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REVIEW ARTICLE

Establishment of a consensus protocol to explore the brain pathobiome in patients with mild cognitive impairment and Alzheimer's disease

Research outline and call for collaboration

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Abstract

Microbial infections of the brain can lead to dementia, and for many decades microbial infections have been implicated in Alzheimer's disease (AD) pathology. However, a causal role for infection in AD remains contentious, and the lack of standardized detection methodologies has led to inconsistent detection/identification of microbes in AD brains. There is a need for a consensus methodology; the Alzheimer's Pathobiome Initiative aims to perform comparative molecular analyses of microbes in *post mortem* brains versus cerebrospinal fluid, blood, olfactory neuroepithelium, oral/nasopharyngeal tissue, bronchoalveolar, urinary, and gut/stool samples. Diverse extraction methodologies, polymerase chain reaction and sequencing techniques, and bioinformatic tools will be evaluated, in addition to direct microbial culture and metabolomic techniques. The goal is to provide a roadmap for detecting infectious agents in patients with mild cognitive impairment or AD. Positive findings would then prompt tailoring of antimicrobial treatments that might attenuate or remit mounting clinical deficits in a subset of patients.

KEYWORDS
Alzheimer's disease, antimicrobial, antiviral, bioinformatics, blood, cerebrospinal fluid, collaboration, dementia, diagnosis, methodology, microbiome, mild cognitive impairment, olfactory neuroepithelium, pathobiome, polymerase chain reaction, protocol, sequencing

1 | INTRODUCTION

Alzheimer's disease (AD) is a slowly progressive brain disorder, principally affecting the elderly, that culminates in devastating loss of memory and cognitive skills (dementia) accompanied by physiological/endocrine impairments. Currently, there is no cure. AD is a major cause of morbidity and mortality but, despite research investment of nearly US$100 billion, its causes are not fully understood and to date there is no effective therapy. One consistent hypothesis over the decades relates to whether microbial infection of the brain and/or periphery might contribute to the pathoetiology of dementias including AD, as highlighted by a panel of experts less than a decade ago.[1] This contention is supported by multiple findings including indications that the molecular and clinical signatures of AD pathology are associated with infection and that diverse microbes are present in *post mortem* AD brains. Moreover, infection is a known cause of some types of dementia. However, there is to date no standard methodology to evaluate patients with early signs of AD (e.g., mild cognitive impairment [MCI]) for potential intracranial or extracranial infectious involvement that would set patients on an appropriate antimicrobial therapeutic path with the aim of arresting or reversing disease progression. In addition, many remain skeptical of the possibility that infection might contribute to AD, further fueled by the technical difficulties inherent in diagnosing and treating brain infections.

This article aims to raise awareness of evidence that microbial infection is a known cause of some types of dementia, and to evaluate the hurdles to be overcome in the development of rapid and accurate diagnostic assays for the assessment of potential brain infections in individuals with or at risk of AD.

1.1 | AD signatures of infection

Although the etiology of sporadic AD remains unknown, brain deposition of the amyloid beta (Aβ) fragment of amyloid precursor protein (APP), resulting in plaque formation, has been recognized for more than a century as a cardinal signature of AD, and the presence of the apolipoprotein E (*APOE*) *ε4* allele is a known susceptibility factor for AD development.[2] Both Aβ and *APOE ε4* may be indicative of an infectious contribution. For many years it was suspected that Aβ might be the toxic cause of AD.[3] Nevertheless, all therapeutic strategies over the past 20 years aimed at removing or blocking Aβ have failed to return any substantial improvement in symptomatic patients. The only potential exceptions are the recent Food and Drug Administration (FDA) accelerated approval of the anti-Aβ antibody aducanumab for asymptomatic Aβ-positive participants, and in January 2023 of a second antibody (lecanemab), although the reported clinical effects were marginal. FDA approval has attracted some criticism.[4]

By contrast, data have recently emerged that the signature protein of the AD brain, Aβ peptide, has a natural and highly conserved physiological role as part of the immune system. Acting as an antimicrobial peptide (AMP), Aβ forms extracellular traps that entrap and inactivate pathogens such as bacteria, fungi, and viruses, and protect host cells from infection.[5–7] These observations provided the foundation for

the antimicrobial hypothesis of AD, as elaborated in a recent review.[8] Expanding on the Aβ cascade hypothesis, the antimicrobial hypothesis postulates that a real (or perceived) pathogenic infection drives Aβ deposition and neuroinflammation as part of an innate immune response to clear the infection. In AD, downstream failure of this immune response or persistent activation from chronic infection leads to sustained inflammation and neurodegeneration. Indeed, bacterial lipopolysaccharide (LPS), herpes simplex virus 1 (HSV1) DNA, and *Porphyromonas gingivalis* gingipains have been reported to colocalize with Aβ in the brain,[9–11] consistent with an antimicrobial role of Aβ aggregation as a mechanism to entrap and inactivate pathogens. The brain also expresses other antimicrobial factors such as chitinases that defend against fungal infection,[12] and these are also upregulated in AD.[13–16] In one study of AD versus control brain, *CHIT1* was the most highly upregulated gene,[17] potentially indicative of local fungal infection. In support, the fungal cell wall component chitin has also been reported in the AD brain,[18–20] but not in brains from patients with multiple sclerosis.[21]

Genetic AD risk factors also point to the involvement of infection. A growing pool of innate immune genes as well as microglial cell activation genes, such as Siglec-3 (*CD33*) and triggering receptor expressed on myeloid cell 2 (*TREM2*), show the expanding importance of immune response and infection in AD.[22,23] In addition to representing the most prominent AD risk factor gene, APOE is an important immune peptide acting as an immunomodulatory protein that presents lipid antigens to the immune system,[24,25] represses inflammation by inhibition of the complement pathway,[26] and binds tightly to Aβ.[27] APOE-derived peptides themselves have direct antimicrobial activity (e.g., Dobson et al.,[28] Siddiqui et al.,[29] and Puthia et al.[30] and references therein), highlighting the possibility that APOE regulates AD risk by modulating the outcome of infection. In support, *APOE* alleles influence viral, bacterial, and parasitic disease.[31] Indeed, the *APOE ε4* allele accelerates human immunodeficiency virus (HIV) proliferation whereas *APOE ε3* is protective,[32] the numbers of *Chlamydia*-infected cells and bacterial load were significantly higher in homozygous *APOE ε4* patients than in *APOE ε2/ε3* carriers,[33] and *APOE ε4* increases susceptibility to cold sores caused by herpes simplex.[34] In COVID infection, *APOE ε4* is a major determinant of severe disease (e.g., Kuo et al.[35] and Lord et al.[36]). By contrast, *APOE ε4* is protective against liver disease induced by the hepatitis C virus[37] as well as against malaria (*Plasmodium falciparum*),[38] whereas *APOE ε2* may predispose to malaria susceptibility,[39] perhaps explaining why different *APOE* alleles persist in the population. Thus, *APOE ε4* appears to be a key driver of AD risk by modulating the outcome of specific types of infection. Furthermore, brain tissue of AD patients displays extensive signatures of infection/inflammation including macrophage infiltration and cytokine upregulation/neuroinflammation[40–42] as well as Aβ deposition. Brain expression levels of C-reactive protein (CRP), a marker of infection, are increased 20-fold in AD brain tissue versus controls,[43] but not in serum (e.g., O'Bryant et al.[44]).

Importantly, this interplay among infection, Aβ's role as an AMP in the innate immune system, and genetics leaves open a large window for the impact of pathogens on AD development. Although it is possible that an infection may initiate sustained inflammation, immune system activation, and Aβ aggregation leading to AD pathology (Figure 1), microbial involvement may take place at any point, thus exacerbating existing pathologies. Together, these observations make a compelling case that the microbes we are exposed to over a lifetime may reach the brain and contribute to the pathobiology of AD.

Research in Context

1. **Systematic review**: The authors reviewed the literature on microbes and dementia and chronic meningitis including research studies, case reports, and research on current diagnostic tests that may be used to characterize the brain pathobiome.
2. **Interpretation**: There is an urgent need for (i) interdisciplinary collaboration and consensus, (ii) testing and validating the best methods to characterize the brain pathobiome, and (iii) identification of the least-invasive biosample(s) that may be collected from living patients to unveil microbial infection(s).
3. **Future directions**: A multi-center study that tests post mortem biosamples using different methods will help develop a consensus diagnostic(s) to reveal most microbes. This can then be used prospectively in patients with mild cognitive impairment and/or Alzheimer's disease to reveal potential microbial infection(s). Precise antimicrobial treatment can be tailored to address infection(s), which according to case reports describing "reversible dementias" may unveil an infectious subset whose symptoms may remit once underlying infections are treated.

1.2 | Microbes in the AD brain

Over recent decades multiple studies on *post mortem* AD brains have reported that diverse pathogens are present, ranging from bacteria to fungi to viruses.[45–53] Archaea, Chloroplastida, and Holozoa have also been reported in the brain.[54,55] There are multiple potential routes of entry to the brain (Figure 2). Although it is possible that pathogen contamination might be introduced during sampling or processing, the accumulated evidence argues against contamination. Brains of germ-free mice, unlike those of conventionally reared mice, were reported to be devoid of microbes,[56] but this awaits confirmation. The presence of bacteria and fungi in the AD brain has been confirmed by multiple methods including DNA- and RNA-based studies, proteomics, immunohistochemistry, and peptidoglycan analysis; moreover, hyphal structures were detected in the brain that are thought to take weeks, months, or even longer to develop,[50,57] thus arguing against contamination. In addition, the observed upregulation of chitinases and the

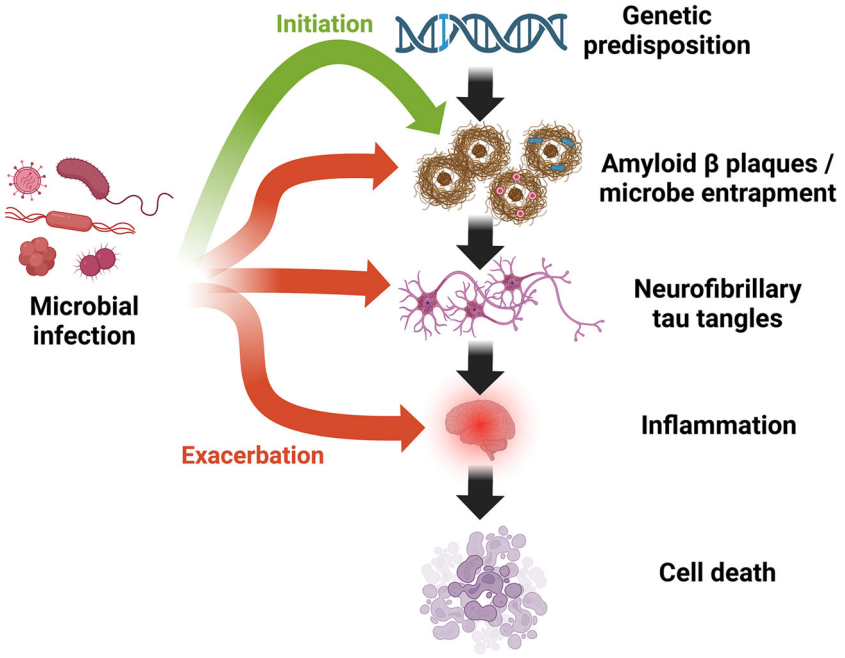

FIGURE 1 The antimicrobial hypothesis of Alzheimer's disease (AD). Genetic and/or physiological predisposition facilitates microbial infection and proliferation, leading to amyloid beta production—a defense mechanism to entrap and inactivate microbes—as well as to aggregation of tau tangles, further local inflammation, and neuronal death, culminating in AD.

presence of chitin in the brain (see earlier) is consistent with in vivo infection rather than with contamination. Bacteria were also detected in surgically resected epilepsy samples of the human brain by in situ hybridization, and peptidoglycan-positive bodies morphologically consistent with bacteria were detected by immunohistochemistry and light microscopy.[48] Moreover, bacteria in the AD brain have been further characterized by direct culture in vitro,[58,59] and bacterial infection could be transmitted by intracerebral inoculation of mice with human brain material.[48] Although each of these studies may be open to challenge, the combined weight of evidence argues that the brain houses its own microbiome, and that infection may contribute to the neuroinflammation and neurodegeneration seen in AD.

However, despite major efforts to date, no specific pathogen has been detected that is present in the AD brain but absent from controls, although this is not an adequate criterion. For example, a microbe can be present but asymptomatic: most HSV1-infected individuals do not develop cold sores or other lesions, and many people infected with *Mycobacterium tuberculosis* do not develop tuberculosis. However, two recent studies identified potential differences in the microbiota of AD and age-matched brains.[53,55] One possible explanation is that dementias such as AD can have diverse etiologies in the same way that pneumonia can be caused by a variety of organisms. For example, viruses (influenza, COVID-19, sometimes herpesviruses), bacteria (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, and others), and fungi (*Aspergillus* spp., *Mucor* spp., *Candida* spp., *Cryptococcus* spp.) sometimes act alone, but often in concert, and can contribute to a complex polymicrobial disorder such as pneumonia that can be difficult to treat without identification of the specific microorganisms responsible. In dementia, our best understanding is that the specific pathogen(s) involved, and even the precise brain regions affected, are likely to differ markedly between individuals based on their exposure and/or genetic predisposition, as well as in the state of activation of microbes (e.g., latent vs. productive infection). This may explain why clinical trials and epidemiological data on the use of antibiotics or antivirals, until now, have had mixed results (see below).

1.3 | Dementias caused by infectious agents

Case reports scattered throughout the medical literature suggest that several different infectious agents can drive dementia development. Rapidly progressive dementia (RPD) can be caused by enterovirus,[60] HIV-associated neurocognitive disorders (HAND) bear similarities to AD,[61] neuroborreliosis can cause secondary dementia,[62] and it is well accepted that syphilis (*Treponema pallidum*) can cause dementia that abates with timely intravenous (IV) antibiotic treatment.[63] Moreover, end-stage syphilis resulting in general paresis is histopathologically indistinguishable from AD.[64] In addition, cognitive impairment may abate with proper anti-infective treatment such as in cases of HIV,[65] syphilis before the development of general paresis,[66] and Lyme neuroborreliosis.[62] Vargas et al.[63] remark that "Neurosyphilis should be a part of the differential diagnosis of every patient showing cognitive deterioration and behavior disturbances," a sentiment supported by other clinicians.[67] The parasitic infection cysticercosis caused by larvae of *Taenia solium* is known to cause dementia in developing countries, and timely initiation of proper treatment results in favorable neurologic outcomes—"reversible dementia."[68,69] Importantly, a handful of cases have been reported in which brain infections with *Cryptococcus* spp. "masqueraded" as AD, but remitted upon appropriate antifungal therapy,[70–73] holding promise that at least some AD cases may be amenable to therapeutic intervention. Clinical reports on dementias caused by infection are summarized in Table 1.

To add to the infectious etiology of neurodegenerative disease, a recent longitudinal study in veterans found that Epstein–Barr virus (EBV) can increase the risk of developing multiple sclerosis by 32-fold[89] and a Mendelian randomization study recently implicated EBV

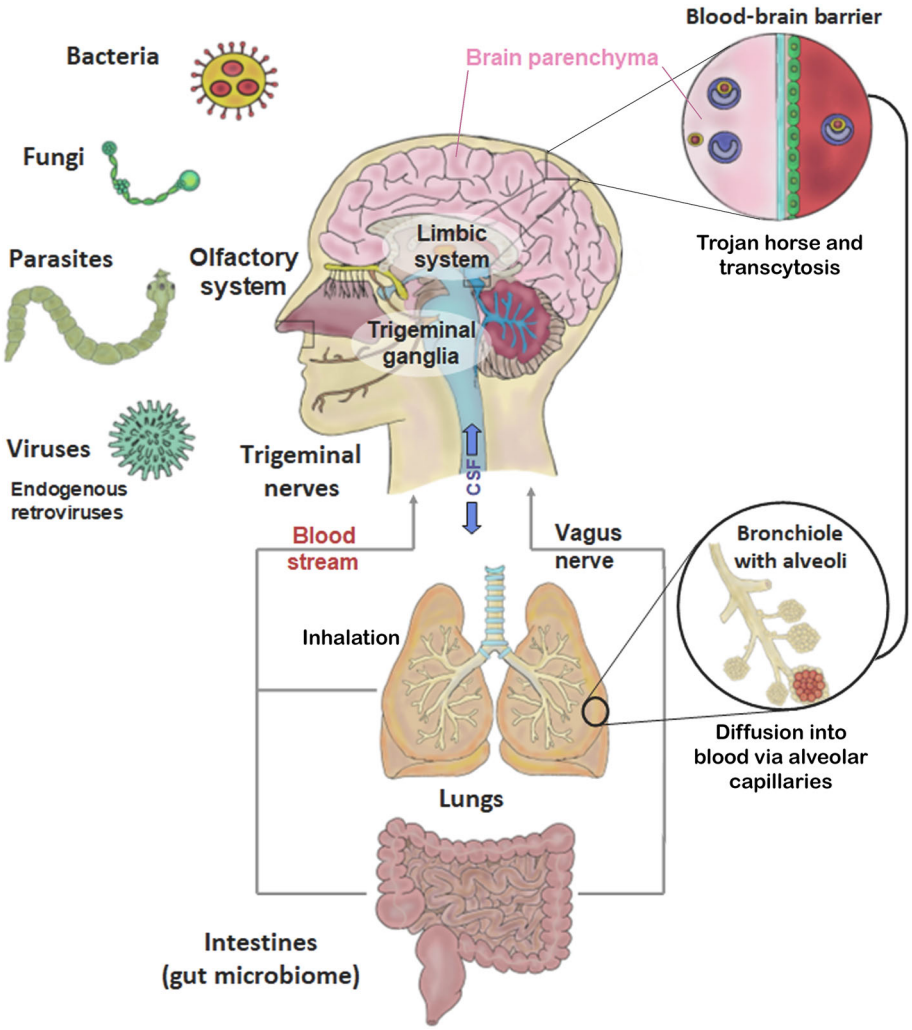

FIGURE 2 Microbes and the brain: routes of entry. Figure courtesy of Noeen Malik.

infection in AD conversion.[90] The EBV study in veterans is compelling and calls for a concerted and strategic effort to untangle the interplay among infection, inflammation, genetics, and dementia as a clinical outcome with aging.

1.4 | Anti-infective trials in AD

Diverse trials of antimicrobial/antiviral agents in AD are summarized in Box 1 (epidemiological studies are not reviewed here). The overall picture is that some studies report strong benefits, whereas others report none. It is possible that variable outcomes reported in these trials relate in part to mismatches between the anti-infective agents used and the organisms present in individual trial participants.

2 | THE NEED FOR BRAIN MICROBIOME STUDIES IN AD

We have highlighted evidence that the human brain contains its own microbiota, and that excesses of some microorganisms may be associated with AD, borne out by case reports of dementias that can be reversed by timely and appropriate antimicrobial treatment. Despite accumulating evidence, the contention that microbes might play a causal role in the neuroinflammation and neurodegeneration in AD remains controversial. To become clinically actionable, formal demonstration of the potential involvement of microbes in the pathoetiology of AD will require a stringent and reproducible test—that *appropriate* antimicrobial medication can attenuate or remit mounting clinical deficits in individuals living with precursors to AD such as MCI or AD itself. However, fundamental issues must be addressed because brain biopsy is unfeasible (with the potential exception of patients undergoing neurosurgery for other conditions), and analysis must therefore be based on peripheral samples. This raises several important questions.

(i) What is the best peripheral tissue(s) for sampling, and when in the course of AD should samples be taken? Specifically, (ii) what is the composition and abundance of pathogens in obtainable biosamples such as cerebrospinal fluid (CSF) and blood from patients living with varying stages of dementia, and is this profile an adequate proxy for the spectrum of microbes present in the brain of the same individual? (iii) Given that traditional methods for microbe detection have largely given way

TABLE 1 Case reports of infectious disease as a primary cause of dementia.

Case report	Symptom duration	Dementia type	Microbe(s)	Treatment	Outcome	Refs
Fungal						
Female 67 years: cryptococcal encephalitis presenting as dementia and myoclonus	2 months	Unspecified dementia	*Cryptococcus neoformans*	Amphotericin B plus flucytosine	Condition deteriorated; died after 18 days	74
Male 73 years: cryptococcal meningitis presenting as AD	3 years	AD	*Cryptococcus neoformans*	Amphotericin B and flucytosine IV for 6 weeks followed by oral fluconazole 400 mg/day 13 months	MMSE score increased from 20/30 to 26/30 (near-complete recovery)	71
Male 57 years: cryptococcal meningitis presenting as VaD	2 years	VaD	*Cryptococcus neoformans*	Amphotericin B IV for 4 weeks, 6 weeks flucytosine, 3 months oral fluconazole	MMSE score increased from 16/30 to 24/30	70
Male 62 years: cryptococcal meningitis presenting as AD	3 years	AD	*Cryptococcus neoformans*	Flucytosine and IV amphotericin B	MMSE score increased from 24/30 to MMSE 30/30 (full recovery)	72
Female 72 years: cryptococcal meningitis, altered mental status, relapsing fevers in a patient with previously diagnosed probable VaD	5 months	VaD with cryptococcal meningitis	*Cryptococcus neoformans*	Flucytosine (250 mg orally q12 h) and amphotericin B liposomal (350 mg IV q12 h). Steroid treatment stopped to prevent impaired infection clearance	Patient developed complications, acute kidney injury (nephrotoxic medicines), tachycardia, and tachypnea, died on day 41 of hospital stay	75
Male mid-70s: cryptococcal meningitis presenting as rapid cognitive decline	2 months	RPD	*Cryptococcus neoformans*	Amphotericin B with high-dose fluconazole (flucytosine preferred but unavailable in this hospital)	Neurological symptoms improved after 3 weeks of treatment	73
Male 64 years: cryptococcal meningitis presenting as cognitive impairment and altered consciousness; memory loss with long-term prednisolone use	2 months	RPD	*Cryptococcus neoformans*	800 mg oral fluconazole, 0.7 mg/kg/day IV amphotericin deoxycholate	Cardiac arrest 5 days after hospital admission, deceased	76
Case series: 19 cases of RPD (2:1.1 ratio male to female) caused by *Cryptococcus neoformans*	Average duration symptoms 7.4 months	RPD	*Cryptococcus neoformans*	Unspecified antifungals	In patients evaluated after treatment MMSE improvement ranged from 4 to 10 points	76
Parasitic						
Female 63 years: racemose neurocysticercosis presenting as reversible dementia (relapsing)	19 years	Unspecified dementia	*Taenia solium*	Antiparasitic therapy, shunt, dexchlorpheniramine (Dxc) 6 mg daily, dexamethasone 20-day taper, Dxc 4 mg/day continuously	MMSE score increased from 24/30 to 30/30 (full recovery)	69

(Continues)

TABLE 1 (Continued)

Case report	Symptom duration	Dementia type	Microbe(s)	Treatment	Outcome	Refs
Viral						
Case series: 45 patients (53% microbial etiology identified) with acute encephalitis, 8 with HSV encephalitis, 7 with herpes zoster	n/a	Encephalitis with new-onset dementia	HSV and herpes zoster	IV acyclovir (30 mg/kg/day) for 11 days given within 3.5–4.5 days of symptom onset	Follow-up at 3.7 years dementia frequency in 40 patients was 12.8%, cognitive performance improved in all but 3 cases; 70% of employed patients returned to previous occupations	77
Male 70 years: enteroviral meningoencephalitis presenting as RPD	4 months	RPD	Enterovirus (echovirus 18)	Steroids for 5 days then at month 6 and 12 post discharge treatment with 2 g/kg IVIG in 5 divided doses	MMSE score increased from 5/30 to 22/30	78
Male: new cognitive decline; young, HIV-positive	Several weeks	HIV-associated dementia	HIV, previously treated neu-rosyphilis	Abacavir, lamivudine, zidovudine, and nevirapine	Memory, attention, and gait improved over the course of 1 year	79
Bacterial						
Male 60 years: severe presenile dementia without clinical signs of meningitis or encephalitis	1 year	RPD	Lyme disease (*Borrelia burgdorferi)*	IV benzylpenicillin 14 days (*Borrelia* titer in CSF increased after treatment)	General condition improved after treatment, 1.5 years later returned to normal social life, car-driving without accidents with only mild forgetfulness	80,81 for commentary
Male 47 years: fatal neuropsychiatric Lyme disease presenting clinically as progressive frontal lobe dementia with pathological severe subcortical degeneration	1 year	Progressive frontal lobe dementia	Lyme disease (*Borrelia burgdorferi)*	IV ceftriaxone 4 weeks followed by oral antibiotics for 6 weeks	Full recovery with treatment followed by relapse 5 months later and death (no repeat antibiotic treatment for probable relapse)	82
Female 63 years: rapid functional and cognitive decline, multiple falls, incontinence	2 months	RPD, suspected CJD	*Mycobacteriur neoaurum*	DNA for *M. neoaurum* found during autopsy, no treatment was initiated. *Pre mortem* cultures of CSF were negative	Deceased	83
Male 56 years: memory loss, reduced attention and concentration, agitation, apathy, anxiety	2 years	Mild dementia	Neurosyphilis (*Treponema pallidum*)	High-dose IV penicillin	MMSE score increased from 23/30 to 26/30 at 6 months, and to 27/30 at 12 months post-treatment	67
Male 33 years: neurosyphilis causing mild to moderate dementia mimicking AD with hippocampal atrophy	18 months	Mild to moderate demen-tia/AD	Neurosyphilis (*Treponema pallidum*)	IV penicillin G 5× 500 000 IU/daily for 20 days	MMSE score increased from 16/30 to 19/30; improvements in daily living and reduced behavioral disturbances	84

(Continues)

TABLE 1 (Continued)

Case report	Symptom duration	Dementia type	Microbe(s)	Treatment	Outcome	Refs
Female 76 years: rapid cognitive decline and headaches	1 year	Subcortical dementia with mild signs of Parkinsonism and Lewy body dementia	Lyme neuroborreliosis (*Borrelia burgdorferi*)	Ceftriaxone (Cftx) 2 g daily 3 weeks	Rapid recovery of cognition within a few weeks	62
Female 71 years: RPD	6 months	Primary dementia	Lyme neuroborreliosis (*Borrelia burgdorferi*)	Cftx 2 g/day	Pathological MMSE score increased to 29/30 within a few weeks (near full recovery)	62
Female 75 years: MCI with short-term memory loss	4–5 months, original infection occurring in 1985 (relapsing repeatedly over a few decades)	RPD	Whipple disease of CNS (*Tropheryma whippelii*)	Cftx 2 g/day, doxycycline 100 mg 2×/day, and hydroxycholoroquine 200 mg 3×/day for 14 days; IV trimethoprim-sulfamethoxazole 320/1600 mg 4×/day for 14 days, followed by oral trimethoprim-sulfamethoxazole 160/800 mg 2×/day	Mild, gradual improvement over 2 weeks	85
Male 75 years: cognitive impairment, deficit of memory, and poor capacity of criticism compatible with degenerative disease	A couple of years with worsening confusion, disorientation, hallucinations and aggressive behavior	Reversible dementia with Lyme disease	Lyme disease *(Borrelia burgdorferi)*	Cftx IV 2 g 2x/day, low-dose prednisone, and doxycycline 200 mg/day for 7 days	Improvement in all symptoms after 6–7 days on antibiotic therapy, MMSE score increased from 22/30 to 29/30 (near full recovery)	86
Polymicrobial						
Male 40 years (immunocompetent): meningitis presenting as reversible dementia	1 year	RPD	Concurrent HSV-2 and syphilis (*Treponema pallidum*)	Acyclovir IV 21 days, penicillin G 14 days	MoCA 13/30 increased to 21/30 at discharge	87
Female 28 years (immunosuppressed): RPD	6 months	RPD	Coxsackie virus B3 (CVB3), *Toxoplasma gondii*	*T. gondii* treatment failed, CVB3 identified in brain with PCR upon autopsy	Deceased	60
Male 59 years: nonopportunistic infection leading to RPD with HIV and CJD	2 weeks	RPD, CJD	HIV/AIDS and *Pneumocystis jroveci*		Deceased	88

Abbreviations: AD, Alzheimer's disease; CJD, Creutzfeldt–Jakob disease; CNS, central nervous system; CSF, cerebrospinal fluid; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IV, intravenous; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination (scores: 24–30, normal; 19–23, mild impairment; 10–18, moderate impairment; 9 or less, severe impairment); n/a, not applicable; MoCA, Montreal Cognitive Assessment (scores similar to MMSE); RPD, rapidly progressing dementia; VaD, vascular dementia.

Box 1. Trials of antimicrobial agents in AD

Several trials have evaluated whether specific antimicrobials might arrest cognitive decline in AD; these are listed in Table A below. This summary does not include epidemiological studies of possible relationships between drug use and AD development, and also does not include trials of anti-infective agents such as anti-leprosy drugs,[91] interferon,[92] and D-cycloserine (e.g., Schwartz et al.,[93] Tsai et al.,[94] and Laake and Oeksengaard[95]).

TABLE A. Antimicrobial trials in AD[a]

Intervention	Number of patients	Microbe testing	Outcome	Refs
Rifampicin (RIF) 300 mg/day and doxycycline (DOX) 100 mg BID vs. placebo	*N* = 101	PCR, IgG, and IgA for *Chlamydia pneumoniae*	6-month study; significant improvement in SADAS-cog score and dysfunctional behavior	96
Omeprazole, clarithromycin, and amoxicillin given to *Helicobacter pylori*-positive patients	*N* = 80	Gastric mucosal biopsy to detect *H. pylori* by histologic analysis and urease test as well as serum anti-*H. pylori*-specific IgG analyzed	2-year study; *H. pylori* eradication was successful in 84.8% of patients, cognitive and functional status parameters improved in the subgroup with successful *H. pylori* eradication (MMSE, CAMCOG, and FRSSD)	97
RIF 300 mg QID, DOX 100 mg BID, DOX 100 mg BID plus RIF 300 mg QID, or placebo	*N* = 305 (*N* = 101 in the RIF+DOX arm)	None	12 months of treatment had no effect on cognition or function	98
Minocycline (MIN) 100 mg BID or 200 mg BID vs. placebo	*N* = 554	None	24-month study; MIN did not delay progress of cognitive or functional impairment	99
Abovir (ribavirin and pleconaril) 600 mg/day reduced to 400 mg/day	*N* = 69	None	9-month study; no significant change in ADAS-cog score vs. placebo (but trend, $P = 0.18$), significant reduction in CSF $A\beta_{42}$	100
Valacyclovir 2 g/daily titrated to 4 g/daily	*N* = 130	Patients positive for HSV-1 or HSV-2 serum antibodies randomized	78-week study; completion anticipated December 2023	101 (protocol)
Atuzaginstat 40 mg or 80 mg (BID) or placebo	*N* = 643	Salivary *Porphyromonas gingivalis* DNA testing	48-week study; no change in primary endpoints (ADAS-cog11 and ADCS-ADL); the subgroup with detectable *P. gingivalis* DNA ($n = 242$) had a statistically significant slowing of ADAS-Cog11 at the high dose (57% slowing)	NCT03823404b Results released at CTAD 2021, so far unpublished
Valacyclovir treatment pilot in AD patients with HSV; 500 mg TID days 1–7, 1000 mg TID days 8–28	*N* = 33	Serum or plasma anti-herpes IgG	4-week pilot; safe, well-tolerated, and MMSE score increased ($P = 0.02$)	102
Rifaximin 550 mg BID; open label pilot	*N* = 10	Fecal microbiota testing	3-month pilot; cognition unchanged	103
COR-588 Investigational Phase 1, dosing range 50–200 mg, targeting gingipains and *P. gingivalis*	*N* = 64	None	10 days, no SAEs, high CNS penetration confirmed	NCT04920903c

[a]Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; ADAS-cog, Alzheimer's Disease Assessment Scale-Cognitive subscale; ADCS-ADL, Alzheimer's Disease Cooperative Study Group-Activities of Daily Living; BID, twice per day; CAMCOG, Cambridge Cognitive Examination for the Elderly; CNS, central nervous system; CSF, cerebrospinal fluid; FRSSD, Functional Rating Scale for Symptoms of Dementia; HSV, herpes simplex virus; IgA, immunoglobulin A; IgG, immunoglobulin G; MMSE, Mini-Mental State Examination; PCR, polymerase chain reaction; QID, four times per day; SADAS-cog, Standardized Alzheimer's Disease Assessment Scale cognitive subscale; SAE, serious adverse event; TID, three times per day.
[b]https://clinicaltrials.gov/ct2/show/NCT03823404
[c]https://www.clinicaltrials.gov/ct2/show/NCT04920903?term=COR588&draw=2&rank=1

to nucleic acid-based techniques (reviewed in Franco-Duarte et al.;[104] direct culture and metabolomics are discussed in section 5), which broad (deep sequencing, metagenomics) and narrow (polymerase chain reaction [PCR]) pathogen-detection techniques will reveal the majority of microorganisms present? (iv) With the ability to gather comprehensive sequence data, what are the best bioinformatic methods for analysis and interpretation?

The current project therefore has three primary objectives.

Objective 1. To determine the best method(s) to characterize the microbiome in tissue samples.
Objective 2. To determine which peripheral tissues are most representative of brain infection.
Objective 3. Combining the first two objectives, to establish a consensus protocol for clinical diagnosis of potential central nervous system (CNS) infections.

There is a clear need for an international, multidisciplinary collaborative study to address multiple issues before the analysis of peripheral biosamples, including blood and saliva, as well as brain-associated specimens such as CSF and olfactory neuroepithelium, could be adopted as a legitimate prospective strategy to understand, and potentially treat, neurodegenerative disorders. Next-generation sequencing (NGS)–based methods have enormous potential to detect pathogens: "NGS represents the next step in an increasingly unbiased approach to diagnosing neurological infections, and it is one of the most exciting translational applications of the genomics revolution for neurologists."[105] However, there are nuances to be considered related to the testing itself and specific features of microorganisms that may evade detection.

This article also aims to reframe our exploration of the MCI/AD microbiome by highlighting methods already being used by infectious disease specialists and medical microbiologists to assess and treat patients with neurological infections that lead to meningitis or encephalitis, as well as in pediatric acute-onset neuropsychiatric syndrome (PANS) and pediatric autoimmune neuropsychiatric disorder associated with streptococcal infections (PANDAS), among others. Although these clinical entities are distinct from MCI/AD, if there is microbial involvement in a subset of neurodegenerative disorders/dementias, it is logical that this should guide treatment. Data from the proposed *post mortem* explorations will enable precise testing and interventions in pilot studies on prospective cohorts. Ultimately, this may provide a model for assessing biosamples in idiopathic neurologic/psychiatric disorders beyond MCI/AD (see Discussion).

3 | PRIORITY ISSUES TO BE ADDRESSED

3.1 | Biosamples for analysis

Of all biosamples that could be secured from individuals living with MCI or AD, CSF may be best for evaluating the resident nervous system microbiome/pathobiome on a per individual basis. CSF is produced primarily by ependymal cells of the choroid plexus in the lateral, third, and fourth ventricles of the brain, from where it flows through the foramen magendie into the subarachnoid space around the spinal cord (although this "unidirectional flow" model is perhaps oversimplified).[106] Because the key limbic brain regions showing early involvement in AD (e.g., hippocampus, cingulate cortex, entorhinal cortex, and hypothalamus) are periventricular in location, a proportion of microbes infecting these brain regions (or the products of these microbes) may be shed into CSF. In addition, the circumventricular organs within the third and fourth ventricles harbor fenestrated capillaries through which pathogens and pathogen-infected immune cells could pass freely.[107,108]

An innovative CSF study by Wilson et al.[109] is informative. They studied a small number of patients with chronic cerebral meningitis of unknown etiology and performed deep sequencing for microbial species in CSF. This study identified some unexpected pathogens such as *T. solium* (pork tapeworm) and *Cryptococcus*, *Histoplasma*, and *Candida* fungal spp.[109] A brain origin was inferred because remedial treatment in most cases brought clinical remission of meningitis. A follow-up report studied a much larger number of patients with likely brain infections, and reached similar conclusions.[110]

3.1.1 | CSF for the investigation of the brain microbiome

CSF sampling is a semi-invasive procedure that involves local anesthesia and lumbar puncture, as well as patient approval. Even so, CSF samples are routinely drawn in clinical centers around the world, particularly in cases of CNS disease of suspected infectious origin, and these are generally inspected by PCR techniques and/or culturing of microorganisms in vitro. Because MCI often shows progression to overt dementia (≈10% per year[111]), CSF sampling does not constitute an insurmountable hurdle if it can guide clinical management. However, potential issues have been highlighted that complicate CSF analysis.[105] First, CSF can be difficult for deep sequencing studies because of its low biomass. This means that precautions must be taken not only in the sampling procedure but also in interpreting the results because low-level sample contamination with environmental microbes might begin to dominate the microbiome.[112] Second, some pathogens may be below the level of detection. The case of West Nile virus has been cited, for which detectable virus may only be present in CSF for a few days after infection,[105] and HSV1 is only detectable in CSF for 1 to 2 weeks after herpes encephalitis. In children with neurological complications associated with adenovirus infection, the virus was detected in respiratory or gastrointestinal samples in 85% of cases, but not in CSF.[113] Third, transient microbial detection in acute infection may not apply directly to putative chronic microbial brain infection, and intermittent shedding may pose a comparable sensitivity problem in the chronic situation. These sampling concerns may need to be addressed by methods that permit pathogen concentration from relatively large volumes.

3.1.2 | Olfactory mucosa and neuroepithelium

The olfactory mucosa comprises a specialized neuroepithelium and an underlying lamina propria that blankets the cribriform plate, dorsal septum, and sectors of the superior and middle turbinates of the upper airway.[114] Nasal brush sampling, as well as biopsies of this mucosa, are relatively non-invasive procedures that have potential to provide key information regarding microbes that may be present in the early stages of AD development. Such samples can be easily obtained from non-sedated subjects in a clinical setting,[115] including vulnerable individuals.[116] The rationale for studying such samples includes the fact that the olfactory mucosa is uniquely exposed to the vagaries of the outside environment, including viral infections, and evidence that smell loss is an early "preclinical" sign of AD and many other neurodegenerative diseases;[117] indeed, olfactory deficits are a common early sign of overall-cause dementia.[118,119] Moreover, the olfactory system is a major entry route of viruses, microbes, and other xenobiotics into regions of the CNS associated with AD-related pathology, including the hippocampus and the piriform and entorhinal cortices.[120,121] Nasal brushings are currently used in the diagnosis of Creutzfeldt–Jakob disease[122] and, along with olfactory mucosal biopsies, are useful in identifying exposures to a range of viruses including SARS-CoV-2.[123]

Routes of viral brain invasion include the olfactory receptor cell neurons that collectively comprise cranial nerve I (CN I), extraneural spaces within the nerve bundles that make up this cranial nerve, and lymphatic channels.[124] Although some viruses and xenobiotics can gain entry to the brain via several cranial nerves, CN I is uniquely vulnerable to such penetration because it comprises 6 to 10 million bipolar receptor cells whose dendritic knobs and protruding cilia provide an exposed surface area conservatively estimated at 23 cm^2. Unlike many other neurons, CN I projects its axons directly into the brain without an intervening synapse, receiving little protection from the blood–brain barrier. However, some protection is afforded by secretions from Bowman's glands and neighboring supporting cells that express a wide range of chemical-metabolizing enzymes, as well as from intracellular detoxification factors, ligand-specific binding proteins that clear agents from the mucosa, immune system cells, and the ability of the receptor cells to degenerate and then promptly regenerate from stem cells within the basement membrane.[125]

Although olfactory sampling may offer a route for the detection of some pathogens, many microbes naturally populate the olfactory mucosa, and discerning CNS from olfactory infection may be challenging. Nevertheless, the accessibility of this anatomical region has significant potential as a diagnostic window.

3.1.3 | Other peripheral biosamples for evaluation of the brain microbiome

Despite the obvious focus on CSF and olfactory neuroepithelium in view of their physical contact with the CNS, it has not been formally demonstrated that either provides an accurate and sensitive picture of microbes present in AD brains, even for circumventricular organs. It is possible that blood and potentially other biosamples close to the CNS (e.g., oral samples) might provide a comparable, complementary (or possibly better) representation of brain microbes. For example, viruses HSV1, human herpesvirus (HHV)6/HHV7, HHV8, varicella-zoster virus, and adenovirus, as well as diverse bacterial pathogens, can be found in saliva,[126–131] and the prominent periodontal pathogen, *P. gingivalis*, has been associated with AD development.[11,132] Furthermore, because all tissues including lungs share a bloodstream with the brain, we recommend comparative investigations using multiple tissues to determine which tissues outside the brain are the most informative for clinical use.

3.2 | Sequence-based versus other approaches

An optimal method for pathogen detection that adequately considers the benefits and limitations of all potential identification methods remains elusive. In choosing to follow a sequence-based approach we are conscious that there is no "one size fits all" solution to the problem of microbe detection. For example, in a pioneering study of CSF from meningitis/encephalitis patients, 19 (33%) of 58 infections were diagnosed by both conventional testing and metagenomics, 26 (45%) by conventional testing only, and 13 (22%) by metagenomics only.[110] In a study of synovial fluid in joint infection, NGS detected 15 pathogens in 10 of 10 culture-negative samples, but seven pathogens identified by culture were not detected by sequencing.[133] However, the use of terms such as "metagenomics" and "NGS" have been used indiscriminately to cover both DNA- and RNA-based techniques, complicating interpretation. Moreover, "conventional testing" has sometimes been widened from direct culture and serology to also include PCR-based methods, and caution is urged in comparisons of "conventional" versus sequence-based technologies. Nevertheless, there is agreement that PCR-based amplification can substantially improve detection rates, although clinical experience shows that nucleic acid–based detection methods sometimes fail (discussed later). The reason for such failures is unknown, and could relate to difficulties in extracting nucleic acid from lysis-resistant microbes.

With regard to "conventional" techniques (discussed later), we underline the necessity of direct culture as an adjunct to nucleic acid–based techniques, and we remain alert to potential methodological advances in the field, notably in culture from CSF or other brain-related tissues. Moreover, although the focus here is on molecular techniques, in the longer term we anticipate that—if key organisms are identified that are likely to play a causal role in the development of AD and related disorders—this will inevitably prompt the commercial development of rapid miniaturized detection methods based on a diverse array of technologies, as we have already seen for COVID (discussed in section 5.4).

3.3 | Biosample extraction protocol

When a promising tissue or fluid has been identified, and biosamples have been obtained, there is uncertainty about how samples should be

extracted for nucleic acid analysis. Until now laboratories have used their individual protocols to obtain data that are difficult to compare from one laboratory to another. The initial nucleic acid extractions are crucial because we need to ensure that we can obtain the inclusive identification of all foreign nucleic acids in human brain and tissue samples. In particular, there are many cases in which microbes, particularly intracellular bacteria and fungi, are recalcitrant to conventional (e.g., protease/lipase/detergent/phenol) extraction techniques (e.g., Moen et al.[134]), and the use of an inappropriate extraction system may miss key microbial species. We, therefore, recommend side-by-side comparison of different extraction methodologies to identify the optimum protocol for these species. In so doing, we should also consider designs that extract DNA and RNA together as well as separate extractions of DNA and RNA.

There are three primary methods for extracting nucleic acids from microbial populations: mechanical, enzymatic, and chemical denaturation with detergents and chaotropic (hydrogen bond-disrupting) agents such as urea and guanidinium, among others. For mechanical disruption techniques, freeze–thawing, bead-beating, grinding, and sonication are principally used to achieve cell lysis (e.g., Fykse et al.,[135] Leuko et al.,[136] Zhang et al.,[137] and Rodríguez and Vaneechoutte[138]). Enzymatic techniques typically try to achieve cell lysis through hydrolysis of cell wall and cell membrane components (reviewed in Salazar and Asenjo[139]). Further cell lysis may be accomplished using chemical detergents for lipid membrane solubilization and strong chaotropic agents for denaturation of cell membranes containing transmembrane proteins (e.g., de Bruin et al.[140]).

After the initial extraction, nucleic acid purification is achieved by washing with organic solutions and detergents, by precipitation through alcohols, and by filtration/chromatography using different types of resins (reviewed in Lever et al.[141]). However, there is no accepted universal extraction kit or technique that works best for all sample types and research goals. Microbes are very variable and diverse, thus necessitating the use of techniques that take into account their specific characteristics. Systematic fine-tuning is difficult because commercially available extraction kits are designed in a proprietary manner. Thus, extraction optimization must cope with variations in both the unique properties of potential microbes as well as sample types. Multiple approaches should be used for the extraction of microbial nucleic acids from the many tissues and fluids that could be evaluated, including *post mortem* brain tissues, CSF, olfactory neuroepithelia, blood, and saliva. In this regard, Lever et al. provide an extensive analysis of extraction protocols[141] that could be applied to establish a consensus technical procedure for infection analysis in MCI and AD. Their protocols were evaluated for microbial analysis from different environmental soil sediments, but they could be adapted to human tissue analysis. It was previously recommended that multiple non-orthologous technologies should be used for microbial lysis to increase the likelihood of capturing all organisms present;[142] nevertheless, this would involve sample splitting and repooling. At a minimum, any extraction protocol used as part of an unbiased analytical approach should contain mechanical, chemical, and enzymatic lytic procedures to maximize recovery of all pathogen classes.

3.4 | Nucleic acid analysis and species identification

Techniques used to characterize the microbiome in clinical samples are generally based either on microbial DNA (already present in the sample) or on cDNA generated by reverse transcription of microbial RNA, and normally use two key techniques: ribosomal RNA (rRNA) or rDNA analysis, and metagenomics (reviewed in Quince et al.,[143] Knight et al.,[144] Osman et al.,[145] Breitwieser et al.,[146] Fricker et al.,[119] Bharti and Grimm,[120] Gao et al.,[121] and Yen and Johnson[147]). However, as noted earlier, there is a widespread tendency to use terms such as "metagenomics" and "NGS" to cover a multitude of methods, and both have been used to cover both DNA-based (unamplified) and deep sequencing of limited-cycle PCR-amplified DNA and RNA-derived cDNA fragments. In the following we use "deep sequencing" to cover random ("shotgun") sequencing either of DNA (DNA-seq) or RNA-derived cDNA (RNA-seq), whereas we restrict "metagenomics" to DNA-seq followed by direct genomic sequence assembly. We discuss these different approaches below.

3.4.1 | DNA-based metagenomics

This technique uses deep sequencing of DNA from tissue samples, removal of human sequences, and assembly of long contiguous DNA sequences (contigs). DNA is far more stable than RNA, and is more likely to resist harsh extraction methods. However, classical metagenomics based on the assembly of genomic sequences requires long-read and expensive sequencing, access to top-level computational facilities, and generates very large amounts of data (often multi-terabyte [TB] volumes; 1 TB = 10^{12} bytes or 10^6 MB). Metagenomic sequencing is useful when all DNAs are present at relatively similar molar levels—this extends to DNAs that differ by up to three to four orders of magnitude difference, in other words 1000- to 10,000-fold differences in starting concentrations. Unfortunately, when sequencing human specimens with a sparse microbiome, even the most highly prevalent microbes will be present at less than 0.01% of the total DNA and the rarer microbes may be present at <0.00001%. Thus, to obtain a single DNA read of the rare microbes would require sequencing more than 10 million reads. Moreover, metagenomics does not easily address differential abundance, and machine-learning tools are essential to unravel the true extent of the microbiome.[148] Nevertheless, accuracy can be very high because large contigs are generated that can precisely identify species. However, microbial DNA is present at one to two copies per cell, and is hence much less abundant than, for example, rRNA.

One alternative is to focus on direct sequencing of multicopy DNA sequences such as rDNA, and in particular on the variable internal and external transcribed spacers (ITS and ETS, respectively). However, the number of rDNA copies per genome can be variable: some microbes may only have one or two rDNA copies, whereas others have hundreds, introducing a significant bias in favor of multicopy rDNA organisms. In addition, given high costs and large data demands, it will be necessary to evaluate whether metagenomics is competitive with other

techniques such as deep sequencing of RNA (RNA-seq) for clinical use.

3.4.2 | DNA-based diagnostic PCR

PCR analysis, the conventional clinical/diagnostic method, involves direct amplification from raw extracted DNA. Selected primers corresponding to specific microbes can be used to determine, using PCR alone (typically using real-time or quantitative PCR using fluorescent tracers), whether a microbe is present or absent. PCR remains the gold standard for many routine clinical diagnostic laboratories, and has advantages in terms of cost. However, clinical diagnostic PCR is limited in that it only inspects a small predetermined subgroup of microbes (typically 20–50). Moreover, variants of those microbes may evade detection if their genomic sequences depart from the standard sequence upon which the PCR primers were based.

3.4.3 | DNA-based rDNA amplification

Diverse methods are available for rDNA amplification, all based on conserved sequences. Ehrlich and other researchers have devised a pandomain assay for specifically amplifying and sequencing rDNAs (16S for prokaryotes; and 18S/28S for eukaryotic microbes) and have demonstrated that the strategy is broadly inclusive, highly sensitive, and species-specific.[53,142,149,150] The general method for 16S pandomain analyses reported by Earl et al.[142] was able to accurately characterize highly complex polymicrobial specimens containing hundreds of different species present at molar input levels that vary by more than three orders of magnitude. In addition, all microbes detected could be speciated in the presence of hundreds of other microbes—even those that are present at very low molar concentrations relative to others. Greathouse et al.[149] demonstrated that sparse microbiomes present in human tissues (mainly the lung) can be characterized to the species level for all members in the presence of a vast excess of human DNA. Finally, Moné et al.[53] reported that the 16S pandomain assay can both identify and speciate all the bacteria found in human brain tissue from both AD patient and age-matched controls. Importantly, differences in the microbiomes of the AD patients and controls were reported, providing further confirmatory data for hypotheses regarding the role of pathogens as etiological agents of AD. The 18S/28S assay serves as a *trans*-pan-domain assay for eukaryotic microbes from fungi and apicomplexan parasites to alveolates. This assay is currently under validation using a large library of pathogens representative of the domains of interest and those implicated in AD (G. Ehrlich, unpublished).

3.4.4 | RNA-based PCR

Reverse transcription (RT) of total cellular RNA into cDNA provides a new substrate for PCR amplification. If primers corresponding to rRNA are used, this method can take advantage of the increased copy numbers associated with rRNA—which can be present in several thousand copies per microbial cell (reviewed in Hu et al.[54]). The method is thus likely to be more sensitive than DNA-based PCR. In support, it was reported that RNA-seq generated 100-fold more microbial "reads" than DNA-seq.[151] Amplification of 16S rRNA sequences has been widely used to characterize bacterial sequences in the human brain.[48,51] However, analysis based on 16S RNA alone omits non-bacterial species (particularly fungi and viruses), and also risks missing key bacterial species that do not precisely match the primer sequences.

3.4.5 | RNA-seq with sequence assembly

Deep sequencing of RNA (RNA-seq) from tissue samples is increasingly available and cost effective. (Sequencing of both strands, which some technologies recommend, is not advocated because it can significantly increase computation time.) In addition, RNA-seq is the only valid approach for RNA viruses, and even DNA viruses make far more transcripts per cell than they make genomes. After an RT step, designer primers are added, limited-cycle PCR is performed, and the material is subjected to deep sequencing. Long reads (500 nt or more) simplify contig assembly but are more expensive and can sometimes be unobtainable if the biosample is partially degraded. Moreover, direct sequence assembly from RNA-seq data faces considerable bioinformatic challenges because of the large excess of human material. Nevertheless, Ramachandran and Wilson emphasize the utility of deep sequencing of CSF in developing "classifiers" (e.g., bacterial vs. viral) to guide treatment.[105] For example, in the real-life cases of patients with meningitis of unknown etiology, 7 of 7 patient CSF microbiomes were sufficiently precise to guide antimicrobial therapy.

3.4.6 | RNA-seq with *k*-mer or eToL methods

k-mer and electronic tree of life (eToL) methods involve probing RNA-seq datasets with shorter probes (30–64 nt) and thus can take advantage of short read lengths (e.g., 100–200 nt), and are therefore substantially less expensive, and moreover are also more reliable when dealing with partially degraded RNA in biosamples. Nevertheless, computational refinements are necessary to exclude human sequences. One rapid analytical technique is to use bioinformatic methods based on short *k*-mers (generally 31-mers) (e.g., Kraken,[152] CLARK,[153] Jellyfish,[154] KrakenUniq,[155] PathSeq,[156] and others) that are specific for particular pathogens, including viruses. The use of short *k*-mers coupled to bioinformatics is a powerful approach that has been widely used for pathogen detection in RNA-seq data.

However, *k*-mer methods tend to be somewhat imprecise at times because the numbers of matches are simply counted to calculate microbe abundance, and this risks generating false positives caused by serendipitous matching to human sequences.[157] Even a single nucleotide difference can lead to a match or non-match. Current databases do not yet encompass the full diversity of the human

genome and transcriptome: each individual typically harbors 4 to 5 million single-nucleotide variants (https://medlineplus.gov/genetics/understanding/genomicresearch/snp/) that are not well characterized. In addition, current sequencing techniques have a significant error rate (typically ≤1%): an abundant cellular sequence (such as rRNA) therefore risks generating multiple "non-human" 31-mers that could potentially be counted as "matches" to microbial sequences.

A recently reported development is the use of longer probes: the eToL method uses a net of much longer (64-mer) probes, pre-filtered against human sequences, to identify (by homology; non-exact matches are detected) all non-human rRNA sequences in a given dataset. The method may be less prone to false positives because it refilters matches against human sequences and is relatively insensitive to single-nucleotide changes,[54,55] and is being explored as a potential diagnostic method on patient samples.

4 | CONSENSUS PROTOCOL DEVELOPMENT: THE ALZHEIMER'S PATHOBIOME INITIATIVE

The different steps in the development of a consensus protocol are summarized in the flowchart in Figure 3. In the following we expand upon the different operational steps.

4.1 | Nucleic acid extraction

The first priority will be to take a single tissue and apply several different commercially available and home-grown extraction protocols to determine (1) what is the best method for "recalcitrant" organisms, and (2) whether aggressive extraction methods (that are perhaps necessary for particular organisms) have a downside in terms of nucleic acid degradation, thus compromising sensitivity for organisms that are more easily ruptured and extracted. Different procedures will be evaluated in parallel from the same tissue(s) (Table 2) to determine which is the most effective for specific organism types. A compromise protocol may need to balance the two factors (aggressive extraction vs. nucleic acid degradation).

In the first instance, buffy coats (total blood leukocytes) may be the ex vivo sample of choice because many of the recalcitrant organisms reside in nucleated immune cells. Ideally, attempts should first be made to secure biosamples from individuals already known to harbor chronic or acute infections with key species.

4.2 | Multisampling and comparison of biosamples

A further priority will be to determine which peripheral biosamples are best in terms of (1) representation of the brain microbiome, (2) ease of sampling, and (3) sensitivity. To this end, it will be necessary to determine how the composition and load of pathogens in peripheral samples, including CSF, compares to brain from the same individual when considering *post mortem* samples. If infection(s) can be detected without exposing patients to lumbar puncture (more costly and invasive than routine bloodwork), this would be very useful information. Blood, olfactory neuroepithelium, sputum/saliva, oropharyngeal tissue, bronchoalveolar lavage, urine, and gut/stool specimens all represent feasible biosamples to compare to *post mortem* brains and CSF when analyzing the microbiome/pathobiome in individuals

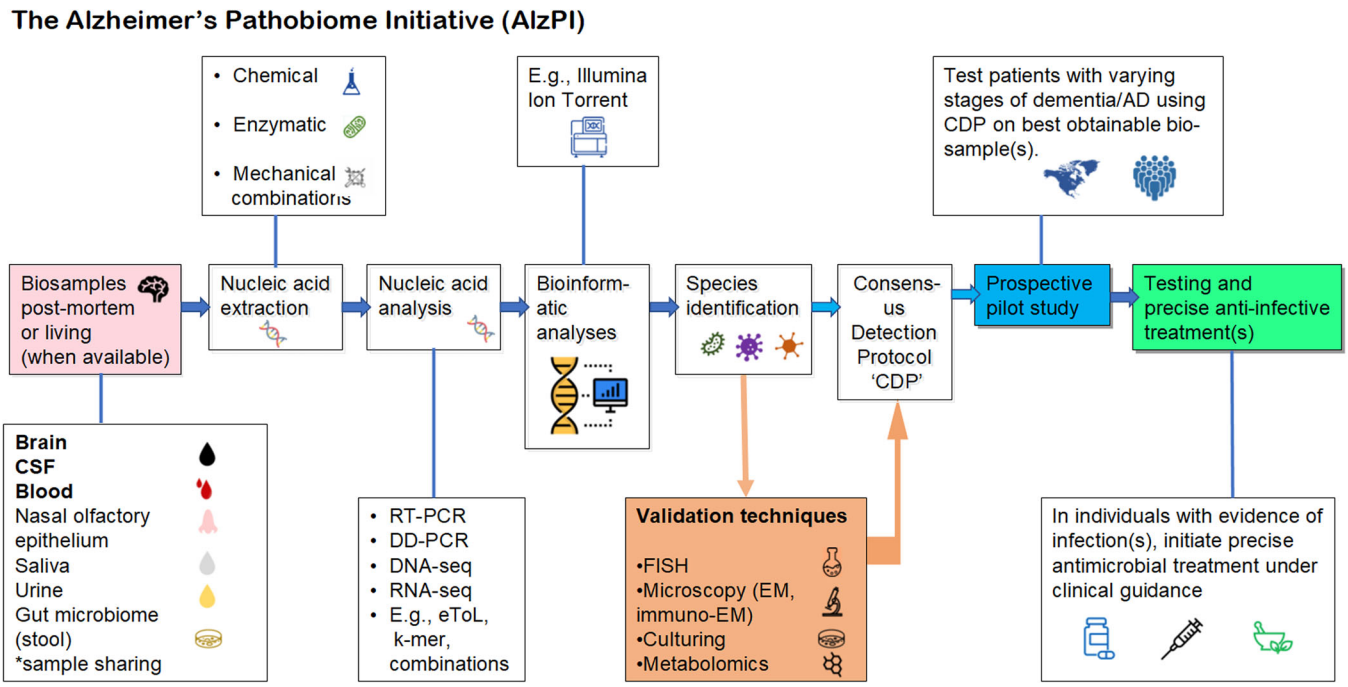

FIGURE 3 Flow chart of the Alzheimer's Pathobiome Initiative. AD, Alzheimer's disease; CSF, cerebrospinal fluid; DD, digital droplet; EM, electron microscopy; eToL, electronic tree of life; FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction; RT, reverse transcription.

TABLE 2 Experimental procedures.

1. Extraction protocol				
Tissue	**Extraction protocol**	**Evaluation 1**	**Evaluation 2**	**Evaluation 3**
(to be determined)	Buffer/treatment A Buffer/treatment B Buffer/treatment C Buffer/treatment D Buffer/treatment E	Total DNA and total RNA by Nanodrop and/or Qubit spectrophotometers and fluorometers Quality of nucleic acid by Agilent BioAnalyzer, Tape Station, or similar	Metagenomics from total DNA[a] Microbiome analyses using long-read, pan-domain rDNA-based assays for prokaryotes and eukaryotes[b]	RNA-seq + eToL and/or *k*-mer[a]
Outcome: identification of consensus protocol.				
2. Tissue selection				
Tissue	**Extraction protocol**	**Evaluation 1**	**Evaluation 2**	**Evaluation 2**
Source A: post mortem individual with known meningitis/encephalitis				
Source B: post mortem individual with confirmed Alzheimer's disease (AD)				
Source C: post mortem individual confirmed without AD (i.e., control unaffected)				
Post mortem brain AMYG Post mortem brain BA24 Post mortem CP Post mortem ERC Post mortem brain HPC Post mortem brain HYPO Post mortem brain LC Post mortem brain LT Post mortem brain PFC Post mortem TL CSF Olfactory neuroepithelium Sputum/oropharyngeal Bronchoalveolar Blood Gut/stool Urine	Consensus	Total DNA and total RNA by Nanodrop and/or Qubit spectrophotometers and fluorometers. Quality of NA by Agilent BioAnalyzer, Tape Station, or similar	Metagenomics from total DNA[a] Microbiome analyses using long-read, pan-domain rDNA-based assays for prokaryotes and eukaryotes[b] PCR according to current clinical guidelines for suspected brain infection	RNA-seq + eToL and/or *k*-mer[a]

Abbreviations: AD, Alzheimer's disease; AMYG, amygdala; BA24, cingulate cortex; CP, choroid plexus; CSF, cerebrospinal fluid; EMBO, European Molecular Biology Organization; ERC, entorhinal cortex; HPC, hippocampus; HYPO, hypothalamus; LC, locus ceruleus; LT, limbic thalamus; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; PFC, prefrontal cortex; TL, temporal lobe.

[a]Sequence libraries to be filed online (NCBI) and different research groups invited to apply their technologies.

[b]Species-specific microbiome data to be filed online (NCBI and/or EMBO).

Outcome: identification of which tissue outside the brain is the best indicator of brain microbe burden.

with varying degrees of cognitive impairment. However, given the vast diversity of microorganisms and their potential locations within the brain, it may be that specific biosamples may be more appropriate for detecting each particular microbe including sensitive multiplex serology assays.

In this work, a key challenge will be to identify clinician researchers who routinely take *post mortem* brain samples (including from individuals with known brain infections), and who are willing to expand their scope to other biosamples from the same *post mortem* individual. In addition, there is a need to establish a reference collection of identical anonymized samples that can be supplied to different researchers for analysis. Our recommendation is that a small number of centers should be tasked with preparing larger samples (e.g., fresh homogenized brain, pooled CSF, blood/buffy coat, other) under sterile conditions (recommendations will be needed for the most appropriate protocol for achieving this) and aliquoting these into at least 100 identical ampoules for storage at −196°C (liquid nitrogen) and distribution to researchers worldwide. This will ensure that we maximize what we learn from these precious and scarce samples generously donated by patients and their families. Some relevant biobanks have already been established

(e.g., NeuroBioBank: https://neurobiobank.nih.gov/) and may be able to contribute to this project.

4.3 | Mapping the brain microbiome

A further issue raised in this work is that the spectrum of microorganisms in the brain may differ according to brain region, as well as as a function of disease status. Efforts will be made to secure samples of multiple brain regions from different individuals, both controls and individuals with AD and related disorders, to determine whether different brain regions have distinct microbiomes.

4.4 | Nucleic acid sequencing of reference samples

For reasons discussed earlier, DNA-based nucleic acid analyses are unlikely to match the sensitivity of RNA-based methods because DNA methods cannot take advantage of the large amplification factor introduced by analysis of highly abundant RNAs such as rRNA. We advocate basing the initial analysis on deep sequencing applied to cDNA generated by RT of total RNA isolated from clinical specimens. Overall, we believe that RNA-based methods and PCR-based methods including whole-locus rDNA amplification from both prokaryotes and eukaryotes are likely to be the most sensitive. Furthermore, it is unavoidable that only RNA-seq–based methods can detect both cellular and viral pathogens in a single screen. Nevertheless, we recommend side-by-side comparison of different methodologies.

For both DNA-seq and RNA-seq, although the use of designer adaptors and limited-cycle PCR is now conventional for the generation of representative libraries, choices must be made in (1) the sequencing methodology applied, including (2) long- versus short-read sequencing. The most popular methods in use today (reviewed in Reuter et al.[158] include the Illumina (http://www.illumina.com/), Ion Torrent (http://www.iontorrent.com/), 454 (http://www.454.com/), Pacific Biosciences (www.pacificbiosciences.com/), and Oxford Nanopore (https://nanoporetech.com/) platforms. Many of these companies offer commercial deep sequencing of biosamples (Table S1 in supporting information).

Our recommendation is that anonymized nucleic acid and/or tissue samples should be sent out to a selection of key commercial companies for deep sequencing, and that the data generated should be presented as sequence read archives (SRAs) and uploaded to the National Center for Biotechnology Information (NCBI) SRA repository (https://www.ncbi.nlm.nih.gov/sra) for open access by researchers worldwide. In parallel, the same samples should be submitted to different clinical diagnostic laboratories, and the results also filed at an appropriate data repository.

4.5 | Bioinformatic analysis

The DNA-seq, RNA-seq, and rDNA data generated from different tissues, using different methods, and filed at NCBI, will be made available for different teams to analyze using their respective methodologies, including recent advances in artificial intelligence. A key objective will therefore be to invite key researchers worldwide to analyze the sequence datasets in different ways and upload their results to an appropriate data repository.

Machine learning tools will allow the construction of models that incorporate multiple variables (microbial profile, detection method, brain region, gender, ethnicity, genomic data, geographic location) to guide clinical management. The database may also provide a reference atlas for other brain diseases.

4.6 | Establishment of a consensus protocol

When all the data have been collected, a panel of expert researchers from across the consortium will be tasked with assessing the following:

1. Which tissues are the most representative and informative regarding the brain microbiome, and which types of microbes are detected using the various biosamples.
2. Which extraction method or combination of methods is best.
3. Which sequencing technique is most appropriate in terms of sensitivity, accuracy, and cost.
4. Which bioinformatic method is best in terms of comprehensive coverage, computation time/cost, low level of false positives, and other relevant factors.

These assessments will enable the establishment of a consensus workflow for clinical sample testing that is simple and reproducible in the laboratory, inexpensive, effective, and suitable for widespread dissemination.

4.7 | Technical developments

There is a clear need to anticipate technical advances. For example, the focus here has been on short-read sequencing that is less expensive than long-read sequencing, but the price of long-read sequencing will certainly fall with time. In addition, miniaturized and portable sequencing devices are under development (e.g., by Oxford Nanopore) that would be useful in every clinic. Furthermore, the field of bioinformatic analysis of sequence data is constantly developing. For these reasons, the design of a consensus protocol for tissue selection, workup, sequencing, and microbial species identification should be open-ended.

4.8 | Pilot clinical study

The final step in the proposed program is to apply the consensus protocol to a series of control, MCI, and AD individuals (number to be determined, Table S2 in supporting information)—for whom detailed cognitive assessments are available—and to determine whether there is a correlation between cognitive status and microbiome profile. Our target is to enroll at least 10 different clinical centers to contribute

to sampling, sequencing, and bioinformatic analysis. The results of the microbiome analysis will be supplied to the clinicians engaged (see below) and will also be posted online in anonymized form.

5 | CONFIRMATION BY DIRECT CULTURE AND OTHER TECHNIQUES, AND IMPLICATIONS FOR CLINICAL DIAGNOSTICS

The overarching objective of this work is to provide a roadmap to determine whether microbial infection might contribute to neurodegenerative disorders such as AD, and to provide a consensus protocol for microbe detection. If the data support such involvement, this opens up the need for independent confirmation as well as consideration of clinical diagnostic protocols. We examine these below.

5.1 | Direct culture

In vitro culture is an established method in clinical microbiology and diagnostics, but many microorganisms such as *Treponema* and *Mycobacterium* spp. are fastidious and refractory to culture.[159] Nucleic acid–based diagnostics for microorganisms, particularly those that use an in vitro amplification-based strategy, could be superior to culture-based methods in terms of sensitivity. In support, in children with possible sepsis, the rate of positive findings was higher with PCR (14.6%) than with blood culture (10.3%);[160] in chronic wound infections molecular testing detected a mean of 14.8 genera whereas aerobic culture detected only 1.8.[161] For polymicrobial infections, deep sequencing was found to outperform standard culture techniques.[162,163] In pulmonary infections the detection rate for bacterial or fungal infections was 95%, but 60% for direct culture.[164] In another study, nucleic acid–based techniques were positive in 68% to 83% of samples, whereas direct culture was only positive in 17%.[165]

There have also been many reports of nucleic acid–based detection where no culturable organisms could be detected by standard techniques.[166–171] In the study of Post et al. it was demonstrated that inoculated live bacteria could be detected by both PCR and culture but, once they were treated with antibiotics, bacteria were only demonstrable via PCR because they adopted a biofilm mode of growth.[166]

However, there is a need to demonstrate that molecular techniques are not detecting dead fragments. In middle-ear infections where sampling is difficult and culture challenging, Rayner et al. demonstrated in children that all culture-negative middle-ear effusions that were PCR positive for *H. influenzae* were also positive for *H. influenzae* mRNA.[167] This is an important finding because bacterial mRNAs are extremely labile (half-lives in minutes), furnishing *prima facie* evidence that live, metabolically active bacteria are present. In addition, PCR-positive but culture-negative middle-ear effusions recovered after antibiotic treatment contained metabolically active bacteria that were able to incorporate radioactively labeled amino acids into proteins synthesized de novo ex vivo.[172] Perhaps the most definitive study found that PCR-positive but culture-negative otitis media patients who failed to resolve their symptoms even after multiple rounds of antimicrobial therapy universally harbored robust bacterial biofilms, as demonstrated by confocal laser scanning microscopy of their middle-ear mucosa.[168]

Nevertheless, in view of skepticism in the field, nucleic acid–based detection methods demand independent confirmation through direct culture. For example, after the detection of *Chlamydia* species in some AD brain samples, Balin et al. found it imperative to confirm this finding by in vitro culture on monocytes,[58,59] followed by whole-genome sequencing of the in vitro isolates.[173] We anticipate that the major classes of microbe detected in this work will require independent validation by techniques such as direct culture from fresh patient samples.

5.2 | Metabolomics

There is growing recognition that microbes, notably fungi, produce atypical metabolites that may be detectable by mass spectrometry (MS)- and nuclear magnetic resonance (NMR)–based techniques ("metabolomics"),[174–176] and such techniques have been used to study CSF from pediatric patients with CNS infections[177] and AD patient samples.[178,179] However, it remains an open question whether metabolomic analysis of CSF and other samples might be sufficiently sensitive to detect potential CNS infections. Additional methodologies on the horizon include Raman spectroscopy[180] and CRISPR-Cas–based technologies.[181] Biosamples from the consortium will be made available to researchers to enable side-by-side comparison of nucleic acid- and metabolite-based analyses.

5.3 | Genotyping

These diagnostic assays can be combined with genotyping to investigate potential correlations among specific genes/alleles, microbial infection, and brain disease, and this approach is likely to increase our understanding of the interplay between multiple contributing factors and dementia development.

5.4 | Implications for clinical diagnostics

An optimal protocol for pathogen detection that adequately considers the benefits and limitations of all potential identification methods remains elusive. However, if organisms are detected that are suspected of potentially underlying AD and related disorders, the project will provide key information on (1) the identities of these organisms and (2) the most promising biosamples in which they can be found.

In addition, we anticipate that this will speed investigation of whether appropriate antimicrobial intervention can delay or remit disease progression.

If successful, we anticipate that this will prompt the development of new clinical diagnostic techniques that are simple, accurate, rapid, and cost effective, notably also for use in resource-poor settings.

We illustrate this through consideration of one such species, *Cryptococcus*, that has been associated with infection-related dementias (Table 1), although we recognize that there are multiple paradigms that could be informative in this context (e.g., neurosyphilis, HIV, among others).

Cases of suspected *Cryptococcus* CNS infection are routinely evaluated by neuroimaging. Subsequent CSF analysis with India ink is rapid and cheap, but rather unreliable. The current gold standard for cryptococcal brain infection is direct culture from CSF.[182,183] However, this is time-consuming (although this may not be a major consideration for slowly progressive diseases such as AD), and has prompted the development of rapid immunoassays (on CSF) for cryptococcus-specific antigen (CrAg), a major advance in the field.[184,185] Nucleic acid–based techniques have also been utilized such as BioFire FilmArray, a multiplex PCR assay for microbial meningitis/encephalitis.[186] This illustrates the potential of new discoveries to drive the development of rapid medical diagnostic tests specific for the target organism(s), which in the case of AD remain unknown.

Recent experience with the COVID-19 epidemic confirms this. Diverse tests developed specifically for SARS-CoV-2 include different types of RT-PCR, isothermal amplification, CRISPR-based assays, microarray hybridization, metagenomics, enzyme-linked immunosorbent assay testing for SARS-CoV-2 antigens, lateral flow immunoassays, and antibody tests including biosensors and luminescence-based assays.[187] Potentially, all these different technologies could be used to develop rapid tests for organisms suspected of contributing to AD and related diseases. However, this will require identification of the most common pathogens found in AD samples, both in post mortem brains and in clinical samples from patients.

6 | DISCUSSION AND CALL FOR COLLABORATION

This ambitious project (the Alzheimer's Pathobiome Initiative) will require multidisciplinary collaborations among neuropathologists, neurologists, specialists in medical diagnostics, microbiologists and molecular biologists, sequencing experts, and bioinformatics and artificial intelligence specialists. Brain banks that can contribute samples are invited to participate, and it is hoped that experts in PCR and deep sequencing (both RNA-seq and DNA-seq), as well as multiplexing/multiomics, will be able to offer their skills. Our recommendation is that sequencing data from brain bank and clinical samples should be filed at openly accessible data resources (e.g., NCBI) such that bioinformatics experts worldwide can apply their favorite analytical tools (including deep neural networks and artificial intelligence) to speed diagnostics and uncover higher-level patterns in the data.

Our principal objective is to determine whether microbiome analysis of peripheral samples can be validated as a means to accurately assess the identity and abundance of microbial species within the brain. A second aim will be to determine, in a pilot study, whether there is a correlation between microbiome identity/abundance and measures of cognition in normal control, MCI, and AD patients. Because some patient samples studied in this work are likely to correspond to individuals who are still alive, there will be a moral obligation to share any positive results (e.g., overabundance of particular microbial species consistent with infection) with the physicians who provided the samples. The decision about whether to treat, or not to treat, any infection detected would therefore be solely that of the clinicians and the patients involved. Conversely, clinicians would be encouraged to share their observations with the researchers with a view to joint publication of any emerging results.

Our ultimate objective is to devise a rapid, inexpensive, robust, and accurate method to determine the extent and nature of the brain microbiome in individual patients. There is also a need to devise protocols that can be applied in low-resource settings. For the future, we envisage an increasing need to extend our methods of analysis to wider neurological and neurodegenerative conditions that have also been associated with infection, including other dementias (e.g., vascular dementia), multiple sclerosis, amyotrophic lateral sclerosis (ALS), Parkinson's disease, epilepsy, schizophrenia, and major depressive disorder.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. Author disclosures are available in the Supporting Information.

CONSENT STATEMENT

This article involved no research on human subjects that would require consent. The work described has not been published previously, it is not under consideration for publication elsewhere, its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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