

# Could a Fungal Infection Cause Some Cases of ALS?

Richard Bedlack MD PhD

Professor of Neurology, Duke University

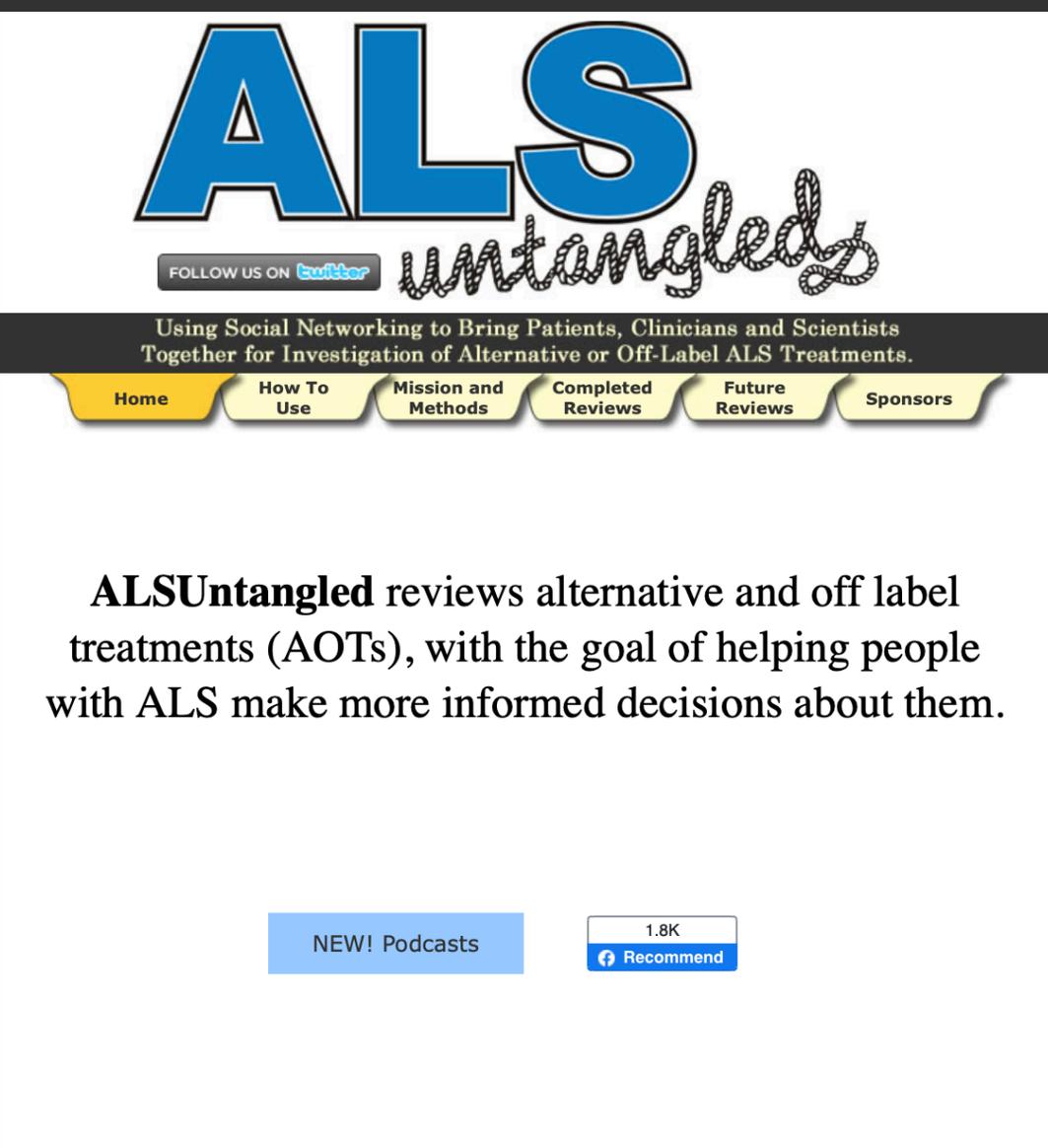
Neurologist, Durham VA Medical Center

# 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)



The screenshot shows the homepage of the ALSUntangled website. At the top, the logo features the letters "ALS" in a large, blue, outlined font, with the word "untangled" written in a black, cursive script below it. To the left of "untangled" is a small black button with the text "FOLLOW US ON" and the Twitter logo. Below the logo is a black banner with white text that reads: "Using Social Networking to Bring Patients, Clinicians and Scientists Together for Investigation of Alternative or Off-Label ALS Treatments." Underneath the banner is a navigation menu with six yellow buttons: "Home", "How To Use", "Mission and Methods", "Completed Reviews", "Future Reviews", and "Sponsors". The main content area contains a bolded paragraph: "ALSUntangled reviews alternative and off label treatments (AOTs), with the goal of helping people with ALS make more informed decisions about them." At the bottom of the page, there are two buttons: a blue button on the left that says "NEW! Podcasts" and a white button on the right that says "1.8K" above a blue button with the Facebook logo and the word "Recommend".

# 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?

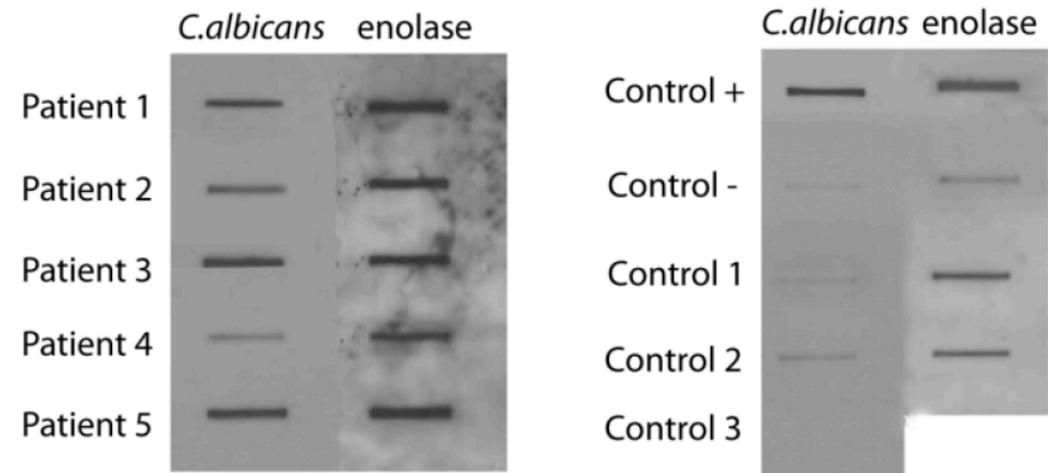
- Dr. Reid treated 5-10 PALS with antifungals, in some cases along with PLEX or IVIG, and reported improved motor function
  - ALSUntangled review noted these improvements were generally small, transient, which can happen spontaneously in PALS
  - ALSUntangled was unable to independently verify the ALS 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.

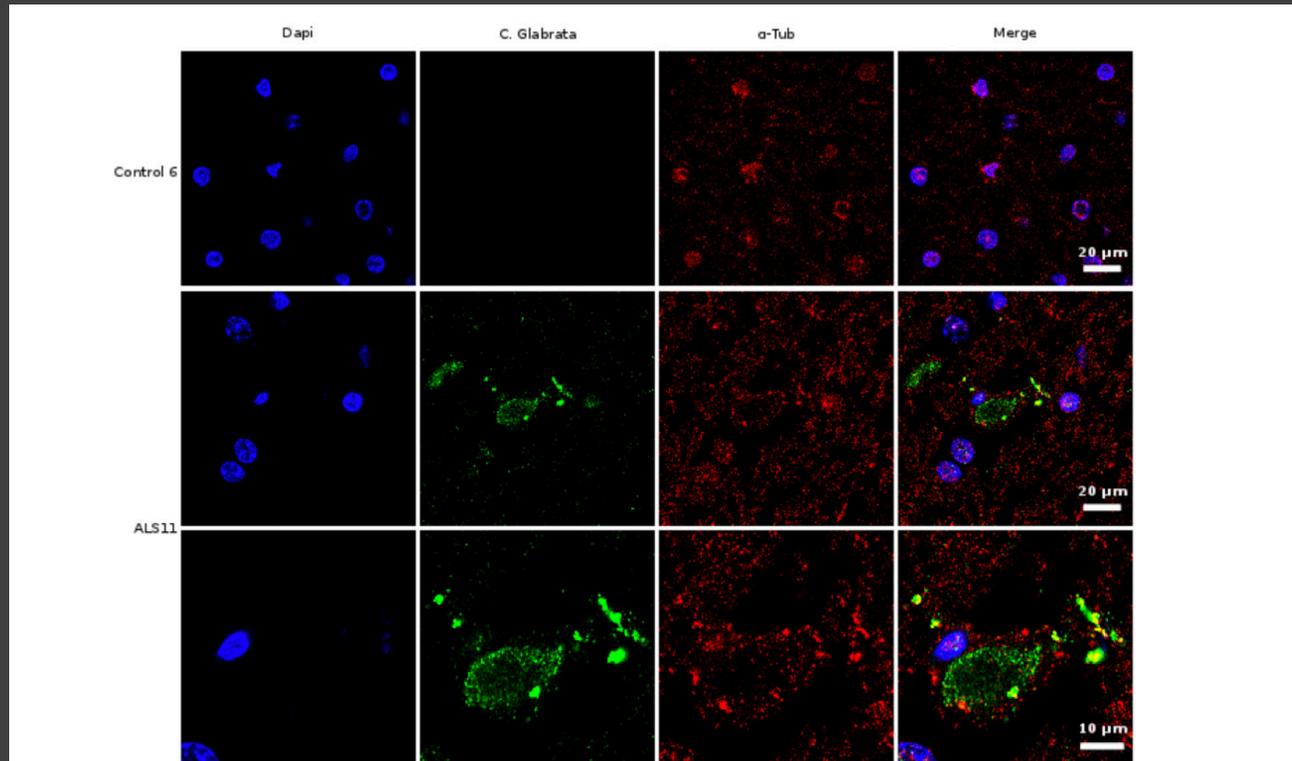
# Evidence-Neuropathology 1

- 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  $\mu$ l CSF samples were diluted with 180  $\mu$ l 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



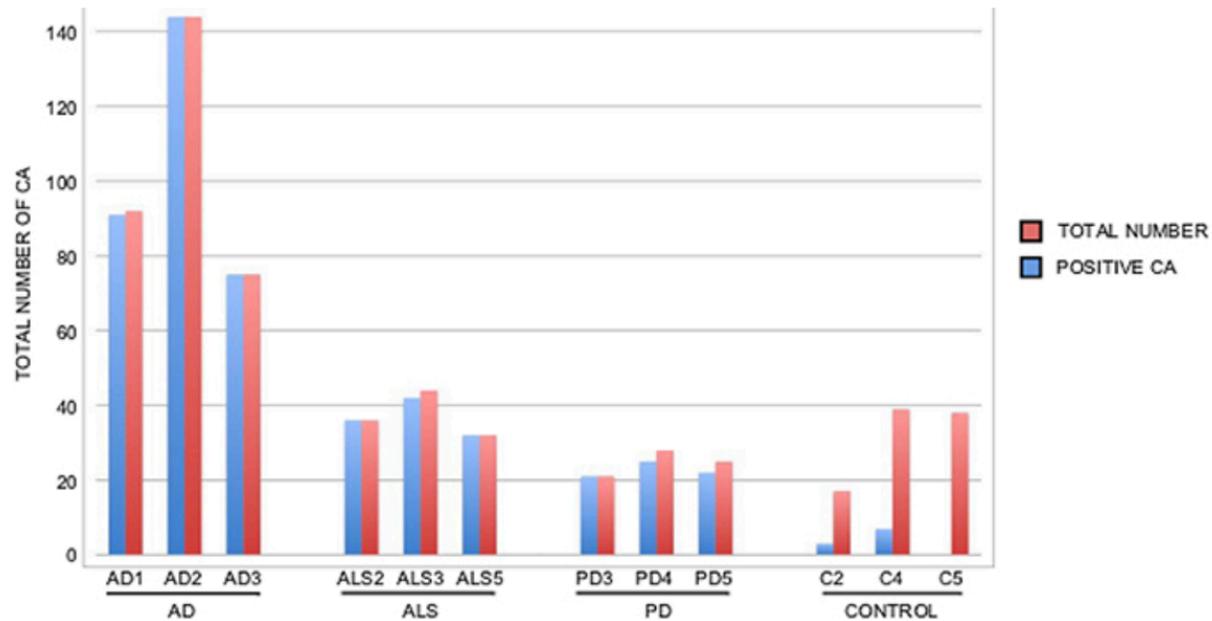
- Immunohistochemistry detected intracellular fungal antigens in frontal cortex of PALS, not healthy controls

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

# 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

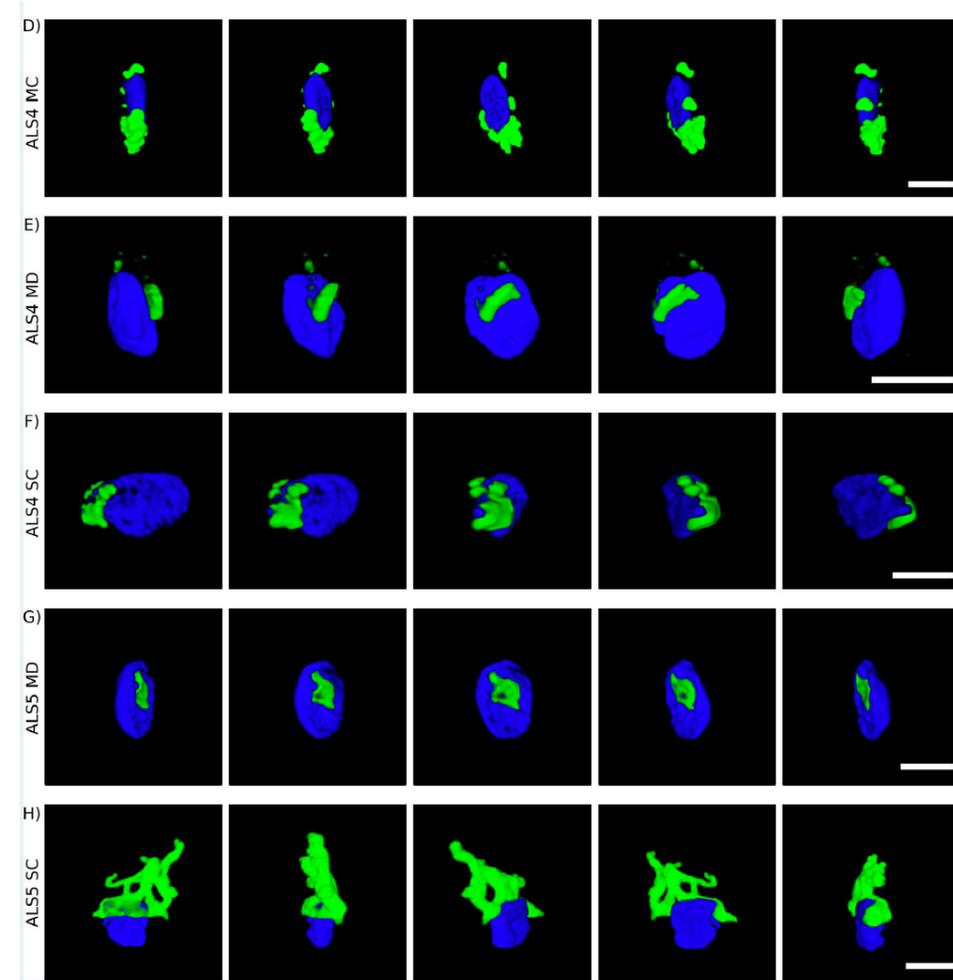


Fig. 3. Orthogonal projections and three-dimensional images. Orthogonal projections (panels A, B and C) and different stacks of a 3D image (D, E, F, G and H) from different ALS patients. Samples were immunostained with a rabbit polyclonal anti-*C. albicans* antibody (1:100 dilution) (green). Nuclei were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

# Evidence-Neuropathology 3

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

- 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

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

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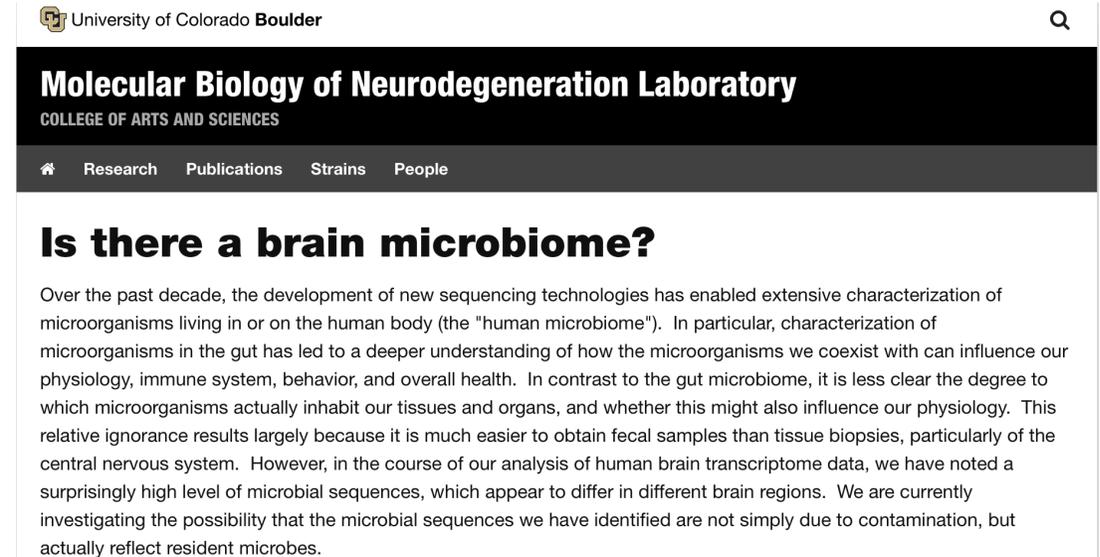
# Critiques of the Neuropathology Studies

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- 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



The screenshot shows the website for the Molecular Biology of Neurodegeneration Laboratory at the University of Colorado Boulder. The page header includes the university name and a search icon. The main title is "Molecular Biology of Neurodegeneration Laboratory" under the "COLLEGE OF ARTS AND SCIENCES". A navigation bar contains links for "Research", "Publications", "Strains", and "People". The main content area features the heading "Is there a brain microbiome?" followed by a paragraph of text discussing the development of sequencing technologies and the characterization of the human microbiome, specifically focusing on the gut and the central nervous system.

University of Colorado Boulder

**Molecular Biology of Neurodegeneration Laboratory**  
COLLEGE OF ARTS AND SCIENCES

Research Publications Strains People

## Is there a brain microbiome?

Over the past decade, the development of new sequencing technologies has enabled extensive characterization of microorganisms living in or on the human body (the "human microbiome"). In particular, characterization of microorganisms in the gut has led to a deeper understanding of how the microorganisms we coexist with can influence our physiology, immune system, behavior, and overall health. In contrast to the gut microbiome, it is less clear the degree to which microorganisms actually inhabit our tissues and organs, and whether this might also influence our physiology. This relative ignorance results largely because it is much easier to obtain fecal samples than tissue biopsies, particularly of the central nervous system. However, in the course of our analysis of human brain transcriptome data, we have noted a surprisingly high level of microbial sequences, which appear to differ in different brain regions. We are currently investigating the possibility that the microbial sequences we have identified are not simply due to contamination, but actually reflect resident microbes.

# 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

