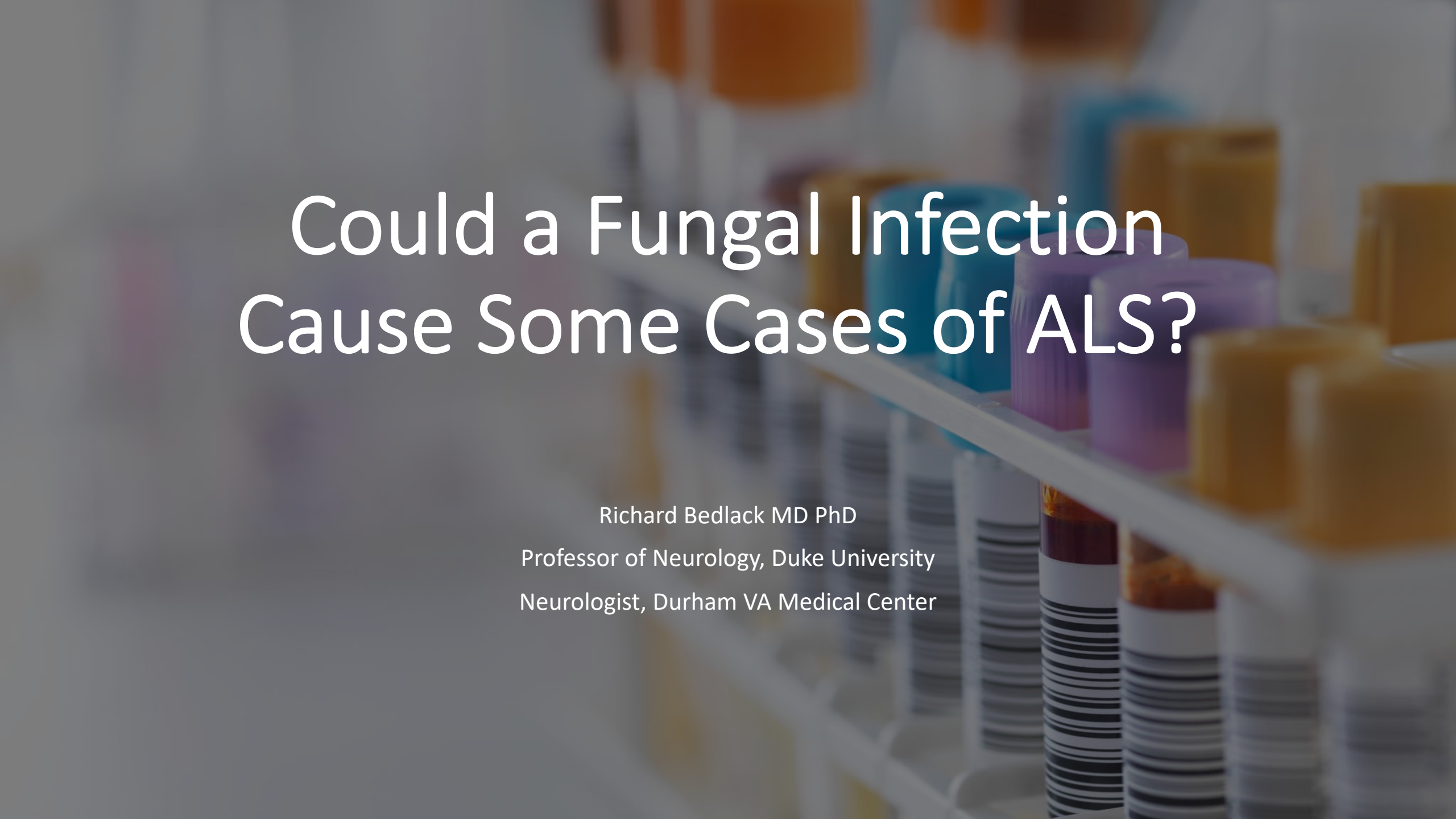

Could a Fungal Infection Cause Some Cases of ALS?

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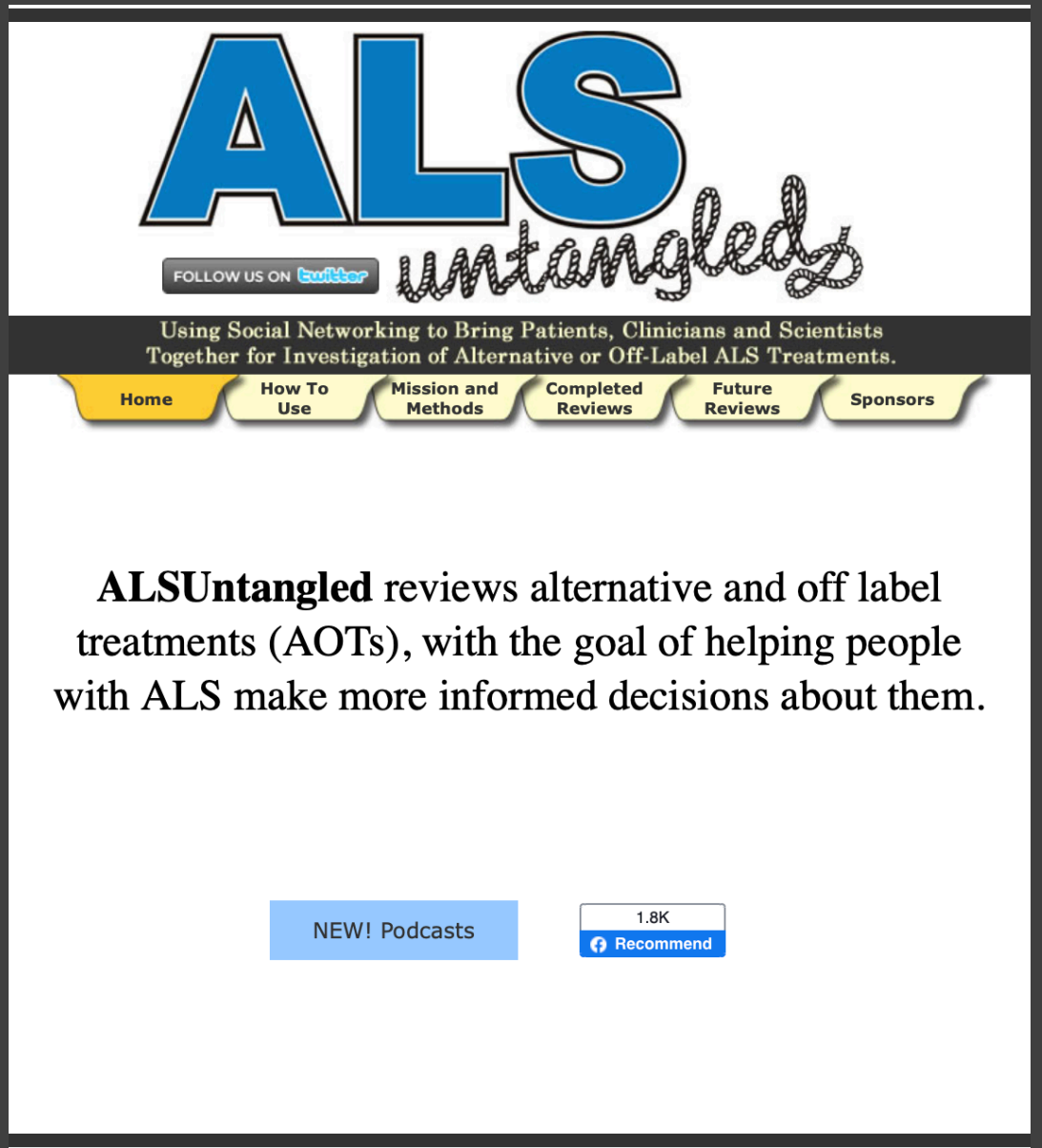
Outline

- Origin of this idea
- Evidence for fungi in people with ALS
- Possible interpretations of the evidence
- Suggested next steps

Origin of This Idea

- ALSUntangled
  - (www.alsuntangled.org)
- By early 2019, >500 requests to review “Anti-fungals” for ALS
- Review published, has nearly 2,000 downloads to date
  - (Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration, 2019; 20: 625–629)



Origin of This Idea

- In 2006, Dr. William Reid filed a patent for treating ALS and other neurodegenerative disease with antifungals
- He hypothesized that people with ALS (PALS) were immunodeficient, colonized with fungi, succumbed to fungal toxins
  - Reid W. Immunosuppression & mycotoxins causing amyotrophic lateral sclerosis. The winnower. 2017. Available at: http://www.webcitation.org/76MCrRWq0

Evidence-Clinical?

- Dr. Reid found some PALS with low IgG levels, lymphopenia, metabolic acidosis, abnormal urine porphyrins, abnormal urine organic acids, abnormal levels of the mycotoxin Trichothecene, all of which he felt supported his hypothesis
  - ALSUntangled review noted most PALS have normal IgG levels and lymphocyte counts

Evidence-Clinical?

- Dr. Reid treated 5-10 PALS with antifungals, in some cases along with PLEX or IVIG, and reported improved motor function
  - ALSUntangled review noted these improvements were generally small, transient, which can happen spontaneously in PALS
  - ALSUntangled was unable to independently verify the ALS diagnoses or the improvements in these patients (no sufficient records sent to us)

Evidence-Neuropathology

- A Spanish group published 3 papers claiming neuropathological evidence of fungi in the brains of PALS
  - 1.Alonso R, Pisa D, Marina AI, Morato E, Rabano A, Rodal I, et al. Evidence for fungal infection in cerebrospinal fluid and brain tissue from patients with amyotrophic lateral sclerosis. Int J Biol Sci. 2015;11:546–58.
  - 2.Pisa D, Alonso R, Rabano A, Carrasco L. Corpora amylacea of brain tissue from neurodegenerative diseases are stained with specific antifungal antibodies. Front Neurosci. 2016;10:86.
  - 2.Alonso R, Pisa D, Fernandez-Fernandez A, Rabano A, Carrasco L. Fungal infection in neural tissue of patients with amyotrophic lateral sclerosis. Neurobiol Dis. 2017; 108:249–60.

Evidence-Neuropathology 1

- CSF from 5 PALS, 3 healthy controls
- Brain tissue from 6 PALS, 4 healthy controls
- Polyclonal antibodies detected various fungal antigens in CSF from PALS, not healthy controls
- PCR analysis detected fungal DNA in CSF and brain tissue from PALS, not healthy controls

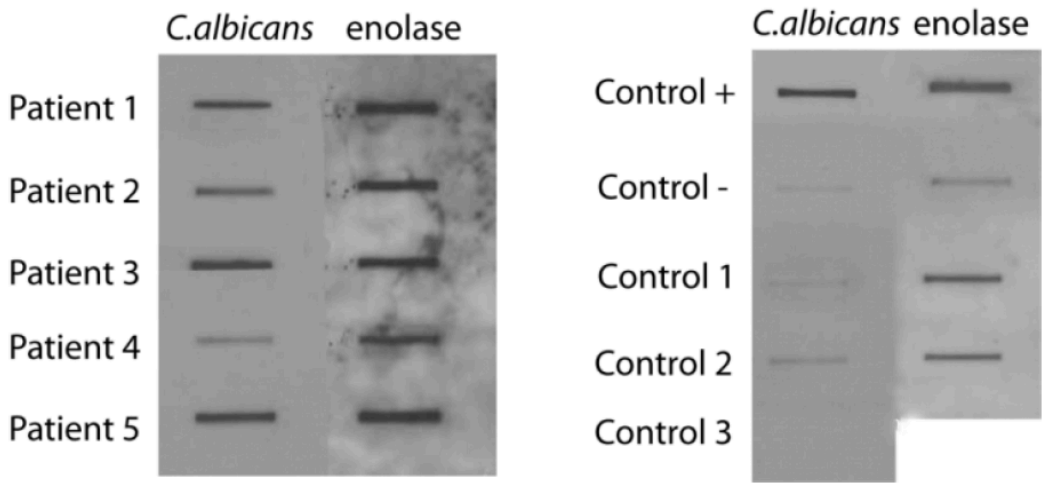
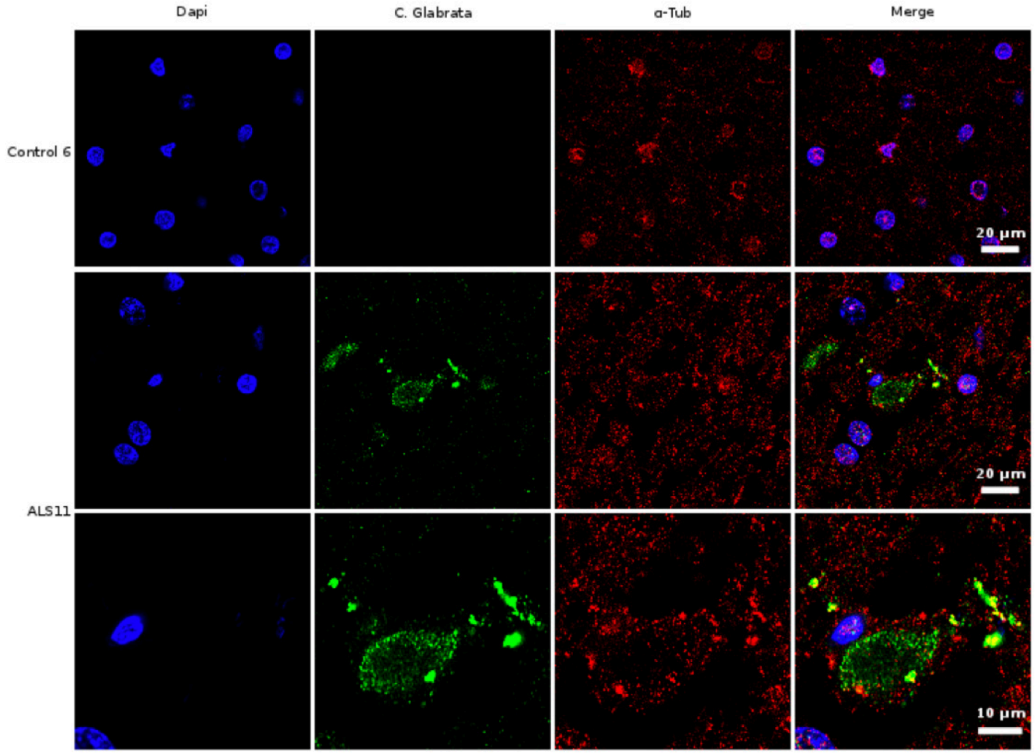


Figure 1 - Analysis of *C. albicans* and enolase antigens in CSF by slot-blot. 20 µl CSF samples were diluted with 180 µl TBS and were blotted onto a nitrocellulose membrane, which was incubated with the rabbit antiserum against *C. albicans* or enolase or recombinant MBP-enolase (primary antibody) as indicated and afterwards incubated with a rabbit anti rat IgG (secondary antibody). Positive control: control + 200 ng yeast protein or purified MBP-enolase. Negative control: control – corresponds to TBS alone.

Evidence-Neuropathology 1



Immunohistochemistry analysis of brain sections from the frontal cortex of an ALS patient and a control. Brain sections (frontal cortex) from AL
and control 6 were observed with a confocal laser scanning microscope. Sections were obtained from fixed tissue and immunohistochemistry analyses were carrie
ble immunofluorescence assay using anti-tubulin and anti-*C. glabrata* antibodies was carried out as detailed in Materials and Methods. DAPI appears in blue and anti-
shown in green. Human tubulin appears in red. The different panels in the figure are indicated.

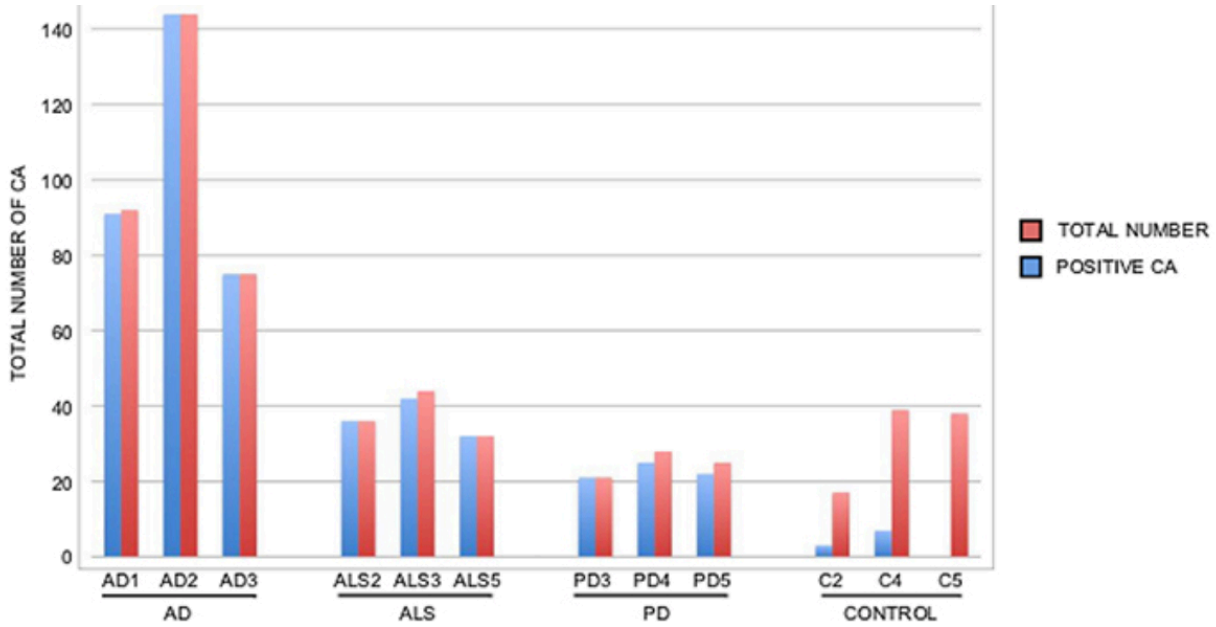
- Immunohistochemistry detected intracellular fungal antigens in frontal cortex of PALS, not healthy controls

Evidence-Neuropathology 2

- Brain tissue from: 6 PALS, 11 patients with AD, 6 patients with PD, 5 healthy controls

- Immunohistochemical analyses of corpora amylacea (CA, glycoproteinaceous inclusions that accumulate in the brain during the course of normal aging and to a greater extent in some neurodegenerative diseases)

Evidence-Neuropathology 2



- Polyclonal antibodies detected several different fungi in CA of patients with ALS, AD and PD but not controls

Evidence-Neuropathology 3

- Brain tissue from 11 PALS, 4 healthy controls
- Immunohistochemistry again showed intracellular fungi in PALS (not controls)
- 3d reconstructions suggested fungi in or on the nucleus of cells from the motor cortex, brainstem, spinal cord

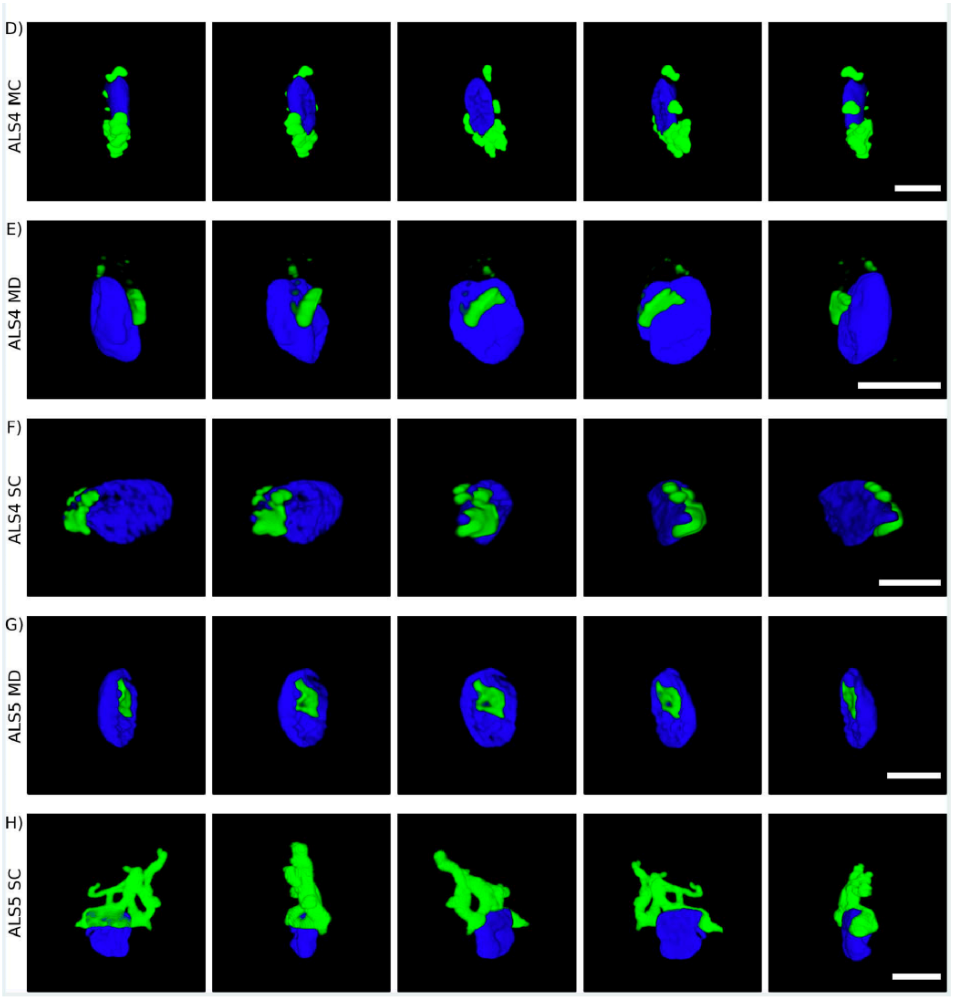


Fig. 3. Orthogonal projections and three-dimensional images.
Orthogonal projections (panels A, B and C) and different stacks of a 3D image (D, E, F, G and H) from different ALS patients. Samples were inmunostained with a rabbit polyclonal anti-*C. albicans* antibody (1:100 dilution) (green). Nuclei were stained with DAPI (blue). Scale bar: 5 μm.

Evidence-Neuropathology 3

REGION ITS1		REGION ITS2	
Species	**Patients**	**Species**	**Patients**
Aspergillus sp	ALS9-MC	*Aspergillus sp*	ALS9-MC
Candida famata	ALS2-SC1 , ALS1-SC1	*Candida albicans*	ALS4-MD,ALS5-MC,ALS8-MD
Cladosporium sp	ALS6-SC1 , ALS2-MC	*Cladosporium sp*	ALS4-MC
Cryptococcus curvatus	ALS7-SC2	*Cryptococcus fonsecae*	ALS7-MD
Cystobasidium sp	ALS8-SC1	*Cryptococcus magnus*	ALS10-SC1
Davidiella tassiana	ALS1-MD ,ALS4-SC1 , ALS5-SC1	*Malassezia globosa*	ALS11-MC
Malassezia globosa	ALS3-SC1,ALS7-MD	*Malassezia restricta*	ALS9-MD,ALS10-MC,ALS3-MC,ALS3-MD,A
Malassezia restricta	ALS3-MC , ALS4-MD		ALS4-MC,ALS6-SC1,ALS7-MD,ALS8-MD
Penicillum sp	ALS5-MD , ALS1-MC	*Penicillum sp*	ALS2-SC1
Rhodotorula mucilaginosa	ALS 4-MC	*Uncultured fungus*	ALS11-MD
		Uncultured malassezia	ALS4-SC1,ALS6-MC,ALS10-MD
Trichoderma sp	ALS3-MD	*Uncultured pezizomycetes*	ALS3-MC
Uncultured basidiomycota	ALS4 MC	*Uncultured pleosporales*	ALS1-SC1
Uncultured fungus	ALS7-SC3	*Uncultured toxicocladosporium*	ALS5-SC1
Uncultured malassezia	ALS5-MC		
Uncultured Sporidiobolales	ALS6-MD		

MC: Motor cortex; MD: Medulla; SC: Spinal cordal

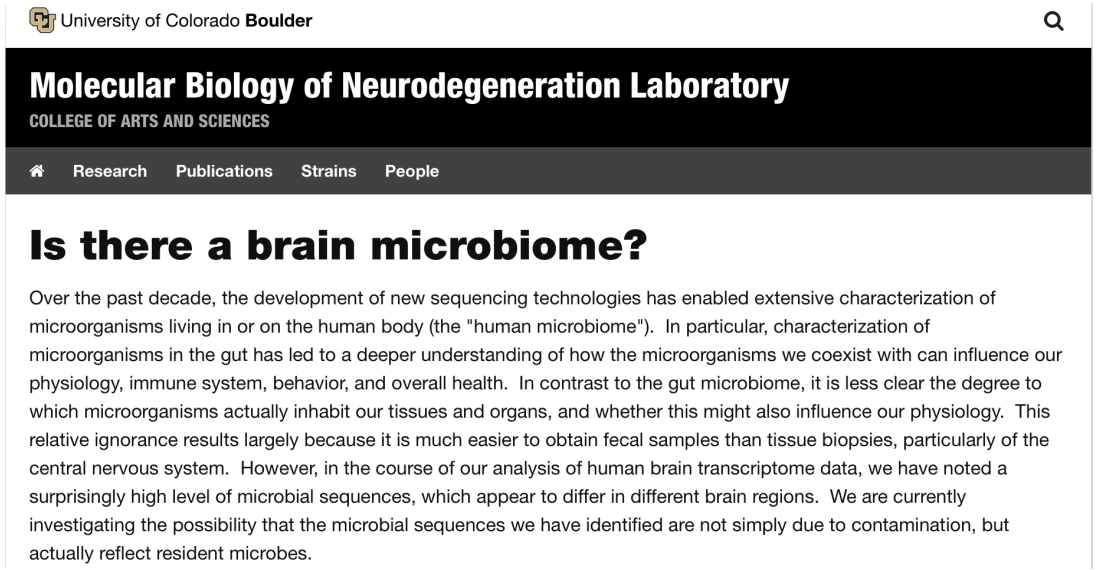
- DNA extracted, nested PCR technique used to amplify specific fungal regions for subsequent DNA sequencing. The genomic regions chosen were the intergenic sequences located between the the ribosomal RNA genes-many specific fungal species identified

Critiques of the Neuropathology Studies

- Small numbers of patients and controls
- Are the same participants being studied in all 3 papers?
- No clinical details on PALS (were they known to have fungal infections in life?)
- Scant details on brain processing methods (contaminants?)
- Polyclonal antibodies (may not be specific for fungi)
- Not all patients' data are included in different analyses
- Not yet independently replicated
- How can this explain the anatomic specificity of ALS (and other degenerative diseases)?

Possible Interpretations

- Artifacts/contaminants?
- Part of the ”CNS Microbiome”?
- Coincidental infection?
- Part of the pathophysiology of ALS (and other degenerative diseases)?
  - Longshot, but even in a subset might have huge implications for treatment



Suggested Next Steps

- I would like to see the VABB try to replicate and extend the neuropathological findings I described today
  - Well-characterized patients and controls
  - Well-described, sound protocols for brain acquisition and prep
  - Immunohistochemistry with monoclonal anti-fungal antibodies (if available)
  - PCR to look for fungal DNA