaining planned experimental parts of the rTMS experiments have been concluded in the two participating centres (Barcelona and Marseille). Analogously, we have proceeded and completed the necessary steps of data management and monitoring and conducted statistical analyses. Data from the rTMS study were verified according to the Good Clinical Practices. Barcelona centre has led the analysis of this challenge both at the behavioural and at the fMRI data level, whereas Foggia/Rome centre has undertaken the EEG data analysis. Results regarding the main achievements of the rTMS challenge are presented in the next section.

WP3

As mentioned above, one of the goals of the project was to identify biomarkers that could be sensitive to pharmacological modulation in healthy young volunteers and help with stratification of patients for future clinical trials. The main goal of WP3 was to test the hypothesis that Donepezil and Memantine have a neuroprotective role in healthy young volunteers as revealed by the EEG/ERP and fMRI markers of brain and cognitive function and the PharmaCog cognitive battery. If the hypothesis were confirmed, new smart drugs for cognition could be compared to Donepezil and Memantine by the WP3 platform. We have focused on neuroimaging biomarkers related to attentional and memory processes that are sensitive to a treatment with Donepezil for 15 days. Three neuroimaging approaches (fMRI with cognitive task and resting state fMRI and PET-FDG) could identify candidate markers of brain functioning sensitive to Donepezil treatment that can be theoretically related to attention and memory systems relevant as biomarkers of AD in patients with both dementia and aMCI.

Regarding resting state EEG module, Foggia/Rome has performed statistical analyses to evaluate whether a chronic (15 day) administration of Donepezil would modify markers of the resting state EEG rhythms in a cohort of healthy young volunteers (Placebo condition as a reference). The results cross-validated those obtained using the same experimental design in another cohort of healthy young volunteers in the WP1 (see the next section).

WP5

Patient Recruitment

Figure 1 summarizes the recruitment and the definitive follow-up of the aMCI patients in all WP5 clinical centres.

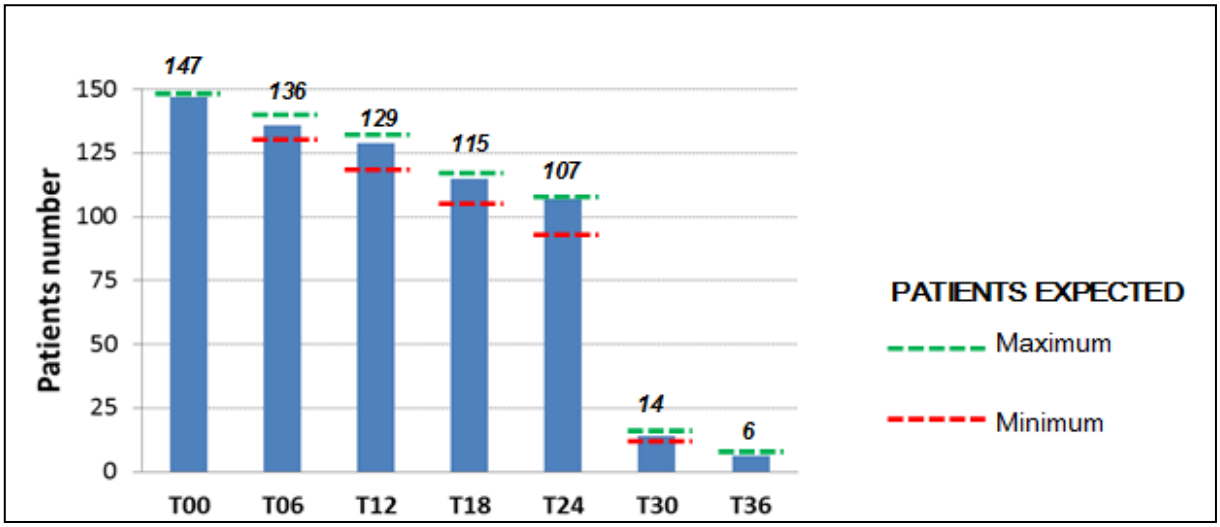

Figure 1. WP5 enrolment and follow-ups of patients with amnesic mild cognitive impairment (aMCI) for each time point (T0= baseline). The numbers "06, 12, 18, 24, 30, and 36" indicate the months from the baseline session.

The loss of the aMCI patients for both dropouts and conversions is in accordance with our expectations (10%/year for both) as reported in the original description of work of the PharmaCog project.

Data Analysis

Final data analysis took place in the last months of the project with the aim to identify/validate candidate human surrogate biomarkers most sensitive to AD progression in aMCI patients.
We examined the ability of commonly used and novel AD biomarkers to explain the variability of cognitive decline in aMCI patients measured by the ADAS-cog score. In particular:

- Brain morphological MRI markers: volumes (hippocampus and its subfields, thalamus, caudate, pallidum, putamen, amygdala) and thicknesses (temporal, parietal and occipital cortex) indexes by Brescia (IRCCS-FBF) centre;
- Brain connectivity MRI markers: microstructural diffusion MRI (fractional anisotropy; axial, radial, and mean diffusivity in several white matter regions) and resting state functional MRI (rs-fMRI; connectivity in and among the nodes of the default mode network, DMN) indexes by Brescia centre; EEG indexes by Foggia/Rome centre (spectral power density of cortical resting state eyes closed EEG rhythms, rsEEG; auditory oddball ERPs);
- Peripheral markers (in the blood): APP metabolites as indexes of disease progression by AlzP centre, transcriptomic indexes by IRCCS-FBF inflammatory indexes of the ADFlag panel by ICDD. The ADFlag® panel was used as a candidate biomarker of disease status and progression with the aim of detecting prodromal AD in aMCI individuals and reflect their cognitive decline across time.

1.5. Scientific and technical results/foregrounds of the project

WP1 SD cognitive challenge results

As described in the study protocol, the investigation of the impact of SD on cognitive (neuropsychological), neuroimaging, and EEG outcomes has been approached into a multi-centric, randomised, placebo-controlled, cross-over design with two parts:

- **Part A** was performed for paradigm validation using a single dose of Modafinil immediately after one SD night as a positive control for the ability to attenuate/reverse cognitive impairments and related biomarkers effects induced by that challenge (Placebo control).
- **Part B** was performed to evaluate the effects of 15-day Donepezil and Memantine at steady state on impairments induced by one SD night on cognitive performance and above biomarkers.

In line with original proposal, the total number of subjects included in the SD study was n=36 male individuals.

Main results on cognition

In the main clinical trial design above (i.e. Part B), we observed that SD had a significant negative impact on the performance of the main cognitive outcome, which was a working memory task (i.e. an n-back task). Hence, we can conclude that the n-back task, as adapted and used in the PharmaCog project, is a sensitive measure to capture the impact of a SD night on working memory capacity. We did not observe an effect on this task following a night of sleep deprivation for experimental Part A, which might be explained by the fact that this n-back task was repeatedly administrated at the screening phase of the study (i.e. before the experimental phase) which could lead to a ceiling effect related to an automatic processing resistant to the effect of SD. Hence in the present WP1 study, using adaptation of experimental designs, tasks and procedures harmonised within the PharmaCog project, we found that the n-back task was globally sensitive to our cognitive challenge model based on one night of SD in healthy young volunteers. This conclusion is in line with mounting previous evidence using comparable protocols amongst on young (age < 35 years) healthy volunteers, which showed a decrease in n-back scores after SD (Thomas et al, Sleep 2006; Groeger et al, Sleep 2008; Vandewalle et al, J of Neurosci 2009; Lo et al, PloS one 2012, Lythe et al, Behav Brain Res. 2012; Reichert et al, J Biol Rhythms. 2014).

As regards the impact of pharmacological compounds administration, a single dose of Modafinil (Part A) was able to significantly improve the cognitive performance of the n-back task when using the percentage of Hits as a dependent variable of the statistical analysis. These findings are aligned with previous evidence showing the beneficial effects of Modafinil on cognitive performance in the SD condition (Baranski and Pigeau, J Sleep Re 1997, Stivalet et al., Hum Psychopharmacol Clin Ex 1998; Caldwell et al., Psychopharmacolog 2000; Wesensten et al., Psychopharmacology 2002; Müller et al., Psychopharmacology 2004) but are amongst the very few ones specifically demonstrating a beneficial effect using a widely employed working memory task such as the n-back.

In the investigation of more typical AD drugs such as Donepezil and Memantine, we observed was that Memantine, but not Donepezil, significantly improved performance following SD, compared to placebo. It is likely that heterogeneous sample sizes in the two Parts of the WP1-SD experiments, linked to dropouts during the experimental activities, may have confounded our findings. However, if we analyse studies with comparable conditions (healthy volunteers, SD, and Donepezil administration), results are heterogeneous with pro-cognitive effects (Fitzgeraldet al., Cogn Behav Neurol 2008; Ginani et al., Journal of Psychopharmacolog 2011; Dodd et al., Hum. Psychopharmacol Clin Exp 2011) or deleterious effects (Beglinger et al., J Psychopharmacol 2004 and 2005). The present findings based on a consistent methodology and cross-over design clarified the lack of clear beneficial protective effects of Donepezil

treatment on cognitive performances after one SD night. In this theoretical framework, we demonstrated the beneficial protective effects of chronic administration of Memantine on working memory after one SD night in healthy young volunteers.

Besides the n-back task, another key cognitive test employed in WP1 studies was the 'PicInOut', an episodic visual memory task entirely developed and used across distinct work packages of the PharmaCog project. In brief, the PicInOut memory task consists of the presentation of emotionally neutral indoor (interiors of apartments or buildings) and outdoor (landscapes) pictures in an Encoding phase. These pictures have to be memorized for a latter recognition demand in a Retrieval phase. In the SD study, we found a deleterious effect of 24h of SD in healthy young volunteers in their ability to encode and recognize the pictures, clarifying that SD affects visual episodic memory on both Encoding and Recognition phases. The literature presents contradictory results related to the effects of SD on episodic memory perhaps because of the diversity of tasks employed (Chee et al, NeuroImage 2010; Kong et al. NeuroImage 2012; Yoo et al, Nature neuroscience 2007; Harrison & Horne Sleep 2000).

As the regards effects of pharmacological agents in this task, in Part A we observed that a single dose of Modafinil has a significant effect regarding the reversibility of SD effects both during the Encoding ($p<0.001$) and the Retrieval phase ($p=0.040$) compared to placebo. This finding is entirely novel in the literature. In contrast we could not evidence a protective impact of any of the tested AD drugs on the PicInOut task performance. This finding underlines the specificity of the beneficial protective effects of the Memantine on working memory (n-back task) when compared to medium term episodic encoding-retrieval processes in the PicInOut task. To our knowledge, there are no other studies in humans testing the restorative cognitive effects of Memantine following sleep deprivation and hence these findings warrant further investigations.

Our findings regarding the secondary cognitive tasks employed in the WP1 SD study can be summarized as follows:

Sleep deprivation had a significant negative impact on the performance of the Rey Auditory Verbal Learning Task (RAVLT) task, both in Part A and B. However, neither Modafinil (Part A), nor Donepezil or Memantine (Part B) were able to counteract the effects of SD in this memory task significantly.

Similar to the RAVLT task, we also observed a deleterious effect of sleep deprivation a Semantic Verbal Fluency task, but no effect of Modafinil, Donepezil, and Memantine in this task.

As a novelty, our SD study includes evaluations of the CANTAB battery (also employed across WP3 and WP5 clinical WPs). For these tasks, SD impacted negatively the performance of the Rapid Visual Information Processing (RVIP) task. Further, Modafinil treatment was able to compensate for the effect of SD when we compare the percentage of RVIP accurate responses. Furthermore, we observed that the speed (reaction time in ms) in the Modafinil condition at *Pos*t-SD has a tendency to be similar to the speed in Modafinil condition at *Pre* -SD ($p=0.88$) and different to the Placebo condition at post SD ($p=0.067$). These results support the hypothesis that Modafinil affects more the visuomotor component than the cognitive component: In reference to the Part B Study, we observed the negative effect of SD on accurate responses of the RVIP under Donepezil, Memantine, and Placebo treatments ($p<0.001$) with no beneficial drug effect

as compared to Placebo ($p>0.05$). However, subjects were faster under Donepezil and Memantine than under placebo treatment after 24h-SD.

In conclusion, present findings validate the Part A Study using Modafinil as a positive control for several cognitive endpoints. They reveal that working memory and visual episodic memory can be improved by Modafinil under SD either at cognitive (accuracy) and visuomotor (response time) level. In Part B Study, results showed that Memantine can potentiate working memory efficiency or speed after SD. However, in our cross-over design, we were not able to conclude about the effect on Donepezil and Memantine on visual and verbal episodic memory and the executive functioning, after 24h-SD.

Overall, our behavioural results demonstrate that although all the present cognitive tasks are sensitive to the SD challenge, only the ones that allow a more refined recording of the behavioural responses, such as those obtained by using button press and computerized recordings (n-back, PicInOut and CANTAB), are significantly sensitive compared to the more classical paper and pencil tasks (i.e. RAVLT and verbal fluency) used in clinical assessments of MCI and AD patients. Hence, the present behavioural results call for the inclusion of such type of cognitive endpoints in further studies where reliable sensitive measures to typical AD compounds will be required as a reference to compare the impact of new treatments.

Main results on Neuroimaging markers

Functional magnetic resonance imaging (fMRI)

In a subsample of individuals (N=19), we acquired a full data set of both structural and functional MRI (fMRI) during the SD module of WP1. fMRI included resting-state as well as task-related acquisitions during an n-back task, both of them believed to be able to reveal relevant changes in functional activity and connectivity meaningful for the AD model..

As regards the resting-state fMRI (rs-fMRI) data, we focused on the analysis of the connectivity of the posterior cingulate cortex (PCC) because it is the main node of the default mode network (DMN) already affected in individuals at the preclinical stages of the AD (Simic et al. CNS Neurosci Ther 2014). Referring to the impact of sleep deprivation, we observed that first, it abnormally increased the rs-fMRI connectivity between PCC and several cortical areas in the frontal and temporal lobes. Second, it decreased the rs-fMRI connectivity between the PCC and cerebellar regions. When we investigated the impact of pharmacological manipulations over this aberrant patterns of brain connectivity induced by SD, we observed that Modafinil counteracted the hyper-connectivity and Memantine reversed the hypo-connectivity of the DMN following SD reported above (all results uncorrected for multiple comparisons $p<0.05$, see Figure 2).

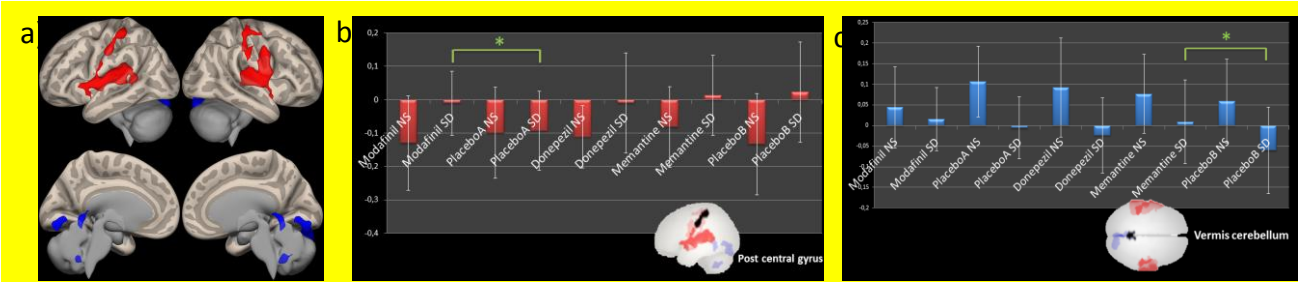

Figure 2. Main SD and pharmacological effects on resting-state fMRI data. The left most panel (a) shows the effects of SD on the rs-fMRI connectivity between the PCC region (seed) and the remaining areas of the brain. SD had the effect of increasing (warm colours in the Figure) and reducing (cold colours in the Figure) the brain connectivity of the PCC differently, depending on the region considered. The middle panel (b) shows the normalizing effect of a single dose of Modafinil on the abnormally increased regions of connectivity induced by SD, whereas the right most panel depicts normalizing effect of a chronic treatment (15 days) of Modafinil on the areas where SD induced a decrease of functional connectivity.

In summary, the present findings suggest that experimentally induced 24 hours of SD results in a temporary alteration of the rs-fMRI connectivity data from a posterior cingulate hub of the DMN, key to early AD pathology. Present results are in agreement with those of recent investigations (e.g. Kaufmann et al., 2015), and validate the SD challenge model developed within PharmaCog for the study of a key brain network dysfunctional (i.e. DMN) early on in AD. As regards our pharmacological results, the findings using Memantine, an AD symptomatic drug, confirm that rs-fMRI is responsive to drug administration and particularly that the effects induced by SD can be partially reversed. Future studies should ascertain if this functional measure can be considered as a putative marker of early hints of efficacy for newly developing drugs aiming to normalize functional brain connectivity amongst AD patients. Up until now, most of the rs-fMRI studies reported the action of Donepezil in AD patients and healthy elderly adults (e.g. Solé-Padullés et al. J Clin Psychompharmacol 2013). Studies with Memantine in AD patients and healthy volunteers are lacking.

As for the fMRI data collected while individuals were performing the working memory (i.e. n-back task), the main results indicated that Modafinil increased brain activity of cortical regions encompassing the parietal, occipital, temporal and frontal lobes, as compared to Placebo. Along with the findings observed in cognition (see above), the results indicate that this drug is able to improve cognition through an optimization of cortical areas involved in working memory performance. Regarding the AD symptomatic drug administration, we could evidence an effect of Memantine, which induced increased parietal activity (as compared to Placebo) during the intermediate level of difficulty of the working memory task, but no effects of Donepezil administration.

In summary, the results obtained using fMRI during the n-back performance are complementary to those of the behavioural analyses alone. First, SD is able to modulate brain networks responsible for this cognitive process in healthy young volunteers proving the validity of this challenge on the task-related fMRI biomarker. Second, pharmacological manipulation affected this fMRI biomarker, interacting with the effects of SD. Specifically, Modafinil is able to partially reverse the effects of SD, validating the pharmacological model. More interestingly, a licensed symptomatic drug used to treat AD such as

Memantine induced greater brain activity during the most demanding working memory condition (n-back) after the SD. Hence, the present task-related fMRI results obtained using the SD challenge and Memantine would indicate the reversibility of a cognitive dysfunction in a neuropsychological domain typically affected in the typical phenotype of AD (i.e. working memory deficits) through the over recruitment of cortical areas critically dysfunctional in that disease. It is concluded that the research model formed by SD, Memantine, and fMRI recordings during a working memory (n-back with several difficulty load) task in healthy young volunteers may be useful in future studies aimed at obtaining any early demonstration of efficacy of developing compounds for AD, both at the behavioural and functional brain integrity levels.

Main results on resting state EEG and ERP markers

In the SD "challenge", resting state eyes-closed EEG rhythms and auditory oddball ERPs were collected for the 36 subjects before and after one night of SD both for Parts A and B Studies. The resting state EEG markers were the cortical sources (estimated neural current) of the scalp EEG power density at the standard delta (2-4 Hz), theta (4-8 Hz), alpha 1 (8-10.5 Hz), alpha 2 (10.5-13 Hz), beta 1 (13-20 Hz), beta 2 (20-30 Hz), and gamma (30-40 Hz) frequency bands. The ERP markers were the cortical sources (estimated neural current) of a late (about +400 ms after target stimuli) positive peak of the auditory oddball ERPs recorded at posterior scalp electrodes (P3b peak).

In the Part A Study, the statistical results ($p<0.05$, corrected) demonstrate that the SD affects both resting state EEG and ERP markers. In the resting state EEG markers, the SD induces an increase and decrease in the posterior source activity at the delta and alpha 1 bands, respectively. In the ERP markers, the SD induces a decrease in the posterior source activity (posterior parietal and PCC) of the P3b peak and poor behavioural performance of the oddball task, namely the percentage accuracy of the subjects' responses given to the target oddball auditory stimuli ($p<0.05$). Noteworthy, these results are interesting in the light of those of the WP5. In the WP5, the aMCI patients positive to the Aβ1-42 and tau levels in the CSF (prodromal AD, aMCI+) showed an increase in the widespread source activity at the delta rhythm and a decrease in the posterior source activity at the alpha 1 in the resting state EEG rhythms. Therefore, it can be speculated that the transient effect of the present SD challenge on the cortical neural synchronization mechanisms underlying resting state EEG rhythms may partially resemble those induced by prodromal AD in aMCI subjects.

As regards the pharmacological manipulation we observed that single dose of Modafinil recovered the SD effects partially on both the resting state EEG and ERP as well as it ameliorated the behavioural performance associated with the ERP paradigm, ($p<0.05$). In the light of the results of the WP5 (Galluzzi et al. J Intern Med 2016; Mar 4. doi: 10.1111/joim.12482), it can be speculated that the WP1 SD- and Modafinil protocol can induce some modulations in the resting state EEG and ERPs that are abnormal due to prodromal AD in the aMCI seniors (WP5). As such, this protocol may be a suitable experimental model to test new drugs acting on brain systems modulating vigilance and alerting (resting state condition) as well as attention and short term memory (auditory oddball task) in healthy young adults, to select the best candidates for clinical trials in aMCI patients with prodromal AD.

In contrast to part A, in the Part B Study, the statistical results ($p<0.05$) reveal that a chronic administration of Donepezil and Memantine protects neither the EEG/ERP markers induced by the SD effects nor the

behavioural performance associated with the ERP paradigm. Therefore, in the present context, the WP1 SD-Donepezil-Memantine protocol can be just considered as a control condition to test the specific effects of Modafinil on the neurophysiological mechanisms of vigilance and short-term memory in the resting state and auditory oddball conditions.

WP1 Transcranial Magnetic Stimulation (TMS) cognitive challenge results

Following a review of the literature (Martin-Trias et al. CNS Neurol Disord Drug Targets, in press) TMS was considered a potentially interesting methodology to be used as a cognitive challenge model. In particular, TMS, in contrast to SD, allows a more refined targeting of specific brain areas/networks in the brain. In WP1 of PharmaCog, we designed a study to test the applicability and reliability of TMS to be used as a cognitive challenge model in healthy young volunteers with three primary aims: 1) to replicate the results published in the literature using the experimental settings and outcome measures developed within and used across the PharmaCog project, 2) to test if the results could be reproduced in at least two centres; and 3) to investigate if the effects obtained were stable if subjects are re-tested 15 days apart (i.e. mimicking the time period to obtain steady state levels in typical symptomatic drug treatment for AD).

Main results on cognition

PicInOut task

High frequency TMS trains were delivered over the left dorsolateral prefrontal cortex (LDLPFC) versus the Vertex (control condition) as an attempt to interfere with subsequent memory retrieval. Experiments included 68 healthy young individuals. Further, a sub sample of individuals was invited to attend the centres 15 days later, during experimental day 2 to test for reproducibility of effects.

The main findings indicated that TMS significantly impaired memory performance compared to Vertex stimulation (i.e. Hits percentage ($t=-4.095$, $p<0.001$) and d'($t=-3.86$, $p<0.001$); but only at experimental day 1 when using real TMS and not when sham TMS was delivered at baseline. The effect size for the impact of TMS over the LDLPFC condition compared to the Vertex was Cohen's $d'= 0.6$, indicating a moderate effect of memory interference (see Figure 3).

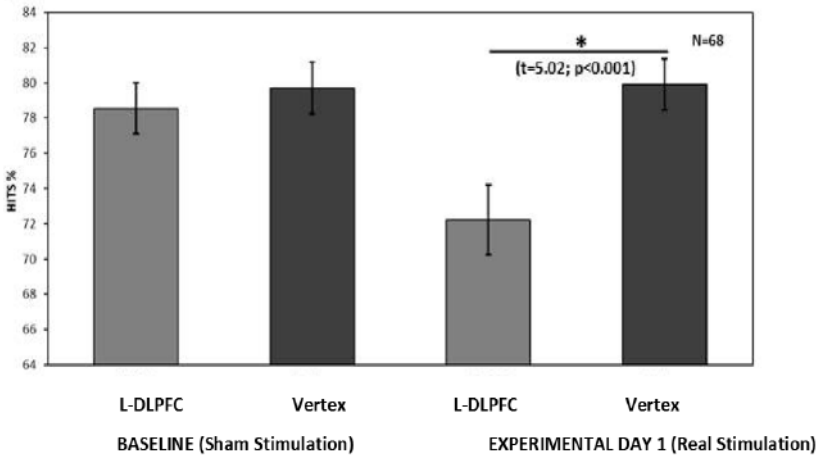

Figure 3. Illustration of the findings in the whole subjects' sample (N=68) comparing the screening Day, where sham TMS was delivered, to the main experimental day when real TMS was delivered over the target area (LDLPFC) and a control region (Vertex). %Hits represents the memory performance outcome variable. As can be seen, only real TMS delivered over the DLPFC resulted in a significant drop in memory performance.

As established in the experimental design, individuals exhibiting a drop of performance of at least 3 items in the LDLPFC compared to the Vertex condition were invited to participate in a second experimental Day 15 days apart. Twenty-five of the 68 subjects (37%) participating in experimental Day 1 were considered to be sensitive to TMS and were invited to additionally participate in Day 2, 15 days later. Results of this analysis (see Figure 4) indicated that whereas there was a clear impact of TMS on the first day that interference was attempted (experimental Day 1), no differences between the LDLPFC and Vertex conditions were found on the Day 2 (mean differences= 1.83, p=0.56).

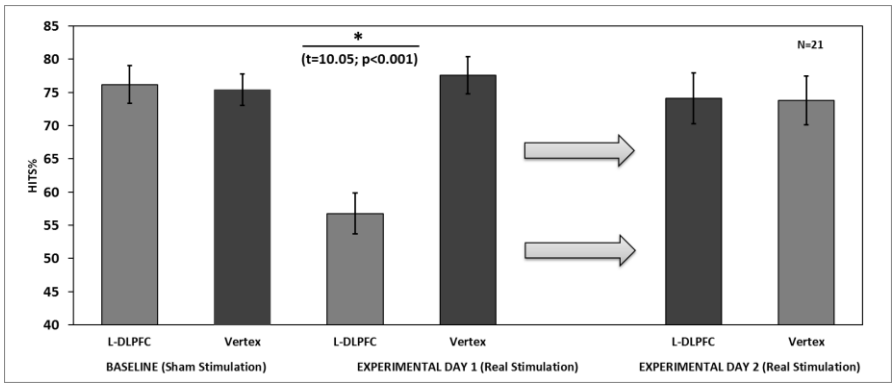

Figure 4. Reproducibility of the rTMS effects across two experimental Days. Mean memory performance (mean correct hits (%)) for the N=21 individuals at screening (baseline), and experimental Day 1 and Day 2. As can be seen, TMS only interfered memory function at experimental Day 1 but this was not reproducible 15 days later (Day 2).

Brain Derived Neurotrophic Factor (BDNF) effects

TMS effects on brain activity are thought to be mediated through modulation of plasticity mechanisms. Hence, we investigated if a natural variation of the BDNF gene, which is tightly associated with inter-individual neuroplasticity responses, interacted with the effects of brain stimulation. Results of the performance analysis comparing the screening day (i.e. sham stimulation) to the main experimental day were memory interference was attempted by DLPFC TMS stimulation showed that only Val/Val individuals exhibit a drop of performance related to TMS interference, suggesting that this genetic subgroup may be more susceptible to disruptive TMS effects over memory performance.

CANTAB tests

Besides the main outcome cognitive measure for TMS experiments, we also investigated the impact of a different form of TMS called "theta burst stimulation" (TBS) on a series of CANTAB tests used across the PharmaCog project. TBS, and in particular, continuous TBS (cTBS) represents a patterned form of TMS leading to a residual decrease of excitability over the cortical area stimulated, which mimics long-term depression (LTD) mechanisms (Huang et al. Neuron 2005). However, the results of real TMS compared to sham stimulation did not reveal any evidence of disruption on cognitive tasks in our study.

fMRI findings

In one of the experimental arms of the TMS study (N=43 subjects), responses during the recognition phase of the PicInOut task were acquired within the fMRI setting, and, hence, we could analyse the patterns of brain activity and connectivity related to the task as a function of the TMS effects.

Preliminary results suggested that during the retrieval phase, there was an increased activation of the posterior cingulate cortex only for brain activity associated to items whose memory encoding was attempted using real TMS vs. control stimulation. Such findings, despite not definitive because they are uncorrected for multiple comparisons, point to the notion of the need to engage compensatory functional mechanisms to counteract TMS interference effects, at least in some of the individuals. We next investigated the impact of BDNF genotype. Results indicated that compared to Met carriers, Val/Val (homozygous) subjects exhibited increases of brain activity including regions involved in memory processes such as the prefrontal cortex, the cingulate cortex, and the hippocampus. These increased areas of brain activity were observed when Val/Val subjects correctly identified previously learnt memory items, despite real rTMS interference having been attempted during the encoding phase. Hence, and in addition to the fact that the Val/Val group seemed to be, in general, more susceptible to behavioural effects of rTMS, this evidence further suggests that a greater amount of brain resources are needed even when they manage to achieve correct performance.

Main results on EEG markers

Statistical results ($p<0.05$) for the impact of TMS on EEG markers indicated that in the retrieval phase, the EEG changes corresponding to the pictures where attempted interference during the encoding condition was performed (i.e. LDLPFC pictures) showed greater desynchronization of widespread beta rhythms (cortical activation) as compared to that of the novel pictures. However, such desynchronization was

indistinguishable from what observed in the control condition (i.e. TMS delivered over the Vertex during encoding). Hence, the present evidence indicates that EEG markers at beta frequencies are sensitive to the general retrieval of episodic information from memory brain networks aspects of the task but the above mechanism is not affected by the specific interfering effects of TMS over LDLPFC during the encoding phase.

In conclusion, the main results obtained using the TMS challenge model in the PharmaCog project can be summarized as follows:

First, rTMS can be used as a reliable challenge to induce episodic memory disturbance by targeting the LDLPFC in healthy young volunteers, even if significant adaptations were done from previous protocols in the literature to fit within the PharmaCog experimental standards.

Second, the effects were observed in both participating centres of the project (Barcelona and Marseille). Altogether, the present results suggest that the rTMS cognitive challenge model developed and tested within PharmaCog is ready and can be used in a reliable manner in future multicentric-multinational European research projects aiming to generate experimental memory interference in healthy young subjects.

Third, the present findings also highlight that individuals that are more likely to exhibit rTMS memory interference are those characterized by 1) low memory performances at baseline and 2) Val/Val BDNF homozygous. These observations may be of interest for enriching study samples in future studies where hints of efficacy for developing compounds are needed, particularly if the mechanisms of action of the compounds are thought to act through modification of (i.e. LTP, LTD) neuroplasticity mechanisms.

Fourth, fMRI data seems to be useful to reveal fast compensatory brain reorganizations enabled as attempts to counteract the impact of brain stimulation over memory performance. Such reorganizations imply key networks and regions for AD pathology, such as the posterior cingulate cortex within the DMN. As such, fMRI responses to TMS may be useful as outcome neurophysiological measures in further pharmacological research, particularly to test if restorative effects of tested compounds on cognitive performance are mediated through optimization of the expression of key functional brain networks affected in AD.

Fifth, in contrast to the main findings of the PicInOut memory task during the first experimental Day, the present study failed to observe TMS interference effects of cognitive function when individuals are tested 15 days apart. The mechanisms enabling this putative ‘protection from stimulation effects’ when subjects are faced the second time to rTMS are under study and will be of high scientific interest. However, for the main aims of the PharmaCog project, these findings indicate that while TMS is a robust technique to experimentally induce transient episodic memory impairments in humans, it appears to be more suitable for future pharmacological studies using parallel rather than cross-over / longitudinal designs.

Finally, in the present study we did not observe any TMS effect on CANTAB tasks. Practice or learning effects associated with repeated assessments may account for the lack of significant results. Alternatively, it might be that for the selected tests the LDLPFC is not a key node where interference would lead to

transient cognitive dysfunction. An alternative explanation is that the present CANTAB memory tasks relied more on DLPFC and networks of right hemisphere than the PicInOut memory task did. A future study should use cTBS interference over bilateral DLPFC as an experimental condition.

Transversal analysis between SD and rTMS challenges: a comparison of size effect relative to cognitive assessment

In line with the aim of WP1, we developed and tested SD and rTMS challenges aimed at modelling (reversible) cognitive impairment in healthy young volunteers. For further studies focusing on the hint of efficacy of a new drug, it is interesting to compare the size effect of the same endpoints obtained in those challenges. At the moment, we performed data analyses from 2 CANTAB cognitive tests: the PicInOut and the RVP. We compared the effect of SD vs. active TMS. In the SD protocol, the effect size was calculated between data before vs. after SD without drug, for 2 dependent variables (% of hits and Reaction Time). In the rTMS protocol, the effect size was calculated between data obtained under Vertex (control condition) vs. LDLPC i.e.: active condition for the above 2 variables. The results are presented with a forest plot (Figure below): The scores in the left of the mean line (for Cohen's d) means that in LDLPC condition and after SD, subjects showed lower percentages of hits than in Vertex condition and before SD. The scores in the right of the mean line (for Cohen's d) imply that in L-DLPC condition and after SD, subjects have higher Reaction times than in Vertex condition (or Before SD). Figure 5 shows that for the PicInOut task, both cognitive challenge models resulted in decreases of cognitive performance with moderate effect sizes and are relatively comparable between them. When we observe the plots of the RVP task, we can clearly see that the performance in this task is only impaired by the SD but not the TMS challenge model.

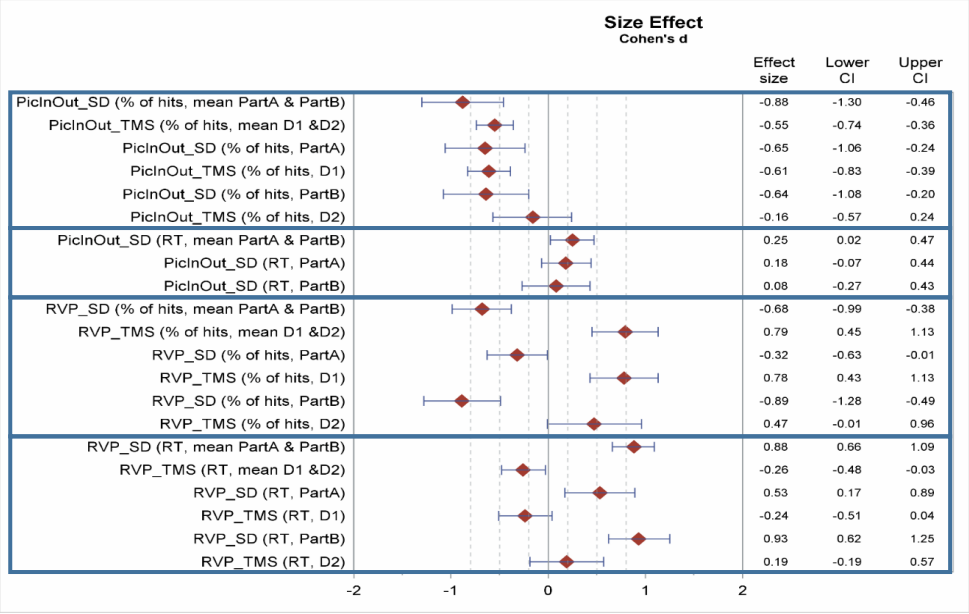

Figure 5. Forest plot representation of the Size effect comparing SD and TMS by the number of hits and the reaction time of the Picinout and the RVP tasks.

WP3

Two studies were designed to test the effects of Donepezil and Memantine in healthy young subjects. The same methodology was used in both, with a difference in treatment used. The first study (WP3-P001) was entitled "Effects of a 15-day Donepezil treatment on biomarkers of AD in healthy volunteers" and is now finalized; the second (WP3-P002) was entitled "Effects of a 15-day Memantine treatment on biomarkers of AD in healthy volunteers" and is currently on going (NCT02288000 (D3.4 & D3.6). End of Enrolment is planned on first semester of 2017. The Toulouse team developed software on xnat to centralize clinical data and compose a PharmaCog database. Computerized data are exported as they become available.

Population of volunteers of WP3-P001 study

Volunteer recruitment and data collection for the study WP3-P001 ("Effects of a 15-day Donepezil treatment on biomarkers of AD in healthy volunteers") was completed in 2013. Thirty healthy young subjects were recruited: 12 in Lille, 10 in Toulouse, and 8 in Marseille. The main characteristics of the volunteers are summarized in the Table 1.

N=30	Mean	Standard deviation	Range
Age (years)	24.6	3.1	19 – 31
Education (years)	15.7	2.2	11 – 20
Handedness (Edimburgh)	94.3	8.9	80 – 100
MoCA	29.3	0.8	27 – 30

Table 1. Main characteristics of the volunteers enrolled for the WP3-P001 study.

Clinical and neuropsychological data analysis

No statistical difference was found between donepezil and placebo for the different neuropsychological parameters ($p>0.05$).

rs-fMRI

In the rs-fMRI study, 30 healthy subjects were randomized to receive Donepezil or Placebo in a double blind design (group A and group B), and they underwent two sessions of rs-fMRI (after drug intake and placebo). Subjects were asked to stay still and relaxed and no particular instructions were given to attend or fixate a particular stimulus.

Based on previous findings (De Pasquale et al, 2013), we performed seed-based functional connectivity analysis of rs-fMRI data, focusing on three main central hubs: the anterior, middle, and posterior cingulate cortex (respectively ACC, MCC and PCC) (Figure 6). Threshold was set at $p<.001$ voxel level and p-FWE<.05 cluster level.

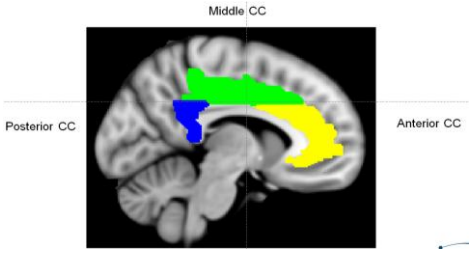

Figure 6. Cingulate Cortex study. Seed-based functional connectivity analysis of rs-fMRI data was focused on three main central hubs: the anterior, middle, and posterior cingulate cortex (respectively ACC, MCC and PCC).

PCC connectivity map

Considering PCC as seeds, the rs-fMRI analysis shows a normal pattern of functional connectivity in the DMN (Fig. 7), consistent with previous studies of functional brain connectivity from rs-fMRI data.

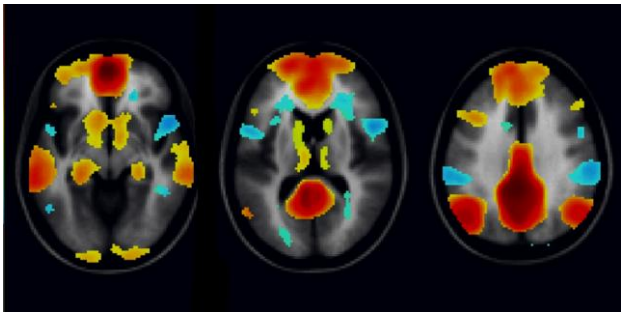

*Figure 7***:** *PCC-related rs-fMRI connectivity maps in the Placebo condition of the Studies A (Donepezil). In red-orange: cortical regions showing a positive correlation of the rs-fMRI signal with that of PCC as a seed. In green-blue: cortical regions showing a negative correlation of the rs-fMRI signal with that of PCC as a seed. Statistical threshold was set at p<.001 voxel level and p-FWE<.05 cluster level.*

Group Effect

When we compare the PCC connectivity maps from the two groups in the second level rs-fMRI analysis, we observe a trend in increase of functional connectivity (correlation coefficient, r-values) in two regions: in somatosensory associative (BA7) and anterior prefrontal (BA 10) areas (Figure 8a and 8b).

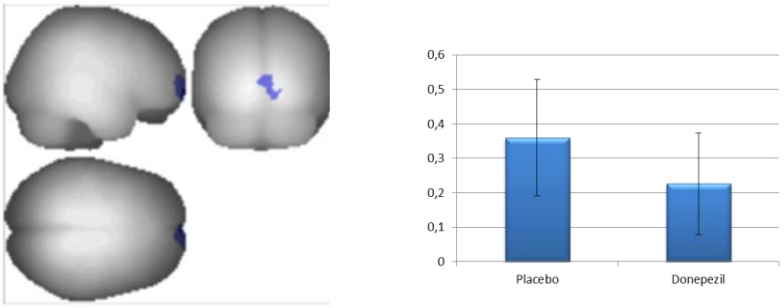

Figure 8a*: Group effect modulation of left PCC connectivity induced by Donepezil. There was a decreased connectivity with the BA 10 anterior prefrontal cortex compared with Placebo (cluster size = 206 voxels; p<.05 corrected for multiple comparison (Family Wise Error, FWE) at cluster level of p-FWE<.05.*

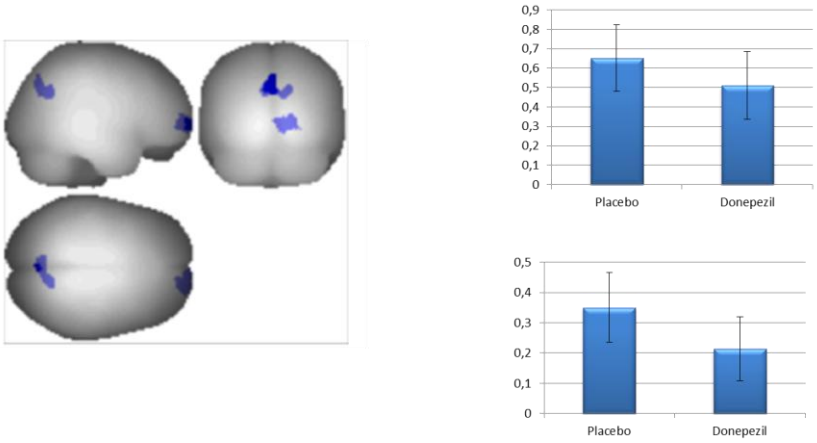

Figure 8b: *Group effect Modulation of right PCC connectivity induced by Donepezil. There was a decreased connectivity with the BA 10 anterior prefrontal cortex compared with Placebo (cluster size = 328 voxels; p<.05 corrected for multiple comparison (FWE) at cluster level) and bilateral BA7 (cluster size = 355 voxels; p<.05 corrected at cluster level).*

When we compare the ACC connectivity maps from the two groups in the second level analysis, we observe a modulation of functional connectivity with premotor cortex (Figure 9a and 9b).

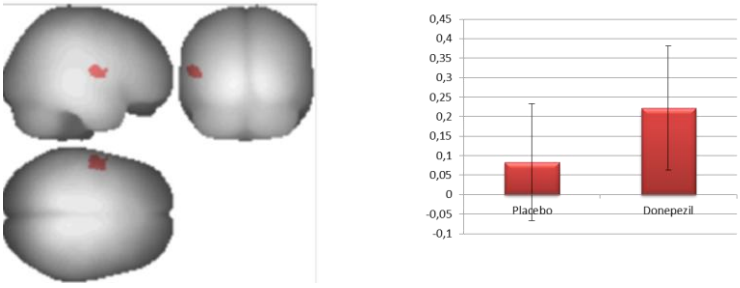

Figure 9a: *Group effect Modulation of left ACC connectivity induced by Donepezil. There was an increase with the Left premotor cortex compared with Placebo (cluster size = 211 voxels; p<.05 corrected for multiple comparison (FWE) at cluster level).*

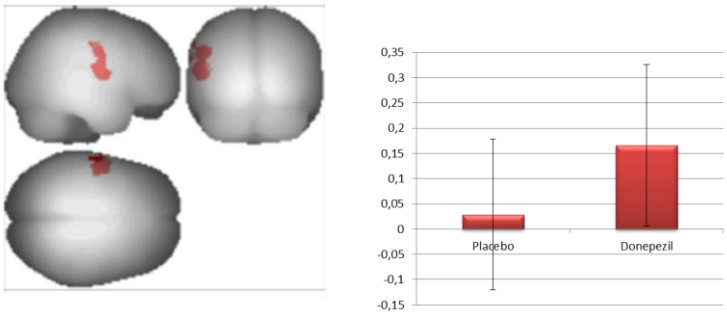

Figure 9b: Group effect Modulation of right ACC connectivity induced by Donepezil. There was an increase with the Left premotor cortex compared with Placebo (cluster size = 610 voxels; p<.05 corrected for multiple comparison (FWE) at cluster level).

fMRI during the PIcInOut task

In the neuroimaging plan of the study, recognition phase of the PicInOut task was performed during the fMRI recordings as a function of pharmacological intervention (Placebo and Donepezil). Compared to Placebo, Donepezil induces a task-related fMRI activation in the left frontal superior cortex (Figure 10) and bilateral occipital gyri, (mostly in the lingual gyrus and the fusiform). To a lesser extent, other activations are observed in the cerebellum, the calcarine, the cuneus, the temporal and the parahippocampus (Figure 11).

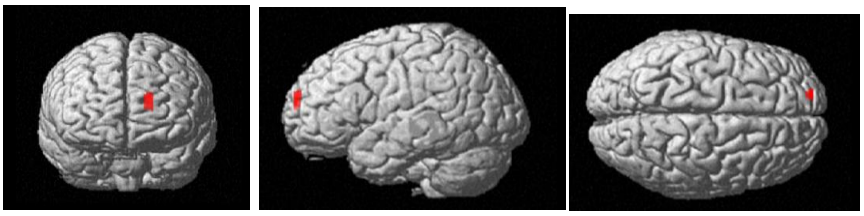

Figure 10. PicInOut Placebo vs Donepezil.

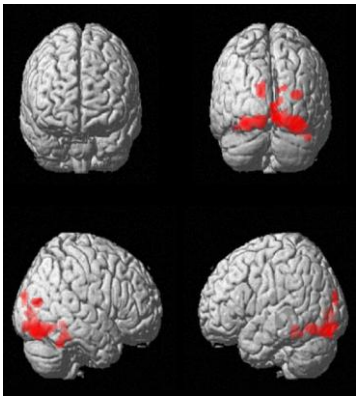

Figure 11. PIcInOut: Donepezil vs Placebo

In the neuroimaging plan of the study, the working memory N-back task was performed during the fMRI recordings as a function of pharmacological intervention (Placebo and Donepezil). The main results of the

fMRI analyses comparing effects of Donepezil vs. Placebo are presented in Table 2. Compared to Placebo, Donepezil induces a task-related fMRI activation in the middle frontal cortex (Figure 12).

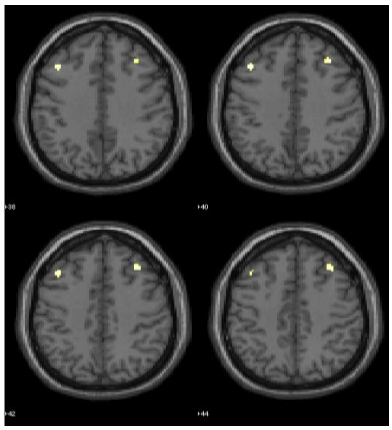

Figure 12*: N-Back vs Fixation: Placebo - Donepezil*

	Donepezil vs Placebo (hyperactivation)		**Placebo vs Donepezil (hypoactivation)**	
	Vs Control	Vs Fixation	Vs Control	Vs Fixation
PicInOut	No difference	Occipital (BAs: 17 18 19 37)	No difference	L Frontal Superior
N-Back	No difference	No difference	R &L Fr Sup. R Fr. Mid. R&L Par Sup L Prec. L Precu	R &L M Fr. (BAs 9, 46)

Table 2: Donepezil effects on PicInOut and N-Back Test activations.

PET-FDG Imaging: statistical parametric mapping (SPM)

The SPM maps reveal mainly a rs-PET-FDG hypometabolism induced by Donepezil (Placebo as a reference) in the left cerebrum, frontal lobe medial frontal gyrus, gray matter, BA6 and, to a lesser extent, in the left cerebrum, parietal lobe, precuneus gray matter, BA7 (Talairach Atlas labels for given coordinates). In contrast, results showed relatively increased metabolic activities (described as hypermetabolism induced by Donepezil) in areas such as left cerebrum, temporal lobe, superior temporal gyrus, gray matter, BA38 or right cerebrum, parietal lobe, postcentral gyrus, gray matter, BA1, right cerebrum, sub-lobar, thalamus, pulvinar and right cerebrum, frontal lobe, medial frontal gyrus, gray matter, BA 9 (Talairach Atlas labels for given coordinates).

Compared to Placebo, Donepezil induces a hypometabolism in the left cerebrum, frontal lobe medial frontal gyrus, gray matter, and BA6. On the contrary, there was a slight increase of metabolic

activities in the right cerebrum, parietal lobe, postcentral gyrus, gray matter, BA 1 and right cerebrum, frontal lobe, medial frontal gyrus, gray matter, and BA 9.

EEG data

Analysis of resting state EEG rhythms

Compared with the Placebo, the Donepezil intervention induces ($p<0.05$) an unselective decrease in the resting state parietal and occipital source activity at the delta, theta, alpha 1, and alpha 2 bands (Fig. 13, top). The visual analysis of these rsEEG markers shows some potential outliers. The Grubbs test ($p<0.005$) unveils 4 outliers (Fig. 13, middle). When these outliers are removed, no statistical difference ($p>0.05$) in the rsEEG markers is observed between the Placebo and the Donepezil intervention Fig. 13, bottom).

The present results suggest that a chronic (15 days) administration of Donepezil is not able to modify the cortical source activity in the rsEEG rhythms in healthy young adults. This lack of effects of the Donepezil treatment on the rsEEG rhythms confirmed and cross-validated what observed in another independent cohort of healthy young adults investigated in the PharmaCog WP1 (Part B Study, data recorded before a sleep deprivation "challenge"). Overall, Donepezil seems not to be active on the cortical neural synchronization mechanisms underlying brain arousal in quiet wakefulness and the generation of resting state EEG rhythms. The lack of effects of this modulation discourages the use of these PharmaCog rsEEG markers in clinical trials using Donepezil as a pharmacological manipulation in healthy young adults.

In the context of the PharmaCog project, the combined results of the WP1-SD and WP3 show that the present rsEEG markers provided reliable, repeatable results in two independent cohorts of healthy young adults under a pharmacological modulation with Donepezil (15 days) contrasted with a Placebo condition.

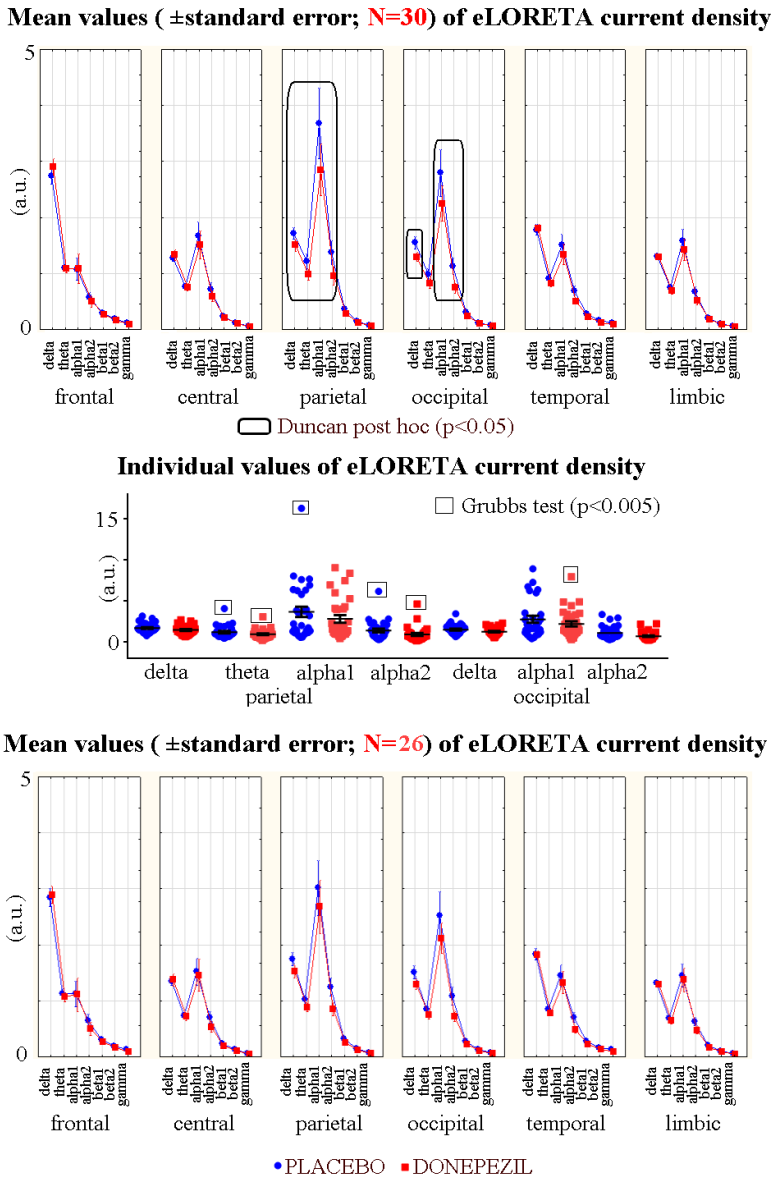

Figure 13. (Top): Mean activity of the eLORETA cortical sources of the rsEEG rhythms in the healthy young subjects (N=30) for the following factors of an ANOVA design (p<0.05): Intervention (Placebo, Donepezil), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2, gamma), and ROI (central, frontal, parietal, occipital, temporal, limbic). An ANOVA design showed a 3-way interaction (p<0.05) of the Intervention, Band, and ROI factors. (Middle): Individual values of the eLORETA cortical sources activity of the rsEEG rhythms showing statistically significant (p<0.05) differences between the Placebo and the Donepezil interventions (i.e. parietal delta, parietal theta, parietal alpha1, parietal alpha 2, occipital delta, occipital alpha 1, occipital alpha 2). Noteworthy, the Grubbs' test (p<0.001) detected 4 outliers from those individual values of the eLORETA solutions. (Bottom): Mean activity of the eLORETA cortical sources of the rsEEG rhythms in the cohort of subjects after the removal of the mentioned 4 outliers (N=26) for the following factors of an ANOVA design: Condition (Placebo, Donepezil), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2, gamma), and ROI (central, frontal, parietal, occipital, temporal, limbic). When these 4 outliers were removed, no statistical difference in the rsEEG markers was observed between the Placebo and the Donepezil intervention (p>0.05).

ERPs and P3 component

ERPs analyses were performed by Lille. They were focused on P3 component of the ERPs. P3 peak amplitude, latency and area (in 250-450 ms interval for auditory task and in 350-600 ms for visual task) were calculated for all subjects, stimuli (i.e., rare, frequent and distractors) and conditions (i.e., Donepezil and Placebo). No significant changes were found between Donepezil and Placebo conditions in terms of peak amplitude, latency and area for Fz, Cz, Pz and Oz electrodes for rare (regarding the both tasks) and distractor (regarding visual task) stimulus (see Table 3).

Electrode	Condition	Mean Peak Latency (ms)	*p value	Mean Peak Amplitude (μV)	*p value	Mean Peak Area (μV*ms)	*p value
Fz	DPZ	331.25 (SD=37.65)	0.64 (NS)	10.90 (SD=6.90)	0.45 (NS)	1203,80 (SD=844.26)	0.75 (NS)
	PLB	325.58 (SD=45)		9.56 (SD=5.20)		1134,7 (SD= 623,80)	
Cz	DPZ	316.58 (SD=37.26)	0.47 (NS)	15.77 (SD=6.70)	0.54 (NS)	2039,60 (SD=968.16)	0.54 (NS)
	PLB	324.42 (SD=37.30)		14.61 (SD=6.16)		1859,10 (SD=1040,5)	
Pz	DPZ	318.25 (SD=33.35)	0.95 (NS)	17.55 (SD=5.46)	0.24 (NS)	2295,80 (SD=878.76)	0.35 (NS)
	PLB	318.83 (SD=31.51)		15.85 (SD=4.40)		2076,70 (SD=739,00)	
Oz	DPZ	309.08 (SD=33.30)	0.55 (NS)	10.29 (SD=4.53)	0.14 (NS)	1214,80 (SD=666.94)	0.17 (NS)
	PLB	314.50 (SD=29.26)		8.50 (SD=3.68)		973,9 (SD=534,50)	

Table 3. Group mean (standard deviation, SD) of latency, amplitude and area of P3 component of the ERPs between Donepezil and Placebo conditions for the oddball rare stimulation (250-450ms) for the auditory oddball paradigm.

In order to reduce the effect of brain volume conduction on the results from time-frequency analyses that were obtained in auditory and visual tasks, Current Source Density (CSD) of ERSPs and ITCs data were calculated.

CSD-ERSPs and -ITCs were analyzed in different time windows (e.g., 0-50, 50-100, …, 1200-1300 ms) and different frequency bands (δ, θ, α, β and γ) for Donepezil and placebo conditions in both oddball paradigms (auditory and visual). However to be concise in our presentation, we will describe here only the topographies from the auditory oddball paradigm.

Among the large number of variables identified by the analysis for CSD-ERSPs and -ITCs, we selected arbitrarily the first six variables explaining the most percentage of variance of data. Figures 14.B and 15.B show the reduction of matrix for scalp topographies for CSD-ERSPs and -ITCs respectively. Topographies of Placebo and Donepezil conditions from PCA analysis were compared for each PCA component using a parametric Student's paired t-test. Color coded significant values were illustrated on a topographic map for each PCA component. Figures 14.C and 15.C show statistical analysis (Student's paired t-test) between Donepezil and Placebo conditions for the rare stimulus.

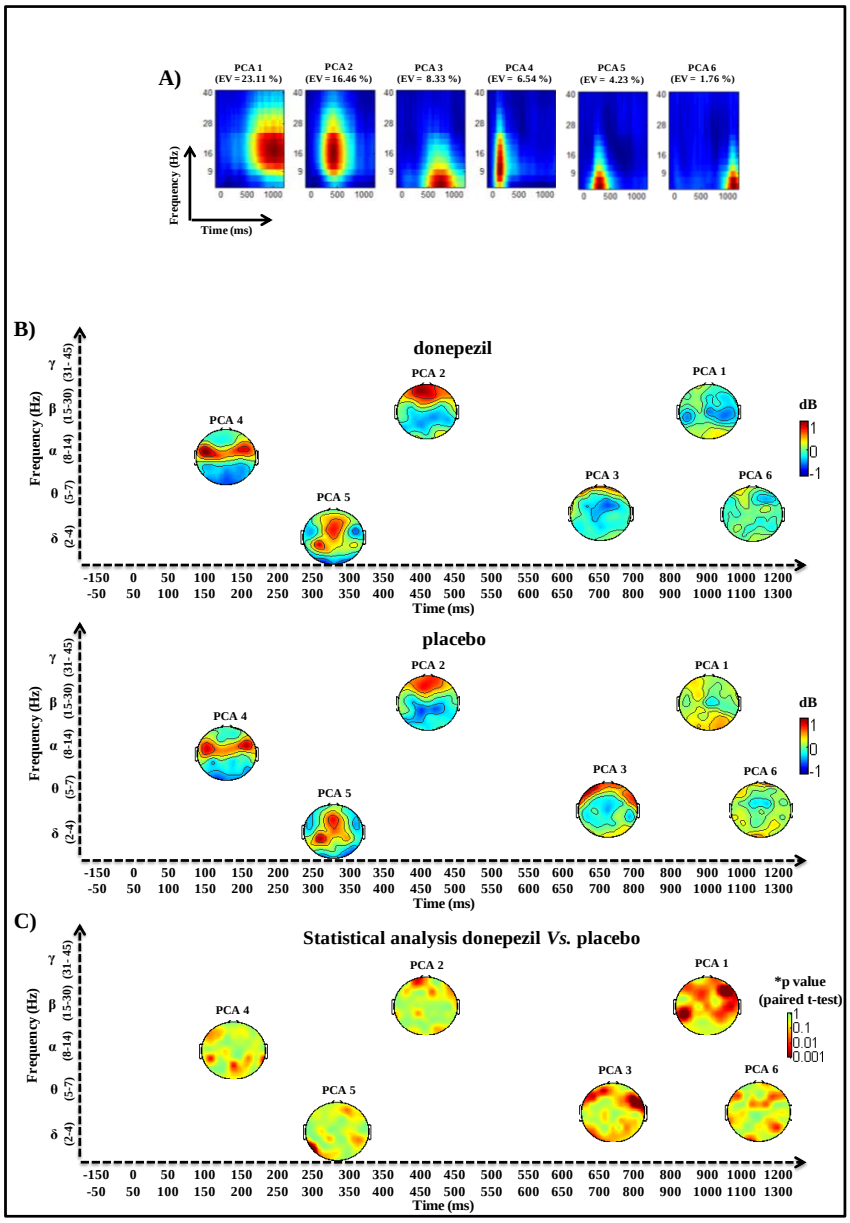

Figure 14. Reduction of CSD-ERSPs results for the rare stimulus from auditory oddball task by Principal Component Analysis. ***A)*** *The 6 time-frequency maps correspond to the 6 first components explaining the most percentage of variance (EV: Explained Variance) of ERSPs data for the rare stimulus (Donepezil and Placebo data combined).* ***B)*** *On the basis of information from principal components, the activity of each of the 58 scalp electrodes was expressed. According to a directional mapping: red color (closed to the value 1) means a positive similarity between the activity of the given electrode and a given component,; blue color (closed to the value -1) means a negative similarity; and an intermediate color (closed to the value 0) means a lack of similarity.* ***C)*** *Topographies of placebo and Donepezil conditions were compared for each component using a parametric Student's paired t-test. Significant values were illustrated on a topographic map with a color coded for each PCA component. Dark red color indicates an important significant difference between the two conditions (p< 0.001). Green color means no significant difference (p = 1).*

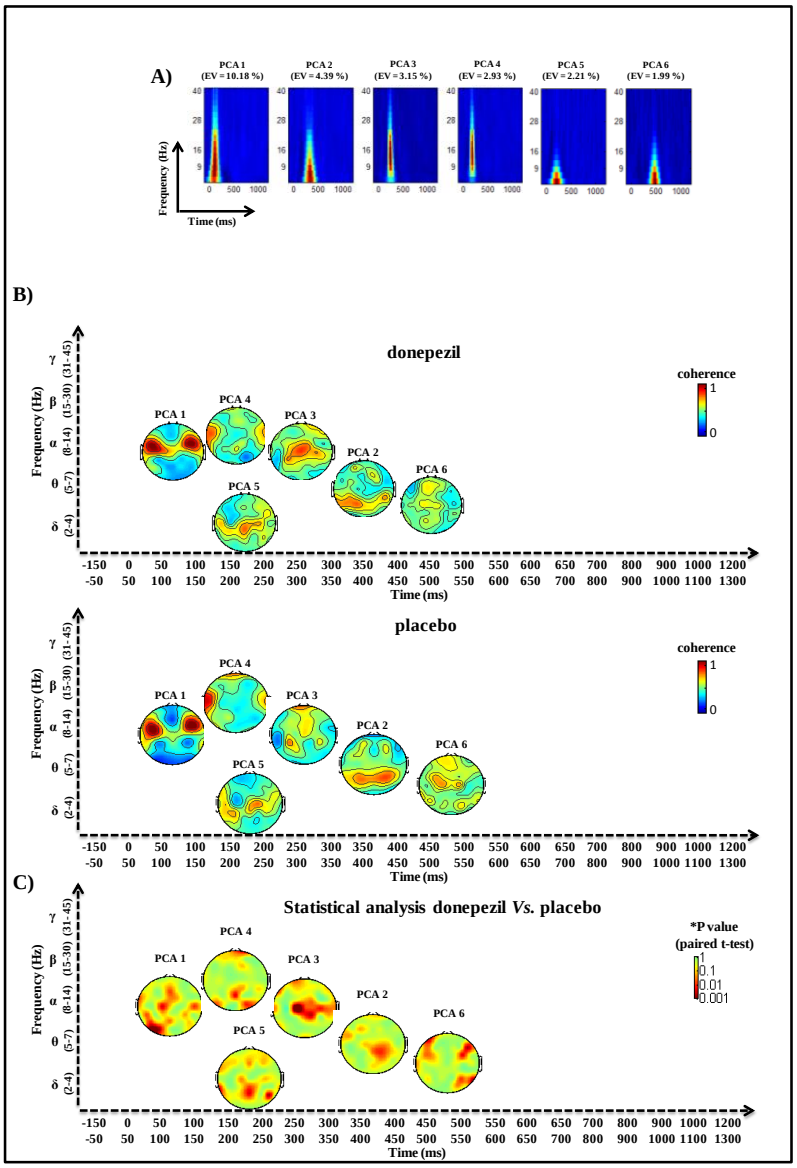

Figure 15. Reduction of CSD-ITC results for the rare stimulus from auditory oddball task by Principal Component Analysis. **A)** The 6 time-frequency maps correspond to the 6 first components explaining the most percentage of variance (EV: Explained Variance) of ITCs data for the rare stimulus (Donepezil and placebo data combined). **B)** On the basis of information from principal components, the activity of each of the 58 scalp electrodes was expressed. According to a directional mapping ITC varies between 0 (blue) and 1 (red). Red color indicates a perfect similarity between the activity of a given electrode and a given component. Blue color means no similarity. **C)** Topographies of Placebo and Donepezil conditions were compared for each component using a parametric Student's paired t-test. Significant values were illustrated on a topographic map with a color coded for each PCA component. Dark red color indicates an important significant difference between the two conditions ($p < 0.001$). Green color means no significant difference (p closed to 1).

Auditory oddball task: EEG data analysed in frequency domain

δ-ERSPs activities are observed in frontal-central-parietal regions at around 300 ms (Figure 14.B, see PCA 5). They are more important in left parietal regions in placebo condition (Figure 14.C, see PCA 5) (p <

0.05). They were higher in frontal regions in Donepezil condition (Figure 14.C, see PCA 5) ($p < 0.05$). Later (between 700 ms and 1200 ms), δ/θ-ERSPs activities in frontal-occipital regions appear more important in Placebo condition (Figure 14.B and C, see PCAs 3 and 6) ($p < 0.05$). In addition, ITCs analyses (Figure 14) show δ-ITCs activities at around 200 ms are observed mainly in central-parietal regions (Figure 14.B, see PCA 5). These activities seem to be more prominent in Donepezil condition ($p < 0.05$) (Figure 15.C, see PCA 5). Then, θ-ITCs activities at about 350 ms in parietal-occipital regions could also be observed (Figure 15.B, see PCA 2). These activities are bilateral but with a more important activation in right parietal-occipital regions for Placebo condition (Figure 15.C, see PCA 2) ($p<0.05$). Finally others δ/θ-ITCs activities appear at around 450 ms in frontal-parietal-occipital regions (Figure 15.B, see PCA 6). These activations are more important in Placebo condition (Figure 15. C, see PCA 6) ($p < 0.05$).

α-ERSPs activities are observed at about 100 ms in temporal regions (Figure 14.B, see PCA 4). Statistical analysis indicates that left temporal α-ERSPs activities are more important in Donepezil condition (Figure 14.C, see PCA 4) ($p < 0.05$).

α/β-ERSPs activities occur at around 400 ms in frontal regions (Figure 14.B, see PCA 2). These activities are more important in Donepezil condition ($p < 0.05$) (Figure 14.C, see PCA 2). Then, other β-ERSPs activities at around 900 ms are observed in frontal-parietal-occipital regions (Figure 14.B, see PCA 1). Donepezil induces changes in rhythmic brain organization that is compatible with the effect on attentional processes in patients.

Visual oddball task: EEG data analysed in frequency domain

For rare stimuli, results are similar to those described for the auditory task (see description above). For distractor stimuli, CSD-ERSPs analyses indicate a α-ERSP higher in Donepezil condition compared to placebo condition. This difference is located in frontal regions of the scalp at about 400 ms ($p < 0.05$). Moreover, there is a δ-ERSP more prominent in Donepezil condition at about 650 ms in frontal-central regions of the scalp ($p < 0.05$). In addition, CSD-ITC analyses show that distractor stimuli in Donepezil condition (compared to placebo condition) are associated with higher α-ITC in occipital regions of the scalp at around 100 ms, higher α/β-ITC in frontal regions (~250 ms), and higher θ-ITC in frontal-central regions (~400 ms). Donepezil induces changes in brain activity related to an increase in attentional processes.

WP5: Identification of new biomarkers for disease-modifier drug assessment

In WP5, biomarkers of prodromal AD sensitive to time (follow ups) and the clinical progression (cognitive decline) were investigated in 147 aMCI patients who underwent clinical, neuropsychological, MRI, rsEEG/ERP, and blood data collection every 6 months for at least 2 years. The CSF was collected at baseline and at 18 months. The number of enrolled aMCI patients (N=147) guarantees the robustness of the final WP5 results:

		T0	T12	T24	T36
Non converted	N	150	120	96	78
	Marker change (%)	---	1.15	1.00	0.90
Converted	N	0	15	27	36
	Marker change (%)	---	1,9 (1,0)	1,9 (1,0)	1,9 (1,0)
Dropouts		0	15	27	36
Alpha (power 0,80)		---	.06	.0014	.00008

All the WP5 data are stored into the neuGRID platform (https://neugrid4you.eu).

Cross-sectional Analyses

The cross-sectional characterization of the WP5 aMCI patients was performed by stratifying them into CSF Aβ positive (prodromal AD) and negative using a fixed cut-off taken from the literature (Galluzzi et al, Journal of Internal Medicine, 2016). Considering that the definition of a single fixed cut-off has recently raised doubts (Villain, Brain 2012; Villeneuve AAIC 2015, Coart AAIC 2015), we applied a data-driven approach to identify CSF thresholds based on the distribution of their baseline CSF Aβ42 levels and Aβ42/t-tau and Aβ42/p-tau ratios. Cut-offs for Aβ42, Aβ42/t-tau and Aβ42/p-tau were established after applying a data-driven approach: the mixture model analysis established three main Gaussian distributions and two cut-offs for each biomarker (Figure 16).

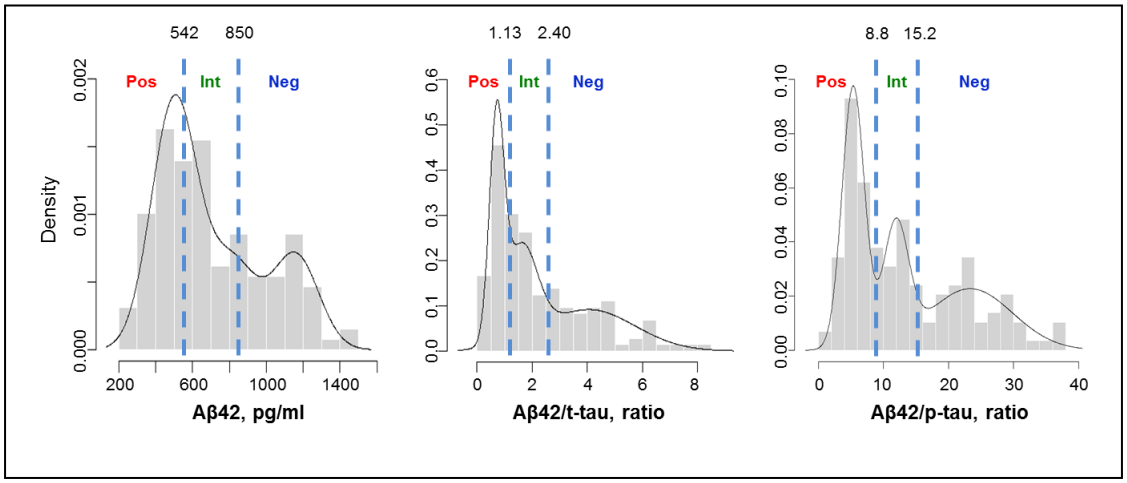

Figure 16. Histograms and corresponding overlaid density curves estimated by mixture models for CSF Aβ42, Aβ42/t-tau, and Aβ42/p-tau. Dotted lines represent the cut-offs established by the mixture model analysis. Legend: For all classifications based on the CSF markers, the aMCI patients were grouped in positive (prodromal AD), intermediate, and negative (non-AD).

For each CSF classification, the resulting aMCI groups (positive as prodromal AD, intermediate, and negative as non-AD) were compared for demographic variables; Apoε4 carriers; global cognition (ADAS-cog); structural, diffusion, and functional MRI; EEG; APP Metabolites; ADFlag® score. The results demonstrated that compared with the negative aMCI groups, the positive aMCI groups (prodromal AD) were globally characterized by: i) lower performance in memory and attention/executive composite

measures; ii) more frequent occurrence of the APOE4 genotype; iii) structural MRI abnormalities in hippocampus and its subfields, parietal, temporal and occipital cortex; iv) alteration of the MRI diffusion in the fornix, splenium, and the cingulum of the hippocampus; v) abnormal DMN rs-fMRI connectivity; (vi) higher global cortical source activity at delta (2-4 Hz) EEG rhythms in the resting-state eyes-closed condition and lower posterior cingulate source activity at auditory oddball P3b peak. These characterizing biomarkers showed the most consistent effects for the CSF Aβ42/p-tau classification, justifying its use to stratify the aMCI patients in the longitudinal study. The APP metabolite biomarkers failed to identify any difference among groups, probably because of the high variability of data.

Finally, the ADFlag® score proved useful to identify pre-dementia subgroups of patients at risk to develop AD. Increasing ADFlag® scores were associated with declining performances of patients in both memory function neuropsychological assessments (RAVLT, CANTAB-PAL, and ADAS-Cog delayed recall subtask), in language function neuropsychological assessments (letter fluency and category fluency tests) and in attention/executive function neuropsychological assessments (TRAIL-making test, WAIS-R, ADAS-Cog attention sub-scores and in the Digit Span forward test). These tests are classical NPSY tasks used to assess MCI/AD patients and have also been used in WP1 (Sleep deprivation and TMS models).

Longitudinal Analysis

The longitudinal results are summarized in Figure 17. The ***biomarker set of structural/functional neural correlates of the global cognitive decline in aMCI patients*** are reported in the second circle of Figure 17. The association between biomarkers and cognitive decline had different strength: the morphological MRI biomarkers (Figure 17, in blue) reported the strongest association, followed by microstructural and functional rs-fMRI and rsEEG connectivity biomarkers (Figure 17, in orange and green, respectively).

The set of biomarkers of disease progression in aMCI patients with prodromal AD (positive CSF Aβ/p-tau aMCI patients) are reported in Fig 17 (inner circle) and represent those with the highest ability in detecting the changes over time in the positive (progressing) relative to negative (stable) aMCI group. In particular:

i) Morphological MRI abnormalities in the hippocampus (with its anterior/inferior subfields), thalamus (decrease volume), lateral ventricles (increase volume) as well as a thinning of the entorhinal cortex (Figure 18, graphs 1-12)

ii) Alteration of the MRI diffusion (structural connectivity) in the fornix (Figure 18, graph 13);

iii) Deranged resting state posterior EEG rhythms (cortical neural synchronization and functional connectivity) in theta and alpha frequencies (4-10 Hz) (Figure 18, graph 14).

Structural and EEG measures, being the most sensitive to change even in a mildly progressing aMCI population, have been considered at the same time to compute a new measure (MATRIX). **This MATRIX of biomarkers of disease progression improved the sensitivity to detect cognitive decline and changes over time compared to the individual biomarkers**:

- stronger correlation with cognitive decline than the hippocampal volume, the individual biomarker with the highest association with ADAS-cog;
- similar ability in identifying aMCI with prodromal AD as the ventricle volumes, the individual biomarker with the highest ability to separate stable aMCI patients from those with prodromal AD.

In addition, we took into consideration the potential of plasma amyloid markers in reflecting AD pathology. Our findings reflect the fact that Aβ plasma measurements, to date, are still affected by technical problems regarding sample storage and quantification (Toledo et al, Alzheimer's Research & Therapy, 2013). The variability of such measures precludes the possibility to clarify their relationship with CSF biomarkers and with AD pathology, underlying the need to standardize the measurement procedure.

Finally, the ADFlag® score was able to segregate non-converter MCI patients from those who progress to AD or other type of dementia and, if higher than 2, significantly increased the likelihood of converting within 2 years. Longitudinal analyses will be carried out next to cross validate the ADFlag® score classification. ADFlag® scoring in pre-dementia AD needs further validation against newly defined IWG and NIA-AA guidelines to evaluate its value as a stratification marker for preprodromal or pre-MCI stages of AD.

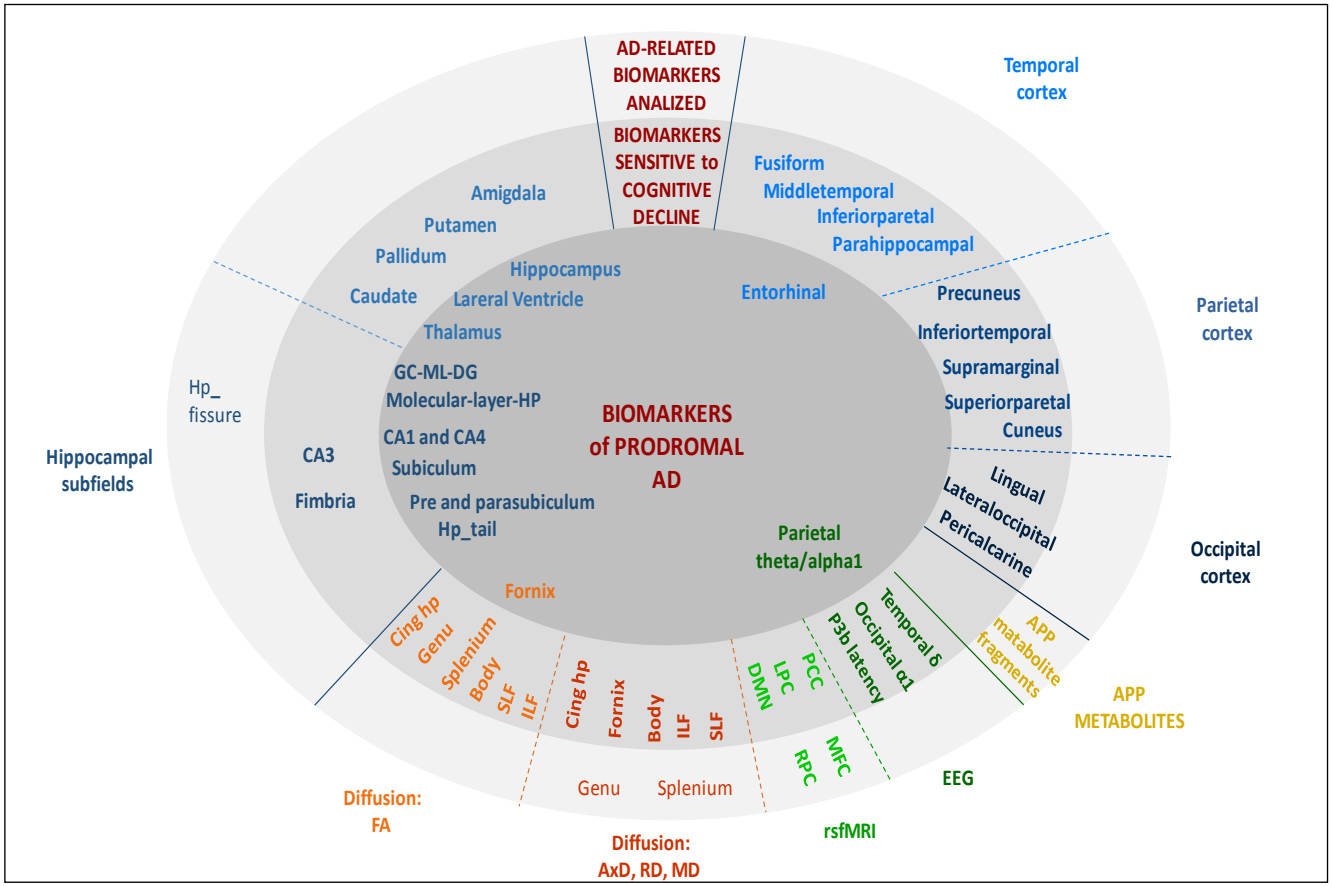

Figure 17. Biomarkers being investigated in aMCI patients, reported according to their sensitivity to cognitive decline (middle circle) and to their ability in separating the aMCI patient groups identified by the CSF Aβ42/p-tau classification (inner circle).

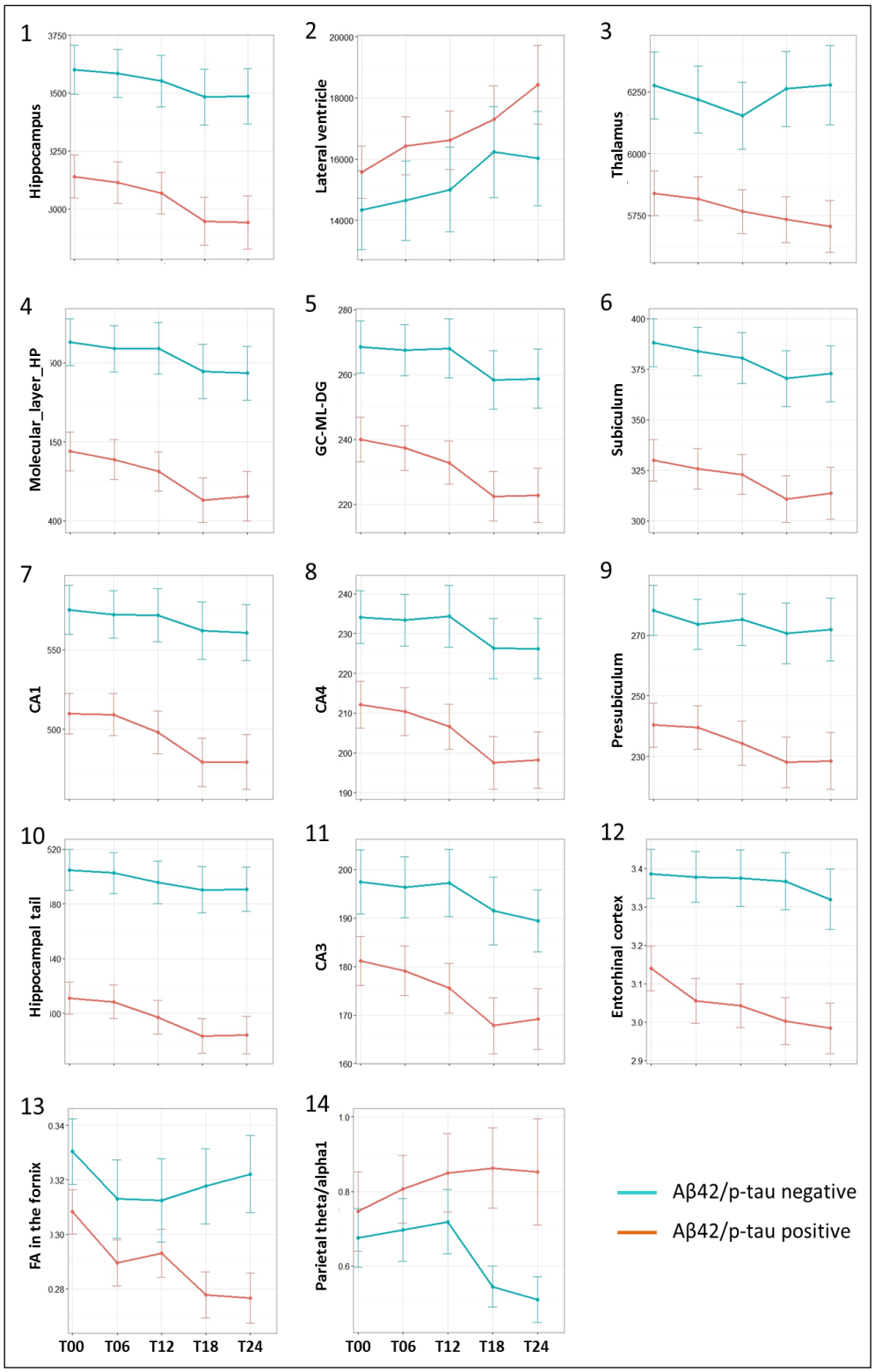

Figure 18. Biomarker progression over time in in Aβ42/p-tau aMCI groups. Error bars indicate the standard deviation on the mean. Morphological markers (only left hemispere was reported but results on the right side were similar): volumes (in mm^3, graphs 1-11) and entorhinal thickness (in mm, graph 12). Connectivity markers: microstructural MRI (FA in the fornix, graph 13) and functional (cortical sources of parietal theta (4-7Hz)/alpha1 (8-10.5 Hz) resting state eyes-closed EEG rhythms, graph 14) markers.

Back-translation to PharmaCog WPs (i.e. WP2, 4, and 6)

From a back-translational point of view, the WP1 and WP2 work led to new standards and scientific consensus in how to perform relevant and robust SD conditions and collect biomarkers and cognitive indexes during this challenge. SD experiments in Lemurs and Octodons have unveiled relevant translational models for future testing of AD drugs (Tarragon et al. 2014; Rahman et al. 2013) while the SD rat model provided too variable effects on cognition. Pharma Companies may not use these models internally due to the practicality of using these species both in terms of drug discovery (e.g. toxicology studies are not routinely performed for these species) and time to age such animal models for optimal experiments. However, the consistency of effects across these species and the phylogenetic proximity between lemurs and humans support the continuation of such further basic research experimental investigations. Concerning the biomarkers, EEG data in lemurs closely resembled that of humans (WP1), i.e. alpha is increased in passive state and decreased in active state. These findings have been confirmed also with the application of SD protocol, indeed the higher alpha activity in the after SD condition could be related to the higher behavioural awake passive state of lemurs. Even though industry may not be able to use an EEG read-out in lemurs to assess effect of compounds, the close resemblance of EEG data between lemurs and humans suggests it would be pertinent to first establish the translation of EEG read-out from lemur to rodent and then use rodent as the decision making assay before entering the clinic. These data are of interest for fundamental research across species.

WP3 used a clinical trial performed in healthy volunteers under chronic administration of Donepezil, a classical symptomatic drug, versus placebo. While there were very few significant effects on neuropsychological tasks, probably due to the healthy volunteers being already at their maximal level of performance, we observed changes on both EEG and neuroimaging (for details, see report of WP3-D3.4). From a translational point of view, donepezil produced effects on EEG activity recorded in rodents in WP4 but further research is required to appreciate the extent to which these effects reflect relevant neurophysiological mechanisms underlying the results observed in the present healthy volunteers.

In WP6, a matrix of biomarkers characterised three lines of genetically engineered mouse models of AD and showed translational biomarkers (e.g. EEG, structural MRI, resting-state fMRI) that reflect aging and/or amyloid pathology. This provided complementary data to changes in the biomarkers seen in MCI patients that were related to CSF Abeta levels (WP5). The profiling of the mouse models of AD and mouse lemur during aging was completed. Age-dependent behavioural and biomarker changes were found in these animals. In mice, these studies unveiled markers potentially useful for the stratification of the transgenic strains based on exhibited metabolic, neurophysiological, neuroanatomical, and behavioural (i.e. learning) abnormal changes in the cerebral cortex and/or other relevant subcortical regions (striatum, hippocampus). Furthermore, most of these biomarkers were able to reveal abnormal cortical changes across time in these mouse strains modelling AD, which mimic the evolution of AD pathology in seniors with amnesic MCI possibly due to prodromal AD (WP5). In these MCI patients, the experimental results with the PharmaCog biomarker matrix were consistent with those observed in mouse models of AD in terms of an alteration of metabolic, neurophysiological, neuroanatomical, and behavioural (i.e. learning) abnormal changes in the cerebral cortex and/or other relevant subcortical regions (striatum, hippocampus).

In lemurs, the best predictor of cognitive impairment was an increase of fasting glycaemia without modification of glucose tolerance. This very interesting finding is consistent with the increased risk of cognitive impairment and AD in diabetes in humans. The drug studies using amyloid lowering agents produced unexpected data regarding non-beneficial effects on the functional consequences of lowering beta amyloid but nonetheless important data to consider when moving these agents forward to the clinic.
Overall, the results of the clinical PharmaCog WPs (WP1-SD, WP3, and WP5) suggest that the matrix of multi-modal biomarkers used in WP2, WP4, and WP6 may represent an ideal platform for a neurobiological, neuroanatomical, neurophysiological, and behavioral characterization of different animal models of AD for the early stage of drug discovery in AD research.

1.6. Potential impact and main dissemination activities and exploitation of results

A new way for drug development in Alzheimers' disease: The PharmaCog project has met the challenge successfully

AD is a progressive brain disorder that causes a gradual and irreversible decline in memory and cognitive abilities. At present, the pharmacological therapy for AD is still limited to symptomatic temporary improvement or stabilization of cognitive performance and the reduction of neuropsychiatric symptoms of the disease. Five drugs are currently marketed for the treatment of AD including four cholinesterase inhibitors – tacrine, donepezil, galantamine, and rivastigmine – and one glutamate antagonist (Memantine). However, owing to the extensive and multifocal nature of neurodegeneration in AD, the effects of transmitter modulators are modest. In recent years, a new therapeutic approach (disease modifying approach) has emerged. Unlike treatments that target symptoms, disease modifying therapies should interact on the natural course of the disease by interrupting early pathologic events thus preventing underlying pathophysiological processes. Although very promising, to date no disease modifying therapies have been clearly shown to be efficacious.

The clinical development of drugs in AD has been confronted with challenging methodological difficulties both to improve symptomatic treatment and validate the disease-modifying strategy. Taking into account the cost involved progressing drug candidates to Phase III of development and the risk of investing time and resources fruitlessly in the evaluation of poor candidate drugs, the crucial decision remains whether to proceed from phase II to phase III (Go/No Go). The aim of phase II studies is to select a molecule likely to be effective in phase III, but also to eliminate candidate-drugs with an inadequate effect. Nevertheless, there is not a real bridge between preclinical development and no consensus currently exists on the best possible design of Phase II studies in AD to inform the Go/No Go decision optimally, neither in terms of patient selection nor in terms of experimental design nor in terms of endpoints. At the present time, neuropsychological-based tools are the most established and approved method of assessing outcomes in AD pharmacotherapy because they are widely available and do not require technological instrumentation. Because of the difficulties in understanding the neurobiological and neurophysiological impact of a candidate-drug using only clinical and

cognitive tools, the challenge of PharmaCog project was to propose a new biomarker battery able to assess the effect on the brain of new symptomatic or disease-modifying strategies that could be integrated in a true translational preclinical and clinical development.

The PharmaCog project proposes a new way for drug development in AD

- ✓ Novel insights into the pathophysiology of AD both in term of biology (complexity of molecular pathways) and brain structure (MRI) and functioning (EEG, FDG-PET, rs-fMRI) associated with cognitive function for the assessment of both symptomatic and drug-disease-modifying drugs;
- ✓ Highlighting the role of EEG in early stage of AD drug development in preclinical and clinical studies;
- ✓ Demonstration of translational usability of the above multimodal biomarker battery in animals, healthy volunteers and patients;
- ✓ Standardization of preclinical (touch-screen, novel object recognition) and clinical (8-items Cantab, E-ADNI battery) cognitive assessment across academic and industrial partners of the consortium;
- ✓ Validation of four animal models useful to select new symptomatic drugs: sleep-deprivation microcebus lemur model; sleep-deprivation octodon degus model; hypoxia mice model; and sleep-deprivation rat model;
- ✓ Validation of the interest of the biomarker battery to assess future new disease-modifying drugs in transgenic mice;
- ✓ Definition and validation of some protocol designs in healthy volunteers coupling drug assessment, challenge model (Transcranial Magnetic Stimulation, sleep deprivation), assessment of cognitive function, and use of biomarker battery;
- ✓ Standardization of EEG and MRI recording across the clinical centres of the consortium;
- ✓ Development of a platforms for testing new molecules in specific animal and healthy volunteers' models, with a standardization allowing the comparison of effect level to reference drugs in multi-centric studies;
- ✓ Long-term follow-up of MCI patients' cohorts with the identification of biological, EEG and imaging criteria to refine the diagnosis of prodromal AD and stratify populations of MCI due to different aetiologies for clinical trials (Figure 19);

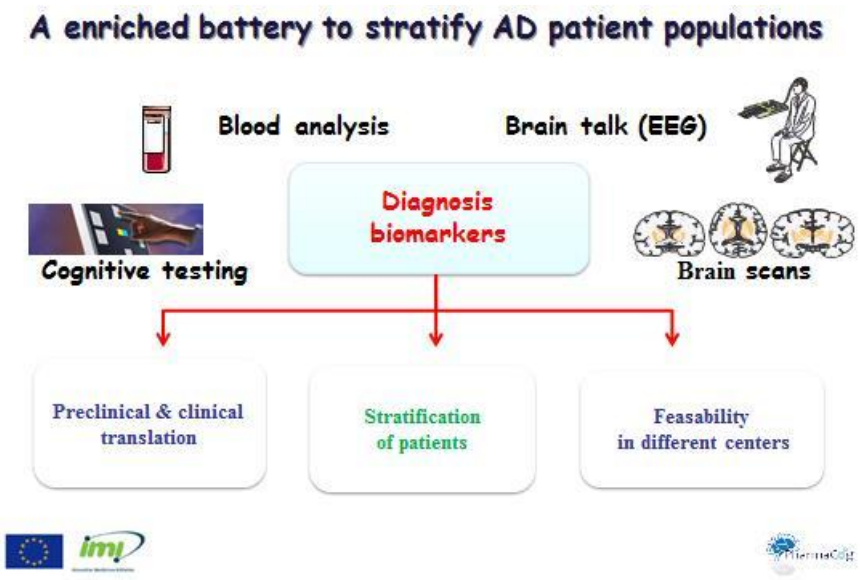

Figure 19. An overview of the utility of the PharmaCog biomarker battery and neuropsychological testing for multicentric clinical trials evaluating the efficacy of new drugs against AD.

- ✓ The PharmaCog project has generated 40 scientific publications with many others in preparation;
- ✓ The PharmaCog project generated new collaborations by: (i) common scientific meetings; (ii) organization of an international associated laboratory supported by Lille University (Lille-Murcia) ; (iii) preparation of several applications to new calls;
- ✓ The PharmaCog project generated a biobank: (i) a plasma and CSF biobank in MCI patients; (ii) a DNA bank in healthy volunteers contributing to the future identification of variability genetic factors in response to drugs;
- ✓ The PharmaCog project has generated a unique pharmacological database sharing all parameters of the different experimental and clinical studies, using the XNAT and Intellimaker techniques,

All these achievements allowed us to model a new paradigm for AD drug (symptomatic and disease-modifying drugs) assessment in the early stages of development for consideration by industrial partners in their GO/NO GO decisions (Figure 20):

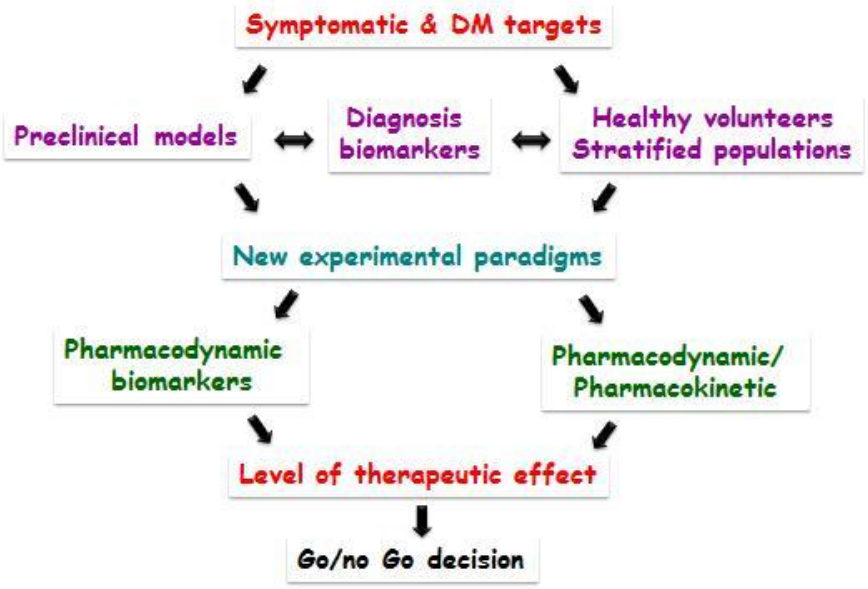

Figure 20. Flow chart of a new paradigm for AD drug (symptomatic and disease-modifying drugs) assessment in the early stages of development for consideration by industrial partners in their GO/NO GO decisions.

This new paradigm is based on the use of an original biomarker battery and cognitive testing that needs to be implemented in animal models, in healthy volunteers or in patients with or without a challenge test to sensitize the assessment of effect level (Fig. 21).

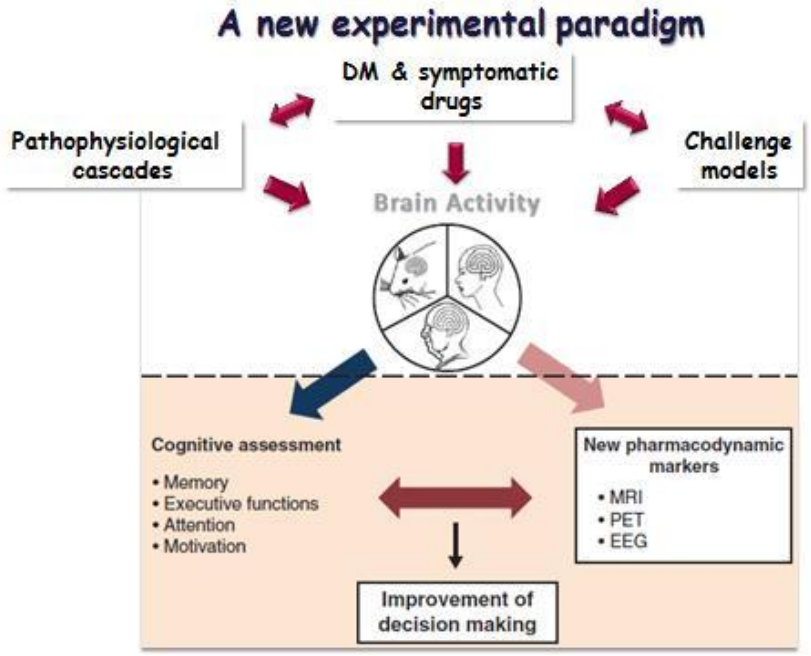

Figure 21. Relationships among pathophysiological cascades, disease modyfing (DM) and symptomatic drugs, and the PharmaCog challenge models.

The PharmaCog project has had and will continue to have socio-economic impact

- ✓ The PharmaCog project has been essential to reconcile both symptomatic and disease-modifying approaches by demonstration that their effects, in particular on brain functioning, are complex, additional or complementary;
- ✓ The PharmaCog project has identified new parameters (EEG, MRI, biology) related to prodromal AD in amnesic MCI patients, improving the early diagnosis of AD and sparing health cost;
- ✓ The PharmaCog project enabled validation of a multiplex test (ADflag) for early diagnosis of AD and developed by a SME;
- ✓ The PharmaCog project should improve the cost of drug development by early predictive identification of molecules that would not reach a sufficient efficacy level, sparing the cost of large clinical trials and reducing the risk of failure;
- ✓ The new paradigm proposed by the PharmaCog project has been already endorsed by a French SME (which was not involved in the PharmaCog project) to assess an original compound with a perspective for application for H2020;
- ✓ Clinical trials should be more sensitive as a result of patient stratification by focusing on potential responders. This will allow the use of relative low groups of patients to test new drugs against AD;
- ✓ Participation to the EMA meeting for revision of the AD guideline draft that is currently under public consultation (from Feb 1 to July 31, 2016). The qualification process for biomarkers will be positioned in line with this guideline (EMA/CHMP/539931/2014).

WP1

The collaboration between Academic and EFPIA members within WP1 has enabled the development of multi-centric technological platforms to test two non-invasive cognitive challenge models able to induce a transient cognitive impairment in cognitive outcomes critically compromised in AD. In building such platforms, we have incorporated the best scientific standards and state-of-the art technologies. This approach contributed to validate multisite optimized protocols, allowing the robust implementation of SD, TMS, and hypoxia technologies for further European research.

Such validation, in particular in the case of SD, where parallel studies have been conducted in humans and animals, will be useful to increase the translational value of using this cognitive challenging paradigm for further pharmacological research. The SD challenge had a relevant merit to induce not only a transient deterioration of the performance to several episodic memory tasks (short term and working memory demands), but also a derangement of brain functions as revealed by practically all biomarkers used in WP1 such as resting state EEG rhythms, auditory oddball ERPs, and task-related fMRI (resting state fMRI showed some derangement with a statistical threshold at $p<0.05$ uncorrected for multiple comparisons, thus requiring a cross-validation study in a larger population of healthy young volunteers). Finally, the SD effects on the biomarkers were corroborated from the fact that a single dose of a psychoactive drug (Modafinil), contrasting the intrusion of sleep after the SD, recovered those cognitive indexes and biomarkers at least partially. That derangement was of particular interest as those memory tasks and biomarkers were able to characterize aMCI

patients with probable prodromal AD in WP5 (see the next section and Galluzzi et al., 2016 J Intern Med 2016; Mar 4. doi: 10.1111/joim.12482). Taking together, the results of the WP1-SD and WP5 led support to the original hypothesis of a back-translation of the cognitive tests and biomarkers sensitive to the SD challenge from aMCI patients with probable prodromal AD to healthy young volunteers. Of note, the SD challenge in the healthy young subjects of WP1 was not substantially recovered by the chronic administration (15 days) of Donepezil and Memantine. Indeed, no effects were observed in the rsEEG/ERP markers and those found in the fMRI markers did not survive to conservative statistical procedures. It can be speculated that one night of SD challenge and the cognitive tasks used were not able to reproduce the derangement of the vigilance and long-term memory brain systems induced by years of AD processes in the human brain. To overcome this limitation, a refinement of the SD challenge may include the recording of EEG and fMRI during a new memory task that seems to be very specifically altered in the AD such as semantic memory as revealed by proactive interference. After SD, a cued recall paradigm could test both proactive and retroactive interference effects while controlling for global memory impairment (i.e., Loewenstein-Acevedo Scales of Semantic Interference and Learning [LASSI-L] procedure; Crocco E, Curiel RE, Acevedo A, Czaja SJ, Loewenstein DA. Am J Geriatr Psychiatry. 2014 Sep;22(9):889-97). Therefore, the SD challenge may require a final research step making Donepezil and Memantine effective in the partial recovery of the cognitive indexes and biomarkers in healthy young volunteers.

In the case of TMS cognitive challenge model, the results confirmed the feasibility of adapting a protocol of the literature and provided positive results in two independent centres. These results represent the first-ever evidence of a multi-centric reproducibility of cognitive effects using this technology. TMS, due to its high spatial and temporal resolution to change brain activity has been previously used to investigate pharmacological manipulations in normal and pathological conditions (i.e. Ziemann et al. Clin Neurophysiol 2015;126:1847-1868). However, so far, outcome measures obtained with TMS have been restricted to those obtained stimulating the sensorimotor system, which may have limited interest in the investigation of neurodegenerative conditions such as AD. Further, the literature so far is dominated by small studies each using different designs and standardization studies. As a step forward, multicentric reports were urgently required to facilitate comparison and establish harmonized procedures increasing the validity of this model (Floel, A. Neuroimage 2014;85:934-947). In the PharmaCog project, we have built and validated a (two centre) multi-centric robust platform conducted on a large sample of participants to transiently impair human episodic memory and related brain networks. This harmonized platform may be useful for future industry/academia/SME cooperative strategies towards research to accelerate AD drug discovery. In this regard, one of the principal advantages of using TMS combined with brain imaging for future drug development might be in studies where evidence of drug effects are not only expected at a behavioural level but particularly in biomarker measures reflective of the integrity of key brain networks for the disease. For example, using TMS over the accessible region of the parietal lobe is possible to induce specific distal brain activity changes in the posteriomedial cortex (i.e. precuneus, posterior cingulate; Eldaief et al. PNAS 2011; 108:2122934), a region critical for AD pathology where changes occur before clinical manifestations. We have even further shown that using this methodology, key brain neurochemical concentrations such as Glutamate and GABA, the main neurotransmitters of the canonical circuit and involved in brain plasticity processes, can be modulated using TMS (Vidal-Piñeiro et al. Brain Stimulation 2015;8:937-44). Hence, TMS may be used

as a method not only to induce transient cognitive impairment but also to interrogate how biological measures reflecting key cerebral processes change due to a given pharmacological intervention.

As regards the results obtained, we have demonstrated that key cognitive functions for AD can be transiently disrupted using both SD and TMS challenges with new sensitive endpoints also validated throughout the course of the project in other human/animal studies. These endpoints also include new scientific consensus of convergent evidence with animal findings (i.e. WP2) identifying endpoints directly comparable between species, such as observations that both the PicInOut task in humans and the analogous NOR task in Octodons and rats, are sensitive to SD and pharmacological manipulation using the same translational experimental designs. New scientific consensus seems to be also supported by the alignment with WP6 animal findings. In PharmaCog, we used the new touch-screen technology for cognitive testing, and in particular tests such as the PAL. Unfortunately, the cognitive tasks of the touch screen technology did not provide the same results in transgenic mouse models of AD in three different PharmaCog preclinical Units, thus warranting a further refinement of the animal training and management procedures as well as validation research. For the others cognitive markers, the harmonization of the variables is not sufficient between preclinical and clinical studies to conduct translational analyses. Along with accompanying findings on EEG and brain imaging biomarkers, cognitive findings in WP1 correspond to tests that can be be back-translated to different animal models (e.g. octodons, lemurs, and mice), helping to increase the predictive validity of WP2 challenge models, as well as having a direct correspondence with measures marking the clinical progression in 'MCI to AD' patients of WP5. For example, TMS and SD challenge models were capable of inducing some changes in EEG/ERP and fMRI biomarkers reflecting resting state functional connectivity of the brain related to quiet vigilance in healthy young subjects. The fMRI evidence emphasized the modulation of DMN nodes as an effect of the WP1 challenges. This finding is relevant as DMN is a circuit sensitive to cognitive impairment progression in AD patients with aMCI and dementia. As the DMN is a central measure of functional brain integrity in aMCI patients with probable prodromal AD, we propose the biomarkers of this brain network as an outcome in the early stages of the evaluation of disease modifying drugs in healthy subjects. Experiments conducted with TMS and SD (prior, during and after the treatment) could be used to interrogate the status of this network. Administration of developing compounds with potential predictive AD modifying activity are expected to partially restore the functional brain connectivity dysfunction induced by the cognitive challenge model.

From the first F2F Preclinical Meeting in Milan (9th November 2010), the need for interaction with clinical researchers of WP1 had been expressed and particularly by the pre-clinical teams (Brunoy (CNRS), Murcia (Univ.), Verona (Univ.)), working on SD models in animals. During the year preceding the start of clinical trials, there were many consultations between the pre-clinical and clinical teams to harmonize the procedures in the WP1 and WP2.

During the First General Assembly, in Marseille, on 17th of January 2012, a round table was dedicated to the definition and harmonization of provocation challenge protocols such as SD in lemurs, mice and octodons compared to healthy volunteers. Important issues were discussed as to the importance of adopting the same terminology, what cognitive assessments are comparable, how to define a comparable SD design in different species. All the academics and private partners were present and for all of us, PharmaCog offered for the first time the opportunity to have a concrete scientific discussion on this topic between pre-clinical and clinical scientists. Thereafter, a SD design

common to lemurs, mice, octodons and healthy volunteers was developed and offered the possibility to have a translational analysis of biomarkers.

Through the PharmaCog project and WP1, we were able to build a network of expertise of harmonized procedures (i.e. devices, neuropsychological tests, data management), of new tools and technologies, identification of new biomarkers, archiving architecture of the Xnat server. We also realized the opportunity through harmonization of procedures to train different partners (medical doctors, neuropsychologists, researchers, post-doctoral fellow and PhD students) across sites to new methods and technologies (EEG, fMRI, TMS and Hypoxia devices). Altogether, these developed platforms should represent a highly attractive network for the biopharmaceutical industry, either EFPIA members of PharmaCog or other pharmaceutical companies, and additionally should continue to provide resources to develop and improve further public/private partnerships. Harmonization of clinical research is a real improvement in the capacity to work more efficiently with more likelihood of demonstrating efficacy in the selection of the patients or volunteers. Furthermore, it has enabled the capacity to obtain data corresponding to the same criteria and allowing the generation of important databases and data banks (see below the E-ADNI). These qualities are essential to test new drugs with a high degree in confidence for their therapeutic potential.

The dissemination activities of WP1 and WP2 were carried out with the systematic presentation of the research results at the most important international conference on AD, organized by Alzheimer's Association (Alzheimer's Association International Conference, AAIC), in the 2013, 2014, 2015, and 2016. Furthermore, WP1 and WP2 members published several articles in the framework of the PharmaCog dissemination activities (see the list of these activities in this report).

WP2

WP2 work led to the establishment of new standards and new scientific consensus in how to perform relevant and robust sleep-deprivation and hypoxia experiments. The lack of a consensus for these models was first realized through publication of a series of reviews (Colavito et al. 2013 ; Deguil et al. 2013 ; Babiloni et al. 2013 ; Tarragon et al. 2013). After performing SD experiments in Lemurs and Octodons, data has now been published supporting that these models may present a translational model for future testing of AD drugs (Tarragon et al. 2014 ; Rahman et al. 2013). Although it is unlikely that industry will use these models internally due to the practicality of using these species both in terms of drug discovery (eg. toxicology studies are not routinely performed for these species) and time to age such animal models for optimal experiments, the consistency of effects across these species and the phylogenetic proximity between lemurs and humans support the continuation of such further basic research experimental investigations.
Discussion between EFPIA and academic partners at our most recent SC meeting concluded that the SD rat model is too variable to be routinely used in drug discovery. Half the rats that have not been sleep deprived cannot distinguish between novel and familiar object. It is important for both the academic and industrial communities that data supporting this conclusion is published and that there is a clear recommendation on the use of SD models supporting AD drug discovery. In addition, this negative data will be included in a supporting report. A review of the models has been presented to the FENS Congress 2014 in Milan (see key dissemination activities) and a written review will be generated for publication.

One of the most significant data generated within WP2 is the EEG data. For example, a novel finding that EEG data in lemurs closely resembles that of humans, i.e. alpha is increased in passive state and decreased in active state, has been achieved. These findings have been confirmed also with the application of Sleep Deprivation (SD) protocol, indeed the higher alpha activity in the after SD condition could be related to the higher behavioural awake passive state of lemurs. Even though industry may not be able to use an EEG read-out in lemurs to assess effect of compounds, the close resemblance of EEG data between lemurs and humans suggests it would be pertinent to first establish the translation of EEG read-out from lemur to rodent and then use rodent as the decision making assay before entering the clinic.
Although difficult to implement in the drug discovery process, industry representatives agree that the experimental models developed in WP2 are of major interest for fundamental research and the data generated should be disseminated, while clearly discussing the pertinence of the different models investigated. The WP2 work allows for clear statements of the use of the models. Investigated.

WP3

Through analysis of the study 1 of WP3, in regards to results of WP1 and WP5, we have demonstrated the reliability (cross-validation of the cognitive indexes and EEG biomarkers in the WP1-pre-SD and WP3) and sensitivity of the PharmaCog biomarker matrix to specific drugs used in AD. This biomarker matrix battery should be now used in the development of new drugs: (i) to contribute to stratification of aMCI patients in those with probable prodromal AD vs. those with other dementing disorders; (ii) to assess the spontaneous AD evolution and placebo effect; (iii) to play the role of end-points of the pharmacological intervention in future clinical studies. We have also demonstrated that the step in healthy young volunteers can be further exploited during the early phase of development in the perspective of Go/No Go decision. Moreover, we have already applied to a call with a start-up working on a project using the PharmaCog platform of WP3 to assess a new original molecule suggesting an added-value for industrial partners.

WP4

From a translational point of view, donepezil produced effects on EEG activity recorded in rodents in WP4 but further research is required to appreciate the extent to which these effects reflect relevant neurophysiological mechanisms underlying the results observed in the present healthy volunteers.

WP5: the European ADNI

The PharmaCog WP5/E-ADNI study is the first pan-European longitudinal investigation in a large cohort of aMCI patients using a unique, comprehensive battery of fluid (CSF, blood), clinical, neuropsychological (paper-pencil and CANTAB), structural and functional MRI, and EEG markers. Noteworthy, EEG techniques were not used in the USA, Australian, and Japanese ADNI. New potential EEG biomarkers reflecting prodromal AD have been identified as a proxy of abnormal neurophysiological mechanisms of cortical neural synchronization for the regulation of brain arousal in quiet wakefulness. Furthermore, the PharmaCog/E-ADNI study has gone beyond ADNI and other international studies with more detailed cognitive (including computerized) tests as well as procedures for the extraction of neuroinflammatory markers from the blood.

The mentioned battery of biomarkers was associated with the development and validation of original standard operating procedures for the collection of blood and CSF samples. MRI (structural and diffusion MRI, rs-fMRI, etc.), neuropsychological, and EEG/ERP data acquisition procedures have been standardized and validated in several European clinical centres as well. The permanent staff and young Ph.D. and post-doc fellows hired at these centres have been trained in the use of these methodological standards. This experience has generated the basis of a permanent, transnational, pre-competitive, public-private network focused on the innovation of biomarkers and clinical trial methodologies for drug discovery in AD.

The PharmaCog/E-ADNI study provided stringent evidence supporting the sensitivity and specificity of a biomarker matrix that may have a marked impact on the design of clinical trials of disease modifiers in AD (using aMCI subjects negative to CSF markers of prodromal AD as a control group) in the following areas:

i) Cohort enrichment, defining patient inclusion criteria by baseline CSF, structural, diffusion, functional, perfusion, neurophysiological (i.e. EEG/ERP), peripheral, and inflammatory biomarkers, with a reduction of the sample size required;

ii) Surrogate outcomes of AD progression, identifying new potential biomarkers of disease progression (i.e. diffusion MRI and EEG/ERP biomarkers) besides those commonly used from morphological MRI (i.e. hippocampal volume). The MATRIX, developed with all these biomarkers combined, is expected to speed up the evaluation of the beneficial effects of new disease modifying drugs for prodromal AD, by facilitating the Go/No Go decision-making in the drug discovery process.

Publications and collaborative research are considered valuable channels for knowledge transfer and commercialisation of research results (see OECD report “Commercialising Public Research. New Trends and Strategies”, DOI:10.1787/9789264193321-en). In this line, the members of the PharmaCog WP5 have developed a careful dissemination plan to maximize the impact of the present findings and procedures. Specifically, the results of the PharmaCog project were disseminated in the most important AD-related scientific conferences (AAIC, Alzheimer's Association International Conference; AD/PD, Conference on Alzheimer's and Parkinson's Diseases and Related Neurological Disorders; CTAD Clinical Trials on Alzheimer's Disease; OHBM, Organization for Human Brain Mapping). Furthermore, 14 articles have already been published in peer-reviewed journals. Thanks to the scientific credibility gained through this big dissemination effort, industry has been persuaded that the procedures of the PharmaCog/E-ADNI study are feasible and useful for the development of clinical studies on prodromal AD. In fact, a follow-up study has recently started, aimed at investigating the incremental diagnostic value of Florbetaben PET Imaging compared with other core biomarkers (Ab42, total and p-tau) for AD in MCI patients. It is a IV phase clinical trial carried out in 10 European centres which have currently active programmes involving the collection of clinical and imaging data, and CSF samples following the ADNI standards. In that study, 8 out of 10 centres are members of the PharmaCog Consortium. Furthermore, the PharmaCog consortium validated biological blood circulating markers that could facilitate future therapeutic clinical trials.

Moreover, synergies have been created with other existing initiatives in the AD field. For example, a Material Transfer Agreement was signed with the European Medical Framework (EMIF) Consortium

(another IMI project) in order to provide them with the samples collected in WP5. Moreover, agreements were signed to use data from WP5 in the Global Alzheimer's Association Interactive Network ("GAAIN"), an initiative funded by the Alzheimer's Association, the world's leading voluntary health organization in Alzheimer's care. The GAAIN project aims at creating a platform for searching and integrating data from AD and other dementia research studies.

WP6

The results of WP5 are an important reference for the preclinical research aimed at translating the most important results from clinical to preclinical research (e.g. the back-translation of biomarkers and cognitive indexes) and *viceversa*. PharmaCog WP6 studies aimed at identifying and validating EEG, neuroimaging, biological, and cognitive behavioural markers sensitive to abnormal brain functions due to accumulation of Aβ-42 and/or Tau peptides in the brain of transgenic mouse models of AD such as those with mutation of APP (i.e. PDAPP), APP+PS1 (i.e. TASTPM), and APP+PS1+Tau (i.e. triple) genes. Most of these markers were adapted from those expected to be sensitive to prodromal AD in the aMCI patients of WP5. The WP6 studies compared these markers between wild type C57 (Wild Type, WT) mice and the transgenic mouse models by cross-sectional and longitudinal designs strictly in line with the WP3 and WP5 experiments. In the case of EEG, 2-DG (FDG-PET in WP3), and assessment of cognitive functions by touchscreen, the studies were performed based on a preliminary harmonization of the data collection in two or more research units (the MRI in mice was performed only in Milan unit)

The main EEG results of WP6 can be summarized as follows: 1) markers of ongoing cortical EEG rhythms in wakefulness showed difference in power (density) across physiological aging in WT mice; 2) these EEG markers also showed differences in power between WT and transgenic mice such as PDAPP and TASTPM; 3) in TASTPM mice, the cortical EEG markers did not normalize after a 4 week treatment with a well-known BACE inhibitor given daily to reduce the accumulation of Aβ-42 in the brain; 4) Compared with WT mice, TASTPM mice were characterized by abnormalities in amplitude and latency of cortical auditory evoked potentials (AEPs) related to target stimuli; 5) Both TASTPM and PDAPP mice were characterized by abnormal cortical EEG power in the sleep stages.

The main neuroimaging results can be summarized as follows: 1) the regional cerebral 2-DG uptake as a measurement of glucose metabolism was significantly reduced in the TASTPM mice compared with WT mice. Furthermore, there was a negative correlation between the Aβ-42 plaque deposition and the 2-DG uptake; 2) in vivo µPET imaging with [18F]-AV45 and [18F]-FDG detected abnormal deposition of fibrillar Aβ-42 and decreased glucose utilization in aged TASTPM mice; 3) In these mice, there was a trend towards increased glucose metabolism in the hippocampus and decreased metabolism in cortical regions compared with age-matched WT controls; 4) Hippocampal atrophy was found in both APP/PSEN1/Tau and TASTPM mice compared with WT and they both displayed an age-related entorhinal cortex thinning and robust striatal atrophy, the latter associated with a significant loss of synaptophysin; 5) In TASTPM mice, the MRI analysis revealed a significant volume reduction of hippocampus, striatum, and a thinning of cerebral cortex in both aged APP/PS2/Tau and TASTPM mice (4 week treatment with the well-known BACE inhibitor do not mitigate that brain atrophy).

The main biological results can be summarized as follows: 1) In male TASTPM mice, Aβ-42 plaque deposition significantly increased along 3, 12, and 18 months of age; 2) TASTPM mice were characterized by a strong Aβ-42 deposition in the cortex, thalamus, and hippocampus, which was associated with a high proliferation of neuroinflammatory cells; 3) Synaptic loss was observed in TASTPM mice and, to a lesser extent, in APP/PS2/Tau mice; 4) Lower glutamate levels in hippocampus and reduction of myo-inositol in the striatum were reported in both TASTPM and APP/PS2/Tau mice after administration of Aβ-42 lowering drugs; 5) There was a reduction of oxinergic neurons in PDAPP, TASTPM, and APP/PS2/Tau mice, with a marked activation of glial cells in the hypothalamus and deposition of Aβ plaques in the brain; 6) 3D6 treatment for Aβ-42 lowering affected oxinergic neurons, which appeared to be vulnerable in TASTPM mice; 7) PDAPP mice were characterized by alteration in intrinsic neural excitability.

The main cognitive behavioural results can be summarized as follows: 1) Exploration behaviour significantly differed in TASTPM compared to WT mice; 2) Touchscreen visual discrimination (VD)-task performance showed no difference in TASTPM and triple-tg mice compared with aged-matched control mice. The same was true in Tg2576 mice; 3) Touchscreen paired associative learning (PAL)-task performance was lower in TASTPM mice compared with aged-matched control mice.

Overall, these preclinical studies of WP6 unveiled markers potentially useful for the stratification of the transgenic mice based on demonstrated metabolic, neurophysiological, neuroanatomical, and behavioural (i.e. learning) abnormal changes in the cerebral cortex and/or other relevant subcortical regions (striatum, hippocampus). Furthermore, most of these markers were able to reveal abnormal cortical changes across time in these mouse strains modelling AD, which mimicked the evolution of prodromal AD pathology in aMCI patients of WP5. For this reason, they may be considered as the back-translation counterpart of the PharmaCog biomarkers battery validated in the WP1, WP3, and WP5 of PharmaCog. As such, the present preclinical biomarker battery should be used for the Go/No Go decision making process in the early phase of AD drug discovery.

1.7. Lessons learned and further opportunities for research

In the last decade, a great amount of literature on SD was published which mainly focused on the consequences of sleep loss for public health. However, the SD paradigm may also be regarded as a cognitive challenge model (Cassé-Perrot et al, 2016). To our knowledge, TMS is more used in academia as a therapeutic method for patients suffering from AD, Parkinson's disease, and depression than as a Cognitive Challenge Model in Healthy Volunteers (HVTs) for drug discovery. Existing literature on hypoxia mostly focuses on the consequences of hypoxia on metabolism and human brain as well as high hypoxic conditions and related pathogenic events (Lanteaume et al, 2016). WP1 has been defined in this way in order *1)* to implement and validate the type of protocols, methods and tools that would be useful to induce transient/reversible cognitive impairments in HVTs, and *2)* to use them by testing new drug-candidates for pharmaceutical companies, in order to speed up the R&D drug development in a framework of an ambitious collaboration of Public/Private partnership. Furthermore, one of the examples of the public-academic collaboration as an added value is the great success of post-doc funded programs by the industry to Academic centres.

Examples include the participation of Dr. Véronique Bragulat (Barcelona) and Dr. Claudio Del Percio (Foggia/Rome) supported by Roche and participation of Dr. Laura Lanteaume (Marseille), supported by Merck Serono). Dr. Bragulat, trained in TMS administration, and with her previous expertise in fMRI, is now a GSK employee and hence the know-how generated during this period in an academic centre could be potential exploited synergistically both in Academia and Industry for future studies. Dr. Claudio Del Percio established a contract agreement in 2016 with one of the Partners of WP5, P33 IRCCS SDN of Naples (Italy), for the exploitation of PharmaCog EEG biomarkers in the clinical management of aMCI and AD patients. Dr Lanteaume acquired expertise as a Project Manager as well as in TMS, Sleep deprivation and hypoxia techniques that will also help facilitate and promote new collaboration with the Pharmaceutical companies. During the lifetime of the PharmaCog project, we realized a lack of anticipation regarding the data management of clinical trials across sites, involving delayed deliverables. In the future, it will be important to clearly determine and harmonize data management at the beginning of the studies: the type and labelling of outcomes measures, the data format. This will ease the transfer of data and reduce the introduction of errors due to reformatting and preparing data. To this end, wherever possible, simple flat column-wise data formats (Figure 22) should be used for storing and communicating data as they are both easier to understand and to read into statistical programs. This approach would be particularly relevant for multicentric studies and to perform transversal comparison across work-packages for the same measures and biomarkers.

	A	B	C	D	E	F
1	day	time	rat	treatment	value	percentage
2	1	4	23	A	10.534	0.131675
3	1	4	18	A	13.421	0.167763
4	1	4	16	A	43.111	0.538888
5	1	6	23	A	50.321	0.629013
6	1	6	15	A	54.327	0.679088
7	1	6	14	A	12.332	0.15415
8	1	4	15	B	43.212	0.54015
9	1	4	12	B	12.422	0.155275
10	1	4	9	B	69.235	0.865438

Figure 22. An example of the electronic sheet defined and used in the PharmaCog consortium to share relevant variables of the experiments in preclinical and clinical WPs.

WP1

In the case of WP1, the next advances for the SD challenge would be to conduct new studies selecting only the endpoints that have been sensitive to the challenge when testing new molecules. As mentioned in a previous section, the original SD platform should be integrated and validated in healthy young volunteers with an additional semantic memory task hopefully able to induce EEG/ERP and fMRI activities partially recovered from Donepezil and Memantine as market benchmarks for the new molecules treating AD. In the case of TMS the obtained results demonstrate that it is a robust challenge model to be used in parallel designs for drug discovery and, hence, it should be tested against pharmacological manipulation in future studies. In general, we can recommend to move the use of cognitive challenge models to the 'pre-clinical AD field', i.e. building new experiments incorporating not only those endpoints that have been proven to be sensitive in the present WP1 experiments but particularly those that are also characteristic of cognitive changes in very incipient stages of the disease, such as learning/memory task and fMRI connectivity markers. As mentioned in

a previous section, the original TMS platform should be integrated and validated in HVTs with specific BDNF genotyping sensitive to the effects of TMS interference and high cognitive performance in "control TMS" condition (Vertex). We expect that at least in this specific population, the effects of TMS challenge on biomarkers should be recovered from Donepezil and Memantine as market benchmarks for the new molecules in development for treating AD. Furthermore, we expect that the use of HVTs with specific BDNF genotyping mitigate the actual issue of possible meta-learning effects influencing the reproducibility of the effects of TMS on cognitive performance in a cross-over design with Drug and Placebo.

WP3

Even though the pharmacodynamics studies in HVTs are not new, WP3 has proven that we need to enrich the assessment of new drugs with biomarkers to refine the design of clinical trials since biomarkers are able to detect more sensitively the pharmacodynamics effects of marketed and likely new compounds. We have created a network between Lille, Marseille, and Toulouse that is able to take on new studies in a harmonized manner. We are able to implement our battery in new French or European centres to develop this new paradigm of drug development. Moreover, this approach has a translational dimension, as for example the position of Dr Julie Deguil. She has been involved in both preclinical and clinical studies and has obtained a position of associate professor in Lille University. The other main lesson is the possibility to organize an informatics tool to share all parameters, bringing a unique pharmacological database that could be incremented and re-used by future studies.

WP5

Population cohorts are invaluable resources. However, the heterogeneity of these cohorts hampers the optimal exploitation of these resources. The definition and implementation of standard operating procedures is essential for maximizing the exploitation of cohorts and for facilitating hypothesis-driven research and data sharing and reuse. The standardization procedures developed in PharmaCog WP5 can bring several benefits to the Community of AD research as it has successfully:

- Enhanced the interoperability of the longitudinal clinical trials in AD by integrating and extending procedures and principles of the ADNI and other major international field studies in a network of more than 10 European clinical units ready to take on new clinical studies in preclinical, prodromal, and dementia stages of AD using the PharmaCog standard operating procedures in a harmonized manner
- Demonstrated that neuropsychological measures (including computerized cognitive testing), structural and functional MRI, and EEG biomarkers can discriminate both disease status and type and rate of progression in aMCI patients with prodromal AD as contrasted to aMCI patients possibly due to other dementing disorders, towards the definition of further sub-classes of AD based on different profiles of abnormal cognitive functions (memory vs. executive vs. language vs. visuospatial) and peculiar neuroanatomical (MRI, rs-fMRI) and neurophysiological (EEG) underpinnings. This definition of AD sub-populations can be an important boost for drug discovery, based on the assumption that different variants of the neurodegenerative processes might be sensitive to different kinds of pharmacological

therapies. Post-hoc analysis of clinical studies investigating the effect of amyloid lowering drugs showed that sub-populations progress more rapidly and may indicate a more positive outcome in response to drug treatment. By enabling a more accurate definition of sub-populations, the signal to noise ratio of the drug effect should be increased leading to a reduction in sample size thus reducing costs and allowing more targets to be investigated.

- Demonstrated that the ADflag® panel, could be used more extensively as a stratification marker to segregate pre-dementia stages of AD and potentially to anticipate disease progression. This marker set, along with the other markers used in PharmaCog, thus maximize the phenotyping of aMCI cohorts in therapeutic clinical trial.
- Enabled the creation of unified databases and the foundation of a network of specialised clinical units that are established for continued collaborations.
- Guaranteed solidity and robustness to research results.

On a more operational level, WP5 activities have demonstrated the importance of having a clear strategy for managing and mitigating cost or schedule issues. Indeed, patient recruitment started later than originally planned mainly due to the delay in ethics committee approvals. Various corrective actions were put in place to recover the delay and boost patient recruitment: 5 additional clinical centres with demonstrated enrolling capacity in clinical trials (P33 SDN, P34 VUA, P35 UCSC, P36 GAARD, P37 UNIPG) joined the consortium as new partners; a monthly motivational newsletter was created; teleconferences with personnel in charge of patient recruitment were made weekly. The most effective corrective action was to make the recruitment phase competitive, by flexibly allocating financial resources according to performance across recruiting centres.

PharmaCog WP5 has paved the way for future research on physiological or biochemical markers sensitive to disease modifying treatments. WP5 successfully characterised biomarkers (neuroimaging and EEG) that were able to identify aMCI patients with the highest probability to convert to AD and were more sensitive to disease progression than the ADAS-Cog rating, one of the most frequently used scales in AD clinical trials to measure cognitive decline.

From a back-translation point of view, the WP5 matrix of multi-modal markers represents an ideal reference for the neurobiological, neuroanatomical, neurophysiological, and behavioral characterization of different mouse models of AD (“stratification indexes”) and the detection of the progression of their brain pathology across time as a function of pharmacological manipulations (“monitoring indexes”), contributing to the enhancement of the efficiency of the early stages of drug discovery.

Finally, PharmaCog WP5 analyses highlighted that the quantification of plasma Aβ isoforms is still confounded by technical problems regarding sample storage, precluding the possibility to clarify their relationship with CSF biomarkers and with AD pathology.

WP7

As outlined in the initial proposal, the implementation of a so-called horizontal research WP was aimed at ensuring optimisation of study protocol design and harmonising data analysis procedures. It was assumed that such objectives would be enablers of subsequent actions in support of the

development of mathematical models to describing the relationships between drug exposure and pharmacodynamic effects.

Among the key deliverables, the use of PKPD concepts envisaged the possibility of establishing infrastructure to support optimal data handling and analysis procedures. Most importantly, it was expected that such concepts would provide efficient and effective collection of blood samples across sites for analysis of drug exposure, assay and data sharing procedures.

The collaboration with partners involved in the pre-clinical and clinical WPs has been very intense. As mentioned above, the review of the literature urged an adaptation of some original working hypotheses and experimental designs in several WPs. For example, the review of the literature suggested the need to test the repetition effects of cognitive tests on fMRI and behavioural indexes in multiple WP1 sessions without the potential confounding effect of a pharmacological intervention. Furthermore, in line with the focus on the behavioural measures as primary endpoints in human WPs, the collection of serial pharmacokinetic data during cognitive testing was considered as a potential confounding variable. As another option, the inclusion of satellite groups or alternative sampling protocols was not viable based on the very tight financial plan of the project and the need to limit the delay in the development of the scientific workplan. After a discussion within the PharmaCog Consortium, we decided not to use anti-AD drugs as an independent variable in the WP5 clinical experiments with mirror effects on the preclinical experiments. Furthermore, data could not be obtained in a longitudinal manner based on serial or sparse sampling procedures as required for an effective PK and PKPD modelling. We removed the risky and too ambitious hypotheses and experiments focused on the study of treatment effects for different doses and at different times from the administration. Such optimized protocols no longer required a more quantitative approach for the characterisation of the exposure-response relationship or any assessment of the predictive performance of biomarkers. As a consequence of this optimization of the general scientific work plan, we reformulated the objectives of the WP7.

From a drug development perspective, the main lesson learned is that the important principles of PK/PD analysis should be taken into account in the design of an ambitious project, we additional resources allocated for the solutions allowing this analysis of the effects of pharmacological interventions on biomarkers and disease progression (dose-response, prediction of therapy response etc.), The future experimental designs should not overlooked these principles when dealing with challenge models or with protocols aimed at diagnostic evaluation (i.e., disease symptoms and signs). Furthermore, screening of candidate molecules and dose projection for subsequent phases of development require longitudinal pharmacokinetic, pharmacodynamics, and biomarker data.