SEVENTH FRAMEWORK PROGRAMME Health

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MultISyn

Multimodal Imaging of rare Synucleinopathies

Instrument: Collaborative Project

Final report

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MultISyn: Multimodal Imaging of rare Synucleinopathies

PUBLISHABLE EXECUTIVE SUMMARY FOR PERIODIC REPORT

1. Summary description of project context and main objectives

Neurodegenerative diseases, such as Alzheimer's disease (AD) or Parkinson's disease (PD) are progressive, devastating and incurable neurologic conditions. In the majority of cases, they are thought to be caused by a complex interplay of genetic and environmental factors. The aggregation of disease-specific abnormally folded proteins, such as the A-beta protein in AD or α-synuclein (αSYN) in PD, and probably their spreading from cell to cell throughout the brain, appears to be the central driver of pathogenesis. The removal of these aggregates holds considerable promise as a therapeutic strategy. However, the relationship between protein misfolding and aggregation on the one hand, and neuronal dysfunction and cell death, on the other hand, is far from being well understood, particularly in the common sporadic forms of these disorders. The consequences of this lack of understanding are dramatically illustrated by the experience from failed vaccination studies in AD that resulted in a reduction of aggregate load but did not translate into clinical improvement. As a consequence, rare genetically defined forms of neurodegenerative disorders are now generally considered to offer substantial advantages for drug development such as a clear cause-effect-relationship and the possibility of diagnosis at an early stage of the disease process when it is still possible to modify its course.

Non-invasive imaging methods such as PET and MRI can be valuable tools to aid diagnosis of neurodegenerative diseases, provide input to differential diagnosis and follow disease progression or therapeutic effects of disease modifying treatments. However, and in contrast to the situation in AD, for many important neurodegenerative diseases including the synucleinopathies and TDP-43 proteopathies, specific PET tracers for the underlying cellular pathology, which would allow tracking the disease burden are still lacking. Moreover, there has been no systematic work performed to link neuronal function as detected by fMRI with molecular information as provided by PET-imaging of aggregates in these diseases. Novel PET/MR imaging systems which could fill this knowledge gap for rodents and humans are available but require the establishment and validation of sensitive and aggregation specific tracers as well as of disease-specific imaging protocols and data analysis tools. Furthermore, the research community faces a major challenge in generating evidence to support that animal models have predictive validity in development of disease modifying therapies for humans. This is especially true for neurodegenerative diseases, including those that involve abnormal handling and deposition of conformationally altered toxic species of proteins. Again, rare genetic forms of these proteopathies, caused for example by mutations in genes for the aggregating proteins Amyloid precursor protein (APP), α-Synuclein (αSYN) or microtubule-associated protein Tau (MAPT), are generally considered to be valuable model diseases for the common neurodegenerative disorders of Alzheimer's disease, Parkinson's disease or Frontotemporal dementia, respectively, and animal models overexpressing the mutated genes have been generated. Among others, the models developed by Partners 2 and 3 of this consortium have now been well characterized and replicate key components of the disease in humans.

From these considerations it becomes evident, that there is an urgent need

(i) to develop novel and to re-evaluate existing PET-tracers that allow to track aggregated proteins and to develop multimodal imaging protocols to link protein aggregation pathology to functional read-outs on the systems level in animal models and in the corresponding human patient populations with rare, but well characterized model diseases, such as genetic proteopathies,

(ii) to validate these tracers in suitable animal models and to test their value as markers to monitor progression in disease-modifying treatment studies, and finally

(iii) to use these tracers to define the distribution and evolution of protein-aggregation pathology in human patients and to provide proof-of-concept that multimodal imaging protocols are suitable for monitoring disease-modifying individualized treatments.

In order to overcome the critical road-blocks described above, we have assembled an interdisciplinary consortium, consisting of world-leading experts in structural biology and ligand development, multimodal neuroimaging, animal models and clinical trials. With this consortium, we are in a unique position to develop a novel imaging system for combined simultaneous molecular and functional imaging (PET-MRI/fMRI) for two rare subtypes of parkinsonism caused by excessive accumulation of misfolded alpha-synuclein (αSYN): multiple system atrophy (MSA) and parkinsonism caused by mutations in the alpha-synuclein gene (αSYN-mut-PD), which will serve as proof-of-principle models for the more common and heterogeneous NDD like AD and PD.

These rare synucleinopathies are uniquely suited for this approach, as they are (i) characterized by a high αSYN load and therefore offer a favorable signal-to-noise ratio, (ii) have a rapid progression and therefore a relatively easily defined endpoint for therapeutic interventions, and (iii) can be modeled in many aspects in rodents.
With the PET/MR technology and novel imaging biomarker development we will pioneer the monitoring of protein aggregation as a surrogate marker for therapeutic effects in the framework of individualized causative treatment.
The central aspects of the work-flow including ligand design, software development and drug trials will be driven by three highly specialized SMEs, while imaging workflow, translation to animal models and clinical use will be implemented by top academic centers.
In this consortium we have the ability to achieve ground-breaking progress and aim to

1. Establish a multimodal imaging workflow based on specific PET tracers and embracing structural and functional MRI methods to yield a tool that sensitively and specifically detects αSYN pathology and associated changes
2. Test this multimodal, molecular neuroimaging methodology in animal models with regard to its potential for diagnosis, monitoring the natural disease course and response to therapy, as well as guide and optimize therapeutic interventions.
3. Translate the workflow including the therapeutic modality (i.e. immunotherapy with PD01A, NCT01568099) to the clinical setting.

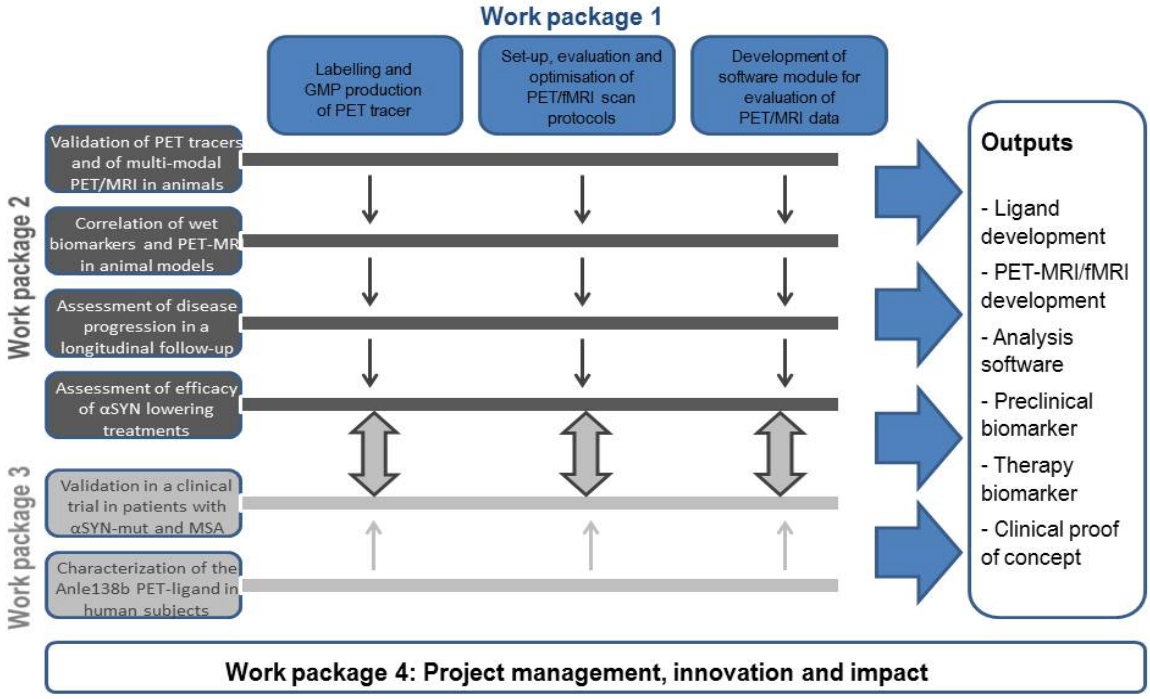


Figure 1: Scheme showing the work flow and interaction within MultiSyn

2. Description of the work performed since begin of the project and the main results achieved

Broken down to aims, the work performed and results achieved can be summarised as follows:

1. Establish a multimodal imaging workflow based on specific PET tracers and embracing structural and functional MRI methods to yield a tool that sensitively and specifically detects αSYN pathology
   <u>Development of labelling strategies for novel PET tracer</u>
   - Synthesis of novel compound library
   - Approach for three 11C-labelled and one 18F-labelled PET tracer candidates developed
   - Compounds anle138b as well as four derivatives were tritiated and used for autoradiography and other binding studies.

   <u>Characterization of binding affinity and specificity of PET compounds in vitro</u>

- Preparation and provision of fibrils from recombinant αSyn and hTau 46.
- Successful quantification of binding affinities for all tritiated compounds
- Demonstration of specific binding of 11C-PIB to αSYN aggregates and competition experiments revealed highest blocking for PIB, anle138b and two candidate compounds.
- Filter binding experiments revealed very high binding affinity for one promising tritiated candidate compound to asyn fibrils and a mixture of fibrils, oligomers and monomers
- Competition binding experiments using one promising tritiated candidate compound revealed a further compound as another interesting candidate with Ki values < 0.1 nM
- Development of a software tool for PET to histology coregistration for AR

PET tracer evaluation
- Set up and evaluation of a blood sampling device to accurately measure tracer and metabolite activity in plasma
- Demonstration of high binding of an 11C-labelled candidate compound to αSYN fibrils in comparison to monomers and oligomers.
- Using human brain tissue, autoradiography studies revealed binding of all tritiated reference compounds to Abeta in AD brain
- One tritiated candidate compoundbinds diffusely to the frontal cortex of LBD patients more than to control brain tissue.

Set-up of dedicated PET/fMRI scan protocols for 18F-FDG and 11C-raclopride in rats

Development of a dedicated PMOD software module
- Inclusion of parametric mapping into PNEURO for synergistic PET/MR analysis
- Extension of PNEURO from human to animal brains.
- Addition of a perfusion mapping plug-in for ASL MRI data and diffusion mapping and tensor calculation plug-in for DTI MRI data.
- R scripts for the statistical analysis of multiparametric outcome data in longitudinal studies.
- Extension to integrated analysis of the multi-modal image data

Further development of one promising candidate compound derived from this project in a Michael J Fox Foundation funded project.

2. Test this multimodal, molecular neuroimaging methodology in animal models with regard to its potential for diagnosis, monitoring the natural disease course and response to therapy, as well as guide and optimize therapeutic interventions.
   - Injection of one promising candidate compound into wild type mice revealed a good BBB penetration, a SUV > 1.5 and fast tracer kinetics in healthy control animals
   - Metabolite analysis of this compound revealed one metabolite, which enters the brain
   - Injection of the fluorinated candidate compound into mice revealed a small uptake into the brain and high signal in the skull, likely due to defluorination making it unsuitable for PET experiments

   - Evidence that AAV-mediated rat model suitable for testing a various PET tracer and imaging protocols
   - AAV-αSYN rat model: positive correlation between 11C-PIB binding and αSYN load as well as dopaminergic cell loss, measured with 11C-methylphenidate and postsynaptic D2 receptor expression changes, measured with 11C-raclopride.
   - AAV-αSYN rat model: Preliminary MR spectroscopy data show reductions of glutamate concentrations
   - Application of the [^{11}C]raclopride PET/BOLD fMRI protocol to the AAV-aSYN rat model of PD using a pharmacological D-amphetamine challenge revealed small differences between the healthy and aSYN overexpressing CPu (more animals needed for a statistical analysis).
   - PD01 and PD03 AFFITOPEs are immunogenic in Sprague Dawley's rats, induced antibodies are able to cross-react with the αSYN original epitope and the recombinant human αSYN protein.
   - Successfully immunization of Sprague Dawley rats against human asyn using two affitope antibodies developed by Affiris AG for use in the clinics.

- Animals with circulating Abs to human alpha-synuclein were not protected from neurodegeneration induced by overexpression of the transgenic protein using AAV viruses.
- There was however a clear reduction in detergent (Triton) insoluble, i.e., aggregated, form of a-syn in the brain suggesting that the Abs reached the target in the brain tissue. Given that the PET imaging agent we sought out to develop in this project would bind specifically the aggregated a-syn species, we think that this model would be suitable to show changes in a-syn load in the brain using non-invasive imaging tools.
- CMA induction via LAMP2A up-regulation represents an effective strategy to mitigate established ASYN pathology in BAC-hu-ASYN rats.

- A30P mouse model: An 11C-labelled candidate compound and 11C-PIB PET showed good uptake kinetics, highest binding in the brain stem and was higher in 73 weeks old tg mice compared to 20 weeks controls.
- The MSA PLP-ASYN tg mouse model demonstrates an increased ASYN burden throughout its lifespan (3- 18 mo) compared to WT, including the accumulation of relatively insoluble oligomeric and phosphorylated species, and is thus a suitable model to assess ASYN-lowering treatments.
- Characterization of the efficacy of anle138b to stop the disease progression in the PLP-α-syn mouse model of MSA
- Characterization of the efficacy of AFFITOPEs to stop the disease progression in the PLP-α-syn mouse model of MSA

- Use of PET imaging to validate a novel AAV-aSYN pig model.
- An 11C-labelled candidate compound showed increased uptake in the striatum and ventral midbrain of pig models on the side of injection of AAV-aSYN.

- Test-retest experiments using a simultaneous [^{11}C]raclopride PET/fMRI protocol showed low between scan variability of 2% (fMRI) and 8% (PET) and a within scan variability of 1-2% (fMRI) and 6-9% (PET)

3. Translate the workflow including the therapeutic modality (i.e. immunotherapy with PD01A, NCT01568099) to the clinical setting:
   - Multimodal PET/MRI protocols in humans have been established and tested in a pilot study in patients with MSA; GBA-PD and controls
   - A53T-PD cohort characterized: longitudinal clinical assessments showed prominent motor decline and deterioration of autonomic and cognitive function during the study period, providing baseline values to inform on future clinical trials.
   - MSA cohort characterized: The midbrain and putaminal volume as well as the cerebellar gray matter compartment were identified as the most significant brain regions to construct a prediction model for the diagnosis of MSA.
   - GBA cohort characterized: Natural history of PD caused by GBA-mutations (GBA-PD) focusing particularly on non-motor symptoms established, as a basis for designing clinical interventional trials and power calculations.
   - *As of April 2016 WP3 ceased to work due to the fact that a novel αSYN binding PET tracer could preclinically not be validated.*

3. Description of the expected final results and their potential impacts and use

Upon completion of WP1 we aim to have established and validated labelled tracers, PET/MR imaging workflows and data analysis tools available to utilize the full potential of temporally correlated functional, molecular and morphological in vivo data to monitor progression of pathology in αSYN aggregation disorders. These tools and workflows will be available for preclinical imaging studies in animals as well as in clinical studies. In addition, approval for clinical studies was planned to obtain.

WP2 is going to establish the experimental basis and present the proof-of-concept for simultaneous PET/MRI imaging as a powerful tool for assessing disease related changes in animal models and provide proof of concept for the use of this method to monitor disease modifying treatments for rare forms of synucleinopathies. At the completion of the work, we will not only be leading the field internationally in how such imaging biomarkers can be implemented to benefit disease staging and define therapeutic benefits in animal models of a specific class of neurodegenerative diseases but will also have established the data set required to take the next step into clinical testing.

In WP3, we planned to fully characterize the signal distribution which, based on our results in animal models, most likely reflects the distribution of the αSYN aggregates, as imaged by using specific tracers such as anle138b-PET-compounds and PIB, as it evolves over time in the brain of human patients with MSA and inherited PD. Secondly, we planned to define the relationship between αSYN deposition and functional connectivity of the brain and the function of the dopaminergic synapse, as visualized by PET/fMRI co-registration cross-sectionally and longitudinally. However, as of April 2016 WP3 ceased to work due to the fact that a novel αSYN binding PET tracer could preclinically not be validated.

4. Final summary report

Project summery work package 1

Title WP1: PET tracers, multimodal PET/MRI technology and PET techniques with simultaneously acquired MRI

1. PET tracer development

One of the central aspects of WP1 included the ^{18}F- and ^{11}C-labeling of optimized derivatives of the lead compound anle138b, which has shown specific binding properties to alpha-synuclein (αSYN) fibrils in fluorescence measurements and therapeutic effects in animal models of synucleinopathies. In the first step, partner MODAG developed the precursor synthesis for the preselected compounds and made them available to the partners in Aarhus and Tübingen for radiolabeling. In all syntheses, we obtained high radiochemical yields of 10-47% and specific activities (SA) of 15-120 GBq/µmol at the end of the tracer synthesis.

After successful radiolabeling, *in vivo* PET experiments were performed in different synucleinopathy animal models in Tübingen and Aarhus. The Thy1-A30P mouse model of PD (partner MODAG) and the PLP mouse model of Multiple System Atrophy (partner Innsbruck) were shipped to Tübingen and Aarhus and the AAV- αSYN overexpression (partner Lund) was established in rats (Tübingen) and in pigs (Aarhus) for PET tracer analysis. Each of the PET ligands entered the brain, however quantitative analysis did not show significant differences in pathological brain regions between transgenic and wild type animals or between the AAV-αSYN-injected and control striatum in αSYN overexpressing animal models. For a higher resolution and better cross validation with immunohistochemistry, *in vitro* autoradiography protocols were established in brain slices of the Thy1-A30P mouse model, the PLP mouse model, mouse models of Alzheimer´s disease (AD) and human cases of dementia with Lewy bodies (DLB), progressive supranuclear palsy (PSP) and AD using different buffer conditions, tracer concentrations and incubation times. In all experiments, unspecific binding of the PET tracer was high due to the high lipophilicity of the compounds (calculated logP = 3.9-5).

After we were not able to show specific binding *in vivo* and *in vitro* with any of the PET compounds selected in the first step, partner MODAG identified in a second step further highly active compounds with regard to inhibition of alpha-synuclein aggregation, which we decided to include in our PET tracer screening to identify compounds, which meet the requirements for PET tracer development. To test the novel compounds from MODAG, we decided to label 5 compounds with tritium (3H) and establish a binding assay based on human recombinant αSYN fibrils for a fast and more effective screening.

A saturation binding assay was established by partners EKUT and MODAG using recombinant human αSYN fibrils and K_d-values were calculated: One of the selected compounds, showed a very high affinity towards oligomeric forms of aSYN in fibril binding assays using a mixture of monomers, oligomers and fibrils (K_D < 1 nM), high affinity to pure aSYN fibrils (K_D < 2 nM), a ~400-fold lower affinity to Aß-fibrils and a ~20-fold lower affinity to tau-fibrils. All tritiated compounds were further applied to human fresh frozen tissue sections of synucleinpathy

cases and control cases in Tuebingen and Aarhus using *in vitro* autoradiography experiments. Blocking with excess of the respective non-radioactive compounds did not lead to a visible reduction of tracer binding, however quantification of specific binding revealed that in most cases at least a partial blocking effect was observed. It is however not clear, if this blocking was related to specific or non-specific binding. Unfortunately, the most obvious specific binding and blocking effects were observed in AD tissue, showing that the compounds were not solely selective for αSYN. However, a comparison to [^{3}H]PIB using *in vitro* autoradiography showed 7-to-10 fold lower binding in brain slices of AD patients and mice, pointing to a reasonable selectivity towards αSYN.
Two compounds were further selected by MODAG, Tübingen and Aarhus for C-11 and F-18 labeling, respectively. Both PET tracer were successfully synthetized and showed high brain uptake and fast wash out from the brain in rats and mice. For the fluorinated compound, we observed an additional high uptake in areas outside the brain, suggesting defluorination of the parent tracer. We therefore continued the *in vivo* evaluation of the [^{11}C]-labelled compound and identified one lipophilic metabolite in the brain, which was the demethylated form of the parent compound, confounding the *in vivo* quantification.
As none of the compounds selected in step one and two seemed to be the ideal tracer candidate, we screen further highly active compounds developed by partner MODAG in *in vitro* competition experiments on recombinant αSYN fibrils. As one compound identified in the previous experiments that showed very high and high affinity to the mixture and fibrils *in vitro*, it was selected as lead structure for competition binding experiments, which was established by partner Tuebingen to determine the inhibitory binding affinity K_i. Partner MODAG selected 13 additional compounds, which were applied to competition experiments. We identified one compound with a very high competitive inhibition towards pure αSYN fibrils (K_i < 0.2 nM) as well as the mixture of αSYN monomers, oligomers and fibrils (K_i < 0.2 nM). Good competitive inhibition was seen for the identified lipophilic metabolite mentioned above (Ki < 1 nM) and another (blinded) compound (Ki < 2 nM) on αSYN fibrils.
We received follow-up funding by the Michael J. Fox Foundation, where we propose to develop the deuterated analog to improve the pharmacokinetic properties and metabolic profile. If the metabolic profile is not improved, we plan to test the metaboliote, in fibril binding experiments and human brain tissue after tritium labeling.

2. Simultaneous PET/fMRI

Pharmacological interventions in animals can be assessed by simultaneous PET/fMRI measurements e.g. by using a infusion protocol of certain PET tracers such as [^{18}F]FDG or [^{11}C]raclopride in combination with BOLD-fMRI imaging. The signal change of these time-activity curves (in PET) or time-signal curves (BOLD-fMRI) can be assessed and used as a degree of influence of the applied challenge on brain metabolism and function. The changes in the PET signal of [^{11}C]raclopride reflect changes of D2 receptor occupancy by dopamine, whereas change in the BOLD-fMRI signal reflect functional changes of cerebral blood flow (CBF), blood volume (CBV) and oxygenation ($CMRO_2$).
One aim of this work package was to evaluate the detection limit of [^{11}C]raclopride PET and BOLD-fMRI in small laboratory animals. For this purpose, test-retest measurements were performed in rats and the variability between and within scans was evaluated for both imaging modalities in several regions of the rat brain. VAR between BOLD-fMRI scans was 7% in the motor cortex, 3% in the midbrain and 2% in the striatum. Within scan VAR was 7% in the motor cortex, 4% in the midbrain and 2% in the striatum. In comparison, VAR between PET scans was 8% in the striatum and within scan VAR was 9%.
Next, a pharmacological intervention was performed using the dopamine releasing drug D-amphetamine using simultaneous PET/fMRI. Our data show, that this pharmacological intervention causes a decrease of [^{11}C]raclopride binding and a simultaneous increase of the BOLD-fMRI signal in the striatum. Further brain regions downstream of the striatum are currently investigated and more animals are scanned to perform a more sophisticated functional connectivity analysis, which requires a higher group size for better statistics.
Our data on dynamic mouse brain imaging using combined PET/fMRI clearly indicate that e.g. a pharmacological intervention causes regional specific changes in glucose metabolism as well as BOLD-fMRI signal. The onset times of these changes are dependent on the region investigated. We found e.g. a negative change of the BOLD-fMRI signal in the striatum, which corresponded to a simultaneously measured increase in the [^{18}F]FDG uptake in the brain area.
In parallel to the establishment of the simultaneous PET/fMRI experiments in rats and mice, partner PMOD developed an integrated analysis tool. After calculating binding potential maps from dynamic PET data in PNEURO with the MR-derived brain area definitions, statistical analysis can immediately be performed. Since the PNEURO tool had been developed for the analysis of only human brain data, the PNEURO tool was extended to animal brains (rats and mice). In addition, a perfusion mapping plug-in was developed for the analysis of ASL

MRI data and a diffusion mapping and tensor calculation plug-in for DTI MRI data. For a more reliable statistical analysis of multiparametric data, PMOD implemented two types of linear models which allows for repeated measures ANOVA and the linear mixed effects model in longitudinal studies.

Project summary - work package 2

Characterization of alpha-synuclein (a-syn) overexpression models towards their use for imaging studies

The first task of the WP2 partners was to qualify two animal models for their suitability to be incorporated into PET and PET/MRI imaging studies. One of the models was related to Parkinson's disease and was constructed based on the use of viral vectors to overexpress human a-syn in the rat midbrain dopamine neurons, while the second model was a transgenic mouse line in which human a-syn was targeted for expression in oligodendrocytes. ULUND partner was in charge of the biochemical and histological characterization of the PD model and collaborated with EKUT teams who performed the imaging studies, while MUI worked closely with BRFAA in characterizing the MSA mouse model. In a separate attempt the ULUND team worked with AU partner in establishing, for the first time, a large animal model of AAV mediated a-syn overexpression in pigs for the purposes of PET studies in this translational species.

The results of the first set of experiments, generated within the collaboration between ULUND and EKUT, showed that the right (injected) hemisphere of rat brains expressing the human a-syn transgene under the chicken b-actin promoter in the substantia nigra was heavily loaded with soluble (soluble in triton) and insoluble species of a-syn protein (requires SDS treatment) and this disease-specific signature was considered to make the model appropriate to incorporate in imaging studies to assess possible binding sites for the asyn PET tracers. The studies included animals that were followed for 1, 3, 5 or 9 months. While, in parallel, the work between AU and ULUND qualified the surgical coordinates, suitability of the vector serotypes and doses were verified in the new pig model. Eight pigs were injected and followed with imaging studies in Aarhus using at the outset existing PET tracers to establish that the overexpression of human a-syn in this model generated a PET signature specific to disease, where the inflammation marker PK11195 demonstrated a specific signal at the site of injection in these animals. Post-mortem analysis confirmed the expression of the transgene in the same area.

For the biochemical determination of a-syn levels in tissue samples of the transgenic PLP-a-SYN (tg) mouse model of MSA, MUI and BRFAA teams worked with animals at 3, 8, 12, and 18 months of age. The analysis of these different age groups was of great interest to determine whether tissue a-syn levels increased with age, or whether the nature of the protein changed with progression e.g. more phosphorylated or aggregated protein might be present in older animals. They found that in the different fractions of the extraction protocol there was a progressive increase with age in the insoluble fraction of a-syn indicating oligomeric aggregate formation. To analyze aggregation pattern of a-syn in more detail, they used a sucrose gradient assay to illustrate the presence of high molecular weight species in the brain extracts.

One further objective of the WP2 was to establish a therapeutic intervention strategy in which an active immunization protocol based on the technology developed by AFFIRIS was incorporated in the rat PD model. This type of intervention was seen as an ideal candidate for imaging studies where the partners would have the opportunity to demonstrate elimination of the pathogenic species of human a-syn changes the course of the disease and this in turn modifies the imaging readouts. Towards this end, we immunized rats and followed their circulating Ab levels over several months. The work was done using two different vaccines that are tested in clinical studies by AFFIRIS where we found that both of them resulted in sero-positivity in rats for the intended antigen. One of the vaccines was then included in a study where rats were injected with AAV vectors encoding the human a-syn gene. The results suggested that the insoluble fraction of the a-syn protein was reduced at least in part and this modification would be potentially measurable in an imaging study.

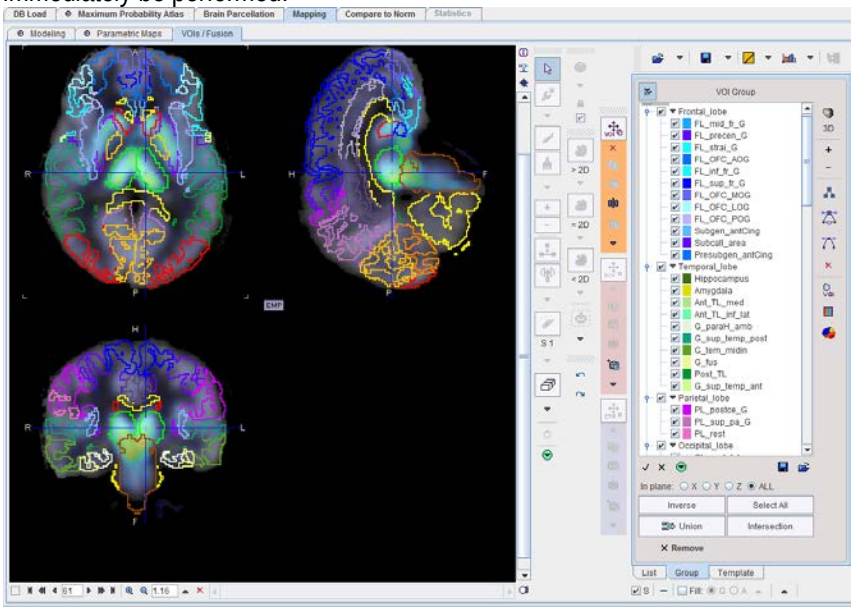
MultiSyn Final Report: WP3
Summary of WP3

The goal of the MultiSyn project was to develop multimodal imaging strategies to link protein aggregation pathology to functional read-outs in synucleinopathies, including most notabliy Parkinson's disease (WP1), and to validate these strategies in suitable animal models (WP2). The specific goal of WP3 was to to use these tracers to define the distribution and evolution of protein-aggregation pathology in human patients and to provide proof-of-concept that multimodal imaging protocols are suitable for monitoring disease-modifying individualized treatments.

Results

1. Development and implementation of multimodal PET/fMRI algorithms

As a preparatory work for project-specific imaging studies, multimodal imaging algorithms combining PET-imaging and fMRI have been developed and implemented
Statistical tools and data management pipelines for the analysis of multimodal imaging have been established. One of the PMOD tools (PNEURO) was developed for the joint analysis of PET and MR data. Its main goal was to apply the structural information in the MRI for generating objective definitions of functional brain areas, which in turn are used for analyzing the PET image in separate programs. In order to facilitate an integrated analysis for MultISyn, parametric mapping was added as a new functionality to PNEURO.
The example below illustrates the situation after calculating the binding potential maps from a dynamic DASB PET in PNEURO with the MR-derived brain area definitions in the overlay, so that statistical analysis can immediately be performed.



2. Regulatory approval

In preparation of the implementation of multimodal imaging methods to monitor alpha-synucleinopathies, approval to use 11C-methylphenidate, 11C-raclopride and 11C-PiB in human patients has been obtained from the relevant regulatory institutions.

3. Pilot study: PiB-PET imaging in MSA-P, MSA-C and GBA-PD

As an anle138-based PET-tracer has not been brought to a developmental stage of sufficient quality to be used in human trials (as described in detail in WP1 and WP2), WP3 developed alternative strategies for aSYN imaging. Pittsburgh-Compound B (PiB)-binding has been shown in vitro to bind to aSYN-containing Lewy-bodies [1,2] with a favorable affinity ratio aSyn versus Abeta [3]. As we had obtained promising preclinical results using the Pittsburgh-Compound B (PiB) as a potential marker for a-synucleinopathy in animal models, as described in WP2, and found elevated PiB binding in the brainstem in a prior PiB-PET study in patients with PD [2], we established a multimodal PET/MRI protocol using 11C-PIB and have demonstrated increased specific binding in multiple affected brain areas in two rare synucleinopathies: multiple systems atrophy (MSA) and Parkinson's disease caused by mutations in the gene for glucocerebrosidase (GBA-PD). We could show increased specific binding potentials and activity concentration ratios in brainstem, substantia nigra, pallidum, thalamus and putamen in three GBA-PD, three MSA-C and three MSA-P patients.

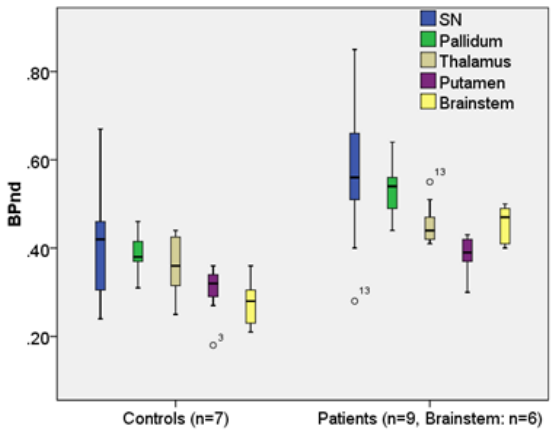


Fig. 1 Non-displaceable binding potentials in seven controls and nine patients (three PD_GBA, three MDS-C, three MSA-P). Binding potential was calculated using a reference tissue model with frontal lobe as reference. Since brainstem atrophy in MSA-C patients violates an assumption of the model, brainstem binding potential is only shown in three PD_{GBA} and three MSA-P patients.

		BP_{nd} (Ref. Frontal Lobe)	BP_{nd} (Ref. Cerebellum)
Brainstem	MSA-C	0.23 ± 0.04	0.41 ± 0.09
	MSA-P	0.46 ± 0.04	0.50 ± 0.06
	GBA	0.45 ± 0.04	0.49 ± 0.10
	Controls	0.27 ± 0.05	0.29 ± 0.05
Substantia Nigra	MSA-C	0.65 ± 0.18	0.91 ± 0.27
	MSA-P	0.45 ± 0.19	0.51 ± 0.18
	GBA	0.63 ± 0.15	0.65 ± 0.16
	Controls	0.41 ± 0.14	0.41 ± 0.11
Pallidum	MSA-C	0.52 ± 0.07	0.77 ± 0.06
	MSA-P	0.57 ± 0.07	0.61 ± 0.06
	GBA	0.52 ± 0.03	0.55 ± 0.09
	Controls	0.39 ± 0.05	0.41 ± 0.05
Thalamus	MSA-C	0.44 ± 0.03	0.70 ± 0.06
	MSA-P	0.49 ± 0.07	0.47 ± 0.08
	GBA	0.44 ± 0.02	0.41 ± 0.03
	Controls	0.36 ± 0.07	0.33 ± 0.05
Putamen	MSA-C	0.37 ± 0.06	0.62 ± 0.13
	MSA-P	0.40 ± 0.04	0.40 ± 0.07
	GBA	0.38 ± 0.01	0.32 ± 0.04
	Controls	0.30 ± 0.06	0.25 ± 0.04

Tab. 1 Non-displaceable binding potentials are shown for all groups and regions (mean ± SD). Binding potentials have been calculated with frontal lobe as reference region and additionally with the cerebellum as reference region. Binding potentials in the brainstem of MSA-C patients as well as binding potentials in MSA-C patients calculated using the cerebellum as reference region are grayed out (due to violation of assumption of reference tissue models).

Group differences between patients and controls were significant and PiB-PET was planned to be performed in a longitudinal multimodal study.

4. Cognition-related metabolic patterns in Parkinson's disease

Parkinson's disease (PD) is a multi-system disorder including primarily motor but also a variety of non-motor symptoms, of which cognitive impairment (CI) in particular is one of the most common and troublesome. Multimodal imaging of correlates of cognitive decline is therefore a major need to establish disease-modifying treatments. Regional cerebral glucose metabolism (rCGM), measured using 18F-fluorodeoxyglucose (FDG) PET

is a marker of neuronal activity and integrity and has been shown to be correlated with cognitive performance in various neurodegenerative disorders. In PD, several studies have shown an association between cognitive dysfunction and expressions of a metabolic pattern, which is characterized by relative hypometabolism in the medial frontal and parietal association regions and relative hypermetabolism in the cerebellum and other predominantly subcortical regions. In the literature, the nature of apparent hypermetabolism in PD is a matter of controversy. Our aim was to analyze hypometabolism and hypermetabolism separately and to evaluate their respective association with CI. The question as to whether hypometabolism and hypermetabolism are independently associated with CI is linked to whether an apparent increase in FDG uptake reflects a true increase in neuronal activity or rather a normalization effect.
At higher stages of CI, increased rCGM was found in the posterior cerebellar vermis and pons, associated with impaired attention, executive function and memory. Reduced rCGM was found in various cortical regions in agreement with the literature. Indices for both, hypo- and hypermetabolism, independently predicted severity of CI, indicating hypermetabolism to be a true feature of PD. These metabolic alterations may represent compensatory activation of cognitive networks including cerebropontocerebellar tracts.
The paper describing this work is publish in the European Journal of Nuclear Medicine and Molecular Imaging (https://doi.org/10.1007/s00259-018-4085-1).

5. Controls-based denoising: a new method for improving statistical image interpretation

Physiologic variance of brain structure is a significant cause of “noise” in imaging studies, resulting in the need for larger cohort sizes. Principal component analysis (PCA) has been explored extensively for analysis of brain image data of patients to identify patterns associated with a particular clinical condition. We here propose a novel approach to apply PCA in a control sample to remove the principal components, assumed to capture physiological variance, from patient data (controls based denoising, CODE). Whereas PCA is usually applied in order to identify a pattern associated with a particular clinical phenotype, we here propose to use PCA to identify patterns that are neither primarily related to such a condition nor orthogonal to the pattern of interest, and to use it for denoising. Particularly, we propose identifying PCs from image data of healthy controls reflecting physiological variance and removing them from patient data before calculating the degree to which a (known) pattern is present. The proposed procedure differs from the usual way of applying PCA in that the PCs that are interpreted as noise and thus removed from the data are not orthogonal to the (known) pattern of interest. As a consequence, these PCs may explain, at least in part, the noise component of the degree to which a pathological pattern is expressed. We provided the theory of CODE and experimental analysis and applied CODE to FDG-PET data from patients with mild cognitive impairment (derived from ADNI database) to improve classification between those who converted to Alzheimer’s disease and those who not. Using Code, we were able to improve classification performance between converter and non-converter: Area under curve AUC = 0.76 with CODE vs. AUC = 0.66 without CODE. The proposed method is not limited to a specific PET tracer or image modality. The paper describing controls-based denoising is currently under review. Another paper focusing on optimizations of CODE is in progress.

6. Characterization of cohorts of patients with rare synucleinopathies

For clinical studies to establish imaging strategies in rare diseases, the development and follow-up of clinical cohorts is a prerequisite. This has been done for all rare synucleinopathies including MSA-P, MSA-C, A53T-PD and GBA-PD. These cohorts of patients who are seen regularly at our centers ensure that patients can be recruited rapidly once clinical cross-sectional, longitudinal or interventional studies are in place.
Patients with MSA and GBA-PD are being followed in Tübingen (Partner 1, EKUT) and Innsbruck (Partner 3, IMU). A cohort of patients with A53T-PD are being followed in Athens (Partner 4, BRFAA) with further clinical characterization, which is necessary for the optimal design of any clinical trial with imaging outcomes.
Cohort recruitments during the reporting period April 1 2015 to Sept. 30, 2016 at the different centers are given in the table.

Center	Patient group		
	MSA	A53T-PD	GBA-PD
EKUT	79	-	40
UBI	15	-	-

BRFAA	-	6	5
Total	94	6	45

7. Establishing the regulatory requirements for longitudinal and interventional studies

Based on the results described above, a protocol for a clinical trial was developed that included an observational and an interventional arm to assess the feasibility of PET-imaging as a read-out in a rare synucleinopathy as suggested in the original proposal. All essential documents were prepared for the submission process to the Ethics Review Committee and the Competent Authority Paul-Ehrlich-Institute, including the clinical study protocol, the Investigator's Brochure, Informed Consent Form, Case Report Form, the Investigational Medicinal Product Dossier and the insurance statement for patients participating in the study.
The study proposal AFF010 to the Tübingen local Ethics Review Committee was submitted by AFFiRiS on April 12, 2016. Additional clarifications were requested on April 27, 2016, which were submitted on May 19, 2016. Approval of the study protocol by the Ethics Review Committee was given on June 16, 2016.
The application to the Paul-Ehrlich-Institute was submitted on April 21, 2016. Additional clarifications were requested on June 20, 2016, which were submitted on September 15, 2016.
Further, the application to the Bundesamt für Strahlenschutz (BfS) was submitted on 22.06.2016.

8. Production of PD01A vaccine batch

The production of the PD01A batch of GMP conjugate vaccine was done as follows: conjugate production (the KLH for the conjugate was delivered in February 2015), formulation and filling, labelling, packaging of the GMP grade batch. The clinical grade material was produced in July 2015, and approved by G.L. Pharma on 20th August 2015. The specific labelling and packaging for the clinical trial AFF010 was done in May 2016, these ampoules cannot be reused. The batch is currently stored and relevant documentation is available. The PD01A batch has an expiration date of December 2016.
The AFF010 study specific costs of the vaccine batch production are 32% of the total batch production costs. The pro rata costs are given in Form C.
The stability analysis of the PD01A batch started in August 2015. The results of the early stability programme demonstrated that the PD01A vaccine complies with predefined stability settings for the Drug Substance (DS, i.e. the active ingredient) and the Drug Product (DP, i.e. a finished dosage form of a therapeutic agent) at the time points assessed.

9. AFF010 clinical trial

In order to initiate safety and efficacy testing of the drug substance AFFITOPE® PD01A in the clinical trial AFF010, a number of essential study documents had to be prepared in this reporting period, as described in WP3, Task 2.
As significant changes had to be made to the originally proposed study due to lack of an anle138-based aSYN PET tracer, the revised protocol was submitted for approval to the EC on July 17, 2016. The EC felt that the revised protocol deviated too far from the original study proposal so that it did not support the suggested changes in the research program. Therefore, as of now, no longitudinal or interventional proof of concept trial could be started.
In due course, AFFiRiS withdrew the clinical study proposal AFF010 from both the local Ethics Review Committee in Tübingen and the Paul-Ehrlich-Institute on September 26th, 2016.

References

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MultISyn web site



www.multisyn.eu

MultISyn logo

4. Participants involved in MultISyn

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