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Tau protein and its variants in the diagnosis of Alzheimer's disease

Tau protein a jeho varianty v diagnostice Alzheimerovy nemoci

Ph.D. thesis

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Statement of authorship

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Abstract

It is accepted that fibrillar aggregated tau is the best histopathological correlate of the onset and progression of dementia. Tau protein was long regarded as an intracellular protein with several functions inside of cells. New evidence suggests tau secretion into the extracellular space. It is plausible that both intracellular and extracellular forms of tau protein contribute to AD neurodegeneration. The truncated/fragmented forms of tau protein are prone to self-aggregate and form soluble oligomers which are now considered the toxic agents that spread the pathology in AD and other tauopathies. In addition, immunologic abnormalities including defective immune regulation and autoimmunity have been demonstrated in AD patients. Therefore, we have studied the role of various extracellular forms of tau protein and antibodies against them in AD.

Firstly, we showed that antibodies isolated from intravenous IgG (IVIG, product Flebogamma) and plasma of older cognitively healthy persons (controls) were reactive with pathological soluble aggregates (oligomers) of tau protein present in the brain of AD patients. On the contrary, isolated antibodies from the plasma of AD patients revealed reactivity with lower molecular weight (LMW, monomeric) tau forms found in brain tissue. Moreover, the antibodies from control subjects showed strong binding to the fragment of tau (155-421 aa). Thus, our findings with the hypothesis of peripheral sink in mind may indicate the participation of blood antibodies in clearance of the aggregated and truncated tau structures from the brain without the need to cross the blood-brain barrier. However, the levels of anti-tau antibodies itself have not proved as suitable biomarkers of AD.

Secondly, we have found tau oligomers in sera of controls and their levels correlated with aging. On the contrary, the serum tau oligomers in the group of patients with mild cognitive impairment due to AD were lowered in comparison to control subjects. This result may be related to elevated serum levels of tau-reactive antibodies found in this study and/or to impaired clearance of tau protein from interstitium to blood and consequent accumulation of tau aggregates in the brain. By western blot, we found that serum of AD patients contained stable higher molecular weight (HMW) oligomers while in the serum of controls the HMW oligomers were unstable and dissociated into LMW oligomers. We suppose extracellular tau proteins are cleared from the brain to the periphery where are subjected to degradation. In some cases as for the AD pathology, this clearance pathway could fail, thus contribute to form oligomers and spread the pathology.

Abstrakt

Vláknité struktury tvořené proteinem tau byly doposud prokázány jako nejvhodnější histopatologický ukazatel nástupu a rozvoje Alzheimerovy nemoci (AN). Tau protein byl zpočátku považován za intracelulární protein regulující výstavbu mikrotubul. Nejnovější nálezy však prokázaly sekreci tau do extracelulárního prostoru. Ukazuje se, že pravděpodobně, jak intracelulární, tak i extracelulární formy tau přispívají k neurodegeneraci. V poslední době se uvažuje o rozpustných agregátech tau (oligomerech) jako o příčině šíření patologie AN a dalších tauopatií. Kromě toho byly u pacientů trpících AN prokázány i změny v regulaci imunitního systému a projevy autoimunitních poruch. Proto jsme se zaměřili na roli různých extracelulárních variant proteinu tau a anti-tau protilátek ve vztahu k AN.

Nejprve jsme studovali výskyt a charakter přirozeně se vyskytujících plasmatických anti-tau protilátek. Zjistili jsme, že anti-tau protilátky izolované z produktu intravenozních IgG (IVIG, Flebogamma) a z plazmy kognitivně zdravých starých lidí vykazují reaktivitu s patologickými agregáty (oligomery) tau proteinu. Naproti tomu protilátky z plazmy pacientů s AN reagovaly především s nízkomolekulárními (monomerními) formami proteinu tau. Anti-tau protilátky od kognitivně zdravých seniorů a z IVIG navíc silně reagovaly se zkrácenou formou tau (155-421). Z literatury i našich experimentů vyplývá, že zkrácené formy tau podléhají snadno agregaci a tvoří reaktivní oligomery. Výše uvedené koreluje s hypotézou „peripheral sink“, která uvádí, že protilátky v krvi mohou podpořit vyplavování a odstranění agregovaných a zkrácených forem tau proteinu z mozku, aniž by prošly přes hematoencefalickou bariéru.

Následně jsme detekovali samotné oligomery tau proteinu v krevním séru. Pomocí námi vyvinuté metody ELISA jsme zjistili, že se vyskytují tau oligomery v krevním séru zdravých starších lidí, kde se tyto hladiny navíc zvyšují s věkem. Oproti tomu jsme zjistili snížené hladiny tau oligomerů u pacientů s mírnou kognitivní poruchou. Snížené hladiny tau oligomerů mohou souviset se zvýšenou hladinou sérových anti-tau protilátek detekovaných u stejné skupiny lidí anebo také s narušeným vyplavováním tau proteinu z intersticia do krve a následným hromaděním tau agregátů v mozku. Kromě kvantitativního měření jsme pomocí Western blot techniky ohodnotili tau oligomery v krevním séru i kvalitativně. V séru pacientů s AN jsme našli stabilní vysokomolekulární tau oligomery, kdežto u zdravých starších lidí jsme anti-tau oligomerní protilátkou

pozorovali výskyt i nízkomolekulárních oligomerů tau proteinu, které pravděpodobně vznikly disociací nestabilních vysokomolekulárních tau oligomerů.

Tyto nálezy nás vedou k hypotéze, že extracelulární tau může být cíleně odváděn do krevního řečiště, kde je dále degradován. U AN mohou tyto mechanismy selhávat a umožnit tak agregování tau a následné šíření patologie.

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List of abbreviations

aa	amino acid
AD	Alzheimer’s disease
AS	Ammonium sulfate
BBB	Blood brain barrier
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CaMK-II	Calcium/calmodulin-dependent kinase II
cdk5	Cyclin-dependent kinase 5
CFA	Complete Freund’s adjuvants
CNS	Central nervous system
CSF	Cerebrospinal fluid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GLM	Generalized linear model
GSK-3β	Glycogen synthase kinase-3 beta
HMW	Higher molecular weight
HRP	Horseradish peroxidase
IFA	Incomplete Freund’s adjuvants
IgG	Immunoglobulins
IMAC	Immobilized-metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVIG	Intravenous immunoglobulins
LMW	Lower molecular weight
MCI	Mild cognitive impairment
MCI-AD	Mild cognitive impairment due to Alzheimer’s disease
MMSE	The Mini Mental State Examination
MT	Microtubule
MTs	Microtubules

MTBR	Microtubule-binding repeats
NFT	Neurofibrillary tangles
NMDAR	N-methyl-D-aspartic acid receptor
nTau-AD	Naturally occurring antibodies isolated from plasma of AD patients
nTau-Ctrl	Naturally occurring antibodies isolated from plasma of older cognitively normal/healthy subjects
nTau-IVIG	Naturally occurring antibodies isolated from IVIG
O.D.	Optical density
OD	Other neurodegenerations / dementias
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	PBS-0.1% Tween-20 buffer
PC	Positive control
PHF	Paired helical filaments
PKA	cAMP-dependent protein kinase
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
PP2C	Protein phosphatase 2C
R	Repeat domain
RT	Room temperature
SD	Standard deviation
TBI	Traumatic brain injury
TBS-T	Tris buffered saline with 0.1% Tween-20
TMB	3,3′,5,5′-Tetramethylbenzidine

1 Introduction

In connection with the global trend of prolonging human life and the increasing number of older persons in the population, age-related neurodegenerative diseases become one of the most serious health and socioeconomic problems. Neurodegenerative diseases are characterized by progressive dysfunction and death of nerve cells in different parts of the human brain. The death of neurons is connected with specific protein inclusions, and the affected areas of the brain define the clinical picture of the disease (Sergeant et al., 2005). This hallmark of age-related neurodegenerative disorders is evident in tauopathies characterized by the presence of abnormally phosphorylated tau aggregates. Alzheimer's disease (AD), the most common tauopathy, is characterized by two main pathological features in the tissue of the brain: firstly, by extracellular amyloid plaques, which are formed by insoluble amyloid-β and secondly, by intraneuronal neurofibrillary tangles (NFT) containing fibrillar tau protein (Brion et al., 2001; Maccioni et al., 2001). Tau protein was discovered in the mid-70s of the 20th century by studying factors necessary for microtubule (MT) formation (Butner and Kirschner, 1991). Tau protein promotes tubulin assembly into microtubules (MTs), one of the major components of the neuronal cytoskeleton that defines the typical morphology and provides the structural support to the neurons (Kosik, 1993). Physiological binding of tau to tubulin is regulated by its phosphorylation state, which is regulated by the coordinated action of kinases and phosphatases on tau molecule (Liu et al., 2005; Mandelkow et al., 1995). In pathological conditions, as in the case of AD, abnormal phosphorylation of tau protein decreases its tubulin binding capacity leading to MT disorganization. Unbound abnormally phosphorylated tau protein self-polymerizes and continually aggregates through oligomeric and fibrillar states to reach the final form of NFTs (Avila et al., 2004; Brion et al., 2001; Buée et al., 2000; Grundke-Iqbal et al., 1986; Iqbal and Grundke-Iqbal, 2008; Lee et al., 1991). We have chosen to study tau protein because neocortical NFT made out of this protein correlates with cognitive decline in AD patients (Nelson et al., 2012, 2009).

1.1 Structure and physiological functions of tau protein

Tau protein appears in the brain as six isoforms (from 352 to 441 amino acids in length) (Goedert and Jakes, 1990). These differ in numbers of 3 or 4 repeat domains of 31 amino acids (3 or 4R) in the C-terminal part of the protein and 1 or 2 N-terminal inserts of 29 amino acids. The occurrence of isoforms depends on alternative splicing of exons 2, 3 that are coding the N-terminal inserts, and exon 10, coding one repeat domain. Each isoform has a different role during the development of the brain and support of neuronal MT structure in the adult brain (Sergeant et al., 2005). For instance, only one tau isoform, characterized by 3R and no N-terminal inserts, is present during fetal stages, while the isoforms with one or two N-terminal inserts and 3- or 4R are expressed during adulthood (Sergeant et al., 2005).

Tau protein belongs to a group of proteins with very low content of secondary structure that are in common resistant to heat and acid treatment without loss of their function (Cleveland, 1977). In fact, a number of biophysical studies revealed that tau is a prototypical "natively unfolded" protein (Dyson and Wright, 2005; Gamblin, 2005; Jeganathan et al., 2008; von Bergen et al., 2005). Primary sequence analysis demonstrates that tau consists of an N-terminal acidic portion followed by a proline-rich region and the C-terminal tail, which is the basic part of the protein. Tau molecule appears, therefore, like a dipole with two domains of opposite charge, which can be modulated by post-translational modifications (Sergeant et al., 2008).

Tau protein is present to a greater extent in axons from neurons, but it also occurs in the oligodendrocytes (Ebneth et al., 1998). Tau has several physiological functions in the brain. The primary function is to support MT polymerization and their stabilization (Brandt and Lee, 1993; Butner and Kirschner, 1991; Mandell and Banker, 1996). Tau stabilizes MT through C-terminal part of the protein, concretely through repeat domains 3 or 4 called microtubule-binding repeats (MTBR). The strength of the bond and the power of MT stabilization depend mostly on a phosphorylation state of tau protein and number of repeat domains. The 4R tau is more potent in MT stabilization than the 3R form (Butner and Kirschner, 1991; Goedert and Jakes, 1990). In general, the more is the protein phosphorylated, the fewer binds to MT, which also plays a role in intracellular trafficking of cargo along the MT in axons. Tau protein in dephosphorylated state regulates the axonal

transport of molecules by tight binding to MTs and probably detaches the cargoes from MT-dependent motor proteins such as the plus-end-directed motor kinesin and its relatives (Brady and Sperry, 1995; Cuchillo-Ibanez et al., 2008; Ebneth et al., 1998; Lippincott-Schwartz et al., 1995). Except MT stabilization mentioned above, tau interacts with several other molecules by its N-terminal part called projection domain and by proline-rich domain. The N-terminal part of protein projects from MT surface and determines spacing between MTs and may increase the axonal diameter (Couchie et al., 1992; Sergeant et al., 2005). As to the interactions with other cytoskeletal components, tau protein binds to spectrin and actin filaments, which may allow tau-stabilized MTs to interconnect with neurofilaments that restrict the flexibility of the MT lattices. Moreover, tau protein through its N-terminal projection domain may interact with intracellular membranous elements such as the mitochondria (Jung et al., 1993) and the neuronal plasma membrane (Brandt et al., 1995; Pooler et al., 2012). Several studies in cell lines revealed that tau protein bound to the plasma membrane is dephosphorylated (Arrasate et al., 2000; Maas et al., 2000). Tau protein is also associated with Src-family kinases, concretely Fyn kinase. Fyn plays a role in protein trafficking and the dynamic relocalization of tau protein in response to its phosphorylation state to the plasma membrane suggest a possible role of this protein in intracellular signaling pathways. It was recently shown that tau binds to the Fyn in dendritic spines, and this interaction regulates N-methyl-D-aspartic acid (NMDA) receptor signaling (Ittner et al., 2010; Pritchard et al., 2011). Pathological tau may participate in the localization of Fyn kinase to the postsynaptic compartment, where it phosphorylates NMDAR subunits, causing increased inward Ca^{2+} conductance and leading to excitotoxicity (Boehm, 2013). The proline-rich part of tau protein also has a regulation function. This part interacts with Pin1 peptidyl-prolyl cis/trans isomerase, which is responsible for conformational changes in the structure of tau protein that restore the ability of phosphorylated tau to bind microtubules and promote microtubule assembly and leads to dephosphorylation by protein phosphatase 2A (PP2A) (Balastik et al., 2007). Some studies suggest that the cis, but not the trans form of pThr231 tau is pathogenic in AD and that the prolyl isomerase Pin1 is protective because it accelerates the conversion from the cis to the trans form of tau (Nakamura et al., 2013a, 2013b).

1.2 Tau protein pathology

As tau protein is involved in several processes in the brain, it is important to characterize pathways that make this protein lose its physiological functions and become a pathological entity. In AD, tau protein is subjected to the cascade of post-translational modifications. These modifications cause conformational changes in the structure of the protein, its aggregation into the paired helical filaments (PHF) and formation of NFT in neuronal cells. This cascade of events leading to NFT was suggested to begin with a toxic PHF-core (Luna-Muñoz et al., 2007) containing aggregates/oligomers of modified tau proteins (i.e. phosphorylated and cleaved tau protein forms) (von Bergen et al., 2006). These toxic oligomers have a high affinity to intact tau molecules, and the cells in an attempt to hide these oligomers may trigger processes like phosphorylation. In AD, these protective mechanisms lead to increased amounts of tau molecules freed for aggregation and further truncations by activated caspases, which in turn re-expose the toxic PHF-core (Flores-Rodríguez et al., 2015). However, this proposed theory is still under debate, because it is not known what causes the creation of toxic PHF-core. It can be a combination of events in neurons including i.e. oxidative stress as well as a wrong interplay of post-translational modifications and degradation processes of proteins.

1.2.1 Post-translational modifications of tau protein

1.2.1.1 Phosphorylation

The phosphorylation of tau should play a significant role because this modification regulates tau's affinity to MTs, transport along them and trafficking of this protein to the plasma membrane. Tau in the healthy brain has 1.9 moles of phosphate per mole of tau, on the contrary, in AD brain, tau carries 6-8 moles of phosphate per mole of tau (Köpke et al., 1993). It was identified around 80 potential phosphorylation sites on the molecule of tau protein as well as a number of kinases that are believed to play the most important role in phosphorylation of tau in the brain (Sergeant et al., 2005). These include GSK-3β, cyclin-dependent kinase 5 (cdk5), cAMP-dependent protein kinase (PKA), and calcium/calmodulin-dependent kinase II (CaMK-II) (Gong and Iqbal, 2008). Most of these potential sites are located in the vicinity of the MTBR in the proline-rich region and the C-terminal extreme of the molecule of tau protein (Buée et al., 2000; Sergeant et al., 2008).

The exceptions are sites of Ser262, Ser293, Ser324, and Ser356 (motif KXGS) in R1, R2, R3, and R4 domains (Dickey et al., 2007; Drewes et al., 1995). The previously mentioned phosphorylation at Thr231 is one of the earliest because it is detected at all stages of NFT maturation (Augustinack et al., 2002; Jicha et al., 1997). This phosphorylation causes local conformational change that allows the access of GSK-3β or other kinases to phosphorylate tau further (Lin et al., 2007; Sengupta et al., 1998). As previously outlined, in the normal state, the phosphorylation of tau is regulated by opposite actions of kinases and phosphatases. Therefore, the phosphorylation is a reversible process. Phosphatases identified to dephosphorylate tau *in vitro* are PP1, PP2A, PP2B, and PP2C (Avila et al., 2004). The activity of PP2A has been found to be reduced in selected areas of the brain of AD patients (Liu et al., 2005). Thus, one theory is that while developing of AD pathology, tau probably becomes hyperphosphorylated, loses its biological activity and detaches from MTs (Alonso et al., 2001; Bancher et al., 1989). The toxic feature of the pathological hyperphosphorylated tau is the ability to detach normal tau from MTs (Alonso et al., 1994). The hyperphosphorylated tau is accumulated in the cytosol where it can be subjected to other modifications.

1.2.1.2 Truncation

The cascade of tau modifications transforming soluble monomeric tau into pathological aggregates is still elusive. It is under debate what comes first, hyperphosphorylation or truncation of tau protein. Several groups proposed that phosphorylation is a protective mechanism because it is known that this phenomenon also occurs during development and hibernation (Arendt et al., 2003; Bretteville and Planel, 2008). Other studies pointed out to hyperphosphorylation of tau as a response to the underlying pathophysiology and that it functions as an adaptive phenomenon in an antioxidant capacity of cells (Castellani et al., 2008; Li et al., 2007; Nunomura et al., 1999). Some studies are showing that tau phosphorylated at few specific sites, especially N-terminally at Thr231 (Luna-Muñoz et al., 2007), is truncated even before the occurrence of hyperphosphorylation (Flores-Rodríguez et al., 2015; Luna-Muñoz et al., 2007; Rissman et al., 2004). The truncation of tau was already proven to occur early in the formation of NFT. The free cytosolic tau can be cleaved by several proteases. Caspases are cysteine aspartate proteases that are critically involved in apoptosis and are very active during

development of AD pathology (Guillozet-Bongaarts et al., 2006). Rissman et al. proved an involvement of Caspase 3 and 7 in the truncation cascade of tau protein (Rissman et al., 2004). Cleaved-tau at Asp421 was described as the first stage of C-terminal truncation during maturation of NFT, which is followed by cleavage at Glu391 (García-Sierra et al., 2001; Jarero-Basulto et al., 2013; Luna-Muñoz et al., 2007, 2005; Mena et al., 1996). Both truncations were described by monoclonal antibodies against these epitopes, TauC3, and MN423, respectively. It has been proved by several different research groups that C-terminally truncated tau is prone to form aggregates in much faster rate than full-length tau molecule *in vitro* (Berry et al., 2003; T Chris Gamblin et al., 2003; Wischik et al., 1988). In the latest study, Flores-Rodriguez et al. proposed a model for the stages of tau assembly into PHFs in pre-tangle neurons in AD (Flores-Rodríguez et al., 2015). The cascade of forming stable PHFs starts with the occurrence of the minimal PHF-tau core unit (truncated at Glu-391) within the cytoplasm. This minimal fragment can capture intact full-length tau molecules, and that is followed by the occurrence of the N-terminal p-tau epitope and TauC3 antibody reactivity. This stage precedes the appearance of fibrillary inclusions, but as oligomers continue to aggregate, it is followed by Thiazin Red positivity that shows up forming beta-sheets in fibrils. New molecules of tau are continually recruited for truncation and phosphorylation on both N- and C-terminals to develop intracellular tangles. The MN423 reactivity has been seen during all stages of NFT formation (Flores-Rodríguez et al., 2015; García-Sierra et al., 2003; Luna-Muñoz et al., 2007; Mena et al., 1996; M Novak et al., 1993). This cascade takes into count mostly truncation on the C-terminal of the protein tau, but tau molecule can be cleaved on the N-terminal as well. There is a study suggesting that the N-terminal truncation of tau at Lys150 leads to mislocalization of tau to the nucleus (Paholikova et al., 2015). Another study showed tau localization in the nucleus using antibody Tau-66 with discontinuous epitope 155-244; 305-314 (Ghoshal et al., 2001). Physiological tau protein can translocate to the nucleus under stress conditions where it protects DNA from fragmentation (Sultan et al., 2011; Violet et al., 2014). On the other hand, an active mislocalization of tau fragment 151-391/4R to nucleus without stress conditions may modify gene expression levels and has a deleterious effect on neuronal cells (Zilkova et al., 2011). Tau fragments localized in the nucleus are phosphorylated to a lesser extent than fragments in the cytosol (Bukar Maina et al., 2016). Therefore, the

phosphorylation could be regulating mechanism to protect cells from toxic truncated forms of tau protein.

1.2.1.3 Acetylation

During neurodegeneration and maturation of NFT tau undergoes conformational changes and modifications that make it unfavorable for successful degradation processes. Recent studies identified reversible lysine acetylation as a post-translational modification of tau as a possible regulatory mechanism of its functions. Min et al. first identified that tau protein in NFT is also acetylated and that the acetylation of tau may directly contribute to the accumulation of phospho-tau (Min et al., 2010). Acetylation of tau protein in KXGS motif of MT domain was described to decrease binding affinity of tau to MT, but also the ability to self-polymerize. The acetylation of lysine residues competes with the ubiquitination of tau that exacerbates the possibility of tau to be degraded by the ubiquitin-proteasome pathway. Interestingly, it has also been shown that phosphorylation counteracts acetylation on KXGS motif (Choudhary et al., 2009; Kouzarides, 2000; Yang and Seto, 2008). All this together could result in tau that could not be degraded appropriately and thereby creates more toxic forms of this protein.

1.2.1.4 Aggregation

The most important step in the evolution of tau pathology is an aggregation of modified forms of tau protein. Aggregation of full-length form of tau protein depends on concentration and must be driven by other molecules that facilitate the aggregation process like polyanions e.g. poly-Glu, heparin and arachidonic acid used *in vitro* (Barghorn and Mandelkow, 2002; King et al., 2000) or amyloid seeds (Lasagna-Reeves et al., 2012, 2010; Morozova et al., 2013). In the normal state, aggregation of tau protein is protected by C- and N-terminals, which are folded in paper clip-like appearance over MTBR (Jeganathan et al., 2008). Post-translational modifications like phosphorylation and truncation make tau molecule prone to aggregation by exposing the MTBR domains containing VQIINK motifs. The 4R isoform of tau containing two VQIINK motifs is much more prone to aggregation than 3R form (Alonso et al., 2001). The formation of tau oligomers is probably facilitated by phosphorylation at Thr231. Lasagna-Reeves et al. showed that this modification precedes the formation of tau oligomers (Lasagna-Reeves et al., 2012). Aggregated tau

forms, soluble tau oligomers rather than fibrillar aggregates (Berger et al., 2007; Brunden et al., 2008; Kayed and Jackson, 2009; Meraz-Ríos et al., 2010; Morsch et al., 1999; Santacruz et al., 2005; Spires-Jones et al., 2011; Sydow et al., 2011; Wittmann et al., 2001) are now thought to be the most toxic agents in the developing pathology of AD and other tauopathies. Oligomers and small soluble aggregates of tau protein form a minimal PHF-core and are proven to propagate pathology in template-like fashion in distally connected areas of the brain (Morozova et al., 2013; Sanders et al., 2014; Falcon et al., 2015; Taniguchi-Watanabe et al., 2016). AD is now considered to be a disorder with the prion-like mechanism of pathology spreading (Ashe and Aguzzi, 2013; Brundin et al., 2010; Clavaguera et al., 2015; Marciniuk et al., 2013; Walsh and Selkoe, 2016; Yin et al., 2014).

1.2.1.5 Propagation of tau pathology

Tau protein was long regarded as an intracellular protein with several functions inside of cells. New evidence suggests tau secretion into the extracellular space (Fá et al., 2016). At first, the occurrence of tau in the interstitial and the cerebrospinal fluid was believed as a consequence of dying neurons and their released cell content. Lately, several proteins lacking signal peptide were shown to be secreted by unconventional pathways (Backhaus et al., 2004; Liu et al., 2014; Yang et al., 2011). This is also evident for tau protein. Several research groups, employing cell cultures and transgenic mouse models, have shown tau to be actively secreted from cells in membrane-free “naked” form (Chai et al., 2012) or included in microvesicles/exosomes (Saman et al., 2014, 2012; Simón et al., 2012). The active secretion of tau could be a physiological response of neurons to increase intracellular amounts of tau during the progression of tau pathology. Therefore, secretion has been proposed as a mechanism to eliminate the excess of tau protein thereby avoiding its toxicity (Simón et al., 2012). Once tau secreted extracellularly, it can be taken up by other connected neurons. Several studies proved propagation of tau pathology trans-synaptically (de Calignon et al., 2012; Dujardin et al., 2014; Liu et al., 2012). Another group showed that modified tau forms are accumulating at the pre and post-synaptic terminals in the AD brain (Tai et al., 2012). It remains unknown which forms of tau can be endocytosed by recipient cells. While some groups have shown uptake of toxic aggregates of tau protein and others even full-length forms of tau, a growing number of studies indicate that only pathological tau can induce the seeded transmission and spread of pathology (Boluda et al., 2015; Guo

and Lee, 2011; Jucker and Walker, 2013). Moreover, a recent study showed *in vivo* and *in vitro* that pathological tau can acquire different conformations that propagate tau pathology and thus these different conformers can cause different tauopathies (Morozova et al., 2013; Sanders et al., 2014). Therefore, AD is considered to be a protein conformational disorder.

Taken together it is plausible that both intracellular and extracellular forms of tau protein contribute to AD neurodegeneration. The current state of research reviewed above demonstrates that tau protein is a ubiquitous, highly dynamic, potentially broad range functional protein whose functions and localization are altered in neurodegenerative disease (for thorough review see (Kolarova et al., 2012)).

1.3 Auto-antibodies in Alzheimer's disease

Over the past decade, there is an ongoing debate if AD can be a consequence of autoimmune processes as well. Immunologic abnormalities including defective immune regulation and autoimmunity have been demonstrated in AD patients (Fillit et al., 1987; Foley et al., 1988; Gaskin et al., 1987; Leffell et al., 1985; Michaelson et al., 1989; Singh et al., 1986; Skias et al., 1985). Numerous reports of the presence of autoantibodies against neuronal and non-neuronal antigens in sera of AD patients were published (Bahmanyar et al., 1983; Bartos et al., 2012; D'Andrea, 2005; Ounanian et al., 1990; Rosenmann et al., 2006; Terryberry et al., 1998; Watts et al., 1981). To date, the research group of Nagele at Rowan University School of Osteopathic Medicine has done the most comprehensive search for auto-antibodies in sera of AD patients and healthy individuals. They have shown that auto-antibodies are surprisingly numerous in human sera regardless of age and disease (Levin et al., 2010; E. Nagele et al., 2011; R. G. Nagele et al., 2011). It remains unknown whether the function of natural antibodies in AD is a protective mechanism or contributes to pathology. While these natural auto-antibodies may play a protective role against microbial infections in individuals with an intact blood-brain barrier (BBB) (Diamond et al., 2009), the same auto-antibodies can enter the brain and bind to the neuronal cells (Levin et al., 2010) if the BBB is impaired. Invasion of these antibodies to the brain parenchyma can activate inflammatory cells, astrocytes and glial cells, which results in nerve damage (D'Andrea, 2005; Diamond et al., 2009) and consequently release of neuronal proteins. There are several reports of immunoglobulins bound to neurons in AD (Clifford et al., 2007;

Franceschi et al., 1989; Loeffler et al., 1997; McRae-Degueurce et al., 1987). In fact, the intact BBB prevents brain antigens to participate in B-cell maturation process. Thereby, B-cells producing antibodies cross-reacting with brain antigens are not silenced by the regulatory mechanism of the immune system as for other self-antigens. As a consequence, the immune system does not have a mechanism to establish tolerance to brain antigens and can not stop the production of antibodies against them when the BBB is disrupted (Levin et al., 2010). Except serum antibodies, cerebrospinal intrathecally synthesized specific antibodies against brain antigens related to AD may be a potential indicator of ongoing pathological processes in the central nervous system (CNS) (Bartos et al., 2012). Moreover, intrathecal synthesis of antibodies accounts for the integrity of blood–CSF barrier function because these antibodies are produced locally within the CNS compartment (Bartos et al., 2012; Deisenhammer et al., 2006).

However, not all of these auto-antibodies must trigger pathological reactions in the brain (Diamond et al., 2009). Naturally occurring antibodies specific against toxic protein aggregates are desired to participate in the removal of these inclusions. This effect is now closely studied as a therapeutical approach to neurodegenerative diseases. Several research groups focus on antibody treatment of AD using monoclonal specific antibodies or polyclonal intravenous immunoglobulins (IVIG) (Castillo-Carranza et al., 2015; Dodel et al., 2004; Gu et al., 2013; Kayed, 2010; Knight and Gandy, 2014; Kontsekova et al., 2014; Sigurdsson, 2009; Steinitz, 2009; Wang et al., 2016). The IVIG products attracted a lot of attention as a possible treatment of neurological disorders (Fuchs et al., 2008) and even AD (Dodel et al., 2010; Kile et al., 2015; Neff et al., 2008; Relkin, 2014) in spite of the fact that the precise mode of its action is not known. Nonetheless, it includes modulated expression and function of Fc-gamma receptor, interference with the complement activation pathway, impact to the cytokine network, and provision of anti-idiotype antibodies, the catabolism of antibodies and neutralization of auto-antibodies (Seite et al., 2008; Stangel and Pul, 2006). However, the clinical trials for treatment of AD failed or missed its primary endpoints for two of the IVIG products, Octagam and Gammagard (Baxter U.S., 2013a, 2013b; Dodel et al., 2013; Relkin et al., 2017). Although, they found a dose-dependent increase in levels of antibodies related to amyloid present in Baxter's IG as measured in the CSF (anti-oligomer and antifibril antibodies) and reduction in plasma

levels of amyloid beta ($A\beta_{1-42}$) (Baxter U.S., 2013b; Relkin et al., 2017). Another two clinical trials with Flebogamma® from Grifols (AMBAR, phase III, NCT01561053) and Sutter Health's IVIG NewGam™ (phase II, NCT01300728) are still under investigations (Grifols Biologicals Inc., 2015; Kile et al., 2015). The NewGam™ IVIG trial reported that a short course of IVIG administered in the MCI stage of AD reduces brain atrophy, prevents cognitive decline in late stage of MCI and delays conversion to AD dementia for at least 1 year (Kile et al., 2015). In the light of these findings, the characterization of specific antibodies against either amyloid beta or tau protein present in these products (Balakrishnan et al., 2010; Dodel et al., 2004; Klaver et al., 2013; Smith et al., 2013, 2014a) could clarify their contribution to the effect of these treatments. Notably, when we consider the use of AD-specific IVIG preparations that would be enriched for antibodies specific for tau, amyloid-beta, complement, cytokines and other factors as was discussed by (Loeffler, 2014).

Nevertheless, naturally occurring antibodies against brain antigens circulating in serum and CSF could be useful biomarkers of developing neurodegeneration.

2 Aims of the thesis

Tau protein is strongly associated with AD because it forms the main part of pathological inclusions called neurofibrillary tangles (NFT) in the brains of AD patients. Moreover, the progress of neurodegeneration and a decline of cognitive functions of AD patients correlate with loads of NFT. Tau protein, conventionally regarded as intracellular, can be secreted in the healthy brain from active neurons into the brain interstitium. Above that tau is released after traumatic brain injury or during AD pathology by dying neurons into the extracellular space in the brain and consequently may appear in the cerebrospinal fluid (CSF) and blood. Once tau secreted extracellularly, it is most probably further modified by truncation and other post-translational modifications, and that can be recognized by the immune system as a toxic antigen. Recent findings showed the occurrence of antibodies against tau protein in the blood of healthy people. They found these naturally occurring antibodies even in intravenous immunoglobulins products.

Here we focus on the immune response to the occurrence of extracellular tau protein.

The aims of this thesis were **1)** to characterize reactivity of naturally occurring auto-antibodies against various forms of tau protein in relation to developing AD pathology. **We hypothesized that patients with mild cognitive impairment and dementia due to AD could have elevated levels of antibodies specific for pathological tau forms**. **2)** We aimed to look into levels of tau modified forms in different biofluids as possible biomarkers of AD. **We expected elevated levels of tau oligomers in biofluids of AD patients.**

The specific aims of this thesis:

- Preparation of various recombinant forms of tau protein
- Preparation and characterization of antibodies against tau protein
  - Rabbit polyclonal anti-tau antibody
  - Polyclonal tau-reactive antibodies isolated from human plasma
- Developing of ELISA method for establishing tau-reactive antibodies levels in different body fluids
- Optimization of ELISA method for measurement of tau oligomers in serum

3 Materials and methods

3.1 Reagents

Most reagents used in this study were obtained from suppliers as described previously (Bartos et al., 2012; Hromadkova et al., 2015; Kolářová, 2011; Kristofikova et al., 2014).

3.2 Preparation of recombinant tau forms

Three recombinant tau proteins were prepared for this study: full-length human tau 1-441 amino acids (tau 1-441) and two truncated human forms (human tau 155-421 and human tau 13-391). Plasmids containing vectors of six-histidine-tagged recombinant human tau proteins were transformed into bacteria Escherichia coli strain BL21 (DE3) and proteins were prepared according to (Carmel et al., 1996; Hromadkova et al., 2015; Kolářová, 2011; Lasagna-Reeves et al., 2010) with slight modifications. Briefly, protein expression was induced with 0.5 mM IPTG at an OD_{600} of 1.0-1.4 for 4 hours. Bacterial cultures were then spun for 10 min at 2,500 × g. Pellets were resuspended in 50 mM phosphate buffer (pH 8.0), 1 M NaCl and sonicated 6 x 30 sec at power setting six using Vibracell Bioblock 72442 (Fisher Scientific, France). Bacterial debris was pelleted for 20 min at 12,800 × g at 10°C and extracted proteins in 50 mM phosphate buffer (PB) pH 8.0 with 1 M NaCl were purified on affinity column with Ni Sepharose 6 Fast Flow resin (GE Healthcare, Stockholm, Sweden). Extraction of the same pellet of bacteria was repeated four times. After thorough washing of column with 50 mM phosphate buffer (pH 8.0), 0.3 M NaCl, 20 mM imidazole and subsequently by 50 mM phosphate buffer (pH 8.0), 1 M NaCl, tau protein was eluted in 10 ml of 50 mM phosphate buffer (pH 8.0), 0.1 M EDTA. The eluted protein molecules were either precipitated by an equal volume of cold methanol overnight on ice (His-tagged tau 155-421) or proceeded to next purification step (His-tagged tau 1-441 and 13-391). The precipitated proteins were pelleted for 30 min at 2,000 × g at 4°C. Pellet was washed with 5 ml of 55% methanol, 2 mM DTT, spun as previously described and stored in 75% methanol and 2 mM DTT in -40°C. The purity and stability of prepared tau forms were evaluated by glycine SDS-PAGE electrophoresis. After dissolving the precipitate in distilled water or PBS buffer pH 7.4, the final protein concentration was determined by the BCA assay (Pierce, Rockford, Illinois, USA) using

bovine serum albumin as standard according to the manufacturer's instructions.

The eluted protein solution from immobilized-metal affinity chromatography (IMAC) was desalted using centrifugal filter unit (AMICON-Ultra 30K) from Millipore (Darmstadt, Germany). The desalted concentrate was diluted with 50 mM phosphate buffer pH 7, 2 mM DTT, 0.05% NaN_3 and loaded onto ion exchange chromatography column Mono-S 5/50 GL from GE Healthcare (Stockholm, Sweden) using 10 ml superloop (GE Healthcare, Stockholm, Sweden) by flow 0.5 ml/min of 50 mM phosphate buffer pH 7, 2 mM DTT, 0.05% NaN_3. The separation included gradient steps of increasing NaCl concentration using 50 mM phosphate buffer pH 7, 1 M NaCl, 2 mM DTT, 0.05% NaN_3 at flow 0.5 ml/min (see Fig. 1). 34 sample fractions (in the volume of 1.5 ml) were collected using FC203 fraction collector from Gilson (Middleton, USA). The samples were analyzed by glycine SDS-PAGE electrophoresis and immunoblotting using specific antibodies.

3.3 SDS-PAGE electrophoresis and Western blotting assays

3.3.1 Quality control of purification process of recombinant proteins

For quality control of ion exchange chromatography separation, the glycine SDS-PAGE was carried out. 30 µl of each fraction was diluted with 30 µl of 2× non-reduction sample buffer (60 mM Tris/HCl pH 6.8, containing 2% SDS, 0.1% bromophenol blue, and 25% glycerol). The samples (20 µl of each) were gel-loaded in parallel with prestained protein molecular weight markers. The separated proteins were electrotransferred to nitrocellulose membrane sheets 0.2 µm (Bio-Rad, California, USA) using a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). To visualize the protein(s) in question, the membrane was processed at room temperature by an immunoblotting procedure coupled to an enhanced chemiluminescence (ECL) detection system. Briefly, the treatment steps included: blocking with 10% non-fat dried milk in PBS-0.1% Tween-20 buffer (PBS-T) for 2 hrs, incubation with Tau 5 or Tau 46.1 antibodies in dilution 1: 60,000 (generous gift from Dr. Francisco Garcia-Sierra, Mexico) in 1% BSA in PBS-T overnight, thorough washing with PBS-T (5 times, each 5 minutes) and incubation with 1:10,000 diluted polyclonal rabbit anti-mouse secondary antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark) in 1% BSA in PBS-T for 1 hr. The membrane was washed five times (each 5 minutes) before adding Western bright ECL substrate (Advansta,

Menlo Park, California, USA) and visualized by Molecular Imager ChemiDoc XRS+ with ImageLab software from Bio-Rad (California, USA).

3.3.2 Characterization of plasma naturally occurring antibodies against native proteins

To characterize the reactivity of isolated plasma antibodies, sections of left hemisphere hippocampi from two AD patients and two controls were homogenized in PBS or PBS containing 2% SDS in 1:3 w/v dilution. 25 µg of total protein/lane was loaded onto the Mini-Protean TGX 10% precast gels, and separated proteins were transferred onto the nitrocellulose membrane. Membranes were blocked with 10% non-fat dried milk in PBS with 0.1% Tween-20 (PBST) and incubated overnight at 4°C with primary antibodies diluted with 1% BSA in PBST; rabbit polyclonal anti-tau antibody (1:250), mouse monoclonal Tau 5 antibody (1:60,000), isolated plasma anti-tau antibodies (1:125), rabbit polyclonal anti-pSer396 tau antibody (1:1,000; GenScript, Piscataway, New Jersey, USA) and phospho-PHF-tau pThr231 antibody (AT180, 1:2,000; Thermo Scientific, Waltman, Massachusetts, USA). After washing steps, incubation for 1 hour at RT with secondary antibodies; anti-rabbit or anti-mouse antibody HRP conjugates (1:15,000; Sigma-Aldrich, St. Louis, Missouri, USA), anti-human IgG HRP conjugate (1:10,000; Novex, Life Technologies, Carlsbad, California, USA) and chemiluminescence detection by WesternBright ECL HRP substrate followed.

3.3.3 Detection of tau oligomers in human serum and cerebrospinal fluid

Western blot analysis of randomly selected CSF and sera samples was carried out to see the distribution of tau forms detected by antibody T22. Each lane was loaded with 5 µg (CSF) or 50 µg (serum) of total protein from one sample on precast NuPAGE 10% Bis-Tris Gels for SDS-PAGE (Invitrogen, Carlsbad, California, USA) and subsequently transferred onto nitrocellulose membrane. After blocking overnight at 4°C with 10% nonfat dried milk in 1 × Tris-buffered saline with 0.1% Tween-20, pH 7.4 (TBST), membranes were probed for 1 h at room temperature with T22 (1:150) and anti-albumin antibody (1:2,000; Bethyl, Montgomery, Texas, USA) diluted with 5% nonfat dried milk. T22 was detected with HRP-conjugated anti-rabbit IgG (1:3,000; GE Healthcare, Stockholm, Sweden) and anti-albumin

antibody with HRP-conjugated IgG anti-goat secondary antibody (1:3,000; Southern Biotech, Birmingham, Alabama, USA). ECL Plus (Pierce, Rockford, Illinois, USA) was used for signal generation. For protein quantification, the densitometry of each band was measured using ImageJ software and normalized with albumin. All densitometry results represent the mean ± SD.

3.4 Preparation of antibodies

3.4.1 Isolation of naturally occurring polyclonal tau-reactive antibodies from human plasma

Antibodies against tau protein from the Flebogamma IVIG product (DIF 50 mg/ml, Grifols Biologicals Inc., Los Angeles, California, USA), from the pool of plasma from seven older cognitively healthy subjects (total protein 108.5 mg/ml) or pool of plasma from four patients with AD (total protein 117 mg/ml) were purified by low-pressure affinity chromatography. Firstly, we isolated tau-reactive antibodies from IVIG (nTau-IVIG-1) using column prepared as follows: 25 mg of His-tagged tau 1-441 protein was reacted with 5 ml of pre-packed resin (Labiomer 300, epoxy-activated, 50 µm bead size (Prague, Czech Republic) in Econo-Pac column of 14 cm length and 1.5 cm diameter (Bio-Rad, Hercules, California, USA)), residual reactive epoxide groups were blocked with 0.2 M ethanolamine overnight at 4°C and then the column was equilibrated with PBS buffer (Hermanson, 2013). Second isolation of natural tau-reactive antibodies (nTau-IVIG-2, nTau-AD and nTau-Ctrl) was performed with new column coated with 4 mg of His-tagged tau 1-441 aa protein that reacted with 2 ml of pre-packed Labiomer 300 resin . A volume of 8 ml (for the first isolation) and 3 ml (for the second isolation) IVIG (Flebogamma) / 3ml of plasma pool diluted to 10 ml with PBS was loaded onto the column by flow 0.1 ml/min, and the flow-through fraction was collected in amounts of 15 ml. The column was washed with 45 ml PBS buffer, and captured antibodies were eluted using 10.5 ml 0.1 M glycine-HCl buffer, pH 2.6. The eluted fraction was immediately neutralized by 1 M Tris-base until the pH reached a value of 8-9. Purified antibodies were then concentrated in centrifugal filter units (AMICON-Ultra 30K) in a final volume of 2 ml. The isolated antibodies from IVIG (first isolation; nTau-IVIG-1) were stored in PBS pH 7.2 with 50% glycerol at a concentration of 0.8 mg/ml at -20°C. The antibodies isolated from IVIG (second isolation- nTau-IVIG-2;

5.1 mg/ml), cognitively healthy subjects (2.51 mg/ml) and AD patients (1.96 mg/ml) were stored in PBS pH 7.2 with 0.05% NaN_3 at 4°C. The amount of protein in the initial fractions, flow-through (F-T) fractions and concentrated eluted fractions (before the addition of glycerol) was determined by BCA assay according to manufacturer's instructions (Thermo Scientific, Waltman, MA, USA).

3.4.2 Production of rabbit polyclonal anti-tau antibody

The polyclonal anti-tau antibody was prepared by immunization of 3 male rabbits (Oryctolagus cuniculus f. domesticus, each 2-3 kg, 10 months old) from an accredited breeding according to §17 Act no. 246/1992 Coll. Immunization protocol was as follow: the first injection was prepared with Complete Freund's adjuvants (CFA) (Sigma-Aldrich (St. Louis, Missouri, USA) and contained 1 mg/ml of partially (IMAC) purified His-tagged full-length form of tau (His-tag tau 1-441). The emulsion of protein (0.5 – 0.7 ml/ animal) was injected s.c. between the shoulder blades of each rabbit. Second and each other boost injection were carried out with Incomplete Freund's adjuvants (IFA) except the last injection when the protein was dissolved in saline. For detailed immunization schedule see Table 1.

Table 1: *Immunization schedule*

Immunization schedule	Injected/ collected volume	Amount of protein
1. injection	28.3.2012 0.5-0.7 ml in CFA/animal	~ 0.5 mg/animal
2. injection	18.4.2012 0.5 ml in IFA/animal	~ 0.5 mg/animal
1. blood collection	24.4.2012 0.5 ml/animal	----------------
3. injection	10.5.2012 0.5 ml in IFA/animal	~ 0.5 mg/animal
2. blood collection	16.5.2012 0.5 ml/animal	----------------
4. injection (final)	31.5.2012 0.5 ml/animal	0.3 mg/animal
Collection of blood (bled)	6.6.2012 70-80 ml/animal	----------------

The rabbits were bled by cardiac puncture; the blood was left to coagulate overnight at 4°C and subsequently spun for 30 minutes at 1,000 × g at 4°C. The supernatant (blood serum) was stored at -80°C until further analysis. Immunization, blood collection and

preparation of sera were implemented at Biology control department, Institute of Physiology of the CAS, v. v. i. The antibody formation during immunization period was analyzed by ELISA assay (Chapter 3.6.1).

3.4.3 Purification of rabbit polyclonal anti-tau antibody

A 40 ml of sera from one rabbit was precipitated with 50% ammonium sulfate (AS) at 4°C overnight, and the precipitate was spun at 2,000 × g for 25 minutes at 4°C. The pellet was resuspended in 40 ml of 50% AS and stored at 4°C. A 5 ml of suspension was spun as above, and the pellet was dissolved in 20 ml of PBS pH 7.4. The concentration of IgG pool was determined spectrophotometrically at an absorbance of 280 nm with PBS buffer run in parallel as a blank. Specific anti-tau antibodies were isolated as previously described in Chapter 3.4.1 with one purification step added. This step was included to remove antibodies against His-tag. Briefly, 20 ml of antibodies was applied to the column (5 mg of protein per ml of resin) by flow 0.1 ml/min and the eluted fraction was collected (11 ml). The eluted fraction of specific anti-tau IgGs was additionally purified on the column containing bacterial proteins from E. coli reactive to IMAC column. The column was prepared as previously described (see Chapter 3.4.1): 1.5 mg of proteins from non-induced bacteria carrying plasmid for tau, which was captured on IMAC column, was reacted with 3 ml of resin. The flow-through fraction (22 ml) and eluted fraction (7 ml) were collected. The flow-through fraction containing anti-tau antibodies was concentrated in centrifugal filter unit (AMICON 30K) to a volume of 1.2 ml and stored in PBS pH 7.4 at a concentration of 1.7 mg/ml at 4°C. In both fractions, the final concentration was determined as described above.

3.5 Dot blot immunoassay for antibody avidity determination

Three different unphosphorylated recombinant tau proteins: the full-length form tau 1-441 and fragments tau 155-421, tau 13-391 and commercial tau 1–441 from rPeptide (for each: 1 μg and 0.5 μg/100 μl 0.1 M PBS pH 7.0) were spotted onto PVDF membrane using a Dot-blot DHM-96 unit manifold and incubated in Opti-4CN kit blocking solution in PBS-T for 1 h at RT. Anti-tau antibodies isolated from IVIG (1:250), plasma of older cognitively normal subjects (1:150), plasma of AD patients (1:150) or rabbit polyclonal anti-tau antibody (1:250) were added in PBS-T with 1% BSA and incubated with the membrane

for 1 h at RT. The unbound IgG molecules were removed by PBS-T washing three times. For avidity evaluation, 5 min incubation step with NH_4SCN in PBS pH 7.0 within molarity range 0–2.1 M was included after washing of unbound IgG molecules. The membrane was washed three times with PBS-T, followed by incubation with goat anti-human IgG antibody HRP-conjugate (Novex, Life Technologies, Carlsbad, California, USA) / goat anti-rabbit antibody HRP-conjugate (Sigma-Aldrich, St. Louis, Missouri, USA) (1:1,000) in PBS-T with 1% BSA for 1 h at RT. Subsequent washing with PBS-T three times followed. The spots were visualized by Opti-4CN kit according to the manufacturer's instructions (Bio-Rad, Hercules, California, USA). ChemiDoc™ XRS+ Imaging System with Image Lab™ Software was applied for documentation and spot density analysis.

3.6 ELISA assays

3.6.1 Verification of rabbit anti-tau antibody production

The wells of Maxisorp NUNC microplates (Thermo Scientific, Waltham, Massachusetts, USA) were coated with 0.5 µg/well His-tagged tau 1-441 form in 0.1 M bicarbonate buffer pH 9.5 overnight at 4°C, then blocked with 1% BSA in bicarbonate buffer pH 9.5 for 2 hours at RT and subsequently washed three times with 0.3 ml/well 0.15 M NaCl, 0.05% Tween-20, 0.0001% Thiomersal. Rabbit serum samples were serially diluted as follow: 1:2,000; 1:8,000; 1:32,000; 1:128,000; 1:512,000; 1:2,048,000 (0.000048-0.05 µl/well) by 1% BSA in PBS pH 7.2. 0.1 ml/well of diluted sera was applied to wells and incubated 2 hours at RT. Microplates were washed five times and secondary swine anti-rabbit antibody conjugated with HRP (Dako, Glostrup, Denmark) at dilution 1:10,000 in 1% BSA in PBS was added for 1 hour at RT. This step was followed by five times washing, and then 0.1 ml/well of TMB substrate was applied for 15 minutes at RT in the dark. The reaction was stopped using 0.1 ml/well 1 M H_2SO_4, and the absorbance was measured at 450 nm with 620 nm as reference wavelength.

3.6.2 Isolation efficacy of natural human plasma anti-tau antibodies

Levels of antibodies against four forms of tau protein (tau 1–441 rPeptide, His-tagged tau 1–441 and truncated tau 155–421 and tau 13–391 protein forms) in both unphosphorylated/phosphorylated states were measured in the initial fraction, flow-through fraction and eluted fraction by ELISA. All samples were measured in duplicates.

Tau antigen in 0.1 M carbonate buffer pH 9.5 was applied to microplate (0.1 µg/50 µl per well) and incubated overnight at 4 °C. Then the wells were blocked with 1% BSA in PBS-T for 1 h at room temperature (RT). Subsequently, the microplate was washed 3 times with 0.1% BSA in PBS-T. Initial fraction, flow-through fraction and eluted fraction were serially diluted 1:100 - 1:72,900 for IVIG-1, 1:100 – 1: 6,400 for IVIG-2, plasma from AD patients and older controls by 1% BSA in PBS-T. The experiment was repeated with adjustment of initial Ctrl plasma pool, F-T and eluted fractions to dilutions 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1,600 by 1% BSA in PBS-T. Diluted antibodies (0.1 ml/well) were added to the plate and incubated 2 h at RT. The unbound molecules were removed by five times washing with 0.1% BSA in PBS-T. Incubation with 0.1 ml/well of goat anti-human IgG antibody HRP-conjugate (Novex, Life Technologies, Carlsbad, California, USA) at dilution 1:10,000 for 30 min at RT followed and then the microplate was washed five times as above. Final incubation with 0.1 ml/well of TMB substrate was performed for 30 min at RT in the dark. The reaction was stopped by 0.1 ml of 1 M H_2SO_4 per well, and the absorbance was measured by Elisa Reader Multiskan EX (Thermo Scientific, Waltham, Massachusetts, USA) at 450 nm and 620 nm as a reference wavelength. The non-specific binding of antibodies to empty blocked wells was evaluated for initial and eluted fractions. There was non-specific binding present in the lowest dilution of initial fractions (highest O.D.= 0.254), but it was negligible in the isolated (eluted) antibodies (O.D.= 0.057). The non-specific signal was subtracted from the signal obtained from coated wells.

3.6.3 Measurement of levels of naturally occurring antibodies in serum and cerebrospinal fluid

Levels of antibodies against various tau antigens (bovine tau protein (Cytoskeleton, Denver, Colorado, USA), recombinant human His-tagged tau 1-441 and truncated tau 155-421 protein forms) were measured by ELISA in serum and CSF samples. All samples were measured in duplicate. Tau antigens in 0.1 M sodium bicarbonate buffer pH 9.5 were coated onto wells of microplate from Gama Group (Ceske Budejovice, Czech Republic) (0.05 ml of 2.5 µg/ml per well) and incubated overnight at 4°C. The content of wells was removed, and all wells were blocked with 0.25 ml/well 1% BSA in PBS-T pH 7.2 for 1 hour at room temperature (RT). Then, the plates were washed three times with 0.3 ml/well of 0.1% BSA in PBS-T. Subsequently, 0.05 ml/well of serially diluted positive control (nTau-IVIG-1),

serum samples and undiluted CSF samples was applied to the wells and incubated 2 hours in RT. The positive control was diluted in 1% BSA in PBS-T in the range from 0.05 to 36 μg/ml (stock concentration was 0.8 mg/ml) for generation of a standard curve. Each serum sample was diluted 1:200, 1:600 and 1:1,800 by 1% BSA in PBS-T. The unbound primary antibodies were removed by five times washing and the incubation with 0.1 ml/well of secondary antibody goat anti-human IgG antibody HRP-conjugate (Novex, Life Technologies, Carlsbad, California, USA) at dilution 1: 20,000 for 30 minutes at RT followed. After five washes as above, the final incubation with TMB substrate for 30 minutes at RT in the dark was carried out. The developing color signal was stopped by 0.1 ml/well 1 M H_2SO_4 and the absorbance was measured during 30 min after stopping by a Multiskan EX ELISA reader (Thermo Scientific, Waltham, Massachusetts, USA) at wavelength 450 nm and with 620 nm as reference wavelength. The concentrations of tau-reactive antibodies were interpolated from the standard curve using GraphPad Prism software and for serum samples adjusted to the dilution factor. The non-specific binding of antibodies to empty blocked wells was evaluated for each sample. The non-specific signal was subtracted from the signal obtained from coated wells.

3.6.4 Determination of levels of tau oligomers in serum and cerebrospinal fluid

In collaboration with M.S. Urmi Sengupta in the laboratory of prof. Rakez Kayed (Department of Neurology, and Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX, USA), we optimized an ELISA assay for measurement of tau oligomers/conformers in human sera using specific antibody T22/TTC-99, respectively. The wells of Polysorp NUNC microplates (Thermo Scientific, Waltham, Massachusetts, USA) were coated overnight at 4°C with 20 μl of 1:20 diluted subject´s sera or cerebrospinal fluid using 0.05 M bicarbonate buffer pH 9.5. The plates were washed one time with 1 × TBST and blocked with 10% nonfat dried milk in TBST for 2 hours at room temperature. Blocked plates were washed one time and treated with T22 (1:100) or TTC-99 (1:100) diluted in 5% nonfat milk in TBST for 1 hour at room temperature. Subsequently, the plates were washed three times and incubated with 100 μl of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (GE Healthcare, Stockholm, Sweden) diluted 1:3,000 in 5% milk TBST for 1 hour at room temperature. Finally, plates were washed three

times with TBST and incubated with 100 µl TMB+ substrate chromogen (Dako, Glostrup, Denmark) for 6 minutes at room temperature in the dark. The reaction was stopped by 100 µl of 2 M HCl, and the plates were read at 450 nm in a POLARstar Omega plate reader (BMG Labtech, Cary, North Carolina, USA). *In vitro* prepared tau oligomers (Lasagna-Reeves et al., 2010) were included on each plate as a positive control and used for generation of the standard curve. However, the precise determination of oligomers concentration was difficult due to variations of dye binding. Therefore, the ELISA is indicated as semi-quantitative with arbitrary units (AU/ml). The interassay variation between plates was calculated from 18 measurements for each antibody. The variation was 10 % and 13 % for T22 and TTC-99 antibody, respectively. All samples were measured in duplicates. In the following statistical analyses, we have dealt with values below the detection limit of ELISA (O.D. 0.107 for T22 and 0.123 for TTC-99) by assigning them a value of the limit of detection (Armbruster 2008). Negative control (primary and secondary antibodies added to non-coated blocked wells) was measured on each plate. Randomly selected samples from each group of participants were probed only with secondary antibody to control non-specific signal. The non-specific signal did not exceed the limit of detection.

3.7 Participants and samples collection

All experiments are in accordance with The Declaration of Helsinki. The research was formally approved by the local ethics commission of the Prague Psychiatric Center, Prague/ National Institute of Mental Health, Klecany, Czech Republic, and is in agreement with Laws 129/2003 and 130/2003.

Serum/plasma and cerebrospinal fluid samples were obtained at AD Center, Charles University in Prague, Department of Neurology or Memory Clinic, Czech Republic. Serum and matched CSF samples for antibody measurements were collected from 134 participants. For tau oligomers and conformers, serum samples from 186 participants, and CSF samples from 118 participants were obtained. The participants were divided into five and four groups for each study, respectively. The first group of non-demented controls consisted of neurological patients with normal the Mini-Mental State Examination (Folstein et al., 1975) and normal basic CSF findings (Deisenhammer et al., 2006). The second group consisted

of patients with MCI not fulfilling the criteria for MCI–AD (MCI) (Petersen et al., 1999), and the third consisted of patients with MCI due to AD (MCI-AD) (Albert et al., 2011). The fourth group consisted of patients with dementia due to AD (AD-dementia) according to the NIA-AA criteria (McKhann et al., 2011). The fifth group comprised demented patients with other types of dementia (OD; frontotemporal dementia, progressive supranuclear palsy, Creutzfeld-Jakob disease, vascular dementia and mixed types of dementia were found in this group). The patients had an established diagnosis of AD from an experienced neurologist (MUDr. Aleš Bartoš). The diagnosis of patients with cognitive impairment was based on objective evidence of a progressive decline in cognition, functional and neuropsychological assessments, hippocampal atrophy seen on brain magnetic resonance imaging (or computer tomography in case of contraindications), temporoparietal hypoperfusion seen using single photon emission computed tomography, or increased total or phosphorylated tau protein and/or decreased amyloid-β concentrations in cerebrospinal fluid using cut-offs established in our previous studies (Bartoš A. et al., 2007; Scheltens et al., 1992) when patients underwent these examinations. We measured concentrations of total tau protein, phosphorylated tau at Thr 181 and $A\beta_{42}$ peptide in CSF of participants using ELISA kits from Fujirebio (Malvern, Pensylvania, USA) according to manufacturer’s instructions and in line with our previous research (Bartoš A. et al., 2012).

Plasma samples for isolation of tau-reactive antibodies were obtained from 11 patients from the Department of Neurology or Memory Clinic of the Charles University, Czech Republic. Their cognitive functions were evaluated using an updated Czech version of Addenbrooke's Cognitive Examination Revised (ACE-CZ) (Bartoš A. et al., 2011; Mioshi et al., 2006). We were then able to derive MMSE scores (range 0-30) from the ACE-CZ (score range 0-100). The normal elderly controls were recruited as in-patients from the Department of Neurology and had normal ACE-CZ (cut-off ≥79) and MMSE scores (cut-off ≥28). They mostly presented with non-inflammatory conditions such as polyneuropathy and peripheral Bell´s facial palsy and the rest presented with a variety of diseases (e.g. a headache, trigeminal neuralgia and transient unconsciousness). AD patients were diagnosed according to the NIA-AA criteria (McKhann et al., 2011).

Serum/plasma and CSF samples were collected, centrifuged, aliquoted in 1 mL polypropylene tubes and stored (on average within 1.5 hours of sampling) at −80° C until

analysis. The specimens were thawed just before measurements.

In total, four human autoptic brains were evaluated for the clinical diagnosis of AD using a silver staining technique in accordance with a study (Krişţofikova et al., 1995). Human brain tissues of two control individuals (two men at the age of 71 and 79 whose cause of death was cancer and myocardial infarct, respectively) and two AD patients (two men at the age of 82 and 83 whose cause of death was cardiac insufficiency) were obtained by autopsy. The control subjects were described as a nondemented and nonpsychotic patients (no marked histological changes indicative of AD pathology). The AD patients had clinically diagnosed dementia with a number of senile plaques and tangles in given areas of the cortex and in the hippocampus higher than would be expected for age. The criteria were consistent with those used in the classification of (Mirra et al., 1991). Hippocampal brain region from left hemisphere used in analyses was dissected on a cold plate. Brain tissue for biochemical examination was wrapped up in aluminum and stored at –80 °C until assayed.

3.8 Statistical analysis

Data were analyzed with GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA) and STATISTICA software (StatSoft, Tulsa, Oklahoma, USA). Nonparametric Kruskal-Wallis test for global and Mann-Whitney test for pairwise comparisons was calculated for group characteristics and results of tau-reactive antibodies measurements and tau conformers levels obtained by ELISA. Generalized linear model (GLM) analysis with the covariate of age and contrast statement for intergroup comparisons was performed for analyzing results of logarithmically transformed tau oligomers levels obtained by ELISA with T22 antibody. Correlations coefficients were calculated using a Spearman correlation. Statistical analysis of Western blot quantification results was performed by using the two-tailed Student's t-test. The results are presented as median with 25th-75th percentiles for clinical variables of groups and results of ELISA assays because the data were non-normally distributed. A value of $p < 0.05$ was considered significant.

4 Results

4.1 Preparation of tau protein forms

Three forms of tau protein were prepared and used as antigens for further analysis of naturally occurring antibodies in different body fluids. The proteins were purified as described above in Chapter 3.2. The full-length form and fragment 13-391 aa of tau protein were purified by affinity and ion exchange chromatography. Fig. 1 shows chromatograms of protein separation. The chromatographic behavior of these two forms of tau protein on Mono S column was similar. Their elution occurred at the same retention time (60. - 70. minute).

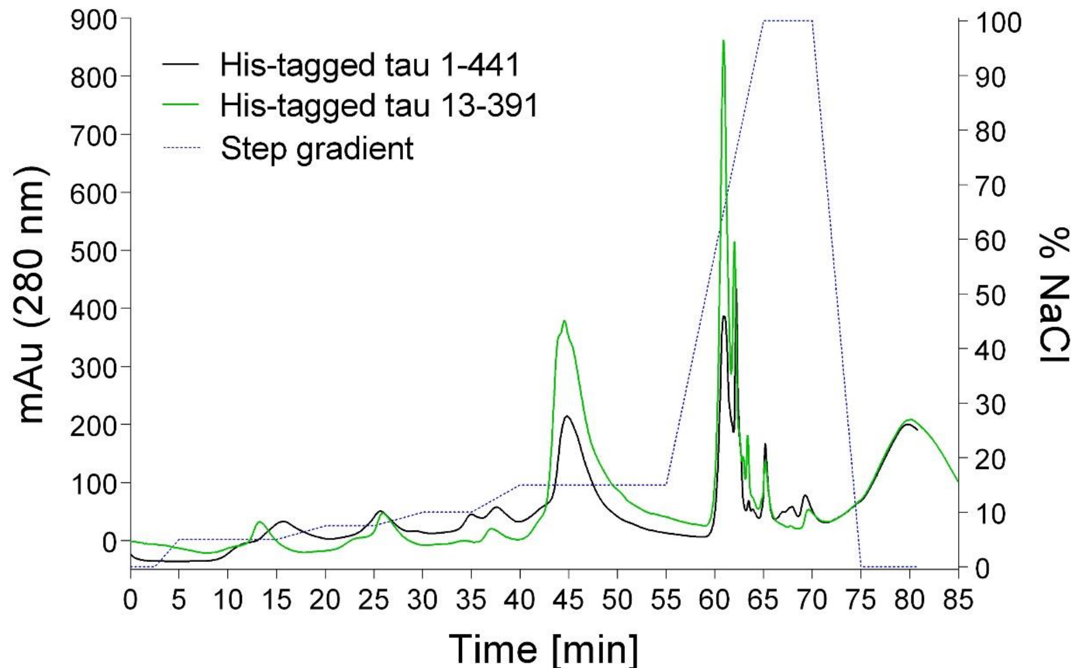

Figure 1: *Purification of two tau protein forms using ion exchange chromatography column Mono S. Elution of both protein forms occurred at 60.-70. minute: fractions 24-28 for His-tagged tau 1-441 aa, and 21-27 for fragment 13-391 aa according to Western blot (Fig. 2).*

The quality of separation and purity of pooled fractions containing desired tau proteins were assessed by glycine SDS-PAGE electrophoresis (Fig. 3) and by Western blot technique (Fig. 2A-D).

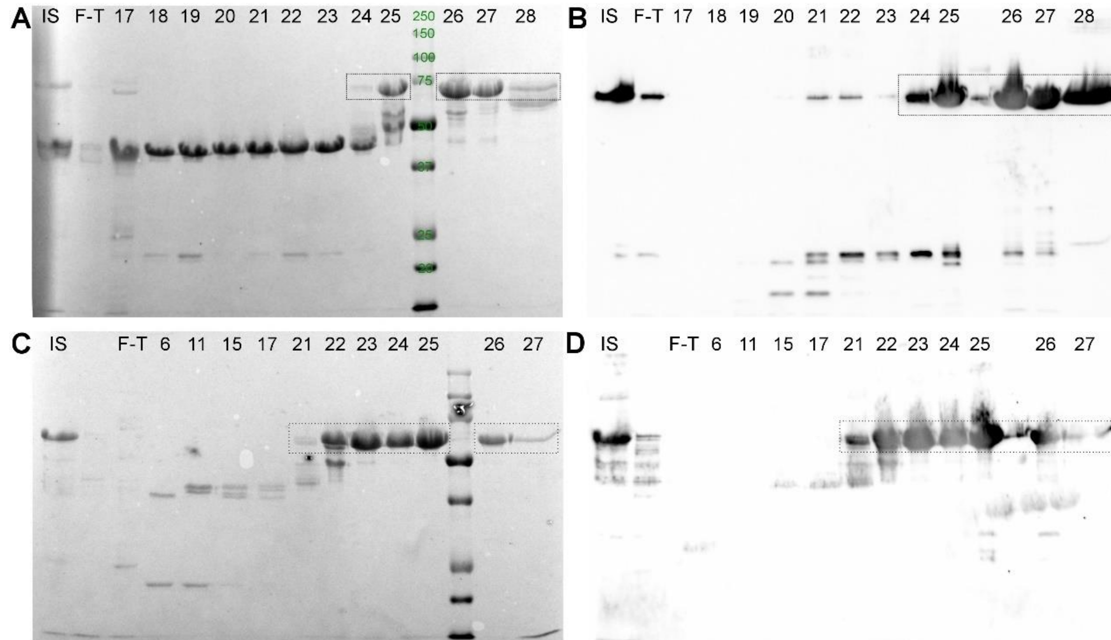

Figure 2: *Western blot analysis of separated protein fractions by ion exchange chromatography. Nitrocellulose membranes containing His-tagged tau 1-441 aa (A) and His-tagged tau 13-391 aa (C) fractions were stained with Ponceau red stain. Subsequently, the reactivity of Tau 46.1 antibody with His-tagged tau 1-441 aa (B) and with His-tagged tau 13-391 aa (D) purification fractions was evaluated. Boxes show fractions containing tau protein.*

The fragment of tau with sequence 155-421 aa was purified only by affinity chromatography to reach the desired purity.

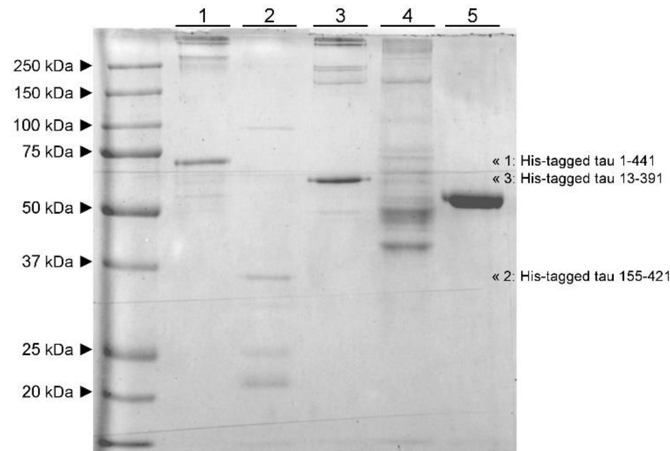

Figure 3: *SDS-PAGE electrophoresis with 10% polyacrylamide gel was carried out for quality control of purified proteins: His-tagged tau 1-441 aa (1), His-tagged tau 155-421 aa (2), His-tagged tau 13-391 aa (3). Native bovine tau (Cytoskeleton, Denver, Colorado, USA) (4) and bovine serum albumin (5) were included for comparison purpose.*

The purity of all three forms was assessed more than 90 %. All recombinant forms of tau protein create aggregates as seen in Fig. 3-lanes 1-3.

4.2 Production and purification of rabbit polyclonal anti-tau antibody

The full-length recombinant form of tau protein (His-tagged tau 1-441 aa) was used as an antigen for immunization of rabbits to obtain rabbit polyclonal anti-tau antibody. The vaccination scheme was described in Chapter 3.4.2. After each boost injection, the titer of formed antibodies was measured by ELISA assay in the sample of blood drawn from each rabbit (Fig. 4).

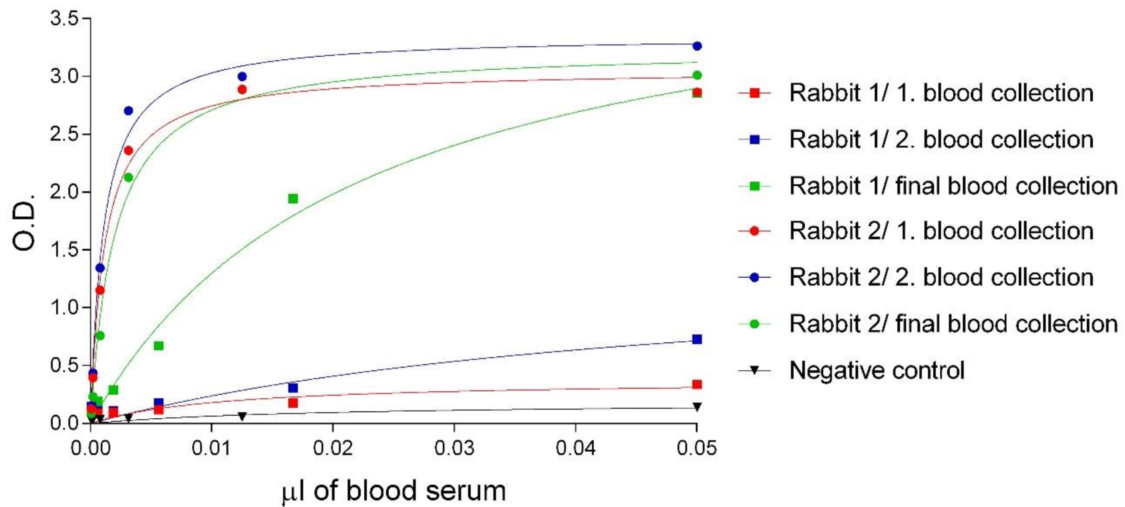

Figure 4: *The production of anti-tau antibodies was evaluated during immunization procedure by an ELISA assay. The rabbit sera samples were serially diluted 1:2,000-1:2,048,000 (0.000048-0.05 μl of sera/well). The serum from non-immunized rabbit served as a negative control.*

After evaluation of the antibody titers in the serum of each of the three rabbits, the serum of rabbit 2 was chosen for isolation of antibodies and their immunopurification. A column containing His-tagged recombinant tau protein 1-441 aa form was prepared as described in Chapter 3.4.1 and used for immunoprecipitation of rabbit anti-tau antibodies. The rabbit serum was at first precipitated by 50% AS and spun to remove serum albumin. 5 ml of precipitate containing IgG was dissolved in PBS, and the concentration was assessed at 280 nm to be 3.5 mg/ml. The rabbit antibody pool was purified against tau protein bound to the resin in the column. The reactivity of isolated antibodies was assessed by Western blot against the same antigen used for purification as well as against recombinant His-tagged 17-beta-hydroxysteroid dehydrogenase 10 (ERAB) and bacterial proteins from E. coli which have co-purified with tau protein on the IMAC column (Fig. 5).

These bacterial proteins with affinity to nickel ions on IMAC column were covalently bound to the copolymer resin and thus, the second column was prepared. The second column was used to remove potential His-tag reactive antibodies from isolated rabbit anti-tau antibodies. After two-step purification procedure using columns containing tau protein and bacterial proteins, it was prepared 1.25 ml of anti-tau antibodies at concentration 1.7 mg/ml. Even after two-step purification procedure, the isolated tau-reactive antibodies showed reactivity to proteins isolated from bacteria (Fig. 5-B). These proteins come from E. coli bacteria carrying plasmid coding tau protein, but without induced expression of the tau protein. However, some small portion of the bacteria might produce little amounts of tau protein without added inducer, and therefore, we can see the reactivity against tau protein on the Western blot. Nevertheless, we depleted the isolated tau-reactive antibodies of the His-tag reactive antibodies as we can not see any signal against His-tagged ERAB (27 kDa) (Fig. 5-B3).

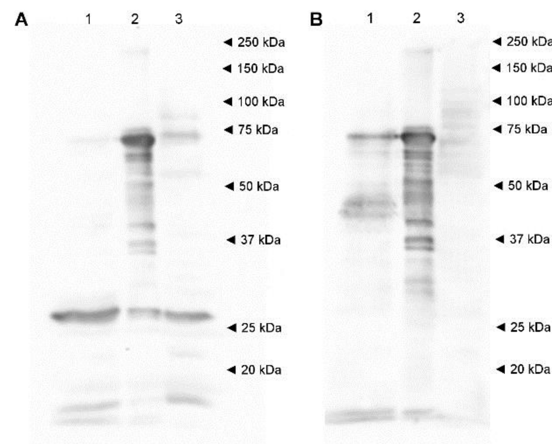

Figure 5: *Western blot was carried out to compare the reactivity of purified rabbit anti-tau antibody (B) and crude rabbit serum (A). The bacterial proteins from E. coli which have co-purified with tau protein on the IMAC column (1), His-tagged tau 1-441 aa as an antigen to which the antibody was raised (2) and a recombinant His-tagged 17-beta-hydroxysteroid dehydrogenase 10 (ERAB; 27 kDa) (3) were used.*

The rabbit anti-tau antibody was subjected to epitope mapping in collaboration with Department of Biological and Biochemical Sciences at the University of Pardubice to identify immunodominant epitopes of tau protein. Briefly, tau protein was cleaved by enzymes clostripain and trypsin, and then fragments were left to react with immobilized rabbit anti-tau antibody on magnetic particles and finally eluted by 0.05% TFA. Captured

immunogenic fragments were analyzed by mass spectrometry (MALDI LTQ Orbitrap XL). This technique identified two epitopes regarding the longest isoform of tau protein: 171-194 aa and 299-317 aa (Jankovicova et al., 2015).

4.3 Interactions between Amyloid-β and tau in cerebrospinal fluid

Partially characterized rabbit polyclonal antibody was used by our collaborator Ing. Krištofiková as a capture antibody in semi-quantitative sandwich ELISA for estimation of levels of amyloid-β-tau complexes in cerebrospinal fluid as a prospective biomarker of AD (Kristofikova et al., 2014). We have observed significantly lower levels of Aβ-tau complexes in the CSF of people with mild cognitive impairment due to AD (MCI-AD; 84.5% of control levels) and patients with dementia due to AD (80.5% of control levels) when compared to cognitively normal controls. No significant changes were found in MCI-others, Frontotemporal dementia and other types of dementia.

4.4 Isolation and characterization of human naturally occurring polyclonal tau-reactive antibodies

We prepared four fractions of tau-reactive antibodies from human plasma samples. The first two polyclonal antibodies were purified from intravenous immunoglobulins product Flebogamma (nTau-IVIG-1 and -2) (Hromadkova et al., 2015). The other two were purified from plasma of cognitively normal older individuals (nTau-Ctrl) and patients with AD (nTau-AD) (Krestova et al., under review).

The IVIG product Flebogamma was applied to the column with the bound recombinant His-tagged 1-441 aa form of tau protein. We isolated 3.2 mg of naturally occurring tau-reactive IgG (nTau-IVIG-1) from 400 mg / 8 ml of total IgG amount contained in IVIG product. The IVIG product contained 0.8 % of tau-reactive antibodies. The isolation efficacy was assessed by ELISA assay against binding ligand in all purification fractions. We concentrated tau-reactive antibodies contained in product Flebogamma in the Elution fraction in comparison to Flow-through fraction (Fig. 6).

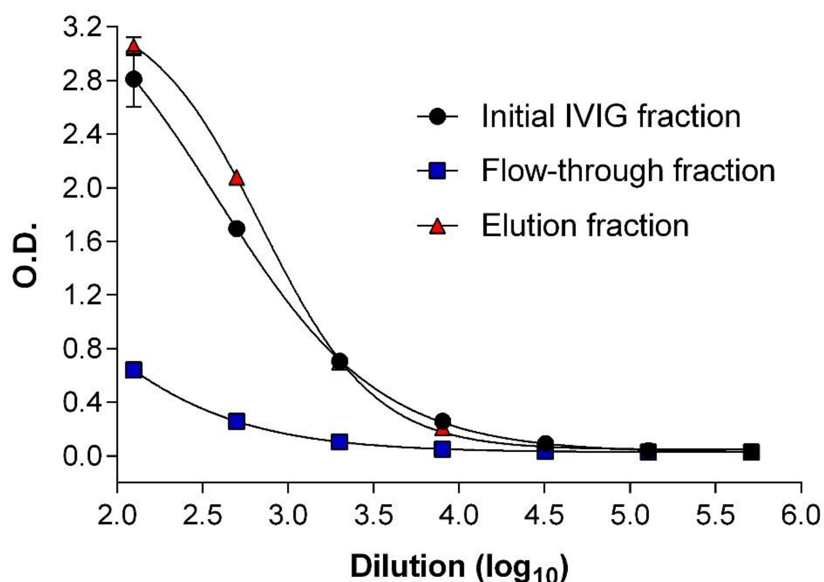

Figure 6: *Isolation efficiency of natural anti-tau antibodies from IVIG estimated by ELISA. Serial dilution of each purification fraction was probed against His-tagged tau 1-441 aa antigen. The detected signal is expressed as absorbance at wavelength 450 nm depending on the logarithm of samples dilution. His-tagged tau 1-441 aa form as an antigen is coated onto a well in concentration 0.1 µg/ 50 µl in 0.1 M bicarbonate buffer pH 9.5. Initial Flebogamma IVIG fraction (50 mg/ml), flow-through fraction (IgG fraction passed through the column with covalently bound antigen (His-tagged tau 1-441 aa) with no retention, 7.9 mg/ml), elution fraction (IgG fraction retained in the column with covalently bound tau ligand, concentrated to final concentration 0.8 mg/ml due to transfer into PBS buffer by Amicon 30K filters) are illustrated.*

The characterization of isolated nTau-IVIG-1 antibodies was firstly carried out against recombinant forms of tau protein and their phosphorylated equivalents and was already published (Hromadkova et al., 2015). The nTau-IVIG-1 antibodies appeared to be most reactive with recombinant fragments of tau with sequence 155-421 aa and 13-391 aa in comparison to ligand used for purification. Comparing the dilution factors of nTau-IVIG-1 antibodies obtained by ELISA at O.D. 0.5 for each antigen, we have found that the phosphorylation of 155-421 aa tau fragment had markedly attenuated its antigenicity (Fig. 7).

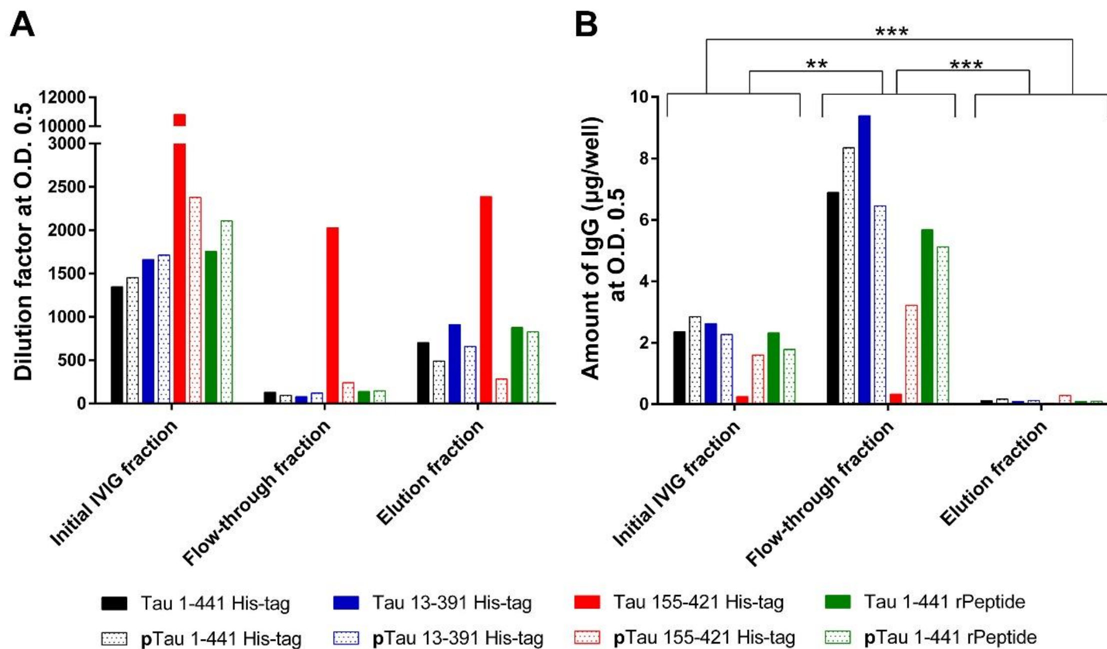

Figure 7: *Reactivity of natural antibodies presented in individual IVIG purification fractions: initial Flebogamma IVIG fraction (50 mg/ml), flow-through fraction (7.9 mg/ml) and elution fraction (0.8 mg/ ml), against different forms of tau protein. The comparison is expressed as a dilution of each fraction (A) and the amount of IgG (B) required to obtain OD 0.5 at wavelength 450 nm. Bars represent a mean value from duplicate wells. Statistical analysis was performed by Student t-test at significant p levels 0.01 (**), 0.001 (***).*

We carried out ELISA and dot blot immunoassay to establish avidity of these antibodies. In the graphical representation, the signal intensity [%] of antibodies represents the percentage decrease in the binding as a function of the molar thiocyanate concentration (Fig. 8). The calculation of avidity index from the log-transformed curve is mentioned in Chapter 3.5 and as a protocol in Natural antibodies- Methods and Protocols (Krestova et al., 2017). The avidity of nTau-IVIG-1 antibodies was low as expected with the exception for the fragment 155-421 aa of tau, where the avidity index was 1.6 M of NH_4SCN (Fig. 8, Table 2). Avidity dot-blot immunoassay was also carried out with polyclonal rabbit anti-tau antibodies raised against His-tagged tau 1-441 aa (Fig. 8-C). The avidity of rabbit polyclonal anti-tau antibodies was higher for all three tau forms in contrast to human nTau antibodies (Fig. 8). Moreover, the signal intensity did not decrease by 50 % in the range of 0 – 2.1 M NH_4SCN to evaluate avidity index for these His-tagged tau forms, tau 1-441 aa (the immunogen) and truncated tau 155-421 aa. The avidity index of rabbit antibodies with truncated form of tau 13-391 was determined as 1.36 M of NH_4SCN.

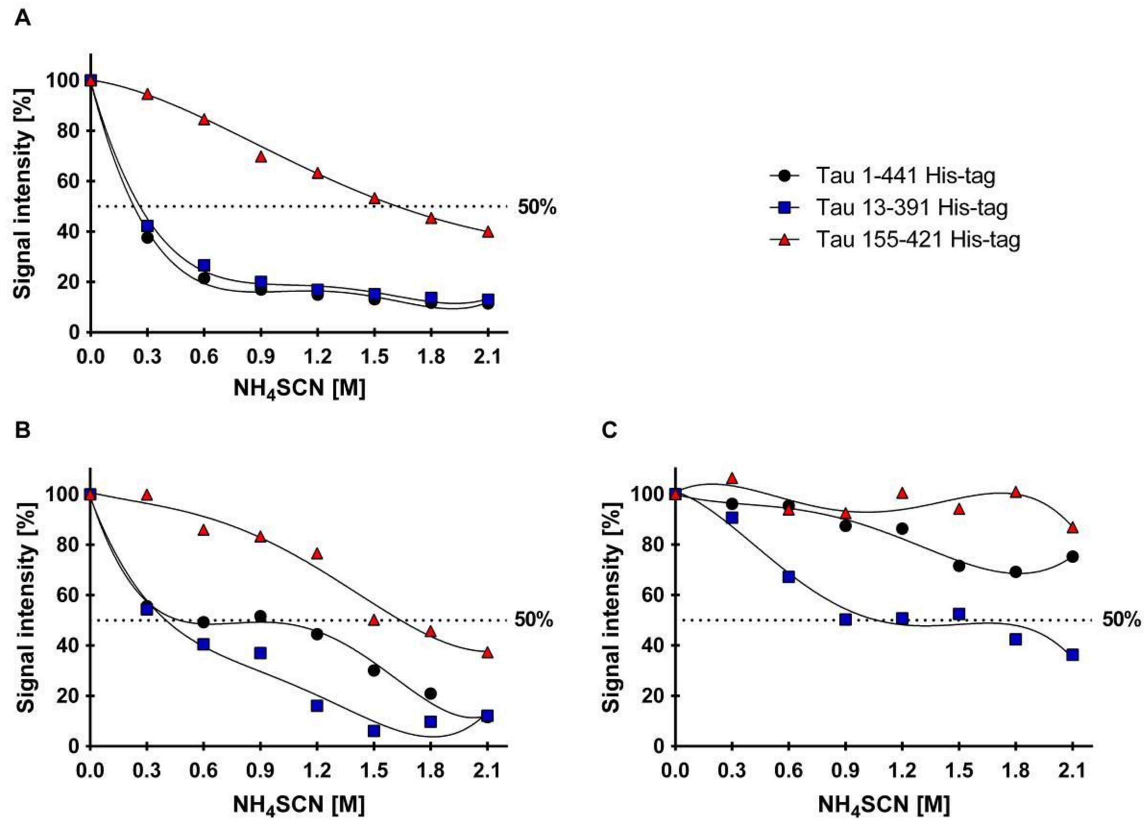

Figure 8: *Graphical representation of avidity index evaluation: expressed as the dependence of signal intensity (%; spot density in dot-blot immunoassay and the absorbance at 450 nm in ELISA) to NH_4SCN molarity. Three tau forms were used: tau 1-441 aa His-tagged, tau 13-391 aa His-tagged and tau 155-421 aa His-tagged. Avidity of isolated natural anti-tau antibodies from Flebogamma IVIG (nTau-IVIG-1) was measured by ELISA (A) and avidity dot-blot immunoassay (B). Additionally, avidity dot-blot immunoassay was carried out with polyclonal rabbit anti-tau antibodies (C). Each point on the graph represents the mean value from wells in duplicates and spots in triplicates. Dotted line shows 50% decrease in signal intensity.*

We performed an isotyping of IgG subclasses in the purified nTau-IVIG-1 antibodies as well. We enriched the IgG3 subclass in the nTau-IVIG-1 antibodies fourfold when compared to initial IVIG product Flebogamma (Fig. 9).

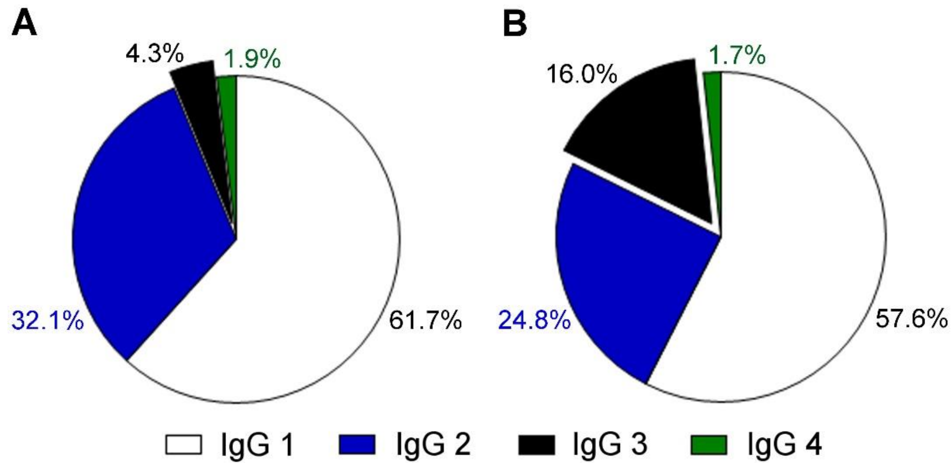

Figure 9: *The IgG subclass distribution in the initial IVIG product (A) and the fraction of antibodies isolated against tau protein (B). IgG subclass percentages were determined by ELISA kit. The IgG1 and IgG4 distribution are comparable in both samples. The proportion of IgG3 increases four times and of IgG2 slightly decreases in the isolated antibody fraction in comparison to the initial IVIG fraction.*

We also characterized the reactivity of nTau-IVIG-1 antibodies against native brain-derived tau protein forms from homogenates of left hemisphere hippocampi of two AD patients and two cognitively normal individuals. The samples were prepared as PBS-soluble protein fractions and SDS-soluble protein fractions from brain homogenate. The reactivity of isolated plasma antibodies from IVIG was compared with other selected antibodies (Fig. 10). Monoclonal Tau 5 antibody and rabbit polyclonal anti-tau antibody showed the typical distribution of tau isoforms in the control brain (39 kDa to 55 kDa) (Boutajangout et al., 2004). On the contrary, the Alzheimer's brain showed an increased occurrence of pathological forms ranging from 55kDa to 74 kDa (Sergeant et al., 1997) as well as higher molecular weight (HMW) protein bands. The nTau-IVIG-1 antibodies revealed reactivity preferentially to HMW proteins. All used antibodies showed differences between control brain and AD brains. We also found the difference between AD brains. The pSer396 antibody stained only the brain homogenate referred as AD1. The staining with pSer396 antibody points to different stages of the disease of these two patients.

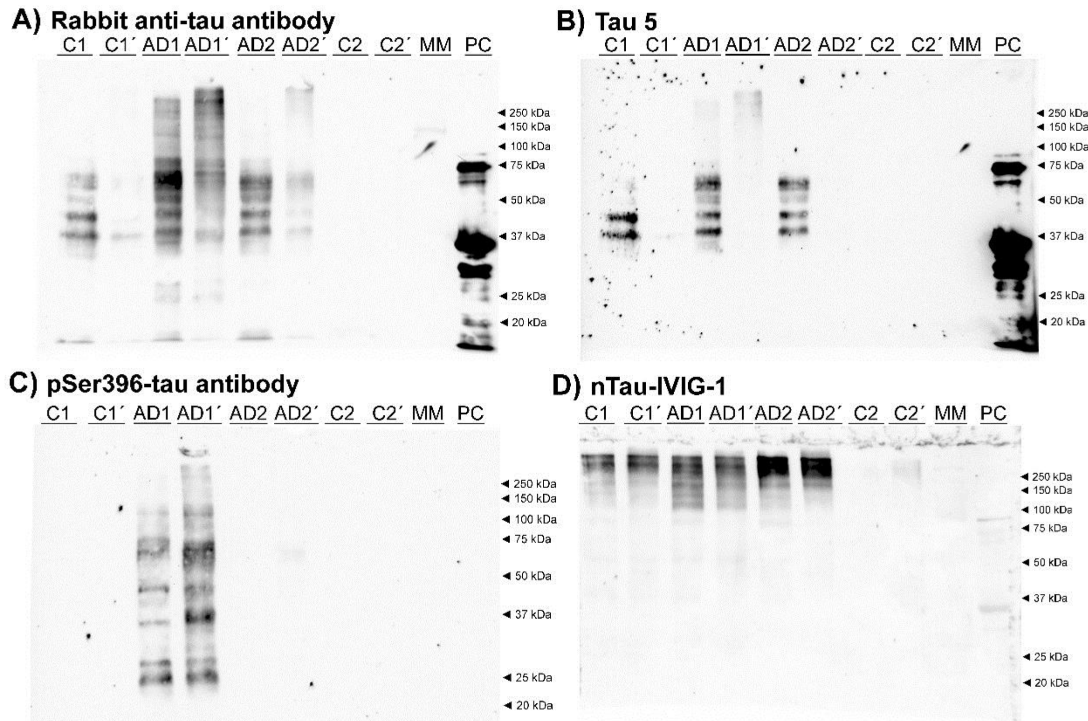

Figure 10: *Western blot analysis of human brain homogenates (25 µg of total protein/lane) was carried out using polyclonal rabbit anti-tau antibody (A); monoclonal Tau 5 antibody (B); polyclonal pSer396-tau antibody (C) and isolated naturally occurring antibodies from IVIG reactive with tau protein (nTau-IVIG-1) (D). Left hemisphere hippocampi of two control brains and two histopathologically proven AD patients were homogenized in PBS buffer (C- control brains and AD- AD brains) or PBS buffer containing 2% SDS (C´-control brains) and (AD´- AD brains), respectively. A recombinant fragment of tau 155-421 aa with the theoretical molecular weight of 30 kDa (2 µg/well) (PC) was included as a positive control. An error occurred in the preparation of control brain homogenate C2 as was proven by staining with anti-tubulin antibody (data not shown).*

Based on these results, we were interested in the reactivity profiles of antibodies present in the plasma of patients with diagnosed AD and age-matched cognitively normal older individuals with non-inflammatory neurological diseases (control subjects). We also repeated the isolation of antibodies from IVIG Flebogamma (nTau-IVIG-2). We pooled plasma samples from four AD patients / seven control subjects that were established to have low, medium and high plasmatic antibody titers according to ELISA performed with the full-length tau and fragment of tau 155-421 aa as antigens (data not shown). The antibodies were purified as described in Chapter 3.4.1 and the protocol (Krestova et al., 2017). We isolated 8.3 mg of natural tau-reactive antibodies from IVIG product Flebogamma (nTau-IVIG-2), 3.8 mg of natural AD plasma tau-reactive antibodies (nTau-

AD) and 5 mg of antibodies reactive to tau protein from cognitively normal older subjects (nTau-Ctrl), determined by BCA test. The isolation process was assessed by ELISA assay in all purification fractions (Fig. 11) and purity of isolated antibodies by SDS-PAGE electrophoresis (Fig. 12).

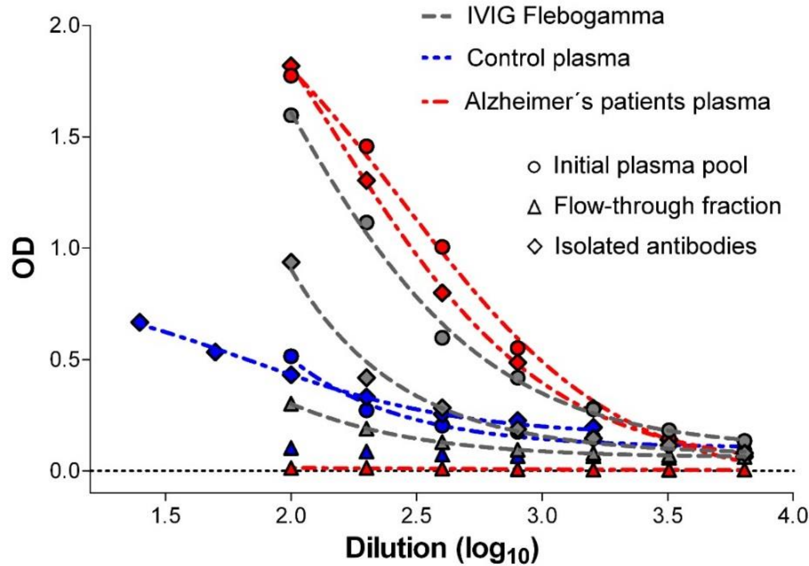

Figure 11: *Isolation efficiency of natural anti-tau antibodies from IVIG, plasma pools of AD patients and cognitively normal older controls estimated by ELISA. Serial dilution of each fraction was probed against His-tagged tau 1-441 aa antigen. The detected signal is expressed as absorbance at 450 nm wavelength depending on the logarithm of samples dilution. His-tagged tau 1-441 aa antigen was coated onto a well at a concentration of 0.1 µg / 50 µl in 0.1 M bicarbonate buffer pH 9.5. Initial plasma pool fraction, flow-through fraction (IgG fraction passed through the column with covalently bound His-tagged tau 1-441 aa antigen with no retention), elution fraction containing isolated tau-reactive antibodies (antibodies fraction retained in the column with covalently bound tau ligand) are illustrated.*

The nTau-AD and nTau-Ctrl antibodies contained impurities or contaminants that represent more than 50 % of total protein content. Therefore, the portion of nTau-AD and nTau-Ctrl antibodies was estimated to be 0.4 % of total protein amount from the initial sample (Fig. 12).

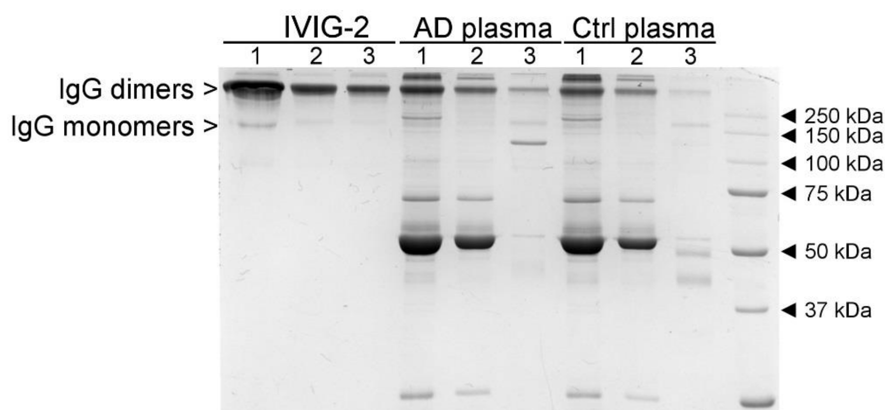

Figure 12: *SDS-PAGE electrophoresis of initial fraction (1), a flow-through fraction (2) and elution fraction containing natural antibodies in PBS pH 7.2 (3) followed by silver staining. Arrowed bands represent >250 kDa band of IgG dimers, 150 kDa band of IgG monomers. Bands under 150 kDa are supposed to be IgG fragments or contaminants representing 0.8 % of nTau-IVIG-2, 55 % of nTau-AD and 58 % of nTau-Ctrl antibodies of total content determined by density analysis.*

We performed an isotyping of IgG subclasses in the newly prepared nTau-IVIG-2, nTau-Ctrl and nTau-AD antibodies (Fig. 13). The pools of plasma samples contained the usual distribution of IgG subclasses (data not shown) (Listì et al., 2006; Lock and Unsworth, 2003). The isolated nTau-Ctrl antibodies were threefold enriched of IgG4 subclass in comparison to nTau-AD antibodies and twofold as compared to the nTau-IVIG-2 antibodies. In agreement with our previous isolation, the newly isolated nTau-IVIG-2 antibodies showed enrichment of IgG3 subclass in comparison to the Flebogamma IVIG product. Although, it formed only 10 % in contrast to first isolation of nTau-IVIG-1 (16 %). This could be the result of differences between batches of Flebogamma product or due to the usage of the newly prepared column with tau protein. On the contrary, we were able to detect mainly IgG1 and IgG2 in the nTau-AD antibodies.

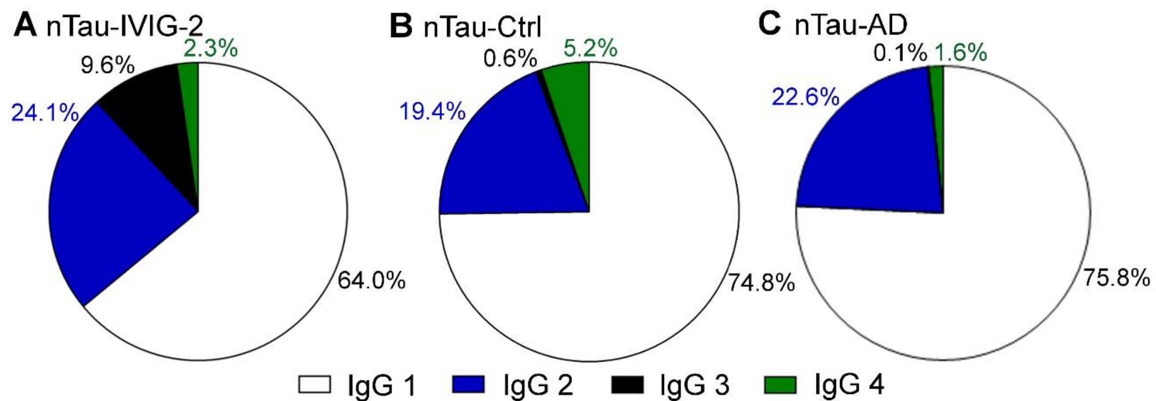

Figure 13: *The distribution of IgG subclasses in isolated antibodies. Isolated antibodies against tau protein from the intravenous immunoglobulin product Flebogamma (nTau-IVIG-2; A), the pool of plasma samples from seven cognitively normal older subjects (nTau-Ctrl; B) and four AD patients (nTau-AD; C). Percentages of IgG subclasses were determined by ELISA. The distributions of IgG1 and IgG2 were comparable in all three isolated antibodies with a small deviation in nTau-IVIG-2. The IgG3 subclass was barely detectable in nTau-AD and nTau-Ctrl in comparison to nTau-IVIG-2. The isolated antibodies from older individuals showed enrichment of IgG4 subclass threefold in comparison to antibodies from AD patients and twofold as compared to nTau-IVIG-2.*

Reactivity of all three isolated antibodies: nTau-IVIG-2, nTau-AD and nTau-Ctrl was verified against recombinant His-tagged proteins; the fragment of tau 155-421 aa and the full-length form 1-441 aa of tau protein and also tau protein forms present in homogenates of brain tissue (Fig. 14). All isolated nTau antibodies reacted similarly with proteins from brain tissue homogenates with a preference towards higher molecular weight (HMW) proteins (Fig. 14-A, B and C) and that was comparable to the reactivity of nTau-IVIG-1 (Fig. 10-D). The nTau-IVIG-2 and nTau-Ctrl antibodies reacted with both tau 1-441 aa and tau 155-421 aa and also produced a strong signal with HMW aggregates. In contrast, nTau-AD antibodies stained only non-phosphorylated tau 1-441 aa both in the monomeric and aggregated form.

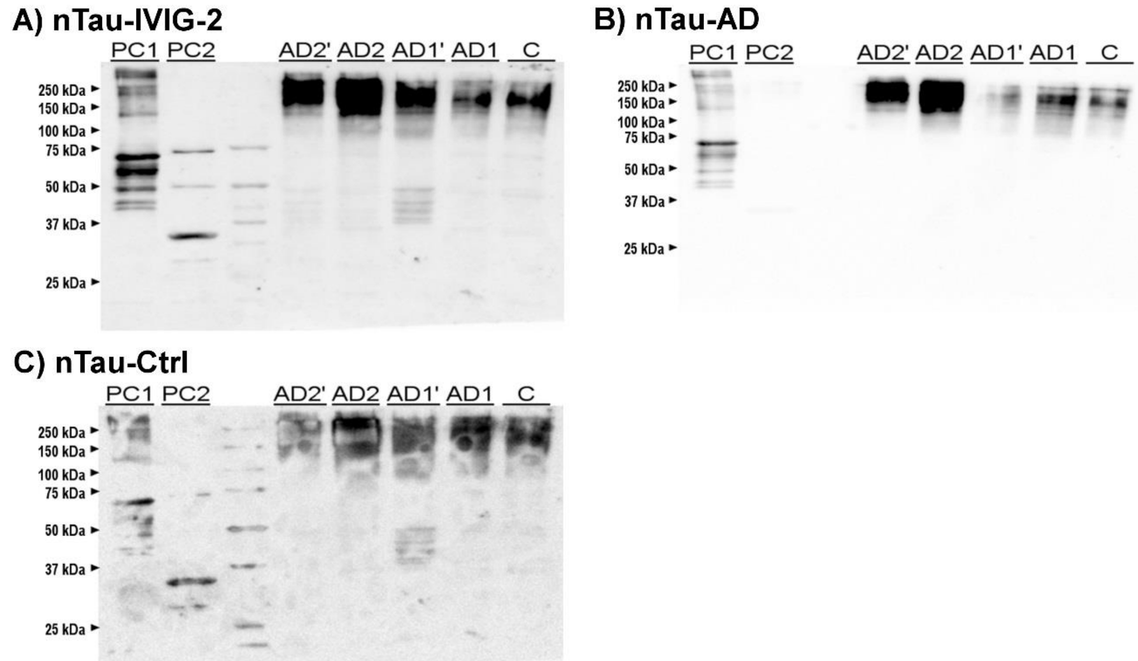

Figure 14: *Western blot analysis of human brain homogenates (25 µg of total protein/lane) was carried out using naturally occurring tau-reactive antibodies isolated from IVIG product Flebogamma (nTau-IVIG-2; A), naturally occurring tau-reactive antibodies from plasma of AD patients (nTau-AD; B), naturally occurring tau-reactive antibodies from plasma of older cognitively normal subjects (nTau-Ctrl; C). Left hemisphere hippocampi of one control brain and two histopathologically proven AD patient's brains were homogenized in PBS buffer (C- control brains and AD- AD brains) or PBS buffer containing 2% SDS (C'- control brains and AD'- AD brains), respectively. A recombinant fragment of tau 155-421 with the theoretical molecular weight of 30 kDa (2 µg/ well) (PC2) and recombinant full-length form of tau protein (1-441 aa) appearing on the gel around 75 kDa (PC1) were included as positive controls. The exposure was kept constant for all plasma antibodies and membranes.*

We conjugated all three purified fractions of human antibodies to biotin to avoid added signal from secondary anti-human IgG antibody alone which we have observed (data not shown). This resulted in an attenuation of the signal against HMW proteins in the isolated antibodies from IVIG Flebogamma and AD plasma and increased staining of lower molecular forms, especially for nTau-AD antibodies (Fig. 15). The biotinylated nTau-AD antibodies showed similar staining pattern as the rabbit polyclonal anti-tau antibody, which was raised against the recombinant His-tagged tau 1-441 aa (Fig. 15-B and E). However, the biotinylated nTau-Ctrl antibodies kept the reactivity against the HMW forms and also recognized the lower molecular weight forms of tau protein. The detection of HMW proteins with nTau-Ctrl antibodies was comparable to monoclonal antibody AT180 which has specificity to phospho-Thr231 of tau molecule. The AT180 antibody confirmed

different stage of neuropathology in the AD brains. It stained mainly sample referred as AD2 brain homogenate (Fig. 15-F).

The avidity of nTau-AD antibodies against the His-tagged Tau 1-441 aa form has been found low in contrast to fragment 155-421 aa (Table 2). However, the nTau-Ctrl and nTau-IVIG-2 antibodies showed comparable high avidity against both forms of tau protein (Table 2).

The quantitative characteristics of purified human plasma naturally occurring tau-reactive antibodies are summarized in Table 2.

Table 2: *The quantitative characterization of reactivity of isolated antibodies with different tau antigens.*

			Isolated natural anti-tau antibodies from human plasma against			
			Tau 1-441 His-tagged	**pTau 1-441 His-tagged**	**Tau 155-421 His-tagged**	**pTau 155-421 His-tagged**
Reactivity ELISA: dilution		**nTau-IVIG-1 Abs**	1:700	1:500	1:2400	1:290
		nTau-IVIG-2 Abs	1:195	ND	1:173	ND
		nTau-AD Abs	1:756	ND	1:595	ND
		nTau-Ctrl Abs	1:67	ND	N/A	ND
Avidity Index[b]	Dot-blots	**nTau-IVIG-1 Abs**	0.74	ND	1.67	ND
		nTau-IVIG-2 Abs	1.54	ND	1.78	ND
		nTau-AD Abs	0.29	ND	1.50	ND
		nTau-Ctrl Abs	1.27	ND	1.56	ND
	ELISA	**nTau-IVIG-1 Abs**	0.20	ND	1.62	ND

The results are expressed as a dilution of antibodies reacting with antigen (0.1 µg/well) to reach OD 0.5 at 450 nm (working concentration of isolated antibodies was 1.96 mg/ml for nTau-AD, 0.8 mg/ml for nTau-IVIG and 2.51 mg/ml for nTau-Ctrl antibodies). Avidity index is expressed as molarity of ammonium thiocyanate (M) that causes the decrease of the initial sample signal (in the absence of thiocyanate) on the value of 50%. ND = not determined, N/A= not applicable.

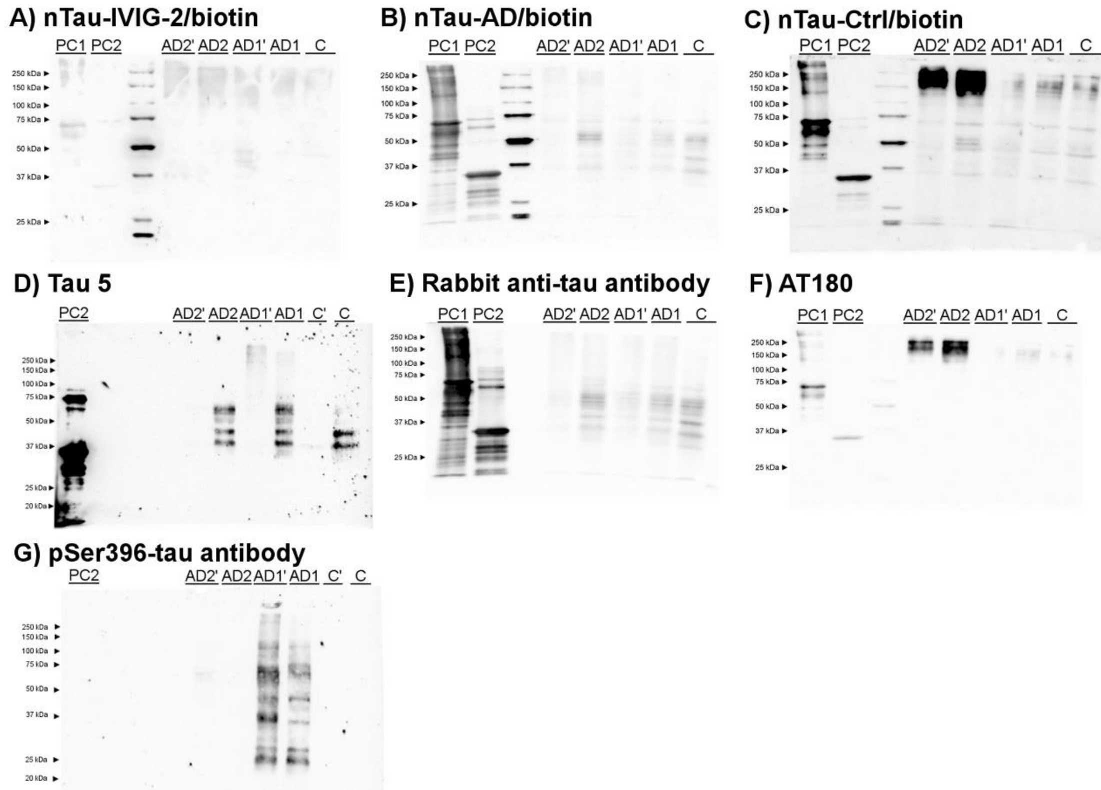

Figure 15: *Western blot analysis of human brain homogenates (25 μg of total protein/lane) was carried out using naturally occurring tau-reactive antibodies isolated from IVIG (nTau-IVIG-2 Abs) product Flebogamma conjugated to biotin (A), naturally occurring tau-reactive antibodies from plasma of AD patients (nTau-AD Abs) conjugated to biotin (B), naturally occurring tau-reactive antibodies from plasma of older cognitively normal subjects (nTau-Ctrl Abs) conjugated to biotin (C) and monoclonal Tau 5 antibody (D), rabbit polyclonal anti-tau antibody (E), monoclonal phospho-tau (pThr231) antibody (AT180, early stage of AD) (F), polyclonal pSer396-tau antibody (late stage of AD) (G) and. Left hemisphere hippocampi of one control brain and two histopathologically proven AD patient's brain were homogenized in PBS buffer (C- control brains and AD- AD brains) or PBS buffer containing 2% SDS (C'- control brains and AD'- AD brains), respectively. A recombinant fragment of tau 155-421 with the theoretical molecular weight of 30 kDa (2 μg/ well) (PC2) and recombinant full-length form of tau protein (1-441 aa) appearing on the gel around 75 kDa (PC1) were included as positive controls. The exposure was kept constant for all plasma antibodies and membranes.*

4.5 Comparison of levels of naturally occurring antibodies against tau proteins in serum and cerebrospinal fluid

Based on our findings of the different reactivity of plasmatic antibodies with tau proteins in older controls and AD patients, we extended the evaluation of humoral immunity associated with tau protein to two different compartments (serum and CSF) and between them (intrathecal synthesis). In total, 134 participants were separated into groups of patients with mild cognitive impairment not related to AD (MCI), mild cognitive

impairment due to AD (MCI-AD), dementia due to AD, patients with other neurodegenerative disorders than AD and cognitively healthy controls. We simultaneously measured antibodies against three forms of tau protein: two His-tagged recombinant forms-full-length form of tau protein (1-441 aa) and one fragment (155-421 aa) and presumably "native" form of tau protein from bovine brain. The native bovine tau protein has more than 80% homology with human tau protein, and its advantage is the presence of post-translational modifications in contrast to recombinant proteins. The nTau-IVIG-1 antibodies were used as a standard for our in-house ELISA assay (Fig. 16).

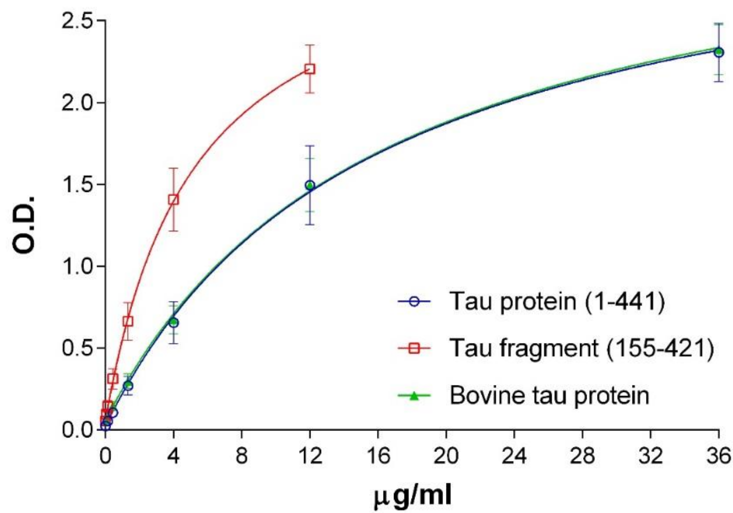

Figure 16: *Reactivity of isolated anti-tau antibodies from IVIG to various tau forms. Serial dilution of tau-reactive IVIG antibodies (0.05-36 µg/ml) was used to create a calibration curve for establishing a concentration of antibodies against tau proteins (0.125 µg/well) in CSF and serum samples.*

The interassay variation between plates was calculated from 25 measurements for each antigen. The variation was 20 %, 21 % and 18 % for the full-length form of tau (1-441 aa), tau fragment 155-421 aa and bovine tau, respectively. All serum samples were measured in duplicates with the use of three serial dilutions to avoid hooking effect, to obtain values best fitting in the linear range of the calibration curve and observe the changes dependent on the dilution of the sample. We have found that dilutions of serum 1:600 and 1:1,800 were optimal for the measurements of most samples because the concentrations corrected for the particular dilution factors corresponded well (Fig. 17). All samples were measured in blocked wells without coated antigen to control non-specific signal. We were able to

measure tau-reactive antibodies in concentrations ranging from 0.3 to 15 µg/ml in CSF and 2 to 300 µg/ml in serum samples of all investigated groups.

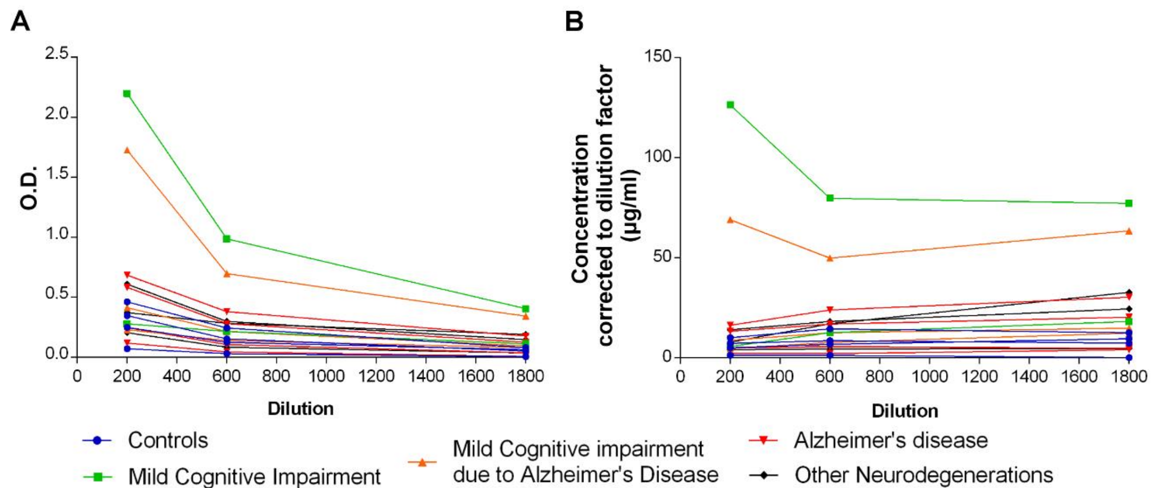

Figure 17: *Effect of serum dilution (1:200, 1:600 and 1:1,800) on the obtained optical densities (A) and final concentration when corrected to dilution factor (B). Samples measured against tau (1-441 aa) protein.*

Table 3 shows basic characteristics of the 134 study participants. The patients in MCI-AD and dementia due to AD groups were older than control subjects. However, the analysis of age covariance did not show any effect of this variable on the analysis of antibody levels or CSF biomarkers. Statistical analysis revealed significant drops in MMSE score in all groups with cognitive impairment when compared to controls. The current CSF biomarkers of AD (total tau, phospho-tau_{181} and $A\beta_{42}$) were measured by commercial ELISA kits (Fujirebio). The CSF biomarkers showed significant differences between groups of MCI-AD, dementia due to AD and the group of patients with other neurodegenerations when compared to control subjects. The group of subjects with mild cognitive impairment not related to AD did not differ from controls for CSF biomarkers specific to AD.

***Table 3**: Demographic, cognitive and cerebrospinal fluid characteristics of subjects enrolled for measurement of tau-reactive antibodies*

	Control subjects	Mild Cognitive Impairment	Mild Cognitive Impairment due to Alzheimer's disease	Dementia due to Alzheimer's disease	Other dementias than Alzheimer's
N per group	*46*	*13*	*19*	*30*	*26*
Age (years)	*65 (71-61)*	*60 (67-59)*	***72 (77-67)*****	***74 (78-70)******	*63 (74-58)*
Female sex	*43%*	*54%*	*37%*	*50%*	*43%*
MMSE score	*29 (29-28)*	***26 (27-24)******	***25.5 (28-23)******	***21.5 (24-17)******	***20 (25-17)******
Total tau (pg/mL)	*195.9 (255.2-155.0)*	*207.6 (263.0-187.0)*	***301.8 (573.0-217.3)*****	***557.5 (687.3-363.5)******	***352.0 (485.4-197.9)*****
Phospho-tau$_{181}$ (pg/mL)	*32.9 (47.3-25.8)*	*43.0 (51.1-37.5)*	***67.2 (106.0-38.9)*****	***60.0 (89.0-47.4)******	*32.0 (42.0-23.0)*
Aβ$_{42}$ (pg/mL)	*928.2 (1121.0-802.1)*	*985.0 (1192.1-852.8)*	***680.5 (1074.1-477.2)****	***578.5 (815.4-467.0)******	***727.0 (950.5-622.0)*****
Total levels of CSF IgG (mg/L)	*37.9 (53.1-23.5)*	*28.3 (40.7-22.9)*	*26.5 (71.0-21.6)*	*29.6 (58.1-22.9)*	*41.1 (56.2-21.5)*
Total levels of Serum IgG (g/L)	*8.9 (10.8-7.9)*	*11.5 (11.8-7.8)*	*11.0 (12.4-7.9)*	*10.2 (11.5-6.7)*	*9.7 (11.7-7.7)*

Data are presented as the median with 25th-75th percentiles (Q_{75}-Q_{25}). Statistical significance (Mann-Whitney test) was calculated with respect to controls ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).*

We did not find any significant differences in levels of CSF tau-reactive antibodies between the group of controls and groups of subjects with impaired cognition for any antigen (Fig. 18-A, C, E). The Kruskal-Wallis test found no significant difference in serum anti-tau antibodies among particular groups, whereas the Mann-Whitney test indicated an elevation of serum antibodies against the full-length form of tau protein in a group of MCI-AD when compared to controls (Mann-Whitney test, p-value 0.020; Fig. 18-B).

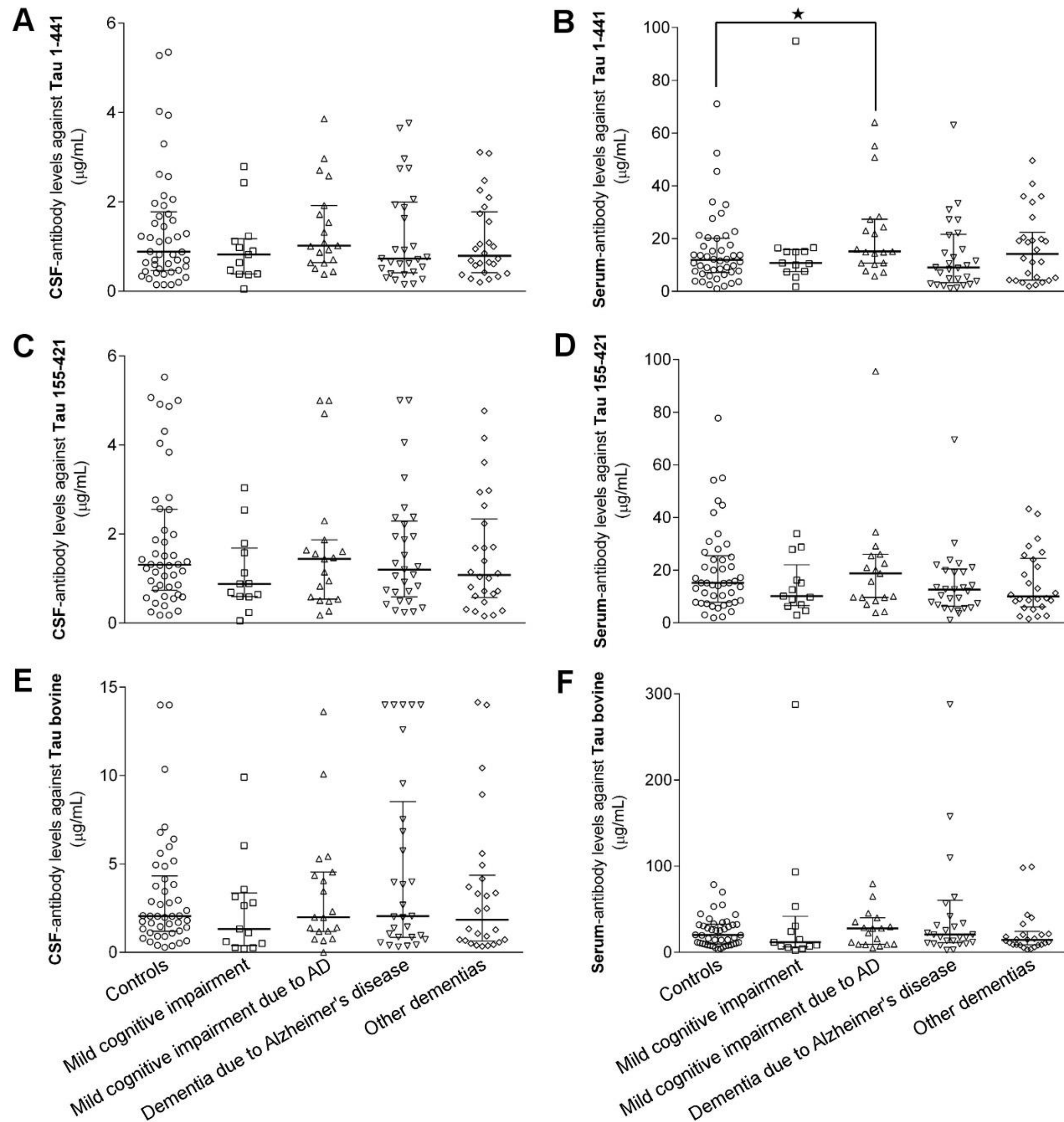

Figure 18: *The concentrations (µg/ml) of tau-reactive antibodies in cerebrospinal fluid and sera samples measured by ELISA assay. The antibodies amounts were established against three antigens (0.125 µg/well): tau 1-441 form (A, B), tau 155-421 fragment (C, D) and native bovine tau (E, F). 50 µl/ well of serially diluted sera samples (1:200, 1:600 and 1:1,800) and undiluted CSF samples was used for analysis.*

We were interested not only in absolute CSF antibody levels but also in the portion of CSF antibodies that were produced locally within the CNS (intrathecal synthesis). We have estimated the relative intrathecal synthesis of specific antibodies in the CSF as follows: ((ratio between CSF and serum specific anti-tau antibodies) / (ratio between CSF and serum concentrations of total IgG))*100 (Terryberry et al., 1998). We did not observe any

significant change in the intrathecal synthesis of specific CSF antibodies against all antigens, although there was a hint of elevation in the MCI-AD group (Fig. 19).

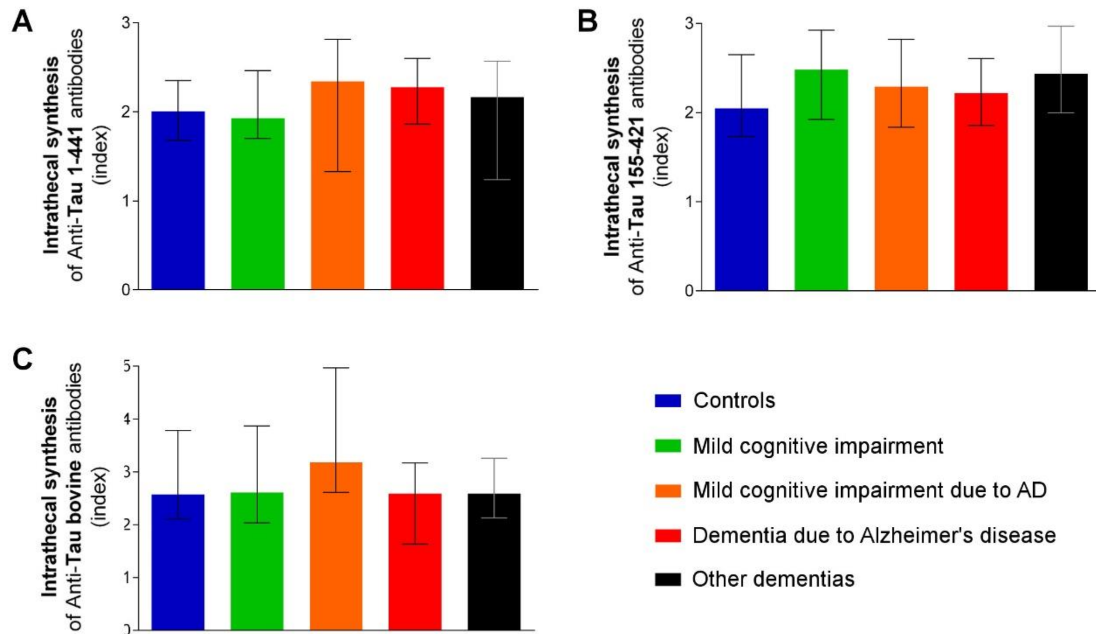

Figure 19: *Intrathecal synthesis of cerebrospinal fluid antibodies reactive with recombinant His-tagged tau 1-441 aa (A), recombinant His-tagged tau fragment 155-421 aa (B) and native bovine tau protein (C) was estimated. The formula was as follows: ((ratio between CSF and serum specific anti-tau antibodies) / (ratio between CSF and serum concentrations of total IgG))*100.*

The CSF and serum levels of tau-reactive antibodies showed high positive correlations for all tau forms (data not shown). Further was the correlation analysis performed only for serum nTau (1-441 aa) antibodies (Table 4). The results indicated a positive link between aging and total IgG levels in CSF in the control and MCI groups. This link was not observed in other cognitively impaired groups suggesting that the production of IgG is disrupted with the progress of neurodegeneration. MMSE did not correlate with any variable. We have observed a negative link between serum nTau (1-441 aa) antibodies and CSF total tau in the MCI-AD group. Serum nTau (1-441 aa) antibodies positively correlated with CSF nTau (1-441) antibodies levels in all groups. Interestingly, we observed negative link of serum nTau (1-441 aa) antibodies to intrathecal synthesis of nTau (1-441) antibodies and positive correlation to total serum IgG levels only in groups of patients with dementia (AD and OD groups). Thus, the elevated levels of serum nTau(1-441) antibodies in the MCI-AD group are not due to the overall increase in IgG levels, but rather due to specific immune response.

Table 4: *Correlation analysis of antibody levels and groups characteristics*

Component	Versus	Controls CC (p)	MCI CC (p)	MCI–AD CC (p)	AD CC (p)	OD CC (p)
Age	Total tau	0.22 (0.151)	0.18 (0.593)	0.33 (0.209)	-0.01 (0.977)	-0.19 (0.361)
	Phospho-tau	0.16 (0.302)	0.10 (0.769)	0.32 (0.231)	0.25 (0.236)	0.07 (0.743)
	Aβ$_{42}$	-0.24 (0.122)	0.11 (0.749)	-0.37 (0.161)	0.26 (0.221)	-0.07 (0.722)
	Total CSF IgG	**0.39 (0.008)**	**0.79 (0.001)**	0.10 (0.694)	-0.04 (0.836)	-0.04 (0.855)
	Total Serum IgG	-0.01 (0.952)	0.32 (0.287)	0.20 (0.401)	-0.13 (0.509)	0.02 (0.913)
	Serum nTau (1-441) Abs	0.13 (0.397)	0.02 (0.957)	0.10 (0.689)	-0.05 (0.785)	0.15 (0.460)
	MMSE	0.10 (0.512)	0.23 (0.465)	-0.26 (0.306)	0.24 (0.229)	-0.09 (0.701)
MMSE	Total tau	0.27 (0.093)	0.09 (0.798)	-0.13 (0.643)	-0.32 (0.148)	-0.24 (0.315)
	Phospho-tau	0.01 (0.931)	-0.30 (0.363)	0.09 (0.752)	-0.33 (0.133)	-0.14 (0.558)
	Aβ$_{1-42}$	0 01 (0.964)	-0.37 (0.265)	-0.14 (0.599)	0.25 (0.253)	0.32 (0.168)
	Serum nTau (1-441) Abs	0.07 (0.683)	-0.54 (0.071)	-0.08 (0.762)	-0.19 (0.355)	-0.34 (0.137)
	Total serum IgG	0.07 (0.676)	0.20 (0.529)	0.42 (0.082)	-0.08 (0.711)	-0.18 (0.460)
	Total CSF IgG	-0.20 (0.193)	-0.08 (0.801)	0.12 (0.643)	-0.10 (0.622)	-0.33 (0.160)
Serum nTau (1-441) Abs	Total tau	-0.18 (0.244)	0.13 (0.709)	**-0.50 (0.049)**	-0.18 0.409	0.14 (0.521)
	Phospho-tau	0.05 (0.769)	0.24 (0.484)	-0.21 (0.431)	-0.37 (0.072)	0.04 (0.835)
	Aβ$_{42}$	-0.08 (0.625)	-0.15 (0.650)	-0.11 (0.680)	-0.33 (0.114)	0.01 (0.945)
	CSF nTau (1-441) Abs	**0.77 (<0.001)**	**0.81 (<0.001)**	**0.48 (0.039)**	**0.89 (<0.001)**	**0.60 (0.001)**
	Intrathecal nTau (1-441 Abs)	-0.17 (0.241)	0.02 (0.957)	-0.43 (0.067)	**-0.61 (<0.001)**	**-0.53 (0.007)**
	Total Serum IgG	0.11 (0.449)	0.22 (0.475)	0.04 (0.856)	**0.60 (<0.001)**	**0.59 (0.001)**
	Total CSF IgG	-0.06 (0.684)	0.27 (0.364)	-0.08 (0.743)	**0.49 (0.007)**	-0.03 (0.898)

Data were collected from 44 controls, 11 MCI, 16 MCI-AD, 24 dementia due to AD patients and 25 patients with other dementias. Correlation coefficients (CC) were evaluated via a Spearman correlation for non-normally distributed data. Finally, permutation analysis was performed to compare groups. Significant correlations are highlighted in bold ($p < 0.05$).

4.6 Levels of tau oligomers and conformation-specific tau forms in cerebrospinal fluid and serum

The levels of tau oligomers and conformers were measured by ELISA assay during my internship at the University of Texas Medical Branch in the Laboratory of prof. Rakez Kayed using unique antibody T22 specific only to oligomeric forms of tau protein (Lasagna-Reeves et al., 2012) and antibody TTC-99 recognizing oligomers and misfolded monomers (conformers; unpublished data). We measured 186 sera and 118 CSF samples in total. Subjects were divided into three groups with different cognitive impairment and one group of cognitively healthy control subjects. The interassay variation between plates was calculated from 18 measurements for each antibody. The variation was 10 % and 13 % for T22 and TTC-99 antibody, respectively. Table 5 shows demographic and cognitive characteristics of individuals in groups including measured levels of current CSF biomarkers (total tau, phospho-tau$_{181}$ and Aβ_{42}) by commercial ELISA kits if they were available.

Table 5: *Demographic, cognitive and cerebrospinal fluid characteristics of subjects enrolled for measurement of tau oligomers.*

	Control subjects	Mild Cognitive Impairment	Mild Cognitive Impairment due to Alzheimer's disease	Dementia due to Alzheimer's disease
N per group (CSF/serum)	*41 / 86*	*14 / 14*	*18 / 18*	*45 / 68*
Age (years)	*63 (68-58)*	*63 (77-59)*	***72** (77-67)****	***76 (81-72)*****
Female sex	*51% / 47%*	*60%*	*41%*	*67% / 68%*
MMSE score	*29 (30-28)*	***26.0 (28.0-24.0)*****	***25.5 (28.0-24.0)*****	***20.0 (23-18)*****
Total tau (pg/mL)	*194.7 (263.0-161.0)*	*207.6 (240.1-185.6)*	***301.8 (458.9-213.3)****	***599.0 (967.0-325.0)*****
Phospho-tau$_{181}$ (pg/mL)	*35.5 (43.0-27.8)*	*39.7 (55.1-29.9)*	***57.9 (80.2-39.2)****	***63.2 (93.4-43.3)*****
Aβ_{42} (pg/mL)	*911.1 (1125.0-729.9)*	*839.6 (988.7-643.3)*	*745.5 (1091.7-459.2)*	***533.0 (814-9-377.9)*****
Total protein (mg/mL)	*0.36 (0.42-0.27)*	*0.36 (0.41-0.32)*	***0.45 (0.68-0.34)****	*0.38 (0.61-0.28)*

*Data are presented as the median with 25th-75th percentiles (Q_{75}-Q_{25}). Statistically significant differences highlighted in bold were calculated with respect to controls (Mann-Whitney test, * p <0.05, ** p <0.01, *** p <0.001).*

Statistical analysis revealed significant drops in MMSE score in all groups with cognitive impairment when compared to controls. The concentration of total tau and

phospho-tau_{181} protein was higher in groups of patients with MCI-AD and dementia due to AD. $A\beta_{42}$ levels were significantly lowered only in the group of patients with dementia due to AD. By comparing the age of subjects in groups we found that individuals in MCI-AD (Kruskal-Wallis test, $p < 0.001$) and dementia due to AD (Kruskal-Wallis test, $p < 0.001$) groups were older than cognitively normal subjects. Therefore, we have analyzed if age is an important variable in the analysis of levels of tau oligomers/conformers. We found that the age of participants correlated with the serum tau oligomers levels (GLM, p= 0.005), but not serum tau conformers (GLM, p= 0.167). The statistical comparison between groups for serum tau oligomers was, therefore, calculated with respect to age as a covariate.

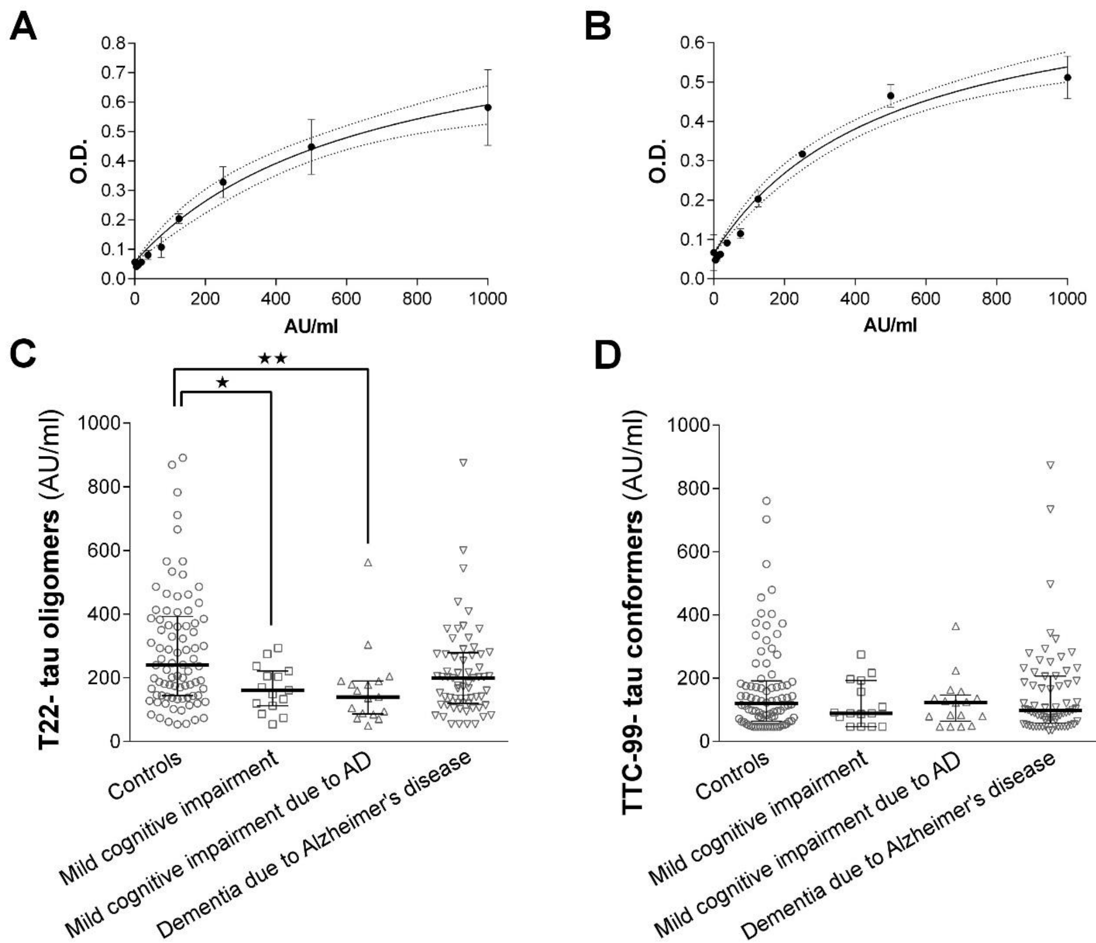

*__Figure 20:__ Serum levels of tau oligomers/conformers measured by ELISA. The standard curves of T22 (A) or TTC-99 (B) reactivity to recombinant tau oligomers were used for interpolation of tau oligomers/conformers concentrations in serum samples. Data are presented as scatter plot of interpolated concentrations of T22- reactive tau oligomers (C) and TTC-99- reactive tau conformers (D). Concentrations are presented as medians with 25th-75th percentiles. Statistical significance was calculated with respect to controls and age as a covariate (Generalized linear model analysis with the covariate of age and contrast statement or Mann-Whitney test, * p<0.05, ** p<0.01).*

The levels of serum tau oligomers were decreased in MCI group (p= 0.033) and MCI-AD group (p=0.006) with respect to cognitively healthy subjects (Fig. 20-C). In sera of patients with dementia due to AD, we observed an elevation of tau oligomers levels in comparison to MCI and MCI-AD groups, but it did not reach statistical significance. Tau conformers did not show any difference between groups (Kruskal-Wallis test, p= 0.486, Fig. 20-D).

The sensitivity of ELISA assay for CSF tau oligomers and conformers was low. We were not able to measure tau oligomers in 29 % of all cases and it reaches up to 50 % in the group of MCI-AD patients (Fig. 21-A and B).

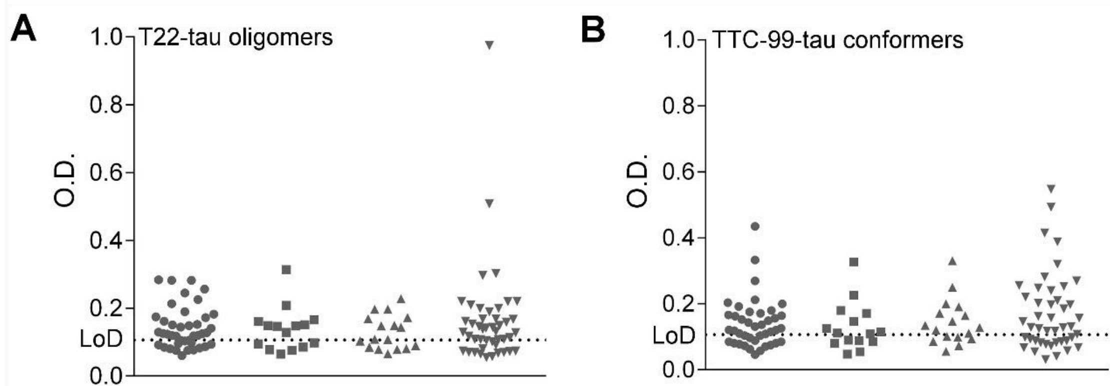

Figure 21: *Scatter plot of optical densities with the marked limit of detection (LoD) for T22-tau oligomers (A) and TTC-99-conformers (B).*

Moreover, we observed a strong positive correlation of tau oligomers (Spearman r 0.46, p <0.001) and conformers (Spearman r 0.40, p <0.001) with total protein levels in CSF. Only those MCI-AD patients with elevated total protein levels had detectable levels of both oligomers and conformers. Therefore, we have decided to normalize the CSF concentrations of tau oligomers and conformers to CSF total protein levels (referred as Index T22 and Index TTC-99, respectively). We have found slightly decreased value of index T22 in the group of MCI-AD subjects in comparison to controls (Fig. 22-A).

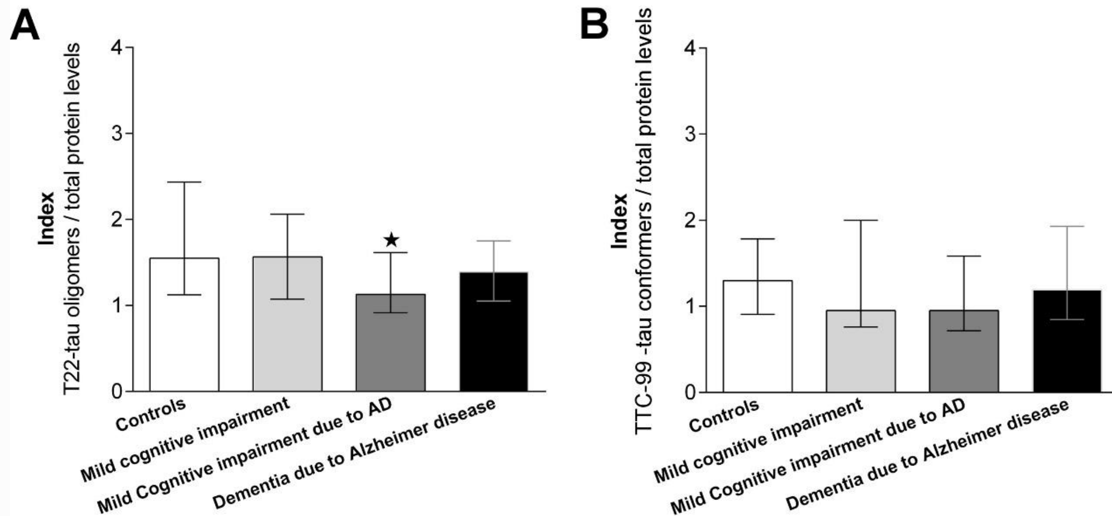

Figure 22: *Normalized levels of tau oligomers (C) and conformers (D) to total protein levels in cerebrospinal fluid. Data are presented as bars showing median with 25th-75th percentiles. Statistical significance was calculated with respect to controls (Mann-Whitney test, * $p<0.05$).*

Further analysis of data was carried out only for tau oligomers due to observed differences between groups. We performed a correlation analysis of measured levels of T22-tau oligomers with basic groups' characteristics (Table 6). The results indicated a positive correlation between aging and serum levels of tau oligomers in the group of controls and index T22 in the group of MCI patients suggesting increasing levels of tau oligomers with aging. From all variables, the MMSE correlated only with total tau levels in the control group. The index value T22 that represents tau oligomers present in CSF negatively correlated with CSF phospho-tau_{181} and $A\beta_{42}$ levels in the group of patients with dementia due to AD.

Next, we looked into the distribution of T22- reactive tau oligomers in CSF and serum of randomly selected samples by Western blot. Representative Western blot detection of tau oligomers in CSF (Fig. 23) and serum (Fig. 24) of three controls and three patients with dementia due to AD is presented. In CSF samples, we have observed bands around 100-150 kDa that could probably be from tau dimer/trimer. There was no aggregated tau detected above and below these dimers/trimers. Student's t-test did not show any statistically significant difference between the density of protein bands in groups of AD patients and control subjects (Fig. 23 B and C).

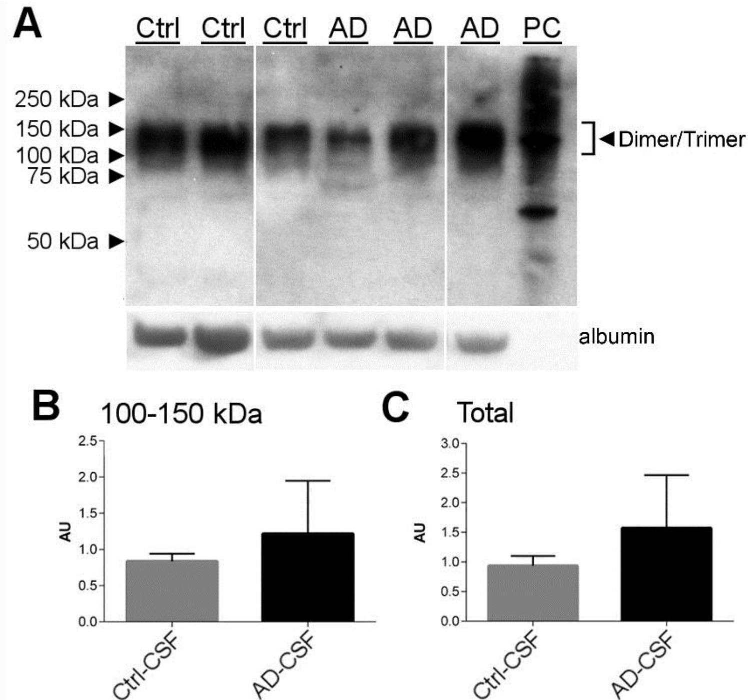

Figure 23: *Western blot analysis of cerebrospinal fluid samples (5 µg of total protein/lane) from three patients with dementia due to Alzheimer's disease (AD) and three cognitively normal controls (Ctrl) (A) was carried out. In vitro prepared recombinant tau oligomers were used as a positive control (PC). Tau oligomers detected by rabbit polyclonal anti-oligomer antibody T22 were quantified by densitometry in the dimer/trimer bands (100-150 kDa; B) and whole lane (C). Densitometric quantifications (AU= arbitrary unit) were normalized to albumin (A), used as loading control. Bars represent mean ± SD.*

T22- immunoreactive tau oligomers higher than 250 kDa were observed in the serum of AD samples, but also in some of the control samples. Densitometric quantifications of higher molecular weight (HMW) bands from the Western blot of serum probed with T22 revealed significantly increased tau oligomers in AD (* $p < 0.05$, Student's t-test; Fig. 24-B). When the density of all protein bands was compared between groups, the AD patients had significantly more of tau oligomers detected with T22 when compared to controls (* $p<0.05$, Student's t-test; Fig. 24-C). We attribute the bands around 120-150 kDa to the tau dimers/trimers, but also probably non-specific immunoglobulins. The lower molecular weight oligomers (under 50 kDa) could be a result of dissociation of less stable HMW oligomers in the presence of SDS in the samples.

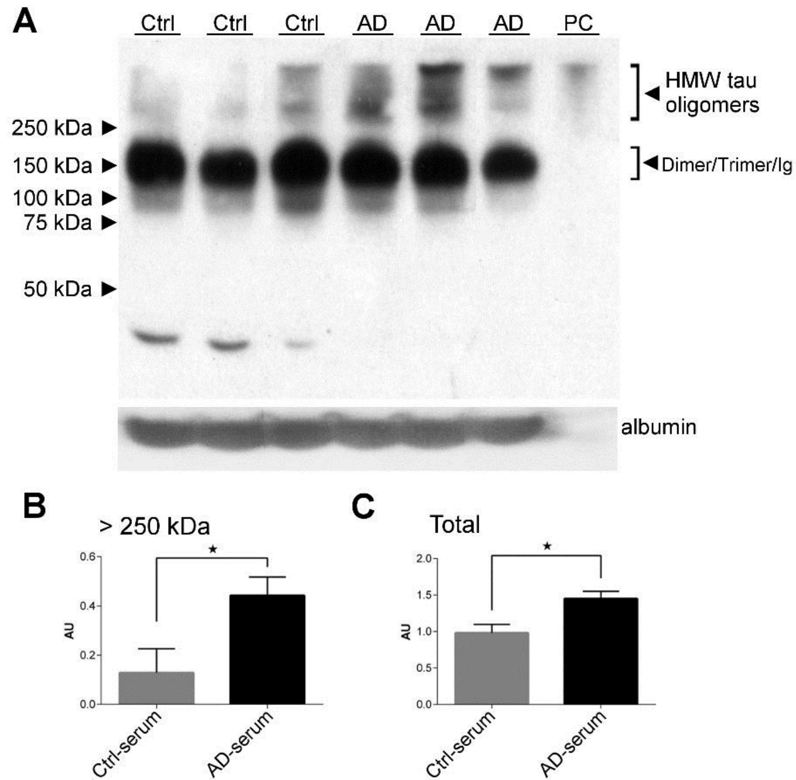

Figure 24: *Western blot analysis of serum samples (50 µg of total protein/lane) from three patients with dementia due to Alzheimer's disease (AD) and three cognitively normal controls (Ctrl) (A) was carried out. In vitro prepared recombinant tau oligomers were used as a positive control (PC). Tau oligomers detected by rabbit polyclonal anti-oligomer antibody T22 were quantified by densitometry in higher molecular weight bands (>250 kDa; B) and in the whole lane (C). Densitometric quantifications (AU= arbitrary unit) were normalized to albumin (A), used as loading control. Bars represent mean ± SD.*

Table 6: *Correlation analysis for tau oligomers and conformers.*

Component	Versus	Controls CC (p)	MCI CC (p)	MCI–AD CC (p)	AD CC (p)
Age	Total tau	0.16 (0.331)	-0.18 (0.565)	-0.07 (0.787)	-0.08 (0.608)
	Phospho-tau	0.02 (0.887)	-0.41 (0.189)	0.18 (0.499)	-0.02 (0.879)
	$A\beta_{42}$	-0.13 (0.426)	0.23 (0.469)	-0.43 (0.094)	-0.01 (0.963)
	Total protein	0.00 (0.982)	-0.53 (0.051)	0.28 (0.268)	0.28 (0.068)
	Index T22	0.23 (0.163)	**0.63 (0.015)**	-0.16 (0.535)	-0.22 (0.153)
	Serum T22 oligomers	**0.26 (0.016)**	0.15 (0.604)	0.16 (0.526)	0.14 (0.271)
	MMSE	–0.04 (0.734)	0.24 (0.424)	-0.33 (0.176)	0.08 (0.521)
MMSE	Total tau	**0.43 (0.008)**	0.11 (0.743)	0.07 (0.802)	-0.21 (0.179)
	Phospho-tau	0.24 (0.144)	-0.38 (0.220)	0.00 (0.987)	-0.29 (0.067)
	$A\beta_{42}$	-0.06 (0.735)	-0.31 (0.324)	-0.03 (0.905)	0.21 (0.187)
	Serum T22 oligomers	0.02 (0.841)	-0.20 (0.517)	0.06 (0.822)	-0.03 (0.784)
	Index T22	-0.00 (0.999)	0.04 (0.903)	0.40 (0.107)	0.05 (0.769)
Total tau	Serum T22 oligomers	0.22 (0.182)	-0.19 (0.529)	-0.26 (0.333)	-0.13 (0.389)
	Index T22	-0.11 (0.511)	-0.35 (0.247)	-0.08 (0.781)	-0.15 (0.331)
Phospho-tau	Serum T22 oligomers	0.17 (0.311)	-0.09 (0.762)	0.01 (0.983)	-0.29 (0.059)
	Index T22	-0.26 (0.108)	-0.50 (0.101)	0.13 (0.648)	**-0.35 (0.023)**
$A\beta_{42}$	Serum T22 oligomers	-0.15 (0.360)	0.56 (0.059)	0.01 (0.957)	-0.19 (0.227)
	Index T22	-0.04 (0.832)	0.48 (0.118)	0.20 (0.467)	**-0.46 (0.002)**

Data were collected from 40 controls, 17 MCI-AD, 11 MCI, 45 patients with dementia due to AD and 20 patients with other dementias. Correlation coefficients (CC) were evaluated via a Spearman correlation for non-normally distributed data. Finally, permutation analysis was performed to compare groups. The index refers to the quotient of tau oligomers and total protein levels in CSF. Significant correlations are highlighted in bold ($p < 0.05$).

5 Discussion

It is accepted that clinical manifestation of dementia in AD is due to the neuronal loss occurring in those areas of the brain associated with cognitive functions of the patients. Fibrillar inclusions are reported to be responsible for cell death. In this regard, a number of reports analyzing the brain of AD patients come to an agreement that fibrillar aggregation of tau is the best correlator with the onset and progression of dementia. It is mostly accepted that abnormal post-translational modifications like hyperphosphorylation, acetylation, glycation, nitration, truncation, and others, are responsible for altered tau structure in AD. Some of these events have been sequentially staged during the formation of NFTs and the evolution of the disease (Binder et al., 2005; García-Sierra et al., 2003). Amino acid site-specific phosphorylation and truncation at both N- and C-termini have been considered as the main post-translation modifications of tau protein in AD (Buée et al., 2000; Ding et al., 2006; Mondragón-Rodríguez et al., 2014; Novák, 1994). These post-translational modifications can significantly change the soluble form of tau protein in a paperclip-like arrangement and promote the aggregation (Kovacech and Novak, 2010; Mandelkow et al., 2007; Wang et al., 2010). It was proven that truncation is a very early event in tangle formation, and that truncated tau phosphorylated at few specific sites even occurs before hyperphosphorylated form (Rissman et al., 2004). Truncation in the C-terminal part of tau protein at cleavage points Asp421 (Fasulo et al., 2000; T. Chris Gamblin et al., 2003) and Glu391 (M. Novak et al., 1993) was well defined and is even considered as a triggering factor in the development of tau pathology (de Calignon et al., 2010; García-Sierra et al., 2001; Mena et al., 1996; Rissman et al., 2004; Zilka et al., 2009). The Asp421 cleavage always precedes Glu391 truncation in NFT formation (Basurto-Islas et al., 2008). To date, some facts are also known about the truncation in the N-terminal part (Arai et al., 2005a; Horowitz et al., 2004; Olesen, 1994).

Thus, tau protein occurs in numerous post-translationally modified forms in the brain tissue, and site-specific phosphorylation and truncation are connected with the balance between physiological and pathological tau function. Therefore, we prepared and studied several tau forms varying in length and phosphorylation state, namely, the recombinant His-tagged full-length form of tau protein (1-441 aa) and two recombinant His-tagged

fragments with sequences 13-391 aa and 155-421 aa. The proteins were characterized by HPLC and immunoassays (Western blot, dot blot, and ELISA) using monoclonal antibodies Tau 5, and Tau 46.1 specific to the middle part of the protein, and to C-terminal, respectively. All forms prepared for this study were showen to form aggregates. At first, we used the recombinant His-tagged full-length form of tau protein for immunization of rabbits to raise specific antibody production. The rabbit serum showed reactivity with unrelated protein ERAB that contained His-tag, although the His-tag is in literature referred as a weakly immunogenic sequence (Gaberc-Porekar and Menart, 2001; Terpe, 2003). The purification process was, therefore, extended for an anti-His-tag antibody depletion. The binding epitopes of thoroughly purified rabbit polyclonal anti-tau antibody have been found in the proline-rich part of the protein (171-194 aa) and the microtubule-binding domain R3 (299-317 aa) that contains sequence ^{306}VQIVYK^{311} with β-structure (Kolarova et al., 2012). This repeat domain was described to promote self-aggregation of tau protein molecules in pathological conditions and *in vitro* experiments (Pérez et al., 1996; von Bergen et al., 2005, 2000). However, the epitope 299-317 aa was identified as a non-specific due to the undesirable adhesiveness of this fragment (Hromadkova et al., 2016). All forms of tau protein prepared for this study contained both sequences and moreover the fragments were C-terminally truncated at sites described in the staging of NFT (Jarero-Basulto et al., 2013). Above that the fragment 155-421 aa was N-terminally truncated at the Arg155, which is a cleavage site of thrombin protease (Arai et al., 2006, 2005b) that is abundant in the blood. As a result of this, the fragment 155-421 aa can represent new epitopes for antibodies circulating in the blood. However, this truncation may occur even in the brain where thrombin is also expressed (Hu et al., 2010; Niego et al., 2011).

Complexes of tau protein with Aβ peptide in the cerebrospinal fluid

The prepared rabbit polyclonal anti-tau antibody was used to study the occurrence of tau-Aβ complexes in CSF of patients with dementia due to AD, cognitively normal controls, people with mild cognitive impairment due to AD and other neurodegenerations (Kristofikova et al., 2014). The drops in levels of complexes to 80 % in the CSF of patients with dementia due to AD in contrast to controls could be interpreted as enhanced interactions between Aβ and tau and subsequent accumulation of the complexes in the brain, in accordance with data in the literature (Guo et al., 2006; Manczak and Reddy, 2013). The

complexes distinguished between controls and dementia due to AD patients with a sensitivity of 68.6 % and a specificity of 73.3 %. Nevertheless, the complexes haven't proved as a significantly better biomarker of AD than current biomarkers (Kristofikova et al., 2014).

Reactivity of naturally occurring plasma antibodies with various forms of tau protein

We utilized prepared recombinant proteins and their phosphorylated equivalents for characterization of isolated naturally occurring anti-tau antibodies; firstly from the commercial product of intravenous immunoglobulins Flebogamma (Hromadkova et al., 2015) and secondly from the plasma of cognitively normal older individuals and patients with AD. The IVIG products containing IgG molecules pooled from several thousand healthy donors are now in the spotlight because of ongoing clinical trials for AD therapeutic interventions. It has been proven that these products contain antibodies against neuronal tau protein (Smith et al., 2013), but their reactive character has been partly established (Smith et al., 2014b, 2013). Therefore, we have characterized these naturally occurring antibodies against recombinant proteins (Hromadkova et al., 2015) and moreover against native brain-derived forms of tau protein. The IVIG product Flebogamma is composed of 99 % of IgG molecules with a distribution of subclasses similar to normal serum values (Ballow, 2009; Jorquera, 2009). The IgG isotyping of isolated nTau-IVIG antibodies revealed that the most and least abundant subclasses were IgG1 and IgG4 as expected. However, the results showed repeatable enrichment of IgG3 subclass. The IgG3 subclass belongs to potent pro-inflammatory antibodies in early response to protein and viral antigens with a shorter half-life (Michaelsen et al., 2009; Vidarsson et al., 2014). It is also suggested that natural IgG antibodies are IgG3 subclass-specific (Panda and Ding, 2015). Unlike the nTau-IVIG antibodies, the isolated nTau-AD antibodies contained mainly IgG1 and IgG2 subclasses with IgG3 at almost undetectable level. This finding could point to the lack of IgG3 subclass due to depletion of these immunoglobulins into complexes with tau or binding to neurons in the case of compromised BBB in patients with AD. Nevertheless, we observed this depletion in the nTau-Ctrl antibodies as well. However, nTau-Ctrl antibodies showed a higher proportion of IgG4 subclass. The IgG4 subclass is referred to be increased after the chronic antigenic stimulation with protein antigens (Jefferis and

Kumararatne, 1990). That is supported by the higher avidity of nTau-Ctrl antibodies against both forms of tau protein.

Interestingly, we have found marked reactivity of all three nTau antibodies to a fragment of tau protein 155-421 aa despite the isolation against the full-length form of tau protein. The strong binding was confirmed by avidity measurements where we observed the highest avidity index of these antibodies (~1.6 M) for fragment 155-421 aa in contrast to other forms (Hromadkova et al., 2015). This antigenicity was partly abolished by phosphorylation of this fragment pointing out to epitopes related to truncation of tau protein rather than phospho-specificity. This finding is interesting because the truncated tau is highly prone to aggregate and forms reactive intermediates (García-Sierra et al., 2008, 2003, 2001; Guillozet-Bongaarts et al., 2005; Mena et al., 1996). Although, the amounts of isolated antibodies against truncated and phosphorylated tau forms could be affected by using non-phosphorylated full-length tau as a ligand and these antibodies may not be sorted out during our isolation procedure. In future experiments, we want to prepare native-like tau protein and repeat the isolation protocol.

Nonetheless, to obtain insight into the reactivity of these isolated antibodies with native proteins, we probed brain homogenates of histopathologically proven AD patients and controls with all three nTau antibodies and compared their reactivity with other selected polyclonal and monoclonal anti-tau antibodies by Western blot. The homogenates contained different forms of tau protein ranging from presumable monomers of different isoforms (characteristic triplet, (Tolnay et al., 2002)) and fragments to HMW protein forms (aggregates/oligomers). The HMW proteins were found mostly in AD brains in contrast to control brain tissue. The phospho-specific anti-pSer396-tau antibody also showed a difference in the staging of Alzheimer's neurodegeneration in the brains of two patients as this antibody is specific to late stage of AD (Augustinack et al., 2002; Götz et al., 2010). The staining of HMW proteins in the AD1 homogenate with the late stage of pathology was stronger in the fraction containing insoluble proteins. This fraction was prepared by homogenization of the brain with 2% SDS confirming the presence of insoluble NFT. This finding was supported by staining of AD2 homogenate with AT180 antibody that binds to pThr231 on the tau molecule that occurs early in the stage of tau aggregation (soluble oligomers) (Lasagna-Reeves et al., 2012). Interestingly, the isolated nTau plasma

antibodies stained the HMW protein bands as did the AT180 antibody. Thus, these antibodies were likely reactive with soluble aggregates of tau protein. To exclude non-specific signal of the secondary antibody, we conjugated all isolated nTau antibodies to biotin. The conjugation resulted in an overall loss of staining for nTau-IVIG antibodies. The conjugation of biotin could interfere with their reactivity because biotin binds to lysine residues present on the Fc fragment and in the hinge region of IgG molecule. This result allows to assume Fc fragment contribution to their ability binding of antigen (Dillon et al., 2016; Stearns and Pisetsky, 2016) or limiting the flexibility of two antigen binding sites through interaction with the hinge region (Michaelsen et al., 1977). On the contrary, the conjugation of nTau-AD antibodies to biotin showed an enhancement of LMW bands staining in the brain homogenates and recombinant fragment 155-421 aa of tau as well. That is in agreement with the high avidity of these antibodies against truncated tau 155-421 aa observed by dot-blot immunoassay. On the contrary, the reactivity against HMW proteins was preserved for the biotinylated nTau-Ctrl antibodies. The latest studies have revealed that aggregated oligomeric tau forms are the toxic species of this protein. They can spread tau pathology and are responsible for cognitive impairment in AD mouse models which can be alleviated by passive immunotherapy specifically targeting tau oligomers (Castillo-Carranza et al., 2015, 2014; Yanamandra et al., 2015). Our findings may refer to immune control of tau truncation and related aggregation in healthy individuals thus providing clearance of these structures from the brain as was suggested by Dr. Castillo-Caranza from the University of Texas Medical Branch (Castillo-Carranza et al., 2014) and others (d'Abramo et al., 2013; Yanamandra et al., 2015, 2013). This explanation would warrant the immunotherapy as a promising approach to AD treatment through the enhancement of the probable insufficient clearance of toxic tau aggregates. However, more experiments are needed to evaluate the character of these HMW proteins.

Levels of naturally occurring anti-tau antibodies in serum and CSF samples

Although AD is a neurodegenerative disorder, the evidence is accumulating that it also involves alterations in humoral immunity and autoimmunity. The immune system involvement in AD is corroborated by the presence or altered levels of antibodies against several self-antigens (Bartos et al., 2012; Bouras et al., 2005; Colasanti et al., 2010; D'Andrea, 2005; Hromadkova et al., 2015; Szabo et al., 2010). Anti-neuronal antibodies

have been detected in sera, CSF and brains of patients with AD (Franceschi et al., 1989; Kumar et al., 1988; Levin et al., 2010; R. G. Nagele et al., 2011; Rosenmann et al., 2006; Soussan et al., 1994; Terryberry et al., 1998). Serum auto-antibodies have also been evaluated as potential diagnostic biomarkers for AD (Bartos et al., 2012; DeMarshall et al., 2015; E. Nagele et al., 2011; Reddy et al., 2011).

In accordance with latest studies and our previous results, we have found naturally occurring antibodies in all investigated groups. We have observed only a slight increase of serum tau (1-441 aa)-reactive antibodies in the group of patients with MCI-AD and their link to total levels of tau protein present in CSF. This finding suggests an activation of the immune system to control higher amounts of tau proteins occurring in the biofluids as a consequence of developing pathology. We did not observe a significant change in the intrathecal synthesis of nTau antibodies, which confirm the observation of increased levels of serum but not CSF nTau antibodies. Our results confirmed that auto-antibodies are ubiquitous in human serum, can be influenced by disease, and are remarkably stable over time (DeMarshall et al., 2015; Lacroix-Desmazes et al., 1999; Mirilas et al., 1999; Nagele et al., 2013). However, these naturally occurring tau-reactive antibodies have not proved to be suitable biomarkers of AD.

Tau oligomers in cerebrospinal fluid and serum of AD patients and aged cognitively healthy persons

There are studies concerning tau occurrence and its modified forms in CSF because this fluid is in direct contact with the brain parenchyma. Lower CSF $A\beta_{42}$ levels and elevated total tau, and phospho-tau_{181} are considered as the core markers of AD pathology. Measurement of other CSF phospho tau proteins like p-tau 231-235,or p-tau 396-404 have also been shown in AD diagnosis (Holtzman, 2011; Sui et al., 2014). However, detection of tau protein forms circulating in the blood could be a valuable diagnostic marker of AD. Several studies have reported plasma and serum levels of tau protein, but the results are contradictory. While one study showed lowered levels of plasmatic tau proteins in AD patients (Sparks et al., 2012), Zetterberg et al on the contrary, found higher plasma levels of tau protein in AD patients using their digital array technology (Zetterberg et al., 2013). The serum levels of tau protein were reported to be elevated after mild traumatic brain injury (TBI) (Bulut et al., 2006) and in acute ischemic stroke (Bitsch et al., 2002).

Moreover, recent studies reported that release of endogenous tau protein from neurons into the interstitial fluid (ISF) is a physiological process which is mediated by neuronal activity (Pooler et al., 2013; Yamada et al., 2014). They suggest that released tau proteins can function as a signal for adjacent cells and may be taken up by post-synaptic neurons. However, the basal levels of tau have to be restored upon release. The secreted tau could be washed out of ISF through glymphatic pathway into the blood where it can be subjected to fast degradation by proteases or targeted by antibodies.

In our study, we detected T22-reactive tau oligomers in the human serum, interestingly their levels positively correlate with aging. However, the exact cause of this aggregation remains unidentified. It is reported that metabolism slows down and coagulation activity in blood increases with aging (Sagripanti and Carpi, 1998). Therefore, the increased tau oligomer burden detected in the serum of cognitively normal persons and patients with dementia due to AD could be a consequence of the higher accumulation of tau, thrombin and clotting agents in the blood. The observation of tau oligomers detected in the serum was also supported by western blot assay. The detection of HMW tau oligomers in serum of AD patients and to some extent in control individuals in our study is in agreement with the study of Neumann et al. and Farías et al. where the researchers observed similar HMW forms of tau protein in the platelets of both AD patients and control subjects (Farías et al., 2012; Neumann et al., 2011). Moreover, we observed LMW bands in the serum of aged controls. The LMW bands could be the products of desired enzymatic degradation of tau protein aggregates in the blood under physiological conditions or unstable HMW aggregates/oligomers that can be dissociated. This observation is supporting the hypothesis that tau can be partly cleared on the periphery. When the clearance pathway is disturbed, the oligomers may accumulate in CSF or brain. In accordance with that we have found decreased levels of tau oligomers in the MCI-AD group. In fact, the impaired clearance of tau protein from the ISF through glymphatic pathway appearing after TBI has recently been described in a TBI mouse model (Iliff et al., 2014), which is an established risk factor for the development of neurodegeneration, including AD. In addition, the physiological efflux of proteins from CSF to blood through the choroid plexus was described as a clearance pathway for Aβ peptide (Crossgrove et al., 2005; Marques et al., 2013; Tarasoff-Conway et al., 2015). No studies describing clearance of tau protein through this barrier have been

published thus far (Tarasoff-Conway et al., 2015). However, pathological changes in the choroid plexus related to neurodegeneration were described as enhancing tau oligomerization (Preston, 2001; Redzic et al., 2005; Serot, 2003) and that may be reflected later on in the course of the disease as an increase of stable tau oligomers in blood. On the other hand, the accumulation of HMW oligomers could correlate to the pathophysiology of the disease in both the CNS and the peripheral blood cells. Nevertheless, the results of decreased levels of tau oligomers in the serum of MCI-AD patients may be related to elevated levels of tau-reactive antibodies found in this study. Thus, the immune system may play a significant role in protection against toxic protein aggregates. This assumption is supported by the reactivity and high avidity of antibodies isolated from plasma of older cognitively normal subjects to various forms of tau protein presented above. Although, the mechanisms involved in the clearance of tau are not completely understood when compared to Aβ clearance, the changes in the levels of tau oligomers with the manifestations of cognitive decline could be a valuable tool for future diagnostics.

We supposed that the levels of tau oligomers would be increased in CSF of AD patients because of impaired clearance pathways, released proteins by dying neurons and decreased turnover of CSF. Recent study of Urmi Sengupta supports this assumption. They optimized the methodic described above for CSF samples and found elevated levels of T22-reactive tau oligomers in the group of patients in the mild and severe stages of AD (Sengupta et al., 2017). On the contrary, we observed reduced index of tau oligomers in the MCI-AD group in comparison to controls. However, this result was most probably influenced by the low sensitivity of the assay for CSF tau oligomers, especially in the group of MCI-AD patients. Therefore, it is difficult to come to any conclusion from the results of our CSF measurements.

Additionally, similar to fibrillar structures, tau oligomers can exhibit different conformers whose aggregation stage might reflect the state of the disease. A recent study has demonstrated that Aβ oligomers from AD brain are characteristically different than those collected from CSF in their seeding property (Fritschi et al., 2014). Therefore, extending our study to better analyze these tau oligomeric species will enhance our existing understanding of the heterogeneity of different tau oligomer conformers from different sources like brain, CSF and blood serum/plasma. Thus, further research on heterogeneous

tau oligomer population will provide more insight into the understanding of tau trafficking and its role in AD.

5.1 Conclusion

The work presented here outlines the role of the peripheral immune system in the protection against toxic tau oligomers early in the development of neurodegeneration. However, our hypothesis of elevated levels of tau oligomers in biofluids of AD patients was confirmed only partly.

We characterized reactivity profile of anti-tau antibodies naturally occurring in plasma of healthy subjects contained in the product of intravenous immunoglobulins Flebogamma (nTau-IVIG), older cognitively normal subjects (nTau-Ctrl) and AD patients (nTau-AD). Interestingly, nTau-Ctrl antibodies showed strong reactivity against higher molecular weight (HMW) forms of tau protein (aggregates/oligomers) present in the brain homogenates of AD patients in comparison to nTau-IVIG were the reactivity was weak. On the contrary, nTau-AD antibodies reacted preferentially with lower molecular weight (LMW, monomeric) forms of tau protein. Moreover, we found elevated levels of serum antibodies against human full-length tau protein (1-441 aa) in the group of patients with mild cognitive impairment due to AD (MCI-AD) when compared to controls (Mann-Whitney test, $p = 0.02$). This finding partly supports our hypothesis stated in the aims. The serum levels of anti-tau (1-441 aa) antibodies negatively correlated with levels of total tau in CSF for this group. These results altogether likely point to the involvement of immune system in protection against the forming toxic tau forms probably via their clearance during normal aging or in the early stage of neurodegeneration, but not in the late stage of pathology.

These assumptions are in agreement with our findings of tau oligomers in the blood. We found tau oligomers in the sera of cognitively normal subjects, and these levels were increasing with age. The amounts of tau oligomers were decreased in the group of MCI-AD in comparison to controls (GLM, $p = 0.015$) and then increased in the group of AD patients. By western blot, we found that serum of AD patients contained stable HMW oligomers while in serum of controls the HMW oligomers could be partly dissociated. Our findings supports the assumption that extracellular tau protein can be partly cleared from

the brain to the periphery. In the case of patients with MCI and MCI-AD, the lower levels of tau oligomers could be the result of impaired clearance of tau protein from interstitium to blood and consequent accumulation of tau aggregates in the brain. However, the mechanisms involved in the clearance of tau from the brain to the periphery or the role of peripheral tau are not completely elucidated. Thus, further research on heterogeneous tau oligomer population and tau trafficking could help us better understand the role of tau protein in AD development.

Nevertheless, our findings stated above support the latest approaches to the treatment of AD via immunotherapy as an enhancement of the probable insufficient clearance of toxic tau aggregates.

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