The relation between human biomarkers and oxidative stress in Alzheimer's disease, novel *in vivo* experimental modelling possibilities

Thesis of the PhD dissertation

Zita Oláh

Szeged

2018

The relation between human biomarkers and oxidative stress in Alzheimer's disease, novel *in vivo* experimental modelling possibilities

Thesis of the PhD dissertation

Zita Oláh

Doctoral School of Clinical Medical Sciences

University of Szeged

Tutor:

Magdolna Pakaski, MD, PhD

Department of Psychiatry

University of Szeged

Publications

Publications related to the thesis:

I. **Olah Z**, Pakaski M, Janka Z, Kalman J Marking the markers of Alzheimer's: too good to diagnose, too bad to use?. Neuropsychopharmacol Hung 2012 Sep;14(3):165-76. Review. PMID: 22987730 **IF: 0**

II. **Oláh Z**, Kálmán J, Tóth ME, Zvara Á, Sántha M, Ivitz E, Janka Z, Pákáski M. Proteomic analysis of cerebrospinal fluid in Alzheimer's disease: wanted dead or alive. J Alzheimers Dis 2015;44(4):1303-12. doi: 10.3233/JAD-140141. PMID: 25428253 **IF: 3.92**

III. **Olah Z**, Bush AI, Aleksza D, Galik B, Ivitz E, Macsai L, Janka Z, Karman Z, Kalman J, Datki Z. Novel in vivo experimental viability assays with high sensitivity and throughput capacity using a bdelloid rotifer. Ecotoxicol Environ Saf. 2017 Oct;144:115-122. doi: 10.1016/j.ecoenv.2017.06.005. Epub 2017 Jun 9. PMID: 28605645 **IF: 3.743**

IV. Datki Zs, **Olah Z**, Hortobagyi T, Macsai L, Zsuga K, Fulop L, Bozso Zs, Galik B, Acs, E, Foldi A, Szarvas A, Kalman J. Exceptional in vivo catabolism of neurodegeneration-related aggregates. Acta Neuropathol 2018 6:6 doi: 10.1186/s40478-018-0507-3 **IF: 3.8, according to the editotor's statement**

The cumulative impact factor of the publications related to thesis: 11.463

Other publications:

I. Sántha P, Veszelka S, Hoyk Z, Mészáros M, Walter FR, Tóth AE, Kiss L, Kincses A, **Oláh Z**, Seprényi G, Rákhely G, Dér A, Pákáski M, Kálmán J, Kittel Á, Deli MA. Restraint stress-induced morphological changes at the blood-brain barrier in adult rats. Front Mol Neurosci 2016 Jan 14;8:88. doi: 10.3389/fnmol.2015.00088. eCollection 2015. PMID: 26834555

II. Várhelyi ZP, Kálmán J, **Oláh Z,** Ivitz EV, Fodor EK, Sántha M, Datki ZL, Pákáski M. Adiponectin receptors are less sensitive to stress in a transgenic mouse model of Alzheimer's Disease. Front Neurosci 2017 Apr 11;11:199. doi: 10.3389/fnins.2017.00199. eCollection 2017.

III. Money KM, **Olah Z**, Korade Z, Garbett KA, Shelton RC, Mirnics K. An altered peripheral IL6 response in major depressive disorder. Neurobiol Dis 2016 May;89:46-54. doi: 10.1016/j.nbd.2016.01.015. Epub 2016 Jan 22. PMID: 26804030

1. Introduction

1.1. Epidemiology and pathology of Alzheimer's Disease

Approximately 44 million patients in the world are suffering from Alzheimer's Disease (AD) or related dementia. By 2050, the prevalence of AD increases will double. Although the exact molecular pathomechanism is still unknown; however, the environmental and genetic factor could influence the initiation of the disease. These factors together may cause neuroinflammation, oxidative stress and neuronal cell death exacerbating the progressive dementia, the impairment of the episodic memory and the decline of executive and verbal functions associated with AD. The pathological hallmarks of the disease are extracellular accumulation of amyloid-beta 1-42 (Aβ1-42) and intracellular deposition of neurofibrillary tangle which consists of hyperphosphorylated tau (p-tau). There are two main pathways dedicated to the cleavage of amyloid precursor protein (APP): protective intermedier is produced by the non-amyloidogenic pathway, and the toxic form derives from the amyloidogenic pathway. The equilibrium shifts to the amyloidogenic pathway during AD progression; therefore, the overproduced Aβ1-42 aggregates in extracellular space and accumulates in senile plaques. The Aβ1-42 influences the progression both directly and indirectly via disturbing the ionic and redox balances and promoting tau pathology via the dysregulation of protein kinases and phosphorylases.

1.2. Invertebrate model of Alzheimer's Disease

The invertebrate models of AD are gaining more importance in toxicological researches, as well as in research of aging associated neurodegenerative disorders. Widely used traditional metazoan models are *Caenorhabditis elegans* and *Drosophila melanogaster*; however, rotifers have been gaining more importance. Rotifers are ideal for investigating evolutionary conserved molecular mechanisms of aging, associated with the control of genome stability; the regulation of proliferation; the differentiation and apoptosis; the modulation of cellular bioenergetics and the damage and repair of macromolecules. These molecular pathways also play an essential role in AD pathomechanism. Our species (*Philodina acuticornis*, PA) has extra advantages compared to other invertebrate models. Rotifers show greater evolutional similarity to humans, more than 10% of their genes have human homologues, which cannot be identified in other invertebrate models, because their genome underwent extensive gene loss. Rotifers and humans share a

phylogenetic common ancestor. In contrast to these advantages, the presence of amyloid producing genes has never been investigated in rotifers; furthermore, only contradictory data is available about the amyloid toxicity in them. Rotifers are the smallest animals (250-300 μm) of the world, their complex body consists of 900-1000 cells. They have distinct and separated ganglia, muscles, photo-, chemo-, and tactile sensory organs; ciliated head-structures, muscles, digestive- and secretory systems. They have primitive eyes sensitive to sharp light, and all transmitter systems are presented in their nervous system. They are extremely resistant to environmental stress. The bdelloid rotifers are ancient asexuals; therefore, their degenerate tetraploid genome provide homogenic background; furthermore, they have short life and generation time, thus they are easy to maintain in controllable environment. The rotifers are adequate models due the ease of their culturing and molecular manipulation.

They have well-established and quantitative physiological and behavioural characteristics that form the basis of our viability assays. Rotifers are widely used in measuring the aquatic pollution in both acute and chronic toxicity. Despite this fact, no standardized and validated behavioural assays and no method for tracking the efficacy in single-housed invertebrates are available. In their natural habitat, rotifers usually have to cope with harsh conditions associated with oxidative stress. Their antioxidant defence system is able to protect them against the harmful molecular effects of reactive oxidative species (ROS). According to literature the exogenic antioxidant agents are able to preserve the mitochondrial function; therefore, extending the life of rotifers. The oxidative molecules remove electrons from the susceptible chemical groups by oxidizing them. The hydrogen-peroxide (H_2O_2) and sodium azide (NaN_3) are usually used as experimental chemical stressors with well-known mechanism of actions; therefore, their physiological effects could be well-defined. These agents promote the ROS production via impairing the function of mitochondrial complex 4. This mechanism is highly similar to the pathomechanism of AD; therefore, rotifers may be *in vivo* models of oxidative stress in AD and related toxicological measurements.

1.3. The risk factors and biomarkers of Alzheimer's Disease

Setting up the diagnosis of AD in prodromal phase is difficult, because the first symptoms are not specific. The aim of biomarker research is to identify a molecule with high specificity and sensitivity which could be indicators of the biological and pathological molecular processes associated with the disease. The cerebrospinal fluid (CSF) is an ideal

source of possible novel markers, because it is a direct environment of the brain; therefore, the molecular composition of CSF is influenced by the intrathecal processes. According to the novel criteria of Dubois, the measurements of core CSF markers are included as an inclusion criteria or as outcome indicators of clinic-pharmacological studies. The decrease of Aβ1-42, the increase of t- and p-tau showed 85% sensitivity and higher than 95% specificity in moderate or severe AD. Despite these facts in mild cognitive impairment, their predictive value is lower than 50%. Further limitations of these core markers, are the facts that 34% of cognitively healthy population and 33% of patients with non-AD dementia showed AD specific molecular pattern.

Numerous risk factors of AD have been identified, but Apolipoprotein E (ApoE) is one of the most important genetic factors. The ApoE4 allele promotes the development of AD, the risk increased 4-fold in heterozygotes, and 12-fold in homozygotes. Although the exact molecular mechanism is unclear, the contributions of ApoE in AD pathology have been described. The ApoE exacerbates Aβ1-42 accumulation (increased aggregation and decreased clearence) and tau hyperphosphorylation. It plays a crucial role in modulation of lipid homeostasis; therefore, it may help the development of lipid abnormalities related to AD. Epidemiological studies presented that the infection and reactivation of *Herpes simplex* virus 1 (HSV1) and *Herpes simplex* virus 2 (HSV2) could also be risk factors of AD. In the course of reactivation, the replicated virus is able to enter the central nervous system, where it could facilitate the AD-related molecular processes and neurodegeneration via damaging the blood brain barrier (BBB).

1.4. Oxidative stress

The oxidative stress is one of the earliest process in the course of AD development. In the affected neurons the impairment of mitochondrial function leads to elevated ROS contributing to the apoptotic pathways which are the molecular basis of pathomechanism. The ROS, as a secondary messenger, is able to influence the neuroinflammation, the synaptic plasticity and memory related processes. Interestingly, the pathogen associated molecular pattern is very similar to mitochondrial damage associated molecular pattern (DAMP), which activates the same inflammatory cascade. As the DAMP exacerbates neuroinflammation, it could facilitate Aβ1-42 production via promoting the amyloidogenic pathway. The Aβ1-42 alone has an essential role as a messenger in forming the specific molecular pattern. The lipids are extremely prone to oxidative stress,

which are considered to be basic pathological signs of AD; therefore, they could be sensitive markers of the pathological processes.

2. Specific aims

The aims of the present study were:

I. to develop a novel, in vivo high-throughput assay system on invertebrates allowing the individual monitoring on various physiological levels of efficacy providing continuous and quantitative indices;

II. to characterize PA in terms of survival, physiology and behavior, named *toxicity and survival lifespan* (TSL), *bright light disturbance* (BLD), *mastax contraction frequency* (MCF) *and cellular reduction capacity* (CRC);

III. to validate the assay system by reactive agents;

IV. to show the relevance of the assay system in AD research;

V. to identify oxidative stress and neurodegeneration-specific protein marker candidates in CSF of AD patients;

VI. to reveal impacts of the HSV1 and HSV2 reaction on lipidomic profile of AD patients;

VII. to investigate the role of ApoE in the modulation of lipidomic changes.

3. Methods

3.1. *In vivo* experimental viability tests

Among the bdelloid rotifers used in our laboratory, the PA was the most adequate for our methodological innovation. The *P. acuticornis* (PA) together with the *Cladophora aegagrophila* (CA) alga, which provides environmental matrix for the rotifers, were obtained from Hungarian aquaristique. The *in vitro* cell culturing methods were adapted and optimized to maintain *in vivo* rotifers. The animals were cultured in cell culturing flasks containing standard medium. The culturing was performed in algae-cotton wool 3D-matrix in semi-sterile environment, since in these cultures 10-times more animals could be observed than in cultures in 2D petri dishes. The elements of matrix were sterilized in ethanol following the optimized protocol. The animals were fed with pasteurized yeast (*Saccharomyces cerevisiae*) in every two days after medium change.

For the experiments rotifers were harvested. As the first step, the animals were placed at -75 °C for a short period of time to dissect the attached animals from the 3D-matrix, then the medium containing the contracted animals was decanted into a sterile petri dish. In

order to re-attach the healthy animals, the dish was kept at room temperature for 30 minutes, then the surface was washed with standard medium to eliminate the unattached dead animals. For genetic studies, the rotifers were used instantly right after harvesting. For viability measurements, the previously isolated population was kept for 24 hours at room temperature without feeding.

3.1.1. The phenotypic and phylogenetic characterisation of our model organism

The homogenic rotifer population was started from one animal. After the ethanol fixation of the animals, the specific markers were used for species-specific and phenotypic characterisation. We determined the size of the specimen (whole body or body parts), its shape, special morphologic markers (e.g. characteristics of trophy, foot, corona, eyes, antennae) and different type of movements (swimming, crawling, the use of the corona, pulsation of mastax, body contraction, etc.).

For the phylogenetic analysis genomic DNA was isolated, then 18S rDNA region was amplified using bdelloid specific primers. The products were separated on gel; the purified fragments were sent to Sanger sequencing. The contigs were analysed by bioinformatics tools, they were compared with 18S rDNA sequence of other rotifer species from the reference database. The evolutionary relations of our species were determined and presented on cladograms.

3.1.2. Adopted method of oil-covered microdrop

Our primary objective was to develop a method that makes possible to observe single rotifers in a microenvironment, thus the oil covered micro-drop method was adopted from the in vitro fertilization. At the bottom of each well 50 µL medium was placed, covered by 1:1 mineral oil mixture of high purity SAGE paraffin oil. The age- and size-preselected animals were transferred into that microenvironment. The oil, as a protective barrier, provided normal gas diffusion between the droplet and its environment; furthermore, it prevented evaporation and hypoxia.

3.1.3. Novel viability tests

Our novel viability assays were validated by oxidative toxins with well-known mechanism of action. The H_2O_2 and NaN_3 were used to increase concentrations in the different assays. Overlapping doses were applied to cross-validate the assays over their dynamic range. In amyloid toxicity tests Aβ1-42 and Aβ25-35 fragments were added to rotifers isolated in the microdrop, where the obligatory presence of amyloids made

possible to evaluate their clear physiological effects. To determine the dose-dependency, bovine serum albumin (BSA) was administered in the same concentrations.

The *toxicity and survival lifespan* (TSL) showed the effects of the administered substances on the lifespan of unfed PA. Various well-defined morphological markers were used for assessing the viability. These markers were the following: (a) normal anatomy and active motility of the body; (b) general movement within the body; and (c) naturally red eyes. The PA was considered dead when the following features were present: the absence of motility after touching the end of the micropipette; loss of telescopic reflex and red colour of eyes; the fragmentation and amorphous granules in the soma within the body.

During the development of *bright light disturbance* test (BLD) the special characters of PA were used, which showed high sensitivity to the treatment agents. The rotifers tend to avoid bright light; therefore, their escape from the illuminated area provided complex information about functional changes of sensory and motor system caused by even sublethal doses. The well was adjusted so that the active animal was in a middle of pre-defined illuminated area. After 30 seconds, if the animal was still active, we elevated the illumination from 20 lux to 40,000 lux. Two reaction parameter was recorded. The *bright light irritation* (BLI) was the total time when the rotifer is contracted or crawling, recapitulating the unfed-state where corona is not wheeling. When the treated animal did not react, i.e. remained in cilia-motile form, it was considered insensitive to the bright light trigger. The *bright light avoidance* (BLA) was the total time spent in illuminated area including the time of leaving the designated area. The BLD index was the percentile ration of BLI and BLA. In healthy untreated animals the index approaches 100%, reflecting the maximal sensitivity and reaction to the light. The treated animals had a maximum of 5 minutes to leave the illuminated area; however, in the case they were unable to escape we considered the value of BLD as 0.

The *mastax contraction frequency* (MCF) showed the contraction per second of the upper part of digestive system, anatomically that organ has a powerful muscular wall and contains tiny, calcified, jaw-like structures, called trophi. The main function of mastax is to cut the food and transmit it by periodic opening and closing. In order to assess the viability of the one-housed rotifer, MCF (contraction/sec) was described, as a quantitative viability marker of organic functionality.

The *cellular reduction capacity* (CRC) assay was developed by optimizing the EZ4U (MTT analogue) widely used in measuring the viability of cell culturing to rotifers.

Diluted XTT solution was added to the control and treated populations; and they were treated in dark for 24 hours (the components are sensitive to light). The colorimetric change of supernatant was measured by spectrometry. The alteration correlated with the additive reduction capacity (NADH content) of population, the reading was normalized to the starting number of animals.

3.2. The investigation of human biomarkers of oxidative stress in cerebrospinal fluid

3.2.1. The enrolment of the patients and sampling

The diagnosis of enrolled AD patients was determined by using DSM-IV and DSM-5, based on an initial evaluation of careful personal and family history and neurological and psychiatric examinations. In each patient computed tomography, magnetic resonance imaging or single-photon emission computed tomography were performed. As obtaining CSF is invasive, the control probands were patients for whom CSF sampling was necessary to confirm their diagnosis; however, their potential disorder did not associate with cognitive decline. Informed consent was obtained from all patients to use their clinical data for research purposes. The study was approved by local Ethics Committee of the Hungarian Investigation Review Board (permit no.: 135/2008 for the control probands, 184/2012 for AD patients in protein array and permit no.: 54/2015 for AD patients in lipidomic-HSV analysis). The CSF samples were obtained from patients undergoing a lumbar puncture in the L4-L5 vertebral interspace and the aliquots were stored at -75 °C until further analysis.

3.2.2. Core markers and risk assessment

The ApoE allele prevalence was determined by using genomic DNA from peripheral blood. In proteomic screening the previously described allele-specific restriction fragment analysis was applied. In the lipidomic study ApoE2, ApoE3, ApoE4 allele specific Taqman probes were used for polymerase chain reaction analysis.

The levels of Aβ1-42, t-tau, p-tau was measured by protein or peptide specific sandwich enzyme-linked immunosorbent assay (ELISA). The international cut-off values were used as references. In Aβ1-42 analysis of rotifers, the prepared homogenates were used with different protein concentration, compared with human CSF samples.

3.2.3. Peptide chip

Master Antibody Microarray was used for the proteomic screening; 656 unique antibodies in duplicate were printed on each slide. Pooled samples of 5 AD patients and age- and gender-matched 5 control probands were pooled and measured on each microarray pair. During the protein isolation, the samples were precipitated as a first step, then they were centrifuged and washed, then purified protein homogenates (2.7 mg) were labelled by Cy3-streptavidine. The local intensity values were normalized to the background. The technical replicates were compared and where the two replicates showed a significant difference they were excluded from the analysis. Ratio of AD and control values of each peptide was determined; below 0.6 meant decrease, while above 1.8 meant increase.

3.2.4. Western blot validation

In order to confirm the results of peptide microarray (methylated-DNA-protein-cysteine methyl-transferase /MGMT/, protein kinase C apoptosis WT1 regulator protein /PAR-4/ and granzyme B /GRB/), semi-quantitative western blot was carried out using 20 or 40 μg protein of the nature pooled CSF samples. Different settings were tested, the optimal one was used in our experiments. To visualize the signs, chemiluminesce was applied.

3.2.5. Determination of HSV reactivation

Nature CSF samples of AD patients were used for determining the HSV1 or HSV2 immunoglobulin G (IgG) titer with specific ELISA. The kit-specific cut-off values were applied in the reactivation-specific IgG titer.

3.2.6. Lipidomic analysis

Mass spectrometry (MS)-based lipidomic analyses were performed in cooperation with the Laboratory of Molecular Stress Biology (Hungarian Academy of Sciences, Biological Research Centre, Szeged, Centre of Excellence of the European Union, Institute of Biochemistry, Membrane and Stress Biology Unit). Lipids were extracted from native CSF based on the optimized Folch extraction. The sphingomyelin (SM) species were detected and quantified using the positive ion mode, while negative ion mode was applied for phosphatidil-serint (PS), ceramide (CER), hexosyl-cermide (hCER), and sulfatide (SULF) species. The measurements were normalized to the added inner lipid standards. The lipid analysis was performed on both class and species level.

4. Results

4.1. Validation of *in vivo* model

The results of 18S rDNA sequencing confirmed that our rotifer belongs to *P. acuticornis* species. Our phylogenetic analysis showed the presence of early divergence in our model due to the isolation of the population.

The developed novel viability assays were validated by dose-dependence studies on H_2O_2 and NaN_3 successfully. The rotifers were treated with different doses of agents, in line with the sensitivity of selected assay method. Both agents reduced the TSL significantly. The BLD was performed after a three-day treatment. Our results showed that the toxins inhibited the light sensitivity and escaping reflex dose-dependently. The administered treatments decreased the MCF, although we observed an interesting phenomenon. The 1μM H_2O_2 increased the frequency, but in CRC assay the same dose triggered oxidative stress in the population. According to literature the molecule potentially has a role in signalisation as well. In our opinion, the enhanced MCF could be a compensatory mechanism, in order to reduce the oxidative stress by elevating the food intake. The three-day treatment caused dose-dependent oxidative stress in our populations indicated by CRC qualitatively. The BLD and CRC predicted TSL, and BLD showed significant correlation with MCF and CRC. The Aβ1-42 was not toxic; furthermore, it extended the lifespan of rotifers as an obligatory energy source compared to the untreated/unfed controls in an isolated environment. The efficacy showed the same kinetic as the BSA applied in the same dose. The artificial Aβ25-35 was toxic, due to the fragment's extreme capability to aggregations. According to ELISA screening the bdelloid rotifers did not produce endogenic Aβ1-42.

4.2. Proteomic screening

As a first step, the core marker levels and ApoE4 allele frequency of enrolled patients were determined in order to validate the clinical diagnosis by neurochemical methods. The core markers showed AD specific pattern in line with the literature, in detail, the Aβ1-42 level was significantly reduced, t- and p-tau significantly elevated compared to cognitively healthy control probands. The proteomic screening showed reduced concentrations of 7 proteins that are the following: DNA polymerase gamma (POLG); MGMT; parkin (PARK); apolipoprotein D (ApoD); PAR-4; GRB and cyclin dependent kinase 5 (CDK5). We were not able to confirm the observed reduction by western blot

as a consequence of the difference between the amount of the used protein and the various sensitivity of methods.

4.3. HSV reactivation caused lipidomic changes

The HSV1 and HSV2 IgG titers were determined by specific ELISA from CSF samples of AD patients. Among them 14% had reactivated HSV1 infection and 25% had reactivated infection by HSV2, 5% patients were in reactivated state of both types of viruses and 56% of patients showed no detectable signs. Patients who showed double-reactivation were excluded from further analysis. Based on reactivity, patients were divided into groups and lipid classes were compared. We found that PS showed significant elevation in the HSV1 group, the concentration of SULF elevated significantly in HSV2 group and there was no significant change in the analysed other classes (SM, CER, hCER). The species analysis showed a reactivation specific lipid pattern. We found species with significant alteration in all measured lipid classes. The changes could characterize the HSV1 reactivation caused apoptosis, and the HSV2 reactivation facilitated compensatory processes. By studying the impact of the ApoE4 allele on lipid changes reactivation specific molecular pattern was observed in SM and SULF classes.

5. Discussion

5.1. *In vivo* model and oxidative stress

In the last 30 years the importance of the invertebrate rotifers in experimental research has increased. The benefits of the rotifer models are: small size, relative short life, high population density, rapid population growing, obligatory parthenogenesis and sensitivity to vast number of toxicants. Surprisingly, standardized behavioural assay using invertebrates as models has not yet been developed. Although the rotifers were used in toxicological assays, but in these experiments the efficacy was followed-up on population level. The novelty of our adopted oil-covered microdrop method was the ease of determination and long-term observation the potential impacts of toxicants on isolated animals. Based on the sequence homology, the 18S rDNA analysis confirmed that our rotifer is a PA. Two toxicants (H_2O_2 and NaN_3) with well-known mechanism of action were used in order to validate our four recently developed, complementary, experimental viability assays with different levels of sensitivity. The TSL, BLD, MCF and CRC showed dose-dependent reaction to the toxins confirming the sensitivity and reliability of the tests; furthermore, these experiments verified the sensitivity of rotifers to oxidative

stress. The markers evaluate various parameters of viability, such as: lifespan, light sensitivity-related behavioural pattern, welfare of the rotifers, need for nutrition and the reduction capacity on the population level. The parameters measured alone or paralelly are able to significantly predict the length of lifespan.

In our experiments we observed survival of rotifers in the presence of Aβ1-42 treatment suggesting partial *in vivo* catabolism that is a novel phenomenon in current literature. The potential explanation to the capability of these animals (metabolizing natural aggregates) might be an evolutionary strategy for adaption and survival. In their natural habitat, bdelloids constantly have to cope with extreme fluctuations in nutrient availability. Our potential results about *in vivo* Aβ catabolism may provide the basis for a new preclinical perspective on therapeutic research in human neurodegenerative diseases.

5.2. Identification of protein markers

By the proteomic screening we searched for biomarkers in CSF of AD patients with neurochemically validated diagnosis using peptide microarray. We found that seven proteins decreased significantly, some of them were first described in CSF.

The POLG is responsible for the mtDNA synthesis, the presence and reduction in CSF were showed first. As for the previous experiments, the decrease of mtDNA was published in CSF of AD patients, which observation was confirmed by our results. The reduced mtDNA replication leads to decreased mitochondrial synthesis. As a consequence, mitochondrial impairment and oxidative stress develop, which play a crucial role in AD initiation. The GRB cleaves PAR-4 that has a role in regulating apoptosis. The presence of GRB in CSF was first identified, previously this molecule had only been introduced in association with neuroinflammation. According to PAR-4, the expression of its mRNA decreased in the brain of AD patients. The MGMT eliminated the harmful alkyl groups from DNA maintaining the proper expression. The reduction in AD was first identified, which could contribute to oxidative stress-caused DNA damage. The PARK has an essential role in ubiquitin dependent protein degradation and the clearence of Aβ1-42. Although it has not been tested in CSF; however, in the transgenic model of AD it was confirmed that the overexpression of PARK could preserve the normal memory function and behaviour. The CDK5 is a multifunctional enzyme-triggering cascade of pathways associated with oxidative stress, cell cycle re-entry, Aβ1-42 plaques and neurofibrillary tangles formation and tau hyperphosphorylation. Normally, it is under strict regulation in neurons. As a consequence of the oxidative stress

and related mitochondrial dysfunctions, the intracellular Ca^{2+} elevates and elicits calpain activation which could cleave p35 to p25, thus forming stable CDK5/p25 complex. The ApoD plays a central role in lipid homeostasis, thus its relation to the AD pathomechanism has arisen. The expression of ApoD in hippocampus and prefrontal cortex is outstanding. Other studies also described its elevation in CSF. The observed decrease in our experiments needs further validation, but these intriguing contradictions in the publications could be explained by the difference between the ApoE4 allele frequencies of the studied populations. The ApoE4 allele increase the expression of the ApoD, but in our population the allele showed lower frequency than in other population from the referred publication.

5.3. HSV reactivation specific lipidomic changes

The consequences of oxidative stress and neuroinflammation are strongly associated with AD initiation and with the integrity of BBB, impaired contribution of the entrance of HSV1 and HSV2 into CNS. According to the pathogen hypothesis, the repeated exogenic stress triggers reactivation leading to enhanced production of toxic Aβ1-42 promoting AD progression. Epidemiologic studies showed that the presence of HSV in the brain significantly elevates the risk of AD progression. In contrast to these results, other experiments suggested that the HSV induced humoral immune response could be protective in the early phase of disease, because it could help in the degradation and clearence of toxic aggregates. The reactivated HSV infection activates microglia and by the expression of proinflammatory cytokines induces inflammatory processes leading to oxidative stress and neuroinflammation. Carrying ApoE4 allele significantly increases the risk of AD progression. The defective protein reduced efficacy in Aβ clearence, in parallel with that it could exacerbate the pathological processes related to HSV reactivation. Our results are the following: 14% of patients showed HSV1 reactivity, while in 26% of them HSV2 reactivation was found which is in line with prevalence in the literature.

The lipids are key molecules of brain function, which are represented by the association between the abnormal lipid homeostasis and neurodegenerative disorders (i.e. AD). As a novel observation it can be highlighted that HSV1 reactivation promotes the progression of dementia, while HSV2 reactivation facilitates the protective processes, which could be characterized by the alterations of lipid panel. In the group of HSV1 reactivation our observations suggested the imbalance in SULF homeostasis. The results showed

decreasing trend and in SM species presented the same change. The pathological alterations could lead to apoptosis, which was confirmed by the increase of apoptosis indicator PS (d18:1/22:6). In HSV2 reactivated group the compensatory elevation of SULF and SM species were experienced compared with non-reactivated AD control. Parallel with that, the levels of toxic degraded CER and hCER showed increase, but it did not associate with the elevation of apoptotic lipid marker, PS (d18:1/22:6). In HSV1 reactivated patients carrying ApoE4 allele, the pathological SM and SULF decrease were more pronounced compared to non-carrying patients. In HSV2 reactivated patients carrying ApoE4 allele, we found increase in the level of SULF species. Our results referred to the complex dual role of ApoE4 allele. Our promising results are based on experiments carried out on a small number of patients, thus they need further validation.

6. Summarization

According to our results we may say that by our invertebrate model we developed a novel approach in aging and AD-related toxicological research. The model is suitable to provide high-throughput, reliable and sensitive results about the oxidative stress and toxicological regulation of aging. These animals are not able to produce endogenic Aβ1-42. The Aβ1-42 was not toxic to them, in contrast with the Aβ25-35 treatment. The Aβ1-42 dose-dependently expanded lifespan of treated rotifers, meaning the treatment agent was used as an obligatory energy source, suggesting a novel *in vivo* catabolic mechanism.

In our human studies, novel AK-specific biomarker candidate proteins were identified related to neuroinflammation and oxidative stress. In association with HSV1 and HSV2 reactivation our results confirmed the ‘inflammatory-infection’ hypothesis of AD. We pointed out that the pathological changes could be specific and trackable on both proteomic and lipidomic levels. Based on our molecular results we are planning to complete our future experiments with non-invasive methods such as electrophysiological recording of gastrointestinal motility or lacrimal sampling.

7. Acknowledgements

I am grateful to my supervisor, Magdolna Pákáski for her contribution and help during the years of my PhD and the thesis preparation. I would like to thank Professor János Kálmán, who provided me an opportunity to join the Research Group of Department of Psychiatry, University of Szeged. I would like to thank Professor Zoltán Janka, former head of Department of Psychiatry, for his prof-reading of my thesis. Special thank goes to Zsolt Datki for his friendship and his contribution and encouragement during the experiments and preparation of my thesis. I am grateful to my colleagues, especially Eszter Ivitz for their work during the experiments in the Department of Psychiatry, University of Szeged. Last but not the least, I would like to thank my family: my parents and to my boyfriend, Bence Gálik for supporting me both in my life and in my experiment.

This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001 'National Excellence Program' and EFOP-3.6.1-16-2016-00008. This study was contributed by grants from Hungarian Ministry of Education and Culture: OTKA (83667), the TÁMOP -4.2.2.A-11/1/KONV-2012-0052 and the Hungarian Brain Research Program - Grant No. KTIA 13 NAP-A-II/16.

SZEGEDI TUDOMÁNYEGYETEM
Általános Orvostudományi Kar
Szent-Györgyi Albert Klinikai Központ
PSZICHIÁTRIAI KLINIKA
igazgató:
Prof. Kálmán János
tanszékvezető, egyetemi tanár
6725 SZEGED, Kálvária sgt. 57.

DEPARTMENT OF PSYCHIATRY
Albert Szent-Györgyi Clinical Center
Faculty of Medicine
UNIVERSITY OF SZEGED
Head of Department:
János Kálmán
MD PhD DSc
57. Kálvária Ave, SZEGED H-6725

Co-author certification

Co-Author Resignation Statement

Hereby I declare, **Zsolt László Datki, PhD** a corresponding author of the manuscript that in achieving the mutually published scientific results, described in the thesis points of **Zita Oláh** (candidate) PhD dissertation the candidate had a decisive role; therefore, these data were not used or intended to be used by any other candidate in the procedure of obtaining PhD degree.

2018.02.01. .....

Co-Author

Jointly published publications related to the candidate's thesis:

Datki Z, Olah Z, Hortobagyi T, Macsai L, Zsuga K, Fulop L, Bozso Z, Galik B, Acs E, Foldi A, Szarvas A, Kalman J. (2018)

Exceptional in vivo catabolism of neurodegeneration-related aggregates.

Acta Neuropathol Commun. 2018 Jan 29;6(1):6.

doi: 10.1186/s40478-018-0507-3. PMID: 29378654

Included results:

Results of amyloid ELISA measurements

3. figure a: Amyloid and BSA dose dependency

3. figure c: (Arteficial amyloid types) Results of Aβ25-35 toxicity