Proteasomal dysfunction

A way to classify FTD subjects?

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Men ought to know that from the brain, and from the brain only, arise our pleasures, joy, laughter and jests, as well as our sorrows, pains, grieves, and tears.

(Hippocrates 460-370 BC)

Preface

Eight months ago, I was very nervous to start my senior internship for a period of 30 weeks. I thought, 30 weeks is such a long time in which I can do so many things. But it is hard to believe the end of my last master year is nearly here. In this period, I acquired new skills and new knowledge, made new friends and I realized that science is not a one man show. Therefore, I would like to take this opportunity to thank some of the people who contributed to my thesis and helped me in becoming a critical, motivated young scientist.

First of all, I would like to thank my promotor Dr. F.W. van Leeuwen for the opportunity to work on such an interesting project. Thank you for your advice and sharing your amazing knowledge about neurodegenerative disorders and proteasomal dysfunction. He helped me realize that neurosciences is the research field I would like to dedicate my career to.

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Abbrevations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
CA	cornu ammonis
CHMP2B	charged multivesicular body protein 2B
CI	cumulative incidence
DAB	diaminobenzidine
DG	dendate gyrus
DNA	deoxyribonucleic acid
DUB	deubiquitinating enzymes
FTD	frontotemporal dementia
FTD-FUS	frontotemporal dementia-fused in sarcoma.
FTD-tau	frontotemporal dementia tau
FTD-TDP	frontotemporal dementia- transactive response DNA-binding protein
FTD-U	frontotemporal dementia ubiquitin
FTLD	frontotemporal lobar degeneration
FUS	fused in sarcoma
HECT	homologous to E6-associated protein C-terminus
MAPT	microtubule-associated protein tau
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NFT	neurofibrillary tangles
NLS	nuclear-localization signals
NMD	nonsense-mediated decay
PGRN	progranulin
PNFA	progressive non-fluent aphasia
RING	real interesting new gene
RNA	ribonucleic acid
RRM	RNA recognition motifs
SD	semantic dementia
TBS	tris buffered saline
TBS-T	tris-buffered saline-triton X100
TDP-43	transactive response DNA-binding protein-43
UBA	ubiquitin-associated domain
UBB	ubiquitin B
UBB^{+1}	ubiquitin B^{+1}
ub-ir	ubiquitin-immunoreactive
UBL	ubiquitin-like domain
UBQ	ubiquitin
UPS	ubiquitin proteasome system
TBS	Tris buffered saline TBS
TBS-T	Tris-buffered saline-Triton X100
sumi	supermix
19S6b	19S regulator ATPase subunit 6b

Abstract

Background: FTD is the most common clinical manifestation of FTLD; therefore, the present study will focus on this subtype. It is pathologically characterized by extensive heterogeneity on the microscopic level with tau-, ubiquitin-, TDP-43- or FUS-positive intraneuronal inclusions. Although, a classification scheme is currently used to subdivide FTD patients based on the expression of tau, ubiquitin, TDP-43 and FUS, not all patients fit perfectly into this diagram. Moreover, researchers expect to discover other, as yet unidentified, proteins present in these inclusions. These findings indicate that FTD is neuropathologically more heterogeneous than currently assumed. Fisher et al. found that out of eight FTD individuals studied, three were ubiquitin B^{+1} (UBB^{+1})-positive. UBB^{+1} is a marker for a dysfunctional proteasome system and expression of this misframed protein has already been shown in tauopathies (e.g. AD) and polyglutamine disorders.

Objectives: To compare the mutual relationship of the markers for FTD (tau, ubiquitin, TDP-43 and FUS) and to add a new marker (UBB^{+1}). The main purpose is to improve the manner in which FTD cases are currently classified based on UBB^{+1} expression. The expression of additional proteins (p62, 19S6b and PGRN) involved in protein quality control and FTD pathology is also examined to achieve an improved classification method.

Hypothesis: There is proteasomal dysfunction in FTD and this dysfunction differs depending on the subtype of FTD.

Methods: In the present study, we examined the expression of 8 different proteins (tau, ubiquitin, FUS, TDP-43, p62, PGRN, 19S6b en UBB^{+1}) in hippocampal brain sections of 47 FTD patients (23 males and 24 females; mean age at death= 66.6 years) and 10 age- and sex-matched non-demented controls (6 males and 4 females; mean age at death= 66 years) by immunohistochemical analysis. The intensity of the stainings was semi-quantitatively scored for each patient. We compared the protein expression between the controls and FTD patients.

Results & Conclusions: We experimentally demonstrated that it is very hard to clearly classify a FTD patient in one of the current known groups: FTD-Tau, FTD-TDP or FTD-FUS. There quite often was an overlap of the different pathologies and therefore we wanted to add a new marker (UBB^{+1}) to reclassify FTD subjects. The results demonstrated that there was cytoplasmic UBB^{+1} expression in CA of the hippocampus which indicates proteasomal dysfunction in FTD. However, we were not able to make an improved classification method of FTD based on the expression of this protein because UBB^{+1} is expressed in CA of all the patients.

Abstract

Achtergrond: FTD is de meest voorkomende klinische vorm van FTLD en daarom zal deze studie zich focussen op dit subtype. Deze aandoening is pathologisch zeer heterogeen op microscopisch niveau met tau-, ubiquitine-, TDP-43- of FUS-positieve intraneurale inclusies. Hoewel er momenteel een classificatiemodel bestaat om FTD patiënten te identificeren aan de hand van tau-, ubiquitin-, TDP-43- and FUS-expressie, kunnen niet alle patiënten onderverdeeld worden via deze classificatie. Onderzoekers verwachten zelfs nog meer proteïnen te ontdekken in de inclusies. Deze bevindingen geven aan dat FTD neuropathologisch nog heterogener is dan tot nu toe verondersteld wordt. Fischer et al. toonde aan dat drie van de acht onderzochte FTD patiënten ubiquitin B^{+1} (UBB^{+1}) immunoreactief waren. UBB^{+1} is een marker voor een dysfunctioneel proteasoom systeem en expressie van dit proteïne is al eerder aangetoond in tauopathies (bv. AD) en polyglutamine aandoeningen.

Doelstellingen: Het vergelijken van de relatie tussen de verschillende gekende markers voor FTD (tau, ubiquitin, TDP-43 en FUS) en het toevoegen van een nieuwe marker, met name $UBB^{+1.}$. Het hoofddoel is om het huidig classifificatiesysteem voor FTD patiënten te verbeteren op basis van UBB^{+1} expressie. Ook de expressie van additionele proteïnen (p62, 19S6b en PGRN) betrokken in proteïne kwaliteitscontrole en FTD pathologie, wordt onderzocht om zo tot een verbeterde classificatie te komen.

Hypothese: Er is proteasomale dysfunctie in FTD en deze dysfunctie verschilt afhankelijk van het FTD subtype.

Methoden: De expressie van 8 verschillende proteïnen (tau, ubiquitin, FUS, TDP-43, p62, PGRN, 19S6b en UBB^{+1}) werd onderzocht in hippocampale hersensecties van 47 FTD-patiënten (23 mannen en 24 vrouwen; gemiddelde sterfteleeftijd= 66,6 jaar) en 10 geslacht en leeftijd overeenstemmende niet-dementerende controles (6 mannen en 4 vrouwen; gemiddelde sterfteleeftijd= 66 jaar) aan de hand van immunohistochemie. De kleuringsintensiteit werd semi-kwantitatief gescoord voor elke patiënt. De proteïne-expressie tussen de controles en de FTD patiënten werd met elkaar vergeleken.

Resultaten & Conclusies: We hebben experimenteel aangetoond dat het onmogelijk is om FTD patiënten onder te verdelen in de gekende subgroepen: FTD-Tau, FTD-TDP of FTD-FUS. Er is vaak overlap tussen de pathologiëen en daarom wilden wij een nieuwe marker toevoegen (UBB^{+1}) om zo tot een nieuw classificatiesysteem te komen. De resultaten toonden aan dat er cytoplasmtische UBB^{+1} expressie is in de CA van de hippocampus, wat wijst op proteasomale dysfunctie in FTD. Maar helaas waren we niet in staat om een verbeterd FTD classificatiemodel op te stellen aan de hand van UBB^{+1} expressie omdat dit protein tot expressie komt in de CA van alle FTD patiënten.

1 Introduction

1.1 Frontotemporal dementia

Frontotemporal lobar degeneration (FTLD) refers to the degeneration in the frontal and temporal lobes of the brain. These areas control behavior, emotions and language. FTLD is an important cause of non-Alzheimer forms of dementia and it is the second most common cause of dementia before the age of 65 (1-4).

FTLD is associated with three clinical subtypes: frontotemporal dementia (FTD), semantic dementia (SD) and progressive non-fluent aphasia (PNFA) (Fig.1). In the FTD subtype, there is damage to the prefrontal and anterior temporal lobe. It is characterized by behavioral and personality changes, lack of social tact, distractibility, loss of insight, emotional blunting and decreased motivation (3, 5, 6). Compulsive, repetitive and stereotypic behavior and reduced speech output are also common features among FTD patients (1). Loss of conceptual knowledge, the major complaint of individuals with SD, is also caused by a damaged temporal lobe. PNFA patients have severe problems producing language fluently, although they mostly have preserved word comprehension. These problems are due to a damaged left frontotemporal lobe (1, 3, 5, 7).

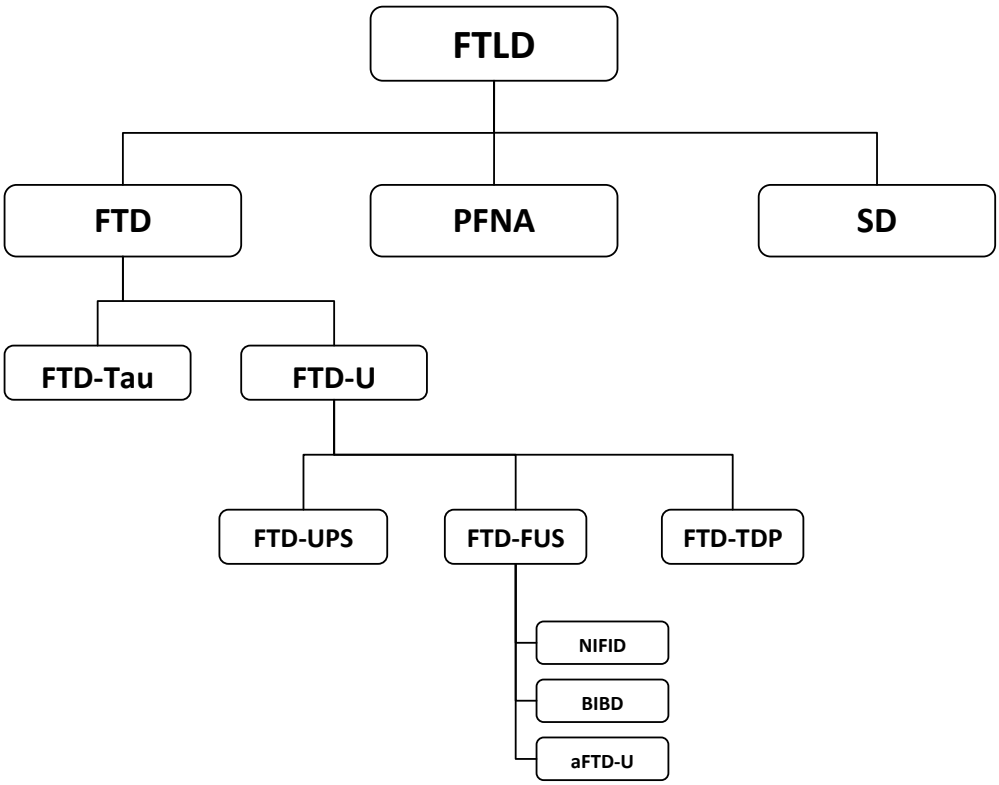

Figure 1: A schematic representation of the FTLD spectrum. FTLD is a spectrum disease which can be subdivided in different subtypes based on clinical symptoms: FTD, PNFA and SD. FTD is the most common clinical manifestation of FTLD and therefore the present study will focus on the FTD part of FTLD. Two histological distinctions can be identified within the FTD group based on (misfolded) proteins present in the inclusions: FTD-Tau and FTD-U. Some cases with ubiquitin-positive inclusions (FTD-U) have a mutation in the PGRN gene. Recently, two new proteins has been found within the ubiquitin-positive inclusions: TDP-43 and FUS. FUS-positive ubiquitin-positive inclusions are negative for TDP-43. Therefore, histologically two distinct entities can be described within the FTD-U group: FTD-FUS and FTD-TDP. Researchers identified pathogenic mutations in both TDP-43 and FUS gene. In other words, some familial cases can have mutations in one of these two genes. Cases without FUS positive or TDP-43 positive inclusions which are only positive for ubiquitin are called FTD-UPS and some of them have a mutation in the CHMP2B gene. Within the FTD-FUS group three subtypes exist: NIFID, BIBD and aFTD-U. NIFID on the one hand refers directly to the pathology: neuronal intermetdiate filament- and- α-internexin-positive inclusions. BIBD on the other hand is characterized by unusual curved or twisted neuronal intranuclear ubiquitin positive inclusions, as well as cytoplasmic inclusions. aFTD-U can be recognized by behavioural problems; a young onset of disease, a high prevalence of psychotic symptoms and a low prevalence of motor symptoms (1-3, 8-11). These findings together demonstrate that FTD is a very heterogeneous and complex spectrum disorder and even more subtypes have been claimed (9). aFTD-U= atypical FTDU; BIBD= basophilic inclusion body disease; CHMP2B= charged multivesicular body protein 2B; FTD= frontotemporal dementia; FTLD= frontotemporal lobar degeneration; FTD-FUS= frontotemporal dementia with fused in sarcoma (FUS) positive inclusions; FTD-Tau= frontotemporal dementia with tau- positive inclusions; FTD-TDP= frontemporal dementia with transactive response DNA-binding protein-43 (TDP-43) positive inclusions; FTD-U= frontotemporal dementia with ubiquitin-positive inclusions; NIFID= neuronal intermediate filament- and α-internexin-positive inclusions; PGRN= progranulin; PNFA= progressive non-fluent aphasia; SD= semantic dementia.

The present study will focus on the FTD subtype because it is the most common clinical manifestation of FTLD and it accounts for 5-10% of all dementia subjects. In individuals younger than 65 years these percentages may be as high as 30-40% (1). Even this may be an underestimation, due to misdiagnosis and non-referral by neurologists, psychiatrists and nursing home physicians. This is because of the complexity and heterogeneity of the disease. A correction for this underestimation would even increase the prevalence of FTD (12).

Due to the lack of methodology for diagnosing FTD subjects, epidemiological data of FTD are scarce (7, 13). Rosso et al. estimated the prevalence of FTD in the province of Zuid-Holland (the Netherlands) in a population-based study. The prevalence estimate for patients aged 45-64 was 4.0 per 100 000 (95% CI, 2.8-5.7)(12). Another, community-based study, conducted by the Cambridgeshire Group (UK), found a prevalence of FTD of 15.1 per 100 000 (95% CI, 8.4-27) at age 45-65 years. This is significantly higher than the study in Zuid-Holland which is probably due to methodological differences or to ethnic differences (14). Mercy et al. found an incidence of FTD of 3.5 cases per 100 000 person-years (95% CI, 2.0-5.7) at age 45-64 years in Cambridgeshire (15).

1.2 Abnormal protein deposits in FTD

FTD is characterized by the presence of abnormal protein deposits in the affected brain. Several distinctions can be made about these inclusions based on immunohistochemistry. The first distinction is based on the presence of tau-positive neuronal inclusions (FTD-tau) (Fig. 1). These inclusions are caused by mutations in the microtubule-associated protein tau (MAPT) gene located on chromosome 17q21 (1, 3, 16, 17). Tau is a protein found predominantly in nerve cells, concentrated in axons in normal brain and has six isoforms. These isoforms exist through alternative mRNA splicing from a single gene. Tau protein initiates and stabilizes neuronal microtubules by binding tubulin in healthy brain. At the moment, about 66 different mutations have been identified in this gene (http://www.molgen.ua.ac.be)(18). When tau is hyperphosphorylated, it causes reduced microtubule assembly and a breakdown of the neuronal cytoskeleton (17, 19, 20).

A second group of inclusions show ubiquitin-immunoreactive (ub-ir) neuronal inclusions (3). Ubiquitin is a polypeptide that can be covalently attached to other proteins. Polyubiquitination targets proteins for degradation via the proteasome system (21). Recently, it has been shown that most subjects with ub-ir inclusions display mutations in the progranulin (PGRN) gene. This gene is a 68.5 kDa multifunctional growth factor located 1.7 Mb upstream of MAPT on chromosome 17q21 (1, 2, 22). This protein is involved in a range of processes including development, wound repair and inflammation (10). A total of 131 different PGRN mutations have been identified (http://www.molgen.ua.ac.be) (18) and the frequency of these mutations is

approximately 5% of the total FTD population. In familial cases these percentages rises to 12-25% (23). The premature termination mutations in the PGRN gene create null alleles. The mutant mRNAs are degraded by nonsense-mediated decay (NMD), which results in loss of functional PGRN, also called haploinsufficiency. This group of patients with cytoplasmic and intra-nuclear ubiquitin-positive tau-negative inclusions is commonly referred to as FTD-U and it is the most common form of FTD (Fig.1) (2, 3, 10, 22, 24).

Recently, transactive response DNA-binding protein-43 (TDP-43), encoded on chromosome 1, was identified as the major component of the cytoplasmic inclusions in amyotrophic lateral sclerosis (ALS) and FTD-U. These two disorders are considered to be closely related neurodegenerative syndromes (11). Half of FTD cases show immunoreactive ubiquitinated TDP-43 aggregates and are called FTD-TDP (Fig. 1) (25, 26). TDP-43 is a highly conserved nuclear protein of 414 amino acids that may act as a regulator of transcription and alternative splicing. This protein is normally localized in the nucleus but, under pathological conditions, TDP-43 is eliminated from the nucleus and accumulates in neuronal cell bodies (11, 27-30). TDP-43 is recognized by two highly conserved RNA recognition motifs (RRM1 and RRM2) which are flanked by the N-terminus and C-terminus. The C-terminal tail, on one hand, includes a glycine-rich motif probably mediating protein-protein interactions. The N-terminal tail, on the other hand, contains two nuclear-localization signals (NLS) and three potential caspase-3 cleavage consensus sites (27). Zhang et al. has shown the expression of 25-kDa C-terminal fragments (TDP-25) of TDP-43 which are similar to those in FTD-U brains (27). This is caused by proteolytic cleavage of TDP-43 by caspases and leads to toxic, insoluble, hyperphosphorylated and ubiquitinated cytoplasmic inclusions (27). It has been shown that both TDP-43 and TDP-25 are cleared by the proteasome and autophagy (28).

Although TDP-43 was initially thought to be “the” pathological protein in ALS and FTD-U , a new neuropathological subtype has been identified: TDP-43 negative ”fused in sarcoma” (FUS) positive FTD-U, also called FTD-FUS (Fig.1) (8). Mackenzie et al. described a subgroup of patients with FTD-U which were negative for TDP-43 and positive for FUS (31). FUS protein co-localized with ub-ir pathological inclusions (32). These unusual cases account for 10%-20% of all FTD-U patients. Seelaar et al. acknowledged that the presence of this new FTD-U subtype can be predicted by the following factors: age at onset < 40 years, a negative family history, psychobehavioural changes and atrophy of the caudate on magnetic resonance imaging (MRI) (32). However, it is not excluded that this pathology can be present in a familial case of FTD (32). FUS is a 526 amino-acids protein which is encoded by a gene located on chromosome 16 and is involved in DNA and RNA metabolism. The C-terminal tail includes multiple domains involved in RNA-protein interactions, whereas the

N-terminus has a role in transcriptional activation. This protein is involved in a range of cellular processes: cell proliferation, DNA repair, transcription regulation, RNA splicing and the transport of RNA between the different cellular compartments. The expression of FUS is proportionally higher in the nucleus of neurons, while its expression in glia is only nuclear. In neurons, FUS has a role in neuronal plasticity and in maintenance of dendritic integrity by transporting mRNA (8). FUS protein shares a high degree of functional homology with TDP-43. Therefore, it is not surprising that FUS is involved in the pathology of FTD and ALS (8, 25, 26). These two proteins are predominantly expressed in the nucleus; however, they also shuttle between the nucleus and cytosol (8). They both have a C-terminal zinc finger motif within a conserved C-terminal region (26). There are pathogenic mutations identified in both genes, most of them are missense mutations in the C-terminus. Mutations in FUS gene cause abnormal relocalization of the protein from the nucleus to the cytoplasm where it forms aggregates of insoluble inclusions. This is a common feature between TDP-43 and FUS (8). As a conclusion: TDP-43 negative FUS-positive FTD-U is thus a distinct entity with a unique neuropathology which is rather unusual within the FTD-U group (8).

However, there are still cases where neither TDP-43 nor FUS are found, these cases are temporally named FTD-UPS (Fig. 1).

1.3 Aim of the study

FTD can be clinically diagnosed during a person's lifetime based on consensus criteria, cognitive tests and brain imaging (5). However, to date, a definitive diagnosis of the different FTD entities is still only possible by postmortem brain examination. Tau, ubiquitin, TDP-43 and FUS can be present in the FTD inclusions; however, there is quite often an overlap of these different pathologies. Moreover researchers expect to discover other, as yet unidentified, proteins present in the inclusions. These findings indicate that FTD is neuropathologically heterogeneous, which even makes the postmortem classification difficult (1).

The scientific relevance of the present study attempts to provide an improved method of subdividing the heterogeneous FTD condition. The group of Fischer showed that, of eight FTD subjects studied, three were UBB^{+1}-positive (33). This study was finalized in 2003 and at that time there were no good markers known for FTD (33). Therefore, the present study aims to determine proteasomal dysfunction (UBB^{+1}) in FTD and a difference in this dysfunction between the different subtypes of FTD. As a second objective, the expression of other proteins involved in protein quality control (p62, 19S6b, ubiquitin) will be analyzed. The expression of FUS, PGRN, TDP-43 and aberrant tau, proteins involved in the FTD pathology, will also be investigated as a part of the second objective. Our goal is

to compare the mutual relationship of the markers for FTD (tau, ubiquitin, TDP-43 and FUS) and to add a new marker (UBB^{+1}). The main purpose is to improve the manner in which FTD cases are currently classified based on UBB^{+1} expression. To achieve this, immunohistochemistry has been performed on the hippocampus of 47 FTD cases and 10 non-demented controls. The hippocampus is a pivotal limbic structure in the brain which plays a role in long-term memory and spatial information. This structure is located inside the medial temporal lobe and consist of two main cell-types: granular cells in the dendate gyrus (DG) and pyramidal cells in the cornu ammonis (CA). FTD can be recognized by variable degrees of frontal and temporal atrophy. There is also reported evidence of hippocampal atrophy, gliosis and neuronal loss in the clinical reports of the FTD patients. These findings are the reason why this study focuses on the hippocampus.

1.4 Ubiquitin proteasome system

The ubiquitin proteasome system (UPS) is an important intracellular pathway which regulates protein turnover. This system plays an essential role in maintaining cellular homeostasis, neuronal functioning, regulation of chromatin structure, DNA repair, transcriptional regulation, cell cycle and cell division, synaptic development, maintaining synaptic connections and ATP-dependent protein degradation(21).

1.5 Ubiquitination cascade and proteasomal degradation

UPS is a pivotal intracellular proteolytic pathway for short-lived, truncated and misfolded proteins. The 76-amino acid ubiquitin protein has a carboxy-terminal diglycine motif which forms an isopeptide bond with an amino group of target proteins. The glycine of ubiquitin forms usually a bond with the ε amino group of a lysine residue present in the targeted protein (34). E1-ubiquitin-acitivating enzyme activates ubiquitin which is transferred to an E2- ubiquitin-conjungating enzyme. A E3-ubiquitin ligase recognizes the proteins (degrons) targeted for degradation. A degron is a specific sequence of amino acids in the protein which indicates an intracellular degradation signal. The E3-ubiquitin ligase transfers the ubiquitin from the E2-conjungating enzyme to the protein substrate. Three different E3 enzymes exist: the Homologous to E6-associated protein C-terminus (HECT) domain E3 enzymes. This type of E3-enzyme binds the E2-enzymes as well as the targeted protein and serves in this way as an intermediate docking station for ubiquitin. A second type of E3-enzyme is the Real Interesting New Gene (RING) finger containing E3-ligases. Ubiquitin is transferred directly from the E2-complex to the targeted protein by the RING-E3 ligase. The third class of E3-enzymes is the U-box containing E3-ligase. The targeted protein undergo several rounds of ubiquitination and in this way a ubiquitin chain is formed. This ubiquitin chain linked with the lysine at position 48 (K48) has to be at least 4 residues long for efficient proteosomal targeting. The

ubiquitinated proteins are transferred to the 26S proteasome for degradation. The 26S proteosome complex consists of a 20S core complex which has 2α and 2β rings and is flanked by 19S activator complexes. The 20S core complex is responsible for proteolytic activity which can be specified in chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolyzing activity. The 19S activator complexes consist of a base and a lid. These complexes recognize, deubiquitinate, unfold and chaperone the targeted protein. The targeted protein is subsequently admitted to the 20S core and degraded into oligopeptides. Degradation of ubiquitin molecules can be prevented by deubiquitinating enzymes (DUB) which cleave the ubiquitin chains from the proteins (Fig.2) (21, 34).

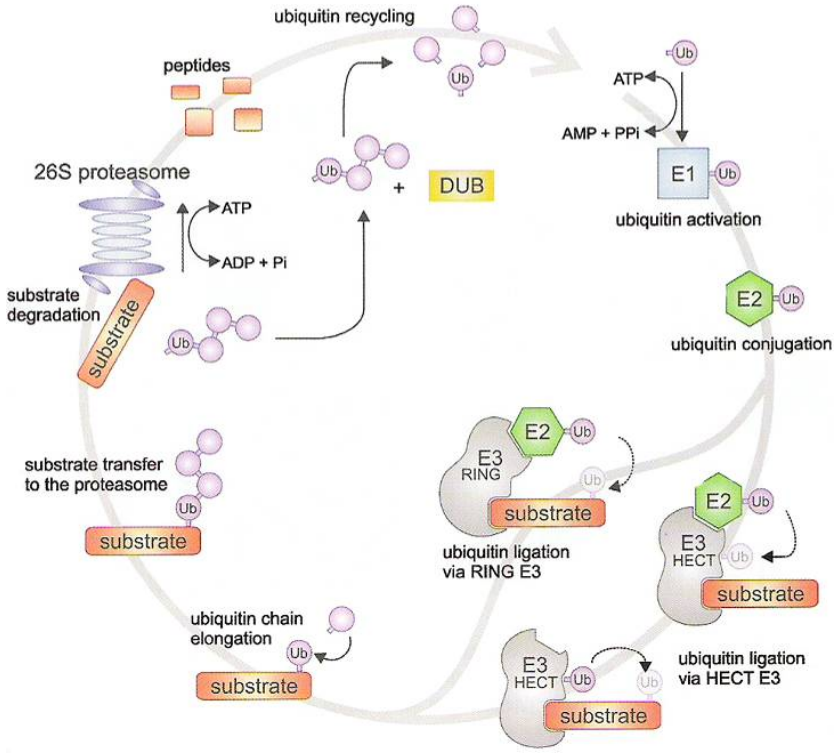

Figure 2: The ubiquitin proteasome system (UPS) (21).

1.6 Ubiquitin proteasome system in FTD

The UPS fulfills an important role in the pathogenesis of FTD and other neurodegenerative disorders. The most important findings are the ubiquitin-positive inclusions and aggregates. Apparently, there is a decreased proteasome function or a failure in transferring the substrates into the 26S proteasome(34). Because of the failure of the UPS, ubiquitinated proteins accumulate intracellularly. Mutant ubiquitin B (UBB^{+1}) is one of these ubiquitinated proteins which is generated by molecular misreading (ΔGA, ΔGU deletions) of simple monotonic repeats (such as GAGAG). These deletions result in mRNA that is out of frame and cause an addition of 20 amino acids to the C-terminus of ubiquitin. The resulting +1 protein has shown in AD brains, other tauopathies and polyglutamine disorders but not in synucleinopathies. UBB^{+1} proteins are not able to bind lysine residues in the targeted proteins (Fig.3). This is because of the lack of a COOH-terminal glycine. This glycine residue is pivotal for multiubiquitylation of the targeted protein and activation of the 26S proteasome. After ubiquitination, the UBB^{+1} form is targeted to the 26S proteasome and eventually degraded. A low expression level of UBB^{+1} is cleared by the proteasome, but a high expression of UBB^{+1} blocks the UPS and results in accumulation of UBB^{+1} and finally in cell death by apoptosis (21, 34-38).

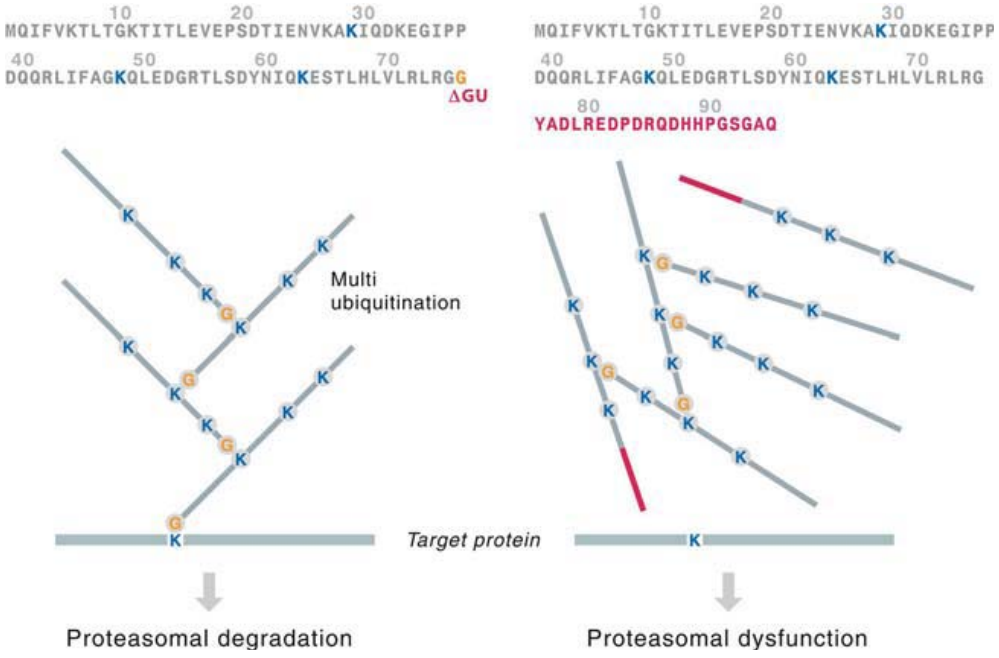

Figure 3: A simplified schematic representation of how UBB^{+1} lacks the C-terminal glycine which is needed for ubiquitination of the targeted protein (38).

1.7 Role of the remaining investigated proteins

The ubiquitin-binding protein p62 binds ubiquitin noncovalently. It has been shown to colocalize in ubiquitin-positive inclusions in FTLD-U. p62 contains ubiquitin-associated (UBA) and ubiquitin-like (UBL) domains. This protein may act as a shuttling factor via these domains to deliver polyubiquitinated proteins to the 26S proteasome. p62 can also form a cytoplasmic complex with proteins, known as the sequestosome, which transfers proteins to the lysosome. It has been shown that long-term autophagy inhibition increases p62. Excess p62 causes decreased clearance of ubiquitinated proteins by the proteasome and therefore, p62 can been seen as a factor which links UPS with autophagy-lysosome pathway (39-42).

The expression of 19S regulator ATPase subunit 6b (19S6b) will also be examined. This subunit is a triple ATP-ase and it is a part of the 26S proteasome. Ubiquitinated proteins are transferred to the 26S proteasome for degradation. The 26S proteosome complex consists of a 20S core complex and 19S activator complexes. The 19S activator complexes recognize, deubiquitinate, unfold, chaperone, and channel the targeted protein into the proteolytic 20S core. It has been shown that neuronal and/or glial inclusions in tauopathies are 19S6b- immunoreactive which indicates an upregulation of this subunit (43).

We want to find an answer on the following research question: Is there proteasomal dysfunction in FTD, and, if so, to what degree in the different subtypes of FTD? The goal of the present study is to subdivide the heterogeneous group of FTD based on these markers. This will enable more accurate postmortem diagnoses which will help the researchers to unravel the mechanism behind FTD.

2 Material and methods

2.1 Autopsy material

Human postmortem brain material of 47 patients (23 males and 24 females; mean age at death= 66.6 years) diagnosed with FTD was obtained from two different sources: the Netherlands Brain Bank in Amsterdam and the University of Antwerp in Antwerp (coordinator Prof. C. Van Broeckhoven). The Netherlands Brain Bank provided brain material of 31 subjects (16 males and 15 females) ranging in age from 40 to 88 years (see Supplemental table S1 for clinico-pathological details). Brain sections of another 8 FTD cases (4 males and 4 females), ranging in age from 52 to 80 years, were also obtained via the Netherlands Brain Bank. This material has already been used by D. Fisher and these patients have an earlier date of mortality (Supplemental table S2). The results of this study were published in the FASEB journal in November 2003 (33) . Brain tissue of the remaining 8 FTD patients (3 males and 5 females), ranging in age from 53 to 88 years, were available via the University of Antwerp (Supplemental table S3). Brain sections of 10 age- and sex-matched non-demented controls (6 males and 4 females; mean age at death= 66 years), which were available via F.W. van Leeuwen, were also included in this study (Supplemental table S4).

After obduction, half of the brain was fixed by immersion in formalin for 4 weeks. Prior to immunohistochemical analysis, the hippocampus and frontal cortex were dissected out, processed into paraffin wax and consecutive sections cut at thickness of 6µm. Several sections of each patient were used for conventional neurohistological prescreenings (e.g. Bodian, silver stains, Congo red and hematoxylin/eosin) and these screenings confirmed that these patients were FTD cases.

2.2 Immunohistochemistry

Six µm thick paraffin-embedded, formalin fixed tissue sections from the hippocampus were examined in all FTD patients. Immunohistochemistry was performed with antibodies (table 1) against UBB^{+1} ($Ubi2^{+1}$ 14-09-94 1:400, Ubi2A 18-03-98 1:500)[homemade, the Netherlands], aberrant tau (MC1 1:100) [generously provided by P. Davies, New York], 19S6b proteasome subunit (PW8175 1:400) [Biomol-Affiniti, UK], progranulin (1:500) [R&D systems, Minneapolis, USA], TDP-43 (1:1000) [Abnova, Taipei City, Taiwan], p62 (1:500) [Biomol, UK], ubiquitin (1:300) [Upstate, Lake Placid, New York], and FUS (1:200) [Sigma-Aldrich, St. Louis MO, USA]. After deparaffination, sections were treated with formic acid [Merck, Damstadt, Germany] for 30 min and washed in Tris buffered saline (TBS pH 7.6) and Tris-buffered saline-Triton X100 (TBS-T pH 7.6) [3,33%, VWR International, Amsterdam, the Netherlands]. Brain sections were incubated with primary antibodies (diluted in supermix (sumi)) in a humid chamber for 1 hour at room temperature and overnight at 4°C.

Hereafter the sections were rinsed in TBS and TBS-T and incubated with secondary antibody (also diluted in sumi) for 60 min at room temperature: biotinylated donkey anti-rabbit (1:400) [Jackson Immunoresearch, Suffolk, UK] against ubiquitin, PW8175, Ubi2^{+1} (14-09-94), Ubi2A (18-03-98), p62 and FUS, biotinylated donkey anti-mouse (1:400) [Jackson Immunoresearch, Pennsylvania, USA] against MC1 and TDP-43, and biotinylated donkey anti-goat [Jackson Immunoresearch, Pennsylvania,USA] against progranulin. After incubation with the secondary antibody, the sections were washed again with TBS and TBS-T and incubated with avidin-biotin peroxidase complex [Vector Laboratories inc, Burlingame, CA] diluted 1:200 in TBS-T for 60 min at room temperature. The sections were washed in between with TBS and Tris-HCl (50 mM, pH 7.6) [Merck, Damstadt, Germany] and incubated with a 3'3 diaminobenzidine (DAB) solution (pH 7.6) (DAB, 25mM Tris-HCl, 8% $NiCl_2$, 0,01% H_2O_2) [Sigma-Aldrich, Saint-Louis, USA]. The staining was blocked by TBS and the sections were dehydrated in graded ethanol [VWR International, Amsterdam, the Netherlands] and ultraclear [Mallinckrodt Baker B.V., Deventer, the Netherlands]. The brain slices on the glass slides [76-26mm, Menzel-Gläser, Germany] were mounted in PERTEX [Histolab, Gothenburg, Sweden] and covered with cover slips [24 x 60mm, Menzel-Gläser, Germany]. Positive controls were included in all of the different immunohistochemical stainings.

Table 1: Information about the different antibodies used in immunohistochemical stainings.

Antibody	Type	Dilution	Company	Specificity
Ubi2^{+1} (anti-UBB^{+1} Ab)	Rabbit pAb	1:400	Homemade	Fischer et al.(33)
Ubi2A (anti-UBB^{+1} Ab)	Rabbit pAb	1:500	Homemade	Fischer et al.(33)
MC1 (anti-aberrant tau Ab)	Mouse mAb	1:100	P.Davies	P.Davies
PW8175 (anti-19S6b Ab)	Rabbit pAb	1:400	Biomol-Affiniti	Zouambia et al.(43)
anti-PGRN Ab	Goat pAb	1:500	R&D systems	see manufacturer's protocol
anti-TDP-43 Ab	Mouse pAb	1:1000	Abnova	see manufacturer's protocol
anti-p62 Ab	Rabbit pAb	1:500	Biomol	see manufacturer's protocol
anti-ubiquitin Ab	Rabbit pAb	1:300	Upstate	see manufacturer's protocol
anti-FUS Ab	Rabbit pAb	1:200	Sigma-Aldrich	see manufacturer's protocol

pAb = polyclonal antibody, mAb= monoclonal antibody

2.3 Immunohistochemical analysis

A semi-quantitative analysis method was used to measure the intensity of the stained brain sections. Each of the stained proteins was scored by two different investigators for each of the FTD patients or controls by using a light microscope: - negative, +/- weak reactivity, + some reactivity, ++ strong reactivity, +++ very strong reactivity. This scoring method enabled the comparison of the controls and FTD cases. The FTD patients were also compared to each other. A score of - or +/- was not regarded as positive so a score of +, ++ or +++ was counted as positive by us.

3 Results

3.1 Immunohistochemical characterization of the hippocampal inclusions

The present study focused on the expression of 8 different proteins in the hippocampus of 47 FTD patients and 10 non-demented controls: UBB^{+1}, aberrant tau, 19S6b, PGRN, TDP-43, p62, ubiquitin and FUS. For this purpose, consecutive brain sections of these FTD patients and controls were incubated with antibodies against the different proteins. The intensity of the stainings were semi-quantitatively scored for both the dendate gyrus and cornu ammonis (hippocampal complex) for each patient. As mentioned above, the consecutive brain sections are obtained from two different sources: the Netherlands Brain Bank and the University of Antwerp. Therefore, the results are shown in different tables depending on the source. Because of possible age-related factors, the patients are ordered by age in each table. Positive controls were included in all our immunohistochemical experiments and there was a high expression of the tested proteins with a score of ++ to +++.

The Netherlands Brain Bank provided brain material of 31 subjects (16 males and 15 females) and the results of the semi-quantitative analysis of the stainings performed on the hippocampus of these FTD patients are shown in two tables (table 2, 3). A different representation of the results is shown in supplemental table S5. Table 2 shows the semi-quantitative results for aberrant tau, ubiquitin, p62, TDP-43, PGRN and FUS, whereas the expression of UBB^{+1} is shown in table 3. The expression of this protein is extremely important for our hypothesis; therefore two different antibodies are used ($Ubi2^{+1}$ and Ubi2A). For FTD patient #05-078, there was no dendate gyrus present in the brain sections and for this reason only a scoring for the CA region is available.

Table 2: Results of the semi-quantitative scoring method performed on the hippocampus of 31 FTD patients (the Netherlands Brain Bank). Hippocampus material is incubated with 7 antibodies against 7 different proteins: aberrant tau, ubiquitin, p62, TDP-43, PGRN, 19S6b and FUS.

	MC1		Ubiquitin		p62		TDP-43		PGRN		19S6b		FUS	
Patient	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA
08-041	+/-	+/-	-	-	+/-	+/-	+	+	++	++	+	++	-	++
05-082	-	+/-	+/-	+	+	++	+	++	++	+++	+	+++	+	++
05-015	+	+	-	+/-	+	+	++	++	++	+++	+	++	+	++
07-036	+	-	-	+/-	+/-	+	+	++	++	++	+	+	+/-	++
07-083	-	-	-	+/-	+	+	+/-	+	+	++	+	+	+/-	+
05-041	+	+/-	-	+/-	+/-	+/-	++	+++	++	+++	+	+	+	+
08-093	+	+	-	+/-	+/-	+	+	+	++	++	+	+	++	+
05-078	abs.	+	abs.	+/-	abs.	+	abs.	++	abs.	++	abs.	++	abs.	+/-
08-025	+	+	+/-	+	++	+	+	+	++	+++	++	++	++	++
07-087	-	-	+/-	+	+/-	+	+	++	+	+++	+	++	+/-	+
08-078	-	-	+/-	+/-	+/-	+	++	++	+	++	+	++	-	-
08-017	+/-	+/-	-	+/-	+	+/-	+	++	++	+++	+/-	+	+	+/-
08-107	+/-	+	-	+	+/-	+	+/-	++	+++	+++	+/-	+	+	+
05-066	-	+	+/-	+/-	++	+	+	++	++	+++	+++	++	++	+
07-004	+/-	+/-	+/-	+/-	+	+	+	+++	+	++	+	++	+	++
07-024	-	+/-	-	-	+/-	+/-	+	+	+++	+++	+	+	+/-	-
05-031	-	+	-	+/-	+	+	++	++	++	+++	+/-	++	+	+
05-037	+/-	+	+/-	+/-	+/-	+/-	+	++	++	+++	+/-	+	+/-	-
05-048	-	-	+/-	+/-	+	++	+	++	+	++	+	++	+/-	+/-
06-023	-	-	+/-	+/-	+	+	++	+++	++	++	+	+	+	+
08-010	-	+	+/-	+/-	+	++	+	+	++	+++	+	++	+/-	+
07-028	-	+/-	+/-	+	-	+	+/-	++	++	+++	+	++	+	+

Table 2: *(continued)*

	MC1		Ubiquitin		p62		TDP-43		PGRN		19S6b		FUS	
Patient	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA
08-039	-	-	-	-	+	+	+	++	++	+++	+	+	+/-	+
07-048	-	-	+/-	+/-	+/-	++	++	++	+	++	+/-	+	-	+/-
08-019	++	++	+	+	+	+	+	+	++	+++	++	++	++	++
05-051	+	++	+	+	+/-	+	++	++	++	++	++	+++	+/-	+
09-013	-	+/-	-	+/-	+/-	+	+	++	++	++	+	+	+	++
08-035	-	+/-	-	+/-	+	+	+	++	+	++	+	++	+/-	+
09-014	-	++	-	+/-	+/-	+/-	+/-	++	++	+++	+/-	+	+	++
06-042	-	+/-	+/-	+/-	+	+	+	++	++	++	+/-	++	+	+
08-082	-	+/-	-	+/-	+	+	+	++	+++	+++	+	+	+	+

-negative, +/- weak reactivity, + some reactivity, ++ strong reactivity, +++ very strong reactivity
DG= dendate gyrus, CA= cornu ammonis, abs. = absent

Table 3: Results of UBB^{+1} staining performed on the hippocampus of 31 FTD patients (the Netherlands Brain Bank).

	UBB^{+1} ($Ubi2^{+1}$)		UBB^{+1} (Ubi2A)			UBB^{+1} ($Ubi2^{+1}$)		UBB^{+1} (Ubi2A)	
Patient	DG	CA	DG	CA	Patient	DG	CA	DG	CA
08-041	+/-	+	+/-	+	05-031	+/-	+	+/-	++
05-082	+/-	+	+/-	++	05-037	-	+/-	+/-	+
05-015	+/-	+	+	++	05-048	-	+	-	+
07-036	-	+	-	+	06-023	+/-	+	+/-	++
07-083	+	+	-	+	08-010	+/-	+	+/-	+
05-041	+/-	+	-	+	07-028	-	+	-	++
08-093	-	+	-	+	08-039	-	+	+/-	++
05-078	abs.	+/-	abs.	+	07-048	-	+	-	+
08-025	+	+	+	++	08-019	+	++	+	++
07-087	-	+	+/-	++	05-051	-	+	+	++
08-078	-	+	+/-	++	09-013	-	+	-	+
08-017	-	+	+/-	+	08-035	+/-	+	+/-	+
08-107	-	+	-	+	09-014	-	+	-	+
05-066	+/-	+	+/-	+	06-042	+/-	+	+/-	+
07-004	+/-	+	+/-	++	08-082	+/-	+	+/-	+
07-024	+/-	+/-	+/-	+					

- negative, +/- weak reactivity, + some reactivity, ++ strong reactivity, +++ very strong reactivity
DG= dendate gyrus , CA= cornu ammonis, abs. = absent

As mentioned earlier, the hippocampus of 8 FTD patients of the Netherlands Brain Bank has already been stained and scored by D.Fischer. Table 4 and supplemental table S6 shows the results of the immunohistochemical stainings performed by D. Fischer. He used 5 antibodies against 5 different proteins (aberrant tau (MC1), ubiquitin, UBB^{+1}, 19S6b and APP^{+1}) and scored the hippocampal complex as a whole. FTD patients #94111 and #99005 are brother and sister which can be interesting keeping their genetic background in mind.

Table 4: Results of the semi-quantitative scoring method performed on the hippocampus of 8 FTD patients (stained and scored by D. Fischer).

Patient	MC1	Ubiquitin	UBB^{+1}	19S6b	APP^{+1}
94075	+/-	+/-	-	+/-	-
92019	+	+/-	+/-	++	-
93036	++	++	-	++	+/-
94111*	+++	++	++	+++	-
99005*	+++	++	++	+++	-
96113	++	++	+	++	-
96498	+/-	+/-	-	+/-	-
94033	+/-	+/-	+/-	++	-

* = brother and sister

- negative, +/- weak reactivity, + some reactivity, ++ strong reactivity, +++ very strong reactivity

We extended the staining of Fischer for 6 of the 8 FTD patients because for 2 cases (#99005 and #96498) there was no more brain material available. For the same reason, we used frontal cortex instead of hippocampus of patient #93036 for 3 of the 4 stainings (p62, TDP-43 and PGRN) while for patient #96113 temporal cortex was used for the FUS staining. Four different proteins are stained and scored by us: p62, TDP-43, PGRN and FUS. The results are shown in table 5 (see supplemental table S6 for a different representation of these results).

Table 5: An extension of the results of D. Fischer. The hippocampus of these 8 FTD patients were incubated with antibodies against 4 more proteins: p62, TDP-43, PGRN and FUS.

	p62		TDP-43		PGRN		FUS	
Patient	DG	CA	DG	CA	DG	CA	DG	CA
94075	+/-	+/-	++	++	+	+++	-	-
92019	-	+/-	+	++	+++	+++	+/-	+
93036	Frontal +/-		Frontal ++		Frontal +++		+	+
94111*	+/-	+/-	+	+	++	++	+/-	+
99005*	n.a.	n.a.	n.a.	n.a.	n.a..	n.a.	n.a.	n.a.
96113	-	+/-	+++	+++	++	++	Temporal -	
96498	n.a.	n.a.	n.a..	n.a.	n.a.	n.a.	n.a.	n.a.
94033	-	-	+	++	++	+++	-	++

* = brother and sister
-Negative, +/- weak reactivity, + some reactivity, ++ strong reactivity, +++ very strong reactivity
DG= dendate gyrus, CA= cornu ammonis, n.a. = not available

The University of Antwerp (coordinator Prof. C. Van Broeckhoven**)** provided brain sections of 8 FTD patients. FTD patients #5693, #5717, #4495, #5746 and #5666 are members of the same Belgian family. The results of the stainings performed on the hippocampus of these cases are shown in table 6. Another representation of the results is shown in supplemental table S7.

Table 6: Results of the semi-quantitative scoring method performed on the hippocampus of 8 FTD patients (University of Antwerp).

	MC1		Ubiquitin		p62		TDP-43		PGRN		19S6b		UBB^{+1} ($Ubi2^{+1}$)		UBB^{+1} (Ubi2A)	
Patient	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA
5610	-	+/-	+/-	+	+	++	+	++	++	+++	+/-	+/-	+/-	+/-	+/-	+
5308	-	-	n.a	n.a	+	++	++	++	++	++	+/-	+/-	+	+/-	+/-	+/-
5693*	-	+/-	+/-	+	+/-	+	++	++	++	++	-	+/-	+/-	+	+/-	+
5717*	+/-	+/-	+/-	+	+	+	++	++	++	++	-	+	-	+	+/-	+
4495*	-	-	+	+	+/-	+	+	++	+	++	+/-	+	-	+	-	+
5746*	+/-	+/-	+	+	+/-	+	+	+	-	+	-	+/-	+/-	+	-	+/-
5666*	-	-	+	+	+	++	++	++	+	++	+/-	+/-	+/-	+	+/-	+
5849	+	+	++	++	+/-	+	++	+++	+++	+++	+/-	+/-	-	+/-	+/-	+

* members of the same Belgian family
- negative, +/- weak reactivity, + some reactivity, ++ strong reactivity, +++ very strong reactivity
DG= dendate gyrus, CA= cornu ammonis, n.a. = not available

Brain sections of 10 non-demented controls were also included in the present study. For control #88307 we used temporal cortex instead of hippocampus for 6 stainings (ubiquitin, p62, TDP-43, PGRN,19S6b and FUS), because there were no more brain sections available containing this region. Hippocampus material of this control was only available for Bodian and UBB^{+1} staining. A Bodian staining was done instead of using a MC1 antibody. Bodian staining visualizes the neurofibrillary tangles (NFT) in brain sections of patients suffering from a neurodegenerative disorder. Paired helical filaments (PHF) are the major component of neurofibrillary tangles and consist of hyperphosphorylated microtubule-associated protein tau. The hippocampus is scored as one complex for the Bodian staining. For the other stainings, the hippocampus is split up in DG and CA. The results are shown in table 7, 8 and supplemental table S8.

Table 7: Results of the semi-quantitative scoring method for 7 different stainings (Bodian, ubiquitin, p62, TDP-43, PGRN 19S6b and FUS) performed on the hippocampus of 10 non-demented controls (F.W. van Leeuwen).

	Bodian Staining	Ubiquitin		p62		TDP-43		PGRN		19S6b		FUS	
Patient	Hippocampal Complex	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA	DG	CA
89003	-	+/-	++	+/-	+	++	++	-	+/-	-	+/-	-	-
81021	-	+/-	+	+/-	+	++	++	++	+++	+	+/-	+	+
94125	-	-	++	+/-	+	+	++	+	++	+/-	+	++	++
88037	+/-	Temporal +/-		Temporal +/-		Temporal ++		Temporal +/-		Temporal -		Temporal -	
90073	-	-	+/-	-	+	+/-	+	-	-	+/-	+/-	-	-
90079	++	+/-	++	+/-	+/-	+	++	++	+++	+/-	+	++	++
91026	++	+/-	++	+	++	+	++	+/-	++	++	+	-	-
91027	++	-	+/-	-	+	-	++	+/-	+++	-	+	-	-
90080	+	+/-	+	-	+/-	+/-	+	+	+++	+	+	+	+
81007	+	+/-	+	+/-	+	++	++	+/-	+	+/-	++	-	+

- negative, +/- weak reactivity, + some reactivity, ++ strong reactivity, +++ very strong reactivity
DG= dendate gyrus, CA= cornu ammonis

Table 8: Results of the UBB^{+1} staining performed on the hippocampus of 10 non-demented controls (F.W. van Leeuwen).

	UBB^{+1} (Ubi2^{+1})			UBB^{+1} (Ubi2^{+1})	
Control	DG	CA	Control	DG	CA
89003	-	-	90079	-	+
81021	-	-	91026	-	+
94125	-	-	91027	-	+
88037	-	-	90080	-	+
90073	-	-	81007	-	+

- negative, +/- weak reactivity, + some reactivity, ++ strong reactivity, +++ very strong reactivity
DG= dendate gyrus, CA= cornu ammonis

In figure 4, 5 and 6 examples of hippocampal sections incubated with various antibodies are shown. Figure 4 shows brain sections of different FTD patients which are stained for aberrant tau, ubiquitin TDP-43 and FUS. Based on the expression of these proteins, FTD cases are currently subdivided in three different pathological groups. Figure 5 shows neuronal UBB^{+1} expression in CA and GD. The expression of this misframed protein in the hippocampus of FTD patients is pivotal for this study, it forms the basis of our hypothesis. Hippocampal sections of different FTD cases positive for PGRN, 19S6b and p62 are shown in figure 6.

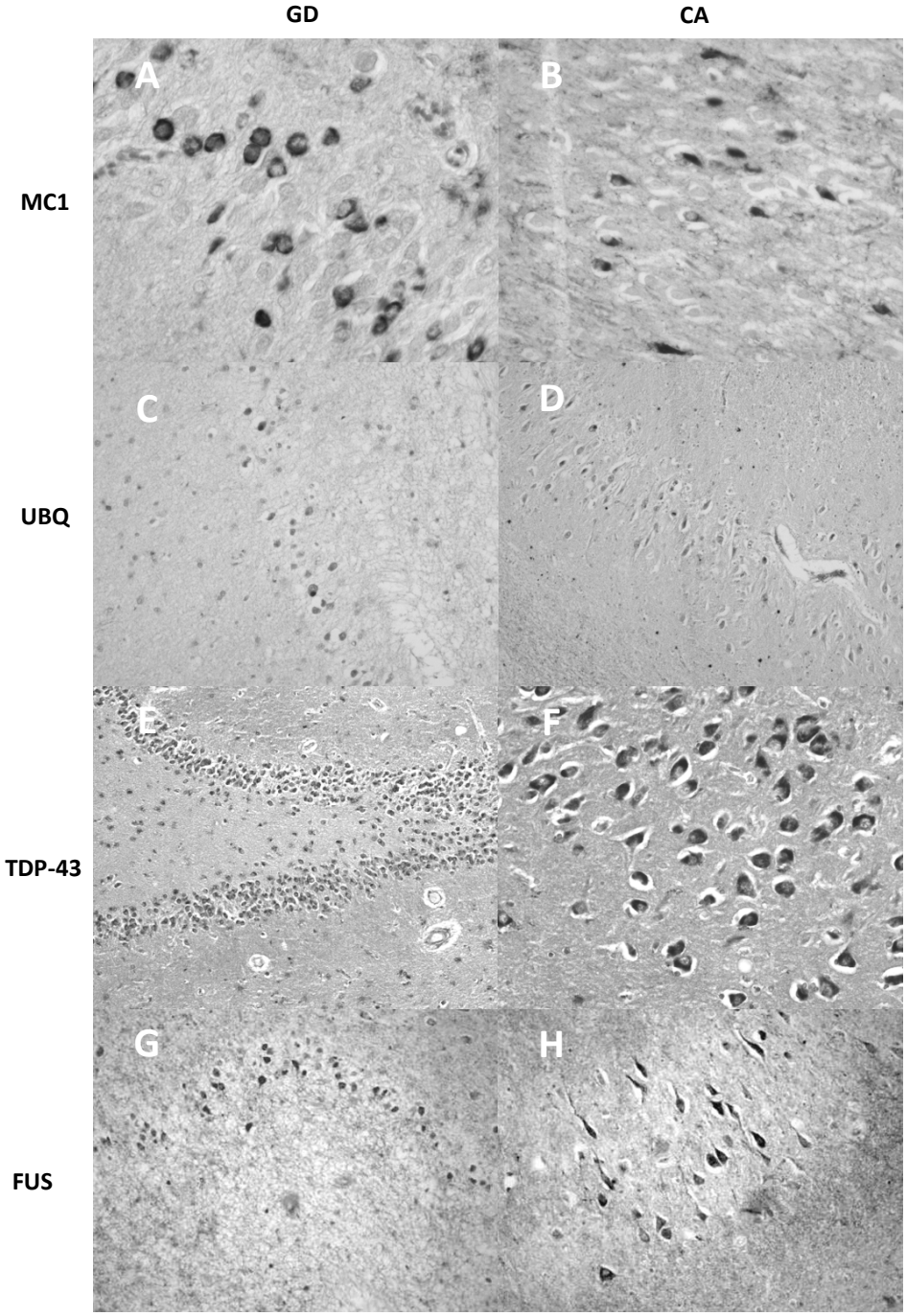

Figure 4: Hippocampal sections of various FTD patients incubated with antibodies against MC1, ubiquitin and TDP-43. *A-B)* FTD patient #08-019. Cytoplasmic staining of aberrant tau in the granular cells of the GD and the pyramidal cells of the CA. *A)* magnification= 40x *B)* magnification= 20x. *C-D)* FTD patient #05-051. Ubiquitin-immunoreactive cytoplasmic inclusions in the neurons of the GD and CA. *C)* magnification= 20x *D)* magnification= 10x. *E-F)* FTD patient #06-023. Abundant cytoplasmic TDP-43 staining can be seen in the granular layer of GD as well as in the pyramidal layer of the CA *E)* magnification= 10x *F)* magnification= 20x. *G-H)* FTD patient #08-025. Expression of FUS protein in granular and pyramidal neurons of CA and GD. *G)* magnification= 20x *H)* magnification= 20x

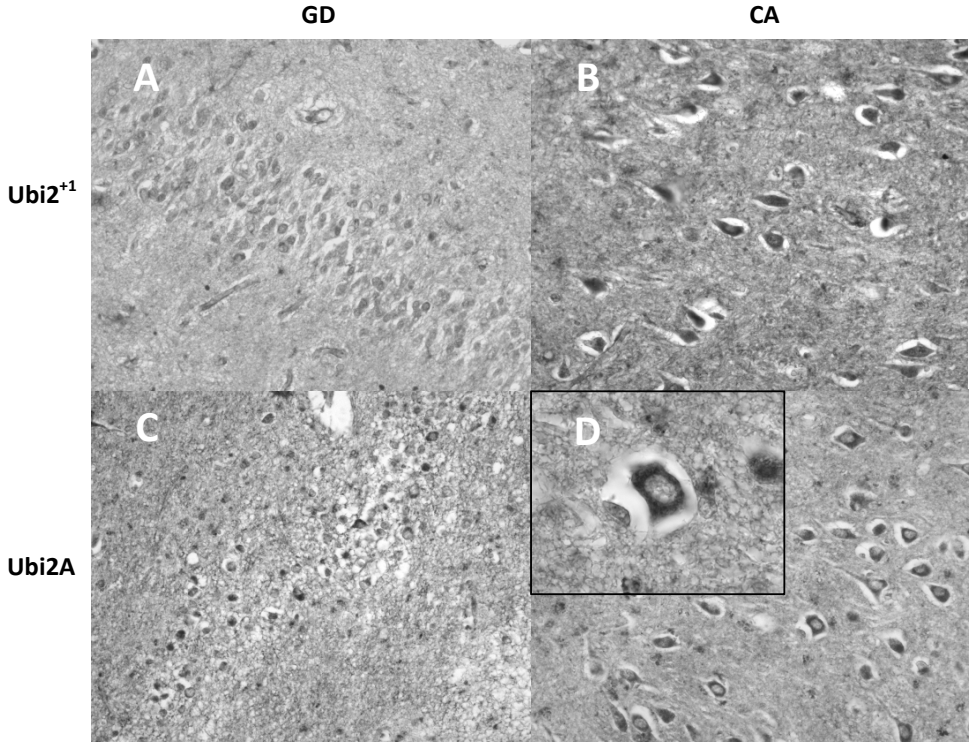

Figure 5: Hippocampal sections of 2 FTD patients incubated with 2 different antibodies against UBB^{+1}. *A-B)* FTD patient #08-019. Cytoplasmic expression of UBB^{+1} in GD and CA when using a Ubi2^{+1} antibody. *A)* magnification= 20x *B)* magnification= 20x. *C-D)* FTD patient #05-051. Abundant presence of UBB^{+1} in the cytoplasm of neurons in GD and CA when using a Ubi2A antibody. *C)* magnification= 20x *D)* magnification= 20x Insert in *D)* shows a neuron in CA which is clearly UBB^{+1} immunoreactive in the cytoplasm. Magnification= 100x

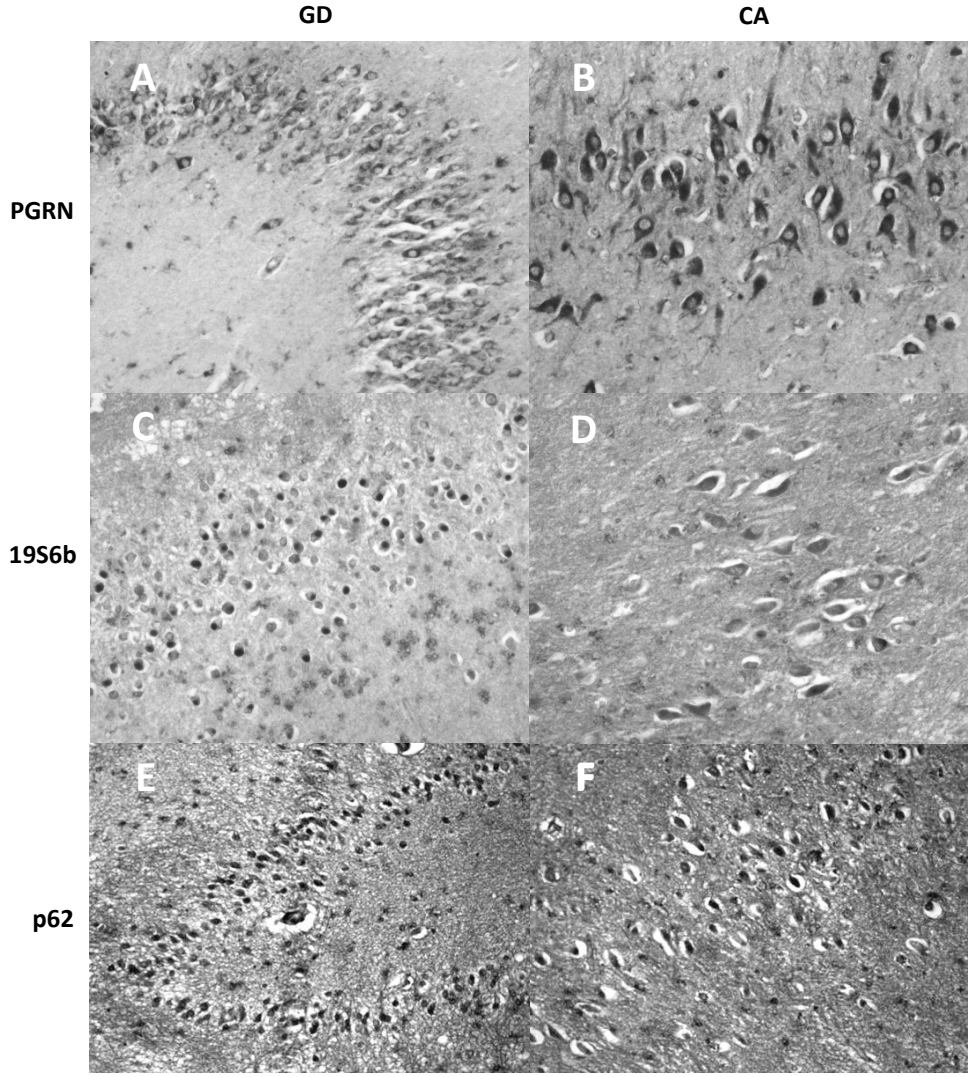

Figure 6: Hippocampal sections of various FTD patients incubated with antibodies against PGRN, 19S6b and p62. *A-B)* FTD patient #07-024. Clear presence of PGRN in the cytoplasm of neurons in GD and CA. *A)* magnification= 20x *B)* magnification= 20x. *C-D)* FTD patient #05-066. Neuronal expression of 19S6b protein in GD and CA. *C)* magnification= 20x *D)* magnification= 10x *E-F)* FTD patient #08-019. Expression of p62 protein in neurons of GD and CA. *E)* magnification= 20x *F)* magnification= 20x

Figure 7 shows consecutive hippocampal brain sections of FTD patient #08-019 stained for 8 different proteins (aberrant tau, ubiquitin, 19S6b, UBB^{+1}, PGRN, TDP-43, p62 and FUS). The red arrows indicate colocalization of the different proteins in the neuronal inclusions.

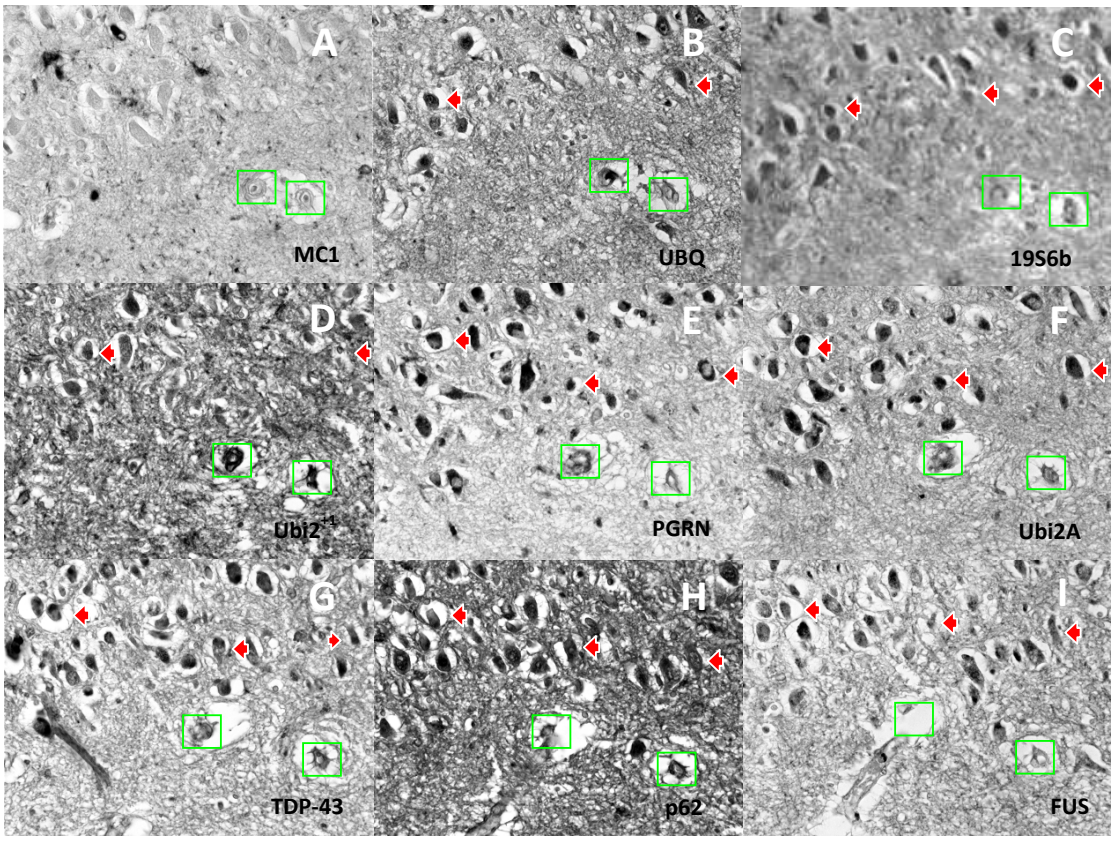

Figure 7: Consecutive sections *(A-I)* of the hippocampus of a FTD patient (#08-019) incubated with 9 different antibodies. *A)* MC1 antibody against aberrant tau*, B)* antibody against ubiquitin, *C)* PW8175 against 19S6b proteasome subunit. *D)* Ubi2^{+1} (14-09-94) antibody against UBB^{+1}, *E)* antibody against PGRN, *F)* ubi2A (18-03-98) antibody against UBB^{+1} *G)* antibody against TDP-43, *H)* antibody against p62, *I)* antibody against FUS. *A-I)* shows neuronal inclusions in the pyramidal cells of the CA region. Each picture is positive for one of the 8 stained proteins. Note there is colocalization of the different proteins in various cells, indicated by red arrows. Green boxes are capillaries used as hallmarks. *(A-I)* magnification= 40x

4 Discussion

4.1 Protein expression profile of tau, ubiquitin, TDP-43 and FUS

FTD is pathologically characterized by extensive heterogeneity on the microscopic level with tau-, ubiquitin-, TDP-43- or FUS-positive neuronal inclusions (Fig.1). A classification scheme for this spectrum disorder was proposed as found in Fig.1 (9, 26). To date, researchers try to divide FTD cases into one of these groups. In an attempt to classify our 47 FTD cases, hippocampal sections of each patient were incubated with antibodies against aberrant tau, ubiquitin, TDP-43 and FUS. As mentioned earlier, a score of - or +/- was not regarded as positive only scores of +, ++ and +++ were counted as positive. If there was expression of one of these 4 proteins in the whole FTD population, it was always expressed in the cytoplasm. Based on the results of the semi-quantitative analysis, we experimentally demonstrated that it is very hard to clearly subdivide a FTD patient in one of these three groups (tables 2, 4, 5 and 6; Fig.4). Several forms of overlap are found in our FTD population. If there is expression of TDP-43 or FUS than the expectations are tau-negative ubiquitin-positive cytoplasmic inclusions. We found several cases which were positive for FUS or TDP-43 but negative for ubiquitin. A second phenomenon shown by the analysis were positive cases for FUS or TDP-43 but they were also immunoreactive for aberrant tau. The simultaneous expression of FUS and TDP-43 was a third form seen in our FTD cases. These three aberrations in protein expression in our FTD population demonstrate that we cannot support the current classification method (9, 26). Of the 31 FTD cases of the Netherlands Brain Bank and the 8 patients, which were previously stained by Fischer, none of the cases could be classified in FTD-Tau, FTD-TDP or FTD-FUS without serious aberrations. The 8 FTD patients obtained via the University of Antwerp could unfortunately not be stained for FUS because no more brain sections were available. It is therefore not possible to draw definite conclusions about these patients keeping the current classification method in mind. However, only one of the FTD cases (table 6; #5849) showed simultaneous expression of aberrant tau en ubiquitin which is not possible according to the current classification method. The other patients of Antwerp were negative for aberrant tau but positive for ubiquitin. Moreover all FTD cases are positive for TDP-43 expression in the hippocampus. Thus, it is possible that 7 of these 8 FTD patients (University of Antwerp) could be classified according to the present method. However, it is also possible that there is simultaneous expression of FUS and TDP-43 which is aberrant.

We also incubated hippocampal sections of the non-demented 10 controls with antibodies against hyperphosphorylated tau (Bodian), ubiquitin, TDP-43 and FUS (table 7). Hyperphosphorylated tau was expressed in the 5 eldest controls which is related to aging. These results are in agreement with

the group of Bancer who showed that hyperphosphorylated tau can be expressed in brains of aged non-demented controls (44).

Under pathologic conditions, TDP-43 and FUS are eliminated from the nucleus and accumulate in the cytoplasm where they form insoluble inclusions. As mentioned earlier, there was indeed cytoplasmic expression of TDP-43 and FUS in our FTD population, but also in all our controls which were positive for one of these two proteins showed cytoplasmic expression besides some nuclear expression. We expected only nuclear expression of these proteins in the controls. The cytoplasmic expression may indicate that TDP-43 and FUS play a role in different disorders besides the neurodegenerative ones the FTD patients suffered from. These findings put the "pathological" cytoplasmic TDP-43 and FUS expression into another perspective and needs further research.

In conclusion, the proposed classification scheme from Cairns et al. and Urwin et al. is hardly applicable on our FTD population (9, 26). The different pathologies frequently overlap and thus a correct postmortem diagnosis is very hard.

4.2 Protein expression profile of UBB^{+1}

To answer the main question whether the possible UBB^{+1} expression in the hippocampus of FTD cases can give more insight into the heterogeneous profile of FTD, two different antibodies against this protein were used (Ubi2^{+1} (14-09-94) and Ubi2A (18-03-98)). Fischer showed that 3 out of 8 FTD patients were positive for UBB^{+1} (table 4) (33). We extended the number of FTD patients (47 cases) to get a clear view of the UBB^{+1} expression in this disorder. The results in tables 3, 4 and 6 show that UBB^{+1} immunoreactivity is more common among FTD cases than initially thought (Fig.5). Of the 31 FTD patients received from the Netherlands Brain Bank, 28 cases were moderately positive for UBB^{+1} expression in CA when using a Ubi2^{+1} antibody (table 3). All cases (31/31) showed moderate UBB^{+1} immunoreactivity in CA when a Ubi2A antibody was used (table 3). There was few to none expression of UBB^{+1} in GD of these 31 cases. Similar results were found for the 8 FTD patients obtained from the University of Antwerp (table 6). A total of 5 out of 8 patients were mildly UBB^{+1} positive in CA when incubated with Ubi2^{+1} antibody while 6 of the 8 cases were immunoreactive when Ubi2A antibody was used. The expression in GD was also negligible in these FTD cases.
In all FTD patients the immunopositive UBB^{+1} structures were cytoplasmic inclusions located mainly in the pyramidal neurons of the CA.

The hippocampus of the 10 non-demented controls was also stained for UBB^{+1} expression which enabled us to compare the FTD cases with the controls. The results of the UBB^{+1} staining performed on the hippocampus of these controls (table 8) showed a cytoplasmic staining of the pyramidal neurons in CA for 5 of the 10 controls. The 5 eldest controls were positive for UBB^{+1}. This is related to aging because it was shown that UBB^{+1} expression is also found in elderly non-demented controls (>51 years)(45).

The hypothesis of the present study is: *There is proteasomal dysfunction in FTD and this dysfunction differs depending on the subtype of FTD.* The results demonstrate that there is cytoplasmic UBB^{+1} expression in CA of the hippocampus which indicates proteasomal dysfunction in FTD. However, we are not able to make an improved classification method of FTD based on the expression of this protein because UBB^{+1} is expressed in CA of all the patients.

4.3 Protein expression profile of p62, PGRN and 19S6b

We investigated the expression of p62, PGRN and 19S6b because these 3 proteins are also related to FTD pathology and protein quality control. It was shown that mutations in PGRN gene are linked to tau-negative ubiquitin-positive inclusions (FTD-U) (2, 10). Another group noticed that all FTD cases with a PGRN mutation seem to have FTD-U pathology, but not all patients with FTD-U have a PGRN mutation (46). Significant elevated PGRN mRNA levels from the normal allele were shown in the affected FTD-U brain of PGRN mutation carriers (23). However, PGRN mutants did not show significant differences in PGRN protein levels compared to controls. Although there are significant differences in PGRN protein expression between PGRN mutation carriers with FTD-U pathology and those without PGRN mutations, those with a mutation show lower levels of protein expression (23). We found a high expression of PGRN protein in the hippocampus of our total FTD population (table 2, 5 and 6; Fig.6) while 25 of the 47 patients are sporadic cases. A total of 7 controls also demonstrated a high PGRN immunoreactivity, mainly in CA (table 7). Therefore, the present study also suggests that there are no great differences in PGRN expression between FTD cases and controls. Remarkably, about half of our FTD cases showed tau immunoreactivity simultaneous with PGRN positivity. Another point of interest is that 11 of 47 FTD cases are carrier of a tau mutation while they are highly positive for PGRN. At this moment, a range of questions about the relation between PGRN expression and mutations in FTD remain unanswered and further research is needed to draw firm conclusions about the expression profile of this very protein.

It was demonstrated that 19S6b and UBB^{+1} can coexist in cell aggregates in tauopathies and that proteasome subunit 19S6b is upregulated in this type of disorders (43). Expression of UBB^{+1} indicates proteasomal dysfunction (33) and it was suggested that the upregulation of 19S6b can be explained as a compensation mechanism to compensate for an increased need to degrade aberrant proteins such as UBB^{+1} (43). In our FTD population, 39/47 FTD cases were positive for 19S6b mainly in CA and 36 of these 39 cases also showed UBB^{+1} expression in the hippocampus (table 2, 4 and 6; Fig.6). These findings support the results found by Zouambia (43). However, 6/10 controls were also 19S6b-positive in their CA region and 5 of these 6 cases were also positive for UBB^{+1} (table 7). This suggests that there is also a compensation mechanism present in aged non-demented controls because it were the eldest controls which showed UBB^{+1} and 19S6b expression.

Finally we also investigated the expression of p62 which links UPS with autophagy-lysosome pathway (39-41). However, based on the expression profile of this protein in the FTD cases and controls we were not able to draw conclusions nor see possible connections (table 2, 5, 6 and 7; Fig 6).

4.4 Colocalization in neuronal inclusions of a FTD patient

Figure 7 showed colocalization of the different proteins in consecutive, hippocampal brain sections of FTD patient #08-019 and red arrows demonstrated the colocalized proteins. Ubiquitin, 19S6b, UBB^{+1}, PGRN, TDP-43, p62 and FUS were colocalized in the same cells. Only aberrant tau expression was less clear in the indicated cells. This suggested that these proteins might be involved with FTD pathology in one way or another.

5 Conclusion

FTD is a very complex, heterogeneous syndrome and the exact mechanisms behind the FTD pathology are not yet known. Recently, a few markers were identified within cytoplamic neuronal inclusions such as tau, ubiquitin, TDP-43 and FUS. A classification scheme (Fig.1) based on the expression of these markers was proposed by two different groups (9, 26). Hippocampal brain sections of 47 FTD patients were available for our study and we also tested the expression of the 4 markers (tau, ubiquitin, TDP-43 and FUS). Unfortunately, we were not able to classify one of our 47 FTD cases according to the proposed scheme. There was quite often an overlap of the different pathologies. We found three main aberrations within our FTD population: cases which were positive for FUS or TDP-43 but negative for ubiquitin, cases positive for FUS or TDP-43 but also immunoreactive for aberrant tau and cases which showed simultaneous expression of FUS and TDP-43. We found it necessary to reconsider the present classification scheme and to search for new FTD markers. In 2003, Fischer et al. found 3/8 FTD cases positive for UBB^{+1} expression (33). The expression of this +1 protein has already been shown in AD brains, other tauopathies and polyglutamine disorders. Therefore we came to the following hypothesis: *There is proteasomal dysfunction in FTD and this dysfunction differs depending on the subtype of FTD.* The results of the semi-quantitative analysis showed that UBB^{+1} immunoreactivity is more common among FTD cases than initially thought. Therefore, we could confirm the first part of our hypothesis but we were not able to reclassify the FTD cases based on UBB^{+1} expression because this protein was expressed in CA of all FTD patients. Our study provided new insights into FTD pathology by demonstrating proteasomal dysfunction in a larger FTD cohort. Further research is needed to gain more information about the role of the UPS, not only in FTD, but also in the broad spectrum disorder FTLD. Future studies will need to focus on collecting more evidence for proteasomal dysfunction in FTD on protein- and mRNA level by western blot en qPCR experiments. We focused on protein expression in the hippocampus, but also expression of markers in the frontal cortex can be measured in the future to confirm our findings.

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Supplement

Table S1: Clinico-pathological information of 31 FTD patients received from the Netherlands Brain Bank.

NBB number	Age (years)	Sex (f/m)	Postmortem delay (hr)	Brain weight (gr)	Cause of death	Clinical diagnosis	Mutation
08-041	40	F	5:50	840	dehydration by pneumonia	FTD	Sporadic
05-082	46	F	6:40	868	dehydration and malignancy	Pick's disease	Sporadic
05-015	49	M	5:10	1248	suffocation (unnatural death)	FTD – Pick's disease	MAPT gene mutation: G272V
07-036	51	M	4:25	1022	pneumonia by choking	FTD-Pick's disease	MAPT gene mutation: G272V
07-083	51	F	n.a.	n.a.	unknown	FTD	MAPT gene mutation: Ser82ValfsX174
05-041	52	M	11:30	1115	cardiac arrest	Pick's disease	MAPT gene mutation: P301L
08-093	56	M	4:05	1437	sepsis, necrosis of ileum and pneumonia	FTD	Sporadic
05-078	57	M	6:40	1102	general deterioration	Pick's disease	Sporadic
08-025	61	F	6:00	820	partial ileus by chronic constipation	FTD	Sporadic
07-087	63	M	6:05	1057	pneumonia and acute heart failure	FTD	Sporadic
08-078	63	M	6:05	1327	cachexia and dehydration	dementia syndrome	Sporadic.
08-017	64	M	8:25	1235	pneumonia and heart failure	morbus AD*	Sporadic
08-107	65	F	4:00	1080	cachexia and dehydration	AD*	Sporadic
05-066	66	M	9:08	1126	acute cardiac decompensation	Pick's disease	Sporadic
07-004	66	M	5:20	1245	pneumonia, dementia	FTD-Pick's disease	Sporadic
07-024	66	F	5:15	934	cachexia and dehydration	Pick's disease	PGRN gene mutation: Gln300X

Table S1: *(continued)*

NBB number	Age (years)	Sex (f/m)	Postmortem delay (hr)	Brain weight (gr)	Cause of death	Clinical diagnosis	Mutation
05-031	68	F	5:40	1218	unknown	Pick's disease tau mutation	MAPT gene mutation: L315R
05-037	68	F	6:20	845	unknown	Pick's disease	Sporadic
05-048	68	F	5:05	837	pneumonia	Pick's disease	Sporadic
06-023	69	M	4:30	1287	pneumonia	FTD-Pick's disease	Sporadic
08-010	69	F	7:30	1055	cachexia and dehydration	FTD	Sporadic
07-028	70	M	5:45	n.a.	sepsis by cholangitis	FTD	Sporadic
08-039	71	F	6:05	965	heart attack	FTD, semantic dementia	Sporadic
07-048	72	M	4:54	1285	urosepsis	Pick's disease	Sporadic
08-019	72	M	5:05	1214	n.a.	n.a.	Sporadic
05-051	75	F	6:46	1135	unknown, probably cachexia and dehydration	Pick's disease	Sporadic
09-013	78	M	5:10	1089	n.a.	dementia syndrome	Sporadic
08-035	79	F	4:55	1010	cachexia and dyhydration	AD*	Sporadic
09-014	81	F	6:58	1005	n.a.	FTD	Sporadic
06-042	87	F	4:25	1100	ileus causing cachexia and dehydration	AD*	Sporadic
08-082	88	M	5:16	1325	dehydration and metastasized prostate carcinoma	dementia syndrome	Sporadic

* = clinical diagnosis was AD, but postmortem diagnosis is FTD based on macroscopic and microscopic examination
sporadic= no mutations reported
n.a.= not available
FTD = frontotemporal degeneration
AD = Alzheimer's disease
MAPT = microtubule-associated protein tau
PGRN = progranulin

Table S2: Clinico-pathological information of 8 FTD patients received from the Netherlands Brain Bank which have already been used by D. Fischer.

NBB number	Age (years)	Sex (f/m)	Postmortem delay (hr)	Fixation duration (days)	Brain weight (gr)	Cause of death	MAPT gene Mutation
94075	52	M	7	97	1087	dehydration,cachexia	P301L
92019	60	M	5	44	1331	n.a.	P301L
93036	66	F	6	45	856	heart failure, cachexia	P301L
94111*	70	M	5	61	1121	lung emboly, cardiac problems	R406W
99005*	71	F	6	33	905	dehydration with respiratory tract infection	R406W
96113	76	F	6	37	1006	dehydration	P301L
96498	76	F	24	38	1120	bronchopneumonia, cachexia	n.a.
94033	80	M	2	74	1145	cachexia	n.a.

* = brother and sister

n.a. = not available

Table S3: Clinico-pathological information of 8 FTD patients received from the University of Antwerp.

IB number	Age (years)	Sex (f/m)	Postmortem delay (hr)	Fixation duration (days)	Brain weight (gr)	Clinical diagnosis
5610	53	F	3:10	84	1288	FTLD
5308	58	M	14	84	943	FTLD
5693*	64	M	8:30	132	1166	FTLD
5717*	69	F	n.a	60	1038	FTLD
4495*	71	M	n.a.	n.a.	n.a.	FTLD
5746*	72	F	2:30	1	828	FTLD
5666*	75	F	3	69	898	FTLD
5849	88	F	2:30	4	1040	FTLD

*members of the same Belgian family

n.a. = not available

FTLD = frontotemporal lobar degeneration

MAPT = microtubule-associated protein tau

Table S4: Clinico-pathological information of 10 non-demented controls available via F.W. van Leeuwen.

NBB number	Age (years)	Sex (f/m)	Postmortem delay (hr)	Fixation duration (days)	Brain weight (gr)	Cause of death
89003	34	M	<17	1124	1348	empyema of pleura, fibrous pleuritis and fibrous pericarditis
81021	43	M	23	53	1260	non-Hodgkin lymphoma
94125	51	M	6	47	1156	sepsis
88037	58	M	24	1088	1797	lung carcinoma, massive hemorrhage
90073	65	F	24	403	1234	pulmonary embolism
90079	72	M	4	126	1330	myocardial infarction, cardiogenic shock
91026	80	F	36	65	1205	cardiogenic shock
91027	82	F	48	38	1100	myocardial infaction, ventricular fibrillation
90080	85	M	5	126	1050	cardiac failure, myocardial infarction, coronary sclerosis, lung emphysema
81007	90	F	2	48	1110	postoperative infections

Table S5: A different representation of the results of the staining performed on the hippocampus of 31 FTD patients (the Netherlands Brain Bank). It shows the number of cases grouped by the different scores.

		-	+/-	+	++	+++
MC1	DG	18	5	6	1	0
	CA	8	11	9	3	0
Ubiquitin	DG	15	13	2	0	0
	CA	3	21	7	0	0
p62	DG	1	13	14	2	0
	CA	0	6	21	4	0
TDP-43	DG	0	4	19	7	0
	CA	0	0	7	21	3
PGRN	DG	0	0	7	20	3
	CA	0	0	0	14	17
19S6b	DG	0	7	19	3	1
	CA	0	0	14	15	2
FUS	DG	3	10	13	4	0
	CA	3	4	15	9	0
Ubi2^{+1}	DG	14	13	3	0	0
	CA	0	3	27	1	0
Ubi2A	DG	10	16	4	0	0
	CA	0	0	19	12	0

Table S6: A different representation of the results of the staining performed on the hippocampus of 8 FTD patients (stained and scored by Fischer). It shows the number of cases grouped by the different scores.

		-	+/-	+	++	+++
MC1	Hippocampal complex	0	3	1	2	2
Ubiquitin	Hippocampal complex	0	4	0	4	0
UBB^{+1}	Hippocampal complex	3	2	1	2	0
19S6b	Hippocampal complex	0	2	0	4	2
APP^{+1}	Hippocampal complex	7	1	0	0	0
p62	DG	3	2	0	0	0
	CA	1	4	0	0	0
	Frontal	0	1	0	0	0
TDP-43	DG	0	0	3	1	1
	CA	0	0	1	3	1
PGRN	DG	0	0	1	3	1
	CA	0	0	0	2	3
FUS	DG	2	2	1	0	0
	CA	1	0	3	1	0
	Temporal	1	0	0	0	0

Table S7: A different representation of the results of the staining performed on the hippocampus of 8 FTD patients (University of Antwerp). It shows the number of cases grouped by the different scores.

		-	+/-	+	++	+++
MC1	DG	5	2	1	0	0
	CA	3	4	1	0	0
Ubiquitin	DG	0	3	3	1	0
	CA	0	0	6	1	0
p62	DG	0	4	4	0	0
	CA	0	0	5	3	0
TDP-43	DG	0	0	3	5	0
	CA	0	0	1	6	1
PGRN	DG	1	0	2	4	1
	CA	0	0	1	5	2
19S6b	DG	3	5	0	0	0
	CA	0	6	2	0	0
Ubi2^{+1}	DG	3	4	1	0	0
	CA	0	3	5	0	0
Ubi2A	DG	2	6	0	0	0
	CA	0	2	6	0	0

Table S8: A different representation of the results of the staining performed on the hippocampus of 10 non-demented controls (F.W. van Leeuwen). It shows the number of cases grouped by the different scores.

		-	+/-	+	++	+++
Bodian	Hippocampal complex	4	1	2	3	0
Ubiquitin	DG	3	6	0	0	0
	CA	0	2	3	4	0
	Temporal	0	1	0	0	0
p62	DG	3	5	1	0	0
	CA	0	2	6	1	0
	Temporal	0	1	0	0	0
TDP-43	DG	1	2	3	3	0
	CA	0	0	2	7	0
	Temporal	0	0	0	1	0
PGRN	DG	2	3	2	2	0
	CA	1	1	1	2	4
	Temporal	0	1	0	0	0
19S6b	DG	2	4	2	1	0
	CA	0	3	5	1	0
	Temporal	1	0	0	0	0
FUS	DG	5	0	2	2	0
	CA	4	0	3	2	0
	Temporal	1	0	0	0	0
Ubi2^{+1}	DG	10	0	0	0	0
	CA	5	5	0	0	0

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Proteasomal dysfunction: a way to classify FTD subjects?

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Voor akkoord,

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Datum: **15/06/2010**