CLINICAL APPLICATION GUIDE

ALZHEIMER'S LINX™

ALZHEIMER'S-ASSOCIATED IMMUNE REACTIVITY

CYREX® Laboratories

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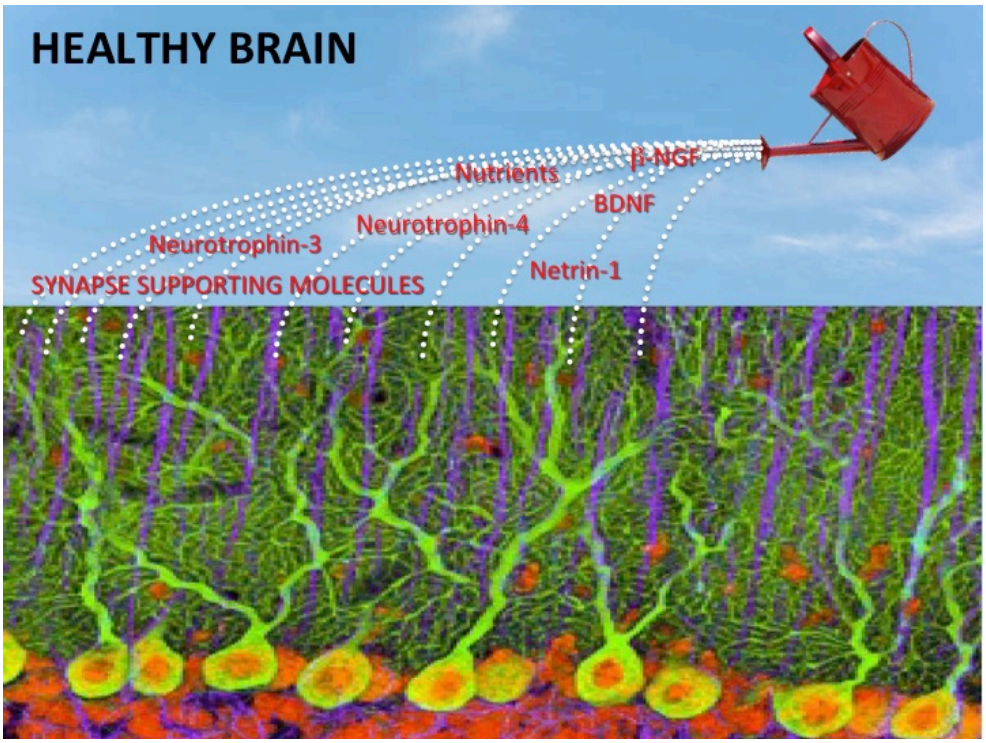
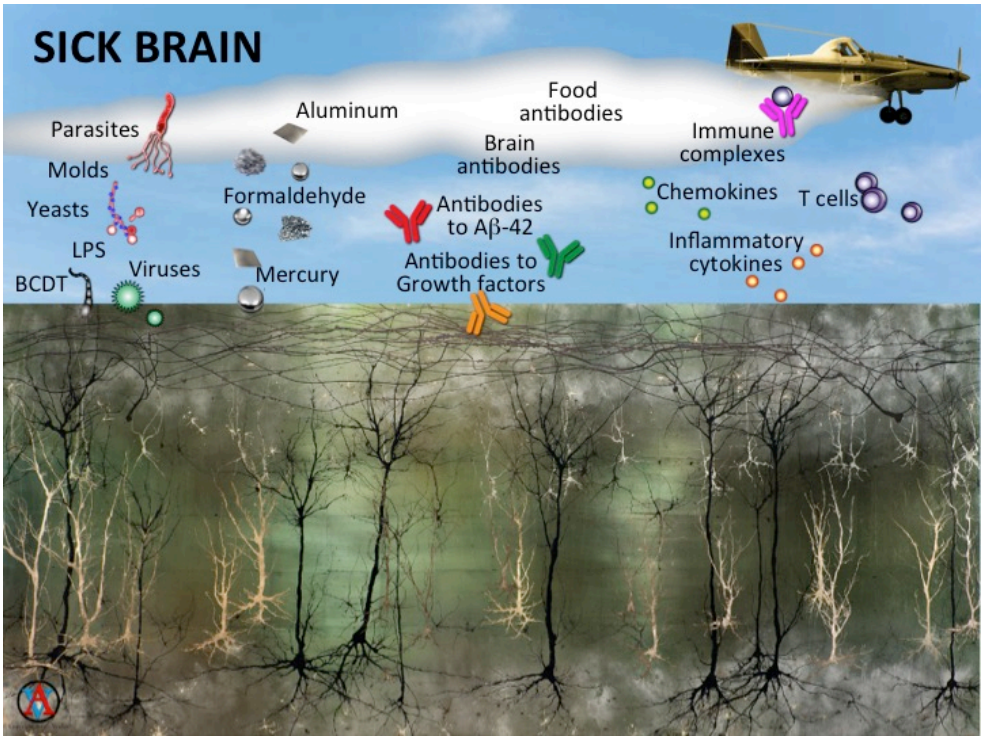




From healthy brain to sick brain. *A healthy brain is nourished and strengthened by the proper nutrients and supporting molecules. However, a barrage of environmental factors can turn the brain towards sickness and neurodegeneration, neuronal death and brain atrophy.*

CLINICAL APPLICATION GUIDE TO ALZHEIMER'S LINX™ ALZHEIMER'S-ASSOCIATED IMMUNE REACTIVITY

OVERVIEW

Neurons are the building blocks of the nervous system, which encompasses the brain, the spinal cord, and the enteric nerve network. Neurodegenerative disorders are a group of conditions that primarily affect the brain and cause a selective loss of neurons in the motor, sensory or cognitive systems. Alzheimer's disease, Parkinson's disease, Huntington's disease, Lewy body dementia, and amyotrophic lateral sclerosis are some examples of neurodegenerative disorders.

Dementia is a degenerative brain disease, in which damage to neurons in parts of the brain involved with cognitive function occurs. It is characterized by a decline in memory, language, problem-solving and other cognitive function of everyday life. The prevalence of dementia ranges from 6% to 10% in adults aged 65 and older; two-thirds of these cases are Alzheimer's disease (AD).[1] AD is characterized as the formation of amyloid plaque deposits in the brain.[2] First identified more than 100 years ago, 70 years passed before AD was recognized as the most common cause of dementia, as well as a major cause of death.[3] It is characterized by cognitive impairment, β-amyloid deposition, neurofibrillary tangle formation, neuroinflammation, neurodegeneration and neurocognitive decline. In AD, the destruction of neurons eventually affects other parts of the brain, which control basic bodily functions such as walking and swallowing. Round-the-clock care is required for people in the final stages of the disease, as they are bed-bound. Alzheimer's disease is ultimately fatal.

Statistically, deaths resulting from stroke, heart disease and prostate cancer decreased in the USA between 2000 and 2013 by 23%, 14% and 11% respectively, while deaths from AD increased 71%.[4]

Before AD takes its toll on the patient's life, there are other tolls to pay. In 2015, an estimated 18.1 billion hours of unpaid, family-member care was given to people with AD and other dementias, which equals about $221 billion.[4] No monetary value can be applied to the emotional cost of AD and other dementias. Paid services for health care, long-term care and hospice services for people aged ≥ 65 years with dementia are estimated at $236 billion in 2016.[4] Someone in the USA develops AD every 66 seconds, but by 2050, due to an aging baby-boomer population that is expected to increase to every 33 seconds, resulting in nearly 1 million new cases of AD per year.[4]

The monetary and emotional cost of AD and other dementias is skyrocketing. Thus, we need to gain control. When neurodegenerative diseases such as Alzheimer's or Parkinson's cause neurocognitive disorder, conditions often worsen as time progresses. It is therefore crucial to determine and deal with the underlying causes of these disorders at the earliest stages possible. Researchers believe that early detection of environmental factors that contribute to the pathogenesis of AD is the most crucial for developing interventional programs that will slow down or stop the progression of the disease.[4-7] Bredesen introduced one such program that included the following: optimization of diet and sleep, reduction of stress, exercise, brain stimulation, prebiotics, probiotics, hormone balance, antioxidants, vitamins, healthy gut, optimization of mitochondrial function, and avoidance of toxic metals.[8-9] This program of personalized protocols for metabolic enhancement showed reversal of cognitive decline in 9 out of 10 patients. These successes of

the Bredesen program show that lifestyle changes can indeed ameliorate and even reverse cognitive decline. Despite this, many people still believe that AD is solely a genetic disorder and therefore there is no solution. This may be true for early-onset AD, but one thing should be clarified: early onset Alzheimer's disease occurs between the ages of the 30s to mid-60s and accounts for much less than 10% of all individuals with AD [https://www.nia.nih.gov/health/alzheimers-disease-genetics-fact-sheet]. Most sufferers of AD have the late-onset form of the disease, which becomes fully active in the mid-60s and beyond. In contrast to early-onset AD, the causes of late-onset AD likely include a combination of genetics, environmental factors (including lifestyle factors), and the integrity of the protective blood-brain barrier (BBB) [https://www.nia.nih.gov/health/alzheimers-disease-genetics-fact-sheet]. This triad of genetic susceptibility, environmental triggers and BBB permeability in AD and other neurodegenerative disorders is shown in Figure 1.

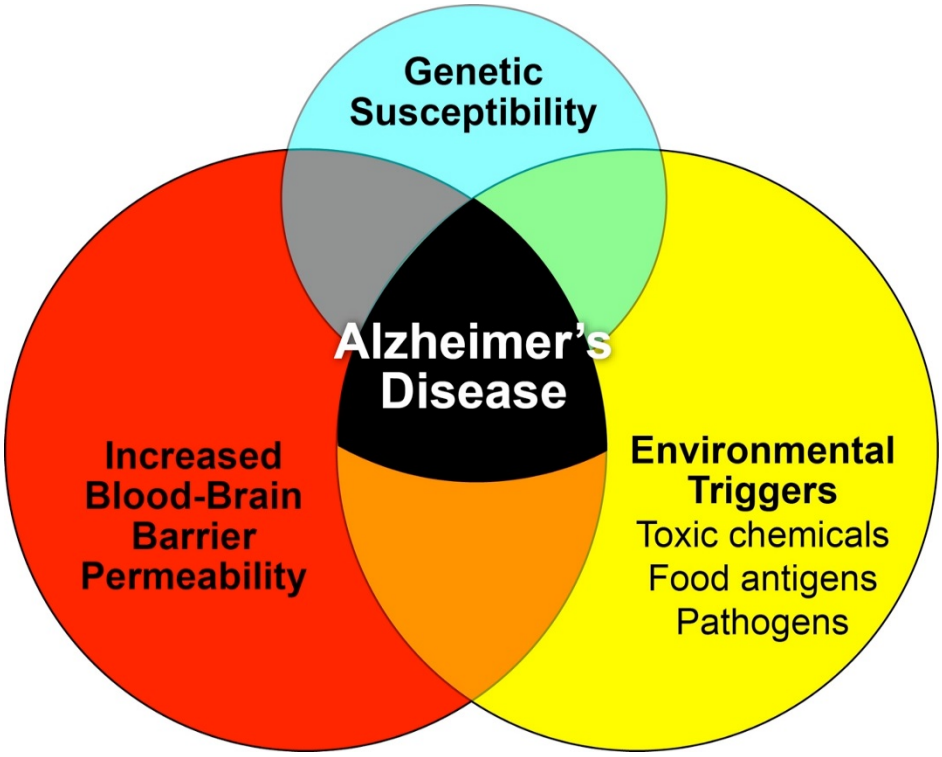


Figure 1. The Triad of Environmentally-Induced Neuroautoimmunity. *Neurological disorders are often environmentally-induced. For the development of AD and other neurological conditions, the three main ingredients are genetic susceptibility, a broken blood-brain barrier and environmental triggers (toxic chemicals, food antigens and pathogens). This combination can be devastating and lead to cognitive decline or other neurological manifestations.*

MECHANISMS LEADING TO ALZHEIMER'S DISEASE, MILD COGNITIVE IMPAIRMENT AND DEMENTIA

The Role of Genetics in Alzheimer's Disease

Researchers have found that the more prevalent form of AD, late-onset AD, is likely attributable to a combination of factors that includes genetics, environmental triggers, lifestyle, and BBB-permeability. As early as 2002, a team from the University of California Irvine proposed that environmental agents such as diet, aluminum and viruses are as important as genetic factors in the etiology of AD.[10] Like other researchers, Grant *et al*. found commonality between the dietary risk factors for AD and those for heart disease; they also cited aluminum's association with neurological damage and the link between apolipoprotein E (ApoE)-ε4 and herpes virus-1 in the brain of AD patients but not in controls. The Pedersen Swedish twin study[11] involving 662 pairs of twins aged 52 to 98 found that only 48% of the variation in liability to AD could be attributed to genetics. In 2006 Stewart *et al*. found strong evidence linking lead exposure to neurodegeneration in former lead workers.[12] More recently, in September of 2018, a Finnish team led by Haapala concluded that genetic risk factors increase the risk of AD but do not actually cause it, citing instead the environmental factor of lifestyle, specifically metabolic syndrome.[13] The Haapala study found that obese girls had a greater risk of developing AD. The BBB's role in AD deserves special mention simply for the overabundance of the literature generated regarding it, more of which is found in a later section of this material. A review citing many of these publications was published by Montagne *et al*. in 2017.[14]

While researchers have not found a specific gene that directly causes the late-onset form of the disease, one of several forms of the ApoE on chromosome 19 has been recognized as increasing a person's risk for AD. The ApoE-ε4 genotype represents the most important genetic risk factor for Alzheimer's disease. It may be useful to evaluate the genotype as part of prevention and early reversal of symptoms.[8] However, some people with an ApoE-X4 allele never get the disease, and others who develop AD have no ApoE-ε4 alleles at all, stressing the point that genetics is not the only contributor to Alzheimer's disease.

One very obvious factor to examine as a harbinger of AD, Mild Cognitive Impairment (MCI) or dementia is, of course, an individual's medical history. Medical history risk factors include:

- Family history of cognitive decline[reviewed in 15-17]
- Genetic susceptibility[reviewed in 15, 17]
- Diabetes[reviewed in 15-17]
- Mid-life obesity[reviewed in 15-16]
- Mid-life hypertension[reviewed in 15-16]
- History of depression[reviewed in 15]
- Sleep disturbance[reviewed in 15]
- Hyperlipidemia[reviewed in 15]
- Traumatic brain injury in males[reviewed in 15]
- Conjugated equine progesterone acetate use[reviewed in 15]
- Periodontitis[18]
- Blood-brain barrier breakdown[reviewed in 19-23]

The Role of Environmental Triggers in AD

Amyloid-β (Aβ) is a protein found in the brain, and tangled aggregates of Aβ are the hallmark of Alzheimer's disease. When highly purified Aβ antigen was used in a study on the stages of AD, researchers found very high levels of Aβ in serum in the mild to moderate AD group compared to healthy controls, and levels decreased in the moderate to severe AD group.[24] Another study found that declining cognitive test scores correlated with increased levels of Aβ-immunoglobulin G (IgG) immune complexes.[25] Although the exact mechanisms for the development of AD are not definitively known, researchers are accumulating evidence that strongly indicates interaction between Aβ and the triad of environmental triggers: infections, reactive foods, and toxic chemicals.

It may not be possible to control a person's genetic makeup, but it is certainly possible for people to control their lifestyles and try to avoid harmful environmental factors. But it is only possible to avoid environmental triggers to the immune system if a patient knows exactly what specific factors personally affect his own unique immune system. The best way to find this out is to determine a patient's antibody signature or immune print, which is a record of the factors to which an individual has reacted or is immunologically sensitive. This is the very reason Cyrex developed the Alzheimer's LINX™ based on three recent research articles by Vojdani.[5-7] See Figure 2.

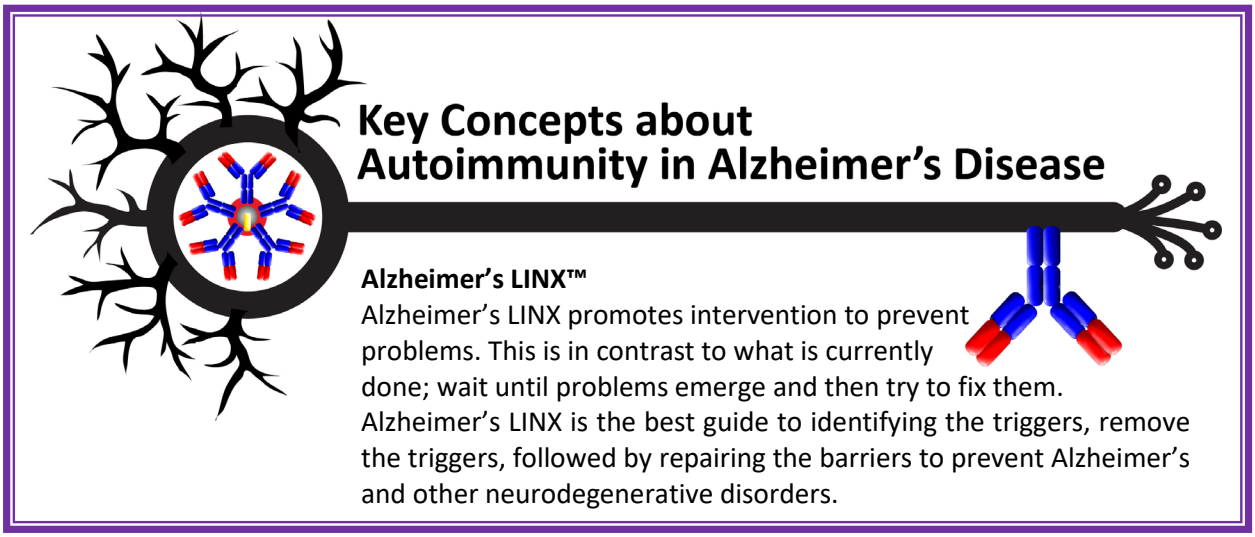


Figure 2. Alzheimer's LINX™. *Using Alzheimer's LINX™ in a clinical setting, provides a comprehensive view of triggers and pathogenesis even before the onset of disease.*

Infections and Their Cross-Reaction with Amyloid-β in Alzheimer's Disease

Infections have been suspected as early as the 1980s of having a role in neurodegenerative disorders.[26] Carter has posited that polymicrobial brain invasion may be a determinant factor in Alzheimer's disease, citing the upregulation of bacterial, viral and fungal sensors/defenders found in the AD brain, blood or cerebrospinal fluid.[27] Aβ appears to be produced by the body as an antibiotic against these pathogenic invaders. Unfortunately, Aβ shares sequence homology or molecular mimicry with the protein sequences of many pathogens, including *Borellia burgdorferi, Cryptococcus neoformans, Chlamydia pneumoniae,*

Helicobacter pylori, Porphyromonas gingivalis, the herpes viruses, and herpes simplex virus-1 (HSV-1). Higher levels of autoantibodies against Aβ are found in AD patients, suggesting that the immune system may have produced antibodies against both Aβ and actual pathogens because of molecular mimicry. These Aβ autoantibodies may weaken Aβ's antimicrobial effects, promoting pathogenic survival and cerebral pathogenic invasion, leading to the activation of neuro-destructive immune/inflammatory processes.[27] Vojdani's study demonstrates, for the first time, direct support for Carter's earlier hypothesis.[5] The study used a specific monoclonal antibody made against amyloid-beta peptide 42 ($A\beta_{42}$), which not only reacted strongly with $A\beta_{42}$, tau protein, and α-synuclein, but also had weak to strong reactions with pathogens such as *Enterococcus faecalis, Escherichia coli, Salmonella,. Campylobacter jejuni,* herpes type-1, oral pathogens or their toxins, some of which have been associated with AD. The homology between peptide stretches of microbial origin and proteins involved in AD could be a mechanism by which antibodies to homologous peptides mount attacks against autoantigens in AD. Vojdani *et al*. concluded that bacterial molecules bind to Aβ protein, forming small oligomers, then encasing pathogens and their molecules to form amyloid plaques, the tell-tale markers of AD. Conversely, these same Aβ peptides induce the production of antibodies to both $A\beta_{42}$ and bacterial molecules, which may inhibit bacterial pathogenesis, but in the process may promote amyloid plaque formation (Figure 3). It should be noted that everyone has a unique personal microbiome and unique personal immune system with its own strengths and susceptibilities. Not everyone will be susceptible to, or react, to the same infections and pathogens. It is therefore reasonable to measure antibodies against these $A\beta_{42}$ cross-reactive pathogens (Figure 4) in both asymptomatic individuals and patients with AD in order to suggest treatment modalities tailored to the individuals that may help delay progression or even provide remission in patients with AD. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the culpable pathogens so that the appropriate treatment can be applied.

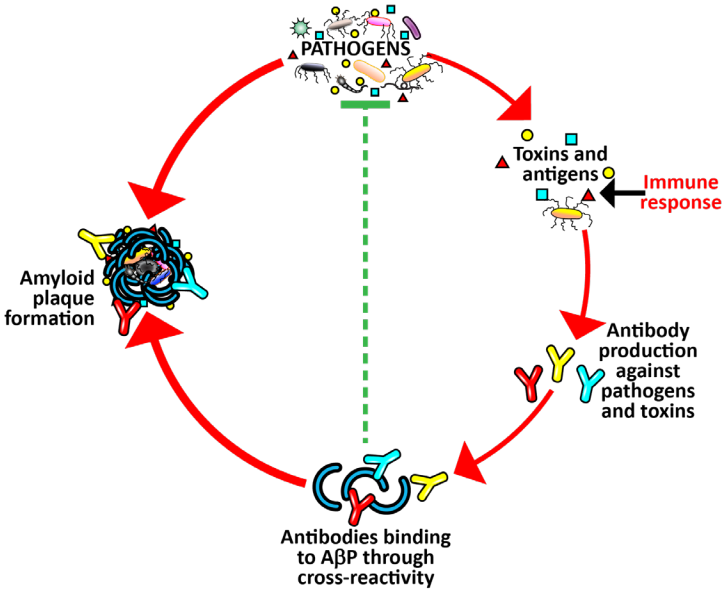


Figure 3. Amyloid plaque formation due to molecular mimicry between pathogenic antigens and Amyloid-Beta peptide (AβP). *Bacteria release bacterial toxins and antigens, eliciting an immune response in the form of antibody production against them. These antibodies bind to AβP through the homology between them, contributing to amyloid plaque formation. It is also possible that these cross-reactive antibodies bind to pathogens and block further pathogenic invasion, or help prevent amyloidogenesis by attacking their corresponding pathogens and toxins.*

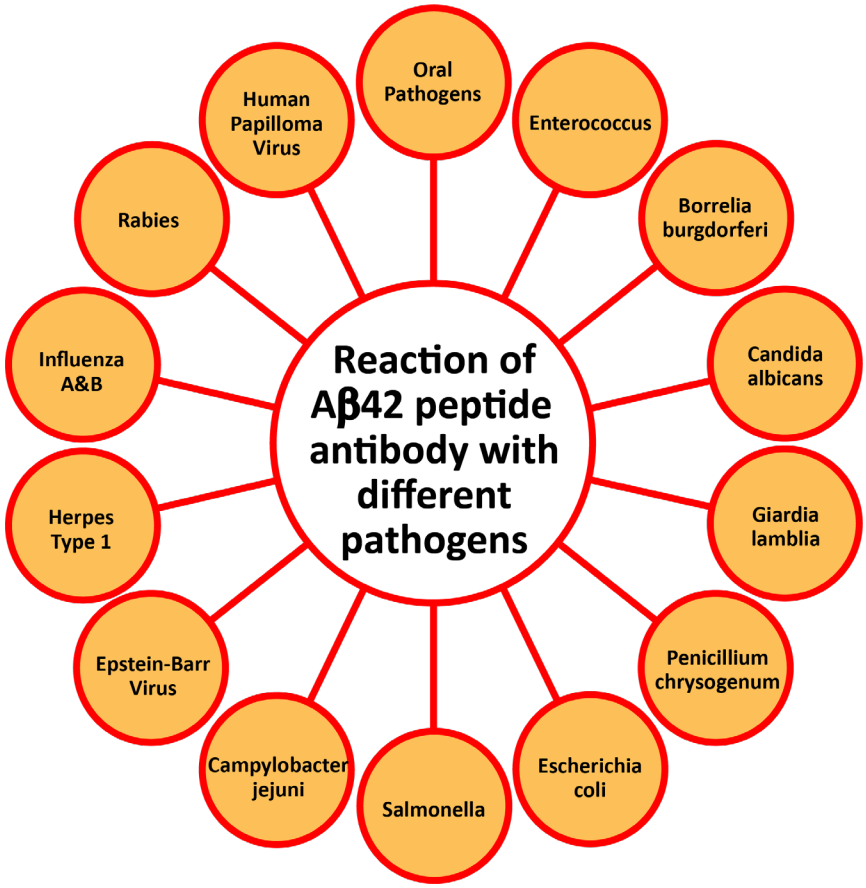


Figure 4. Pathogens associated with Alzheimer's disease with which Aβ42 shares homology or molecular mimicry. *Not everyone will be susceptible to or react to the same infections and pathogens. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the culpable pathogens so that the appropriate treatment can be applied.*

Food Antigens and Their Cross-Reaction with Amyloid-β in Alzheimer's Disease

Carter found that Aβ peptide also shares similarity with some food proteins.[28] Vojdani's study provided the first direct corroboration for Carter's earlier work.[7] Vojdani and Vojdani tested 208 food antigens and found that 19 of the foods reacted with $A\beta_{42}$. This is not surprising as earlier studies have already shown significant homology between food and brain antigens, such as wheat peptide with cerebellar, synapsin, casein, butyrophilin, myelin basic peptide, myelin oligodendrocyte glycoprotein, and plant aquaporins with human aquaporin.[29-32] This is how it is believed that some of these foods may be involved in such diseases as gluten ataxia, multiple sclerosis, and neuromyelitis optica.[33-35] The Vojdani study also showed a very strong reaction between $A\beta_{42}$ antibody and canned tuna, but not with raw tuna. Again, this is not to be unexpected, as earlier research by Vojdani has already shown that there can be vastly different reactivities between raw foods and cooked/processed foods, as the cooking/processing can affect the molecular structure of the food.[36] Similar to infections and the immune system, everyone has a different composition and balance of digestive peptides and commensal bacteria within their gut. Different people will be sensitive or immune-reactive to different foods, so that one person can happily eat peanuts and another can actually die from the same thing. It is therefore reasonable to measure antibodies against these $A\beta_{42}$ cross-reactive

foods (Figure 5) in both asymptomatic individuals and patients with AD in order to remove the specific $A\beta_{42}$ cross-reactive foods from the individual's diet, which may prevent, delay progression or even reverse the course of the disease in patients with AD. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the reactive foods.

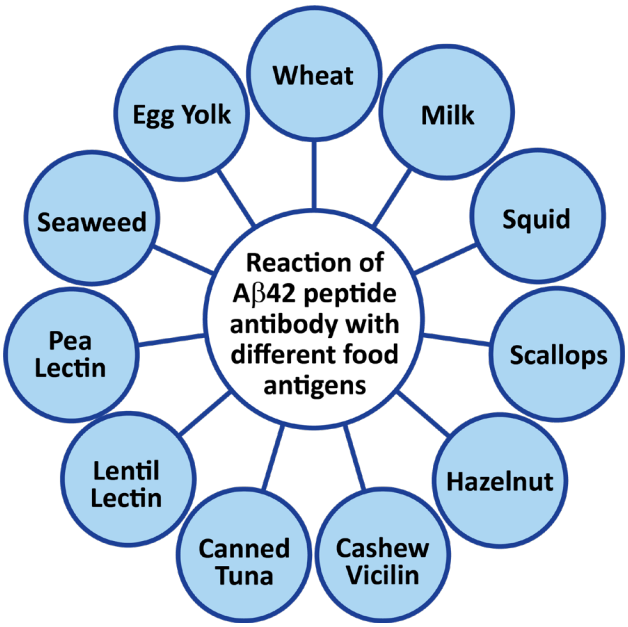


Figure 5. Foods with which Aβ42 shares homology or molecular mimicry. *Not everyone will react to the same foods. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the culpable foods so that the appropriate treatment can be applied.*

Chemicals and Their Effect on Protein Structures in Alzheimer's Disease

In a recent study, Vojdani and Vojdani[7] found that monoclonal anti-$A\beta_{42}$ has moderately to strong reactions with several chemicals bound to human serum albumin (HSA), but not to many other chemicals bound to HSA, or to HSA alone. Autoantibodies against amyloid-β protein and peptides are commonly detected in AD and even in some non-demented members of the ageing population.[37] The exact source of these anti-Aβ antibodies is not clear, but they could be derived from immune response to aggregated forms of amyloid-β, or from protein misfolding induced by aluminum, mercury, and plasticizers. Aluminum, phthalate and dinitrophenyl are three chemicals bound to HSA that reacted significantly with anti-$A\beta_{42}$. Toxic metals such as aluminum[38-42] and mercury,[43-45] are among the few that are known to cause toxicity to the brain and other organs and have been linked to numerous neurodegenerative disorders, including AD.

For many years, exposure to aluminum was suggested to favor an abnormal immune response in different diseases, including autoimmune conditions.[46] Aluminum accumulates in the skeletal system and the brain, and a link with diseases such as osteomalacia, encephalopathy, Alzheimer's and Parkinson's diseases has been reported. Internal accumulation of aluminum may be particularly relevant to Crohn's disease since it has been identified not only within macrophages of Peyer's patches, but also around dilated submucosal lymphatics and in MLN. The low percentage of oral bioavailability of aluminum is actually misleading. In fact, after oral administration, 40% of the ingested aluminum accumulated within the intestinal mucosa, affecting barrier function.[47]

Moreover, aluminum is a well-established neurotoxin, and is suspected of being a factor in the development of neurodegenerative disorders. This is because aluminum accumulation in the brain affects the memory and cognition, alters synaptic activity, activates microglia, and promotes Aβ and neurofilament aggregation, all of which are hallmarks of neurodegenerative disorders.[48] Exposure to aluminum and such metals has been followed by aggregation of amyloid-β protein on neuronal cells,[49] as well as, AD-like pathologies, which have been shown in animals as well as in human subjects. Aluminum-induced neurotoxicity includes oxidative stress, mitochondrial dysfunction, inflammatory response, and neurofibrillary degeneration, possibly through amyloid-β oligomerization.[38,40] In vitro studies have shown that aluminum together with other metals is involved in the formation of Aβ protein aggregation, which leads to amyloid fibrils and the formation of amyloid-like plaque structure.[42]

A review in 1998,[50] showed that such accumulations of amyloid and extracellular tangles act as irritants, resulting in inflammatory reactions that lead to the production of potentially neurotoxic products that contribute to neuronal loss. And as recently as 2018, Yumoto *et al*. found that aluminum and iron had colocalized in the nuclei of nerve cells in the AD brain. They theorized that this colocalization might lead to neurodegeneration and the development of AD.[51] Aluminum is one of the factors that accelerate $A\beta_{42}$ monomer aggregation by cross-linking anionic amino acids contained in the $A\beta_{42}$ sequence to form $A\beta_{42}$ aggregates.[40,52] This may be one explanation as to why high levels of antibodies to $A\beta_{42}$ and other amyloid proteins are detected in patients with AD.[53-54] Similar mechanisms of action may be applied to the aluminum binding to human albumin, where the aluminum may affect the functional properties of albumin or other proteins, leading to the formation first of dipoles and then of clusters that may mimic $A\beta_{42}$ oligomerization or protein misfolding similarities (see Figure 6).[55]

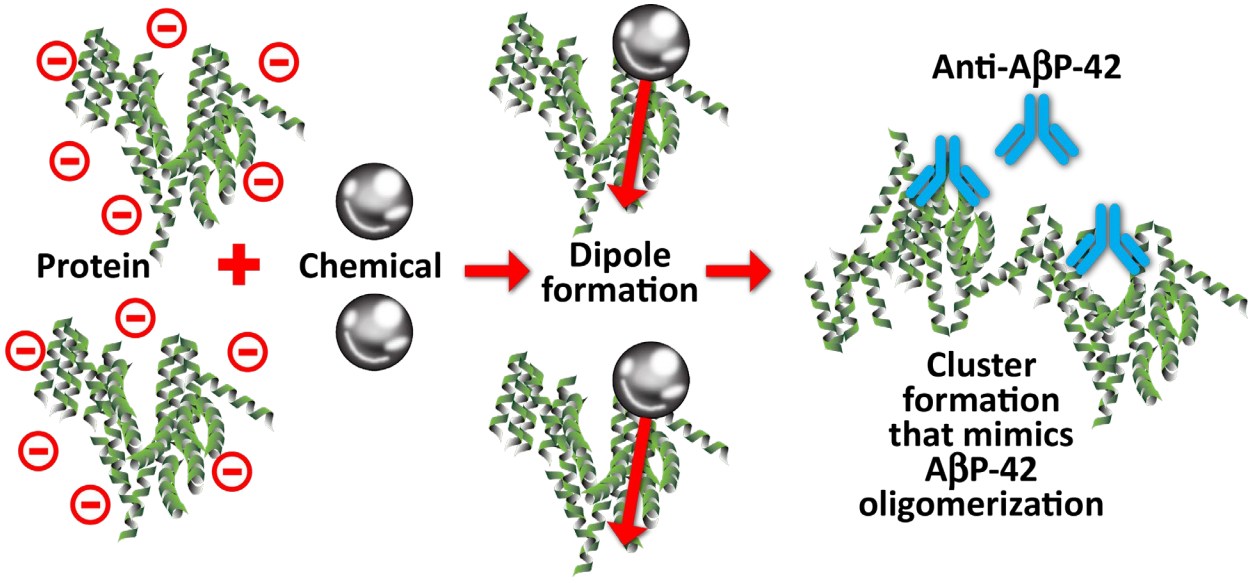


Figure 6. Chemicals, cluster formation and induction of protein misfolding. *Chemicals may bind to human albumin and other tissue proteins, affecting their properties and leading to the formation first of dipoles and then of clusters that mimic $A\beta_{42}$ oligomerization or protein misfolding similarities [Modified from Saha].*[52]

Mercury has also been reported as a risk factor for AD due to its well-known neurotoxicity. Mercury ions bind to tubulin, inhibiting guanosine triphosphate (GTP) nucleotide binding capacity and reducing its biological activity, leading to microtubule degeneration.[43] In vitro and animal studies have shown that mercury causes hyperphosphorylation of tau protein and increased formation of Aβ protein aggregation.[44]

Phthalates and bisphenol A (BPA) are used as plasticizers in water bottles, food cans, and many other products. As such, they can leach or migrate into food and water, and hence through estrogenic activity or epigenetic modification may affect human health.[56-57] If BPA crosses the blood-brain barrier, it can bind to a target enzyme called protein disulfide isomerase (PDI).

PDI is a stress protein found in the endoplasmic reticulum of many cells, including neural tissue, and is involved in protein folding. Normally this enzyme effectively inhibits α-synuclein fibril formation, but the S-nitrosylation of PDI by chemicals leads to a loss of enzymatic activity and the enhancement of protein misfolding and α-synuclein aggregation that are found in AD and Parkinson's disease.[58-59] This explanation is supported by the findings that numerous age-related disorders are now recognized to be related to the accumulation of different misfolded proteins that result in the production of autoantibodies called anti-oligomer antibodies.[60]

Not everyone will be susceptible to or react to the same toxic chemicals which, after binding to human protein, can cause misfolding similar to Aβ protein. It is therefore reasonable to measure antibodies against these chemicals (Figure 7) in both asymptomatic individuals and patients with AD in order to suggest treatment modalities tailored to the individuals that may help delay progression or even provide remission in patients with AD. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the culpable toxins so that the appropriate treatment can be applied.

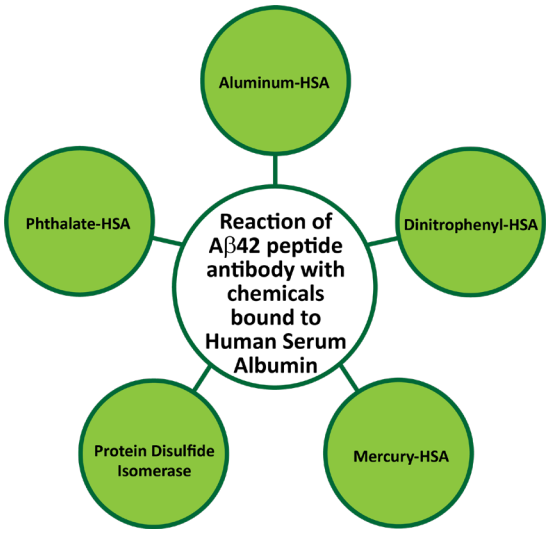


Figure 7. Chemicals associated with Alzheimer's disease which, after binding to Human Serum Albumin (HSA), can induce the formation of protein clusters that mimic Aβ protein misfolding. *Not everyone will be susceptible to or react to the same infections and pathogens. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the culpable pathogens so that the appropriate treatment can be applied. This can lead to autoimmune disorders, including AD.*

Lifestyles and Their Role in Alzheimer's Disease

Environmental risk factors for AD, cognitive decline or dementia include:

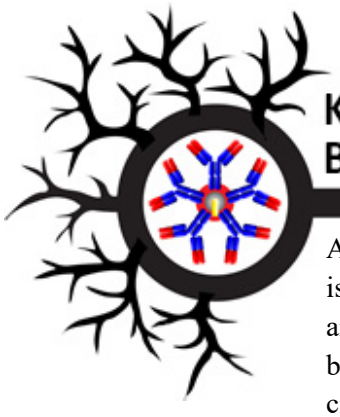
- Smoking[reviewed in 15-17]
- Poor diet[reviewed in 15-17]
- Lack of social engagement[reviewed in 15-17]
- Lack of cognitive stimulating exercise[reviewed in 15-17]
- Lack of physical exercise[reviewed in 15-17]
- Fewer years of formal education[reviewed in 15,17]
- Military service (PTSD)[19,21]
- Professional athletics in head impact sports (CTE)[reviewed in 19,20,22]
- Physical and/or emotional stress leading to the breakdown of the blood-brain barrier[61,62]

The Role of the Blood-Brain Barrier in Alzheimer's Disease

The blood-brain barrier (BBB) acts as a gatekeeper, preventing neuroinvasion of autoreactive agents circulating in the bloodstream. For full details on the function of the BBB and its role in health and disease, please see our Clinical Application Guide for the Array 20 - Blood-Brain Barrier Permeability Screen. The basic concept of the BBB is that circulating autoreactive agents are relatively harmless unless they breach the BBB. If the BBB is broken, then the more autoreactive agents there are in the bloodstream, the greater the neuronal tissue damage.[63]

Some researchers are pointing to BBB breakdown as an early stage in the pathogenesis of mild cognitive impairment (MCI) and AD.[64, reviewed in 65-67] Indeed, cognitive decline was associated with stronger BBB breakdown in both patients with MCI and with early AD.[64] Using dynamic contrast-enhanced MR imaging with dual-time resolution, the BBB leakage was shown to be via the tight junctions and was globally found throughout the brain, rather than localized to a single tissue class.[64] The breakdown of BBB tight junctions can lead to the toxic accumulation of substances in the brain. These autoreactive agents can trigger neuroautoimmunity. The BBB can become compromised as a person ages, and inflammatory agents in the bloodstream, such as lipopolysaccharides (LPS) and other bacterial toxins can accelerate the BBB breakdown allowing for the transport of Aβ into the cerebrum leading to AD.[reviewed in 67] The loss of BBB tight junctions and BBB breakdown in patients who died with diagnosed AD has been confirmed by more than 20 independent postmortem human studies showing brain capillary leakages and perivascular accumulation of blood-derived autoreactive agents such as fibrinogen, thrombin, albumin and IgG.[reviewed in 14,66]

Protecting the BBBs in patients with a family history of AD, MCI or dementia should be incorporated into a therapy for the prevention of neurodegeneration in healthy individuals. Assessing and addressing BBB breakdown in patients already exhibiting early signs of cognitive decline should be a first-line step in recovering the patient. BBB protocols can be implemented in patients already in later stages of cognitive decline as a means to slow down the pathogenesis.



Key Concepts
Breach in Blood-Brain Barrier

A breach in the BBB is dangerous. While the BBB is intact, even multiple serological autoreactive antibodies are harmless, but when there is a breach in the BBB, the more autoreactive antibodies there are in circulation, the greater the risk that they will reach the brain through the breach and cause neurological tissue damage. Thus, in the context of BBB compromise, the cross-reactive circulatory antibodies become pathogenic.

It appears that $A\beta_{42}$ is an important and prolific factor in the development of AD; it cross-reacts with infectious pathogens and dietary peptides, and binds with toxic chemicals to mimic misfolded protein, thus causing the generation of autoantibodies that directly or indirectly lead to neurodegeneration. How is it, then, that these harmful and destructive autoantibodies can be found in the blood of apparently perfectly healthy individuals? In fact, antibodies against a variety of neural antigens and various proteins are detected in the sera of both patients with AD and healthy subjects.[6] Brain-reactive autoantibodies have been found to be nearly ubiquitous in human sera.[23,68]

In another recent study, Vojdani and Vojdani[6] investigated this seeming contradiction of destructive autoantibodies in healthy individuals. Theorizing that the key could be the BBB, they examined the immune reactivity of monoclonal anti-$A\beta_{42}$ with neuronal antigens, BBB proteins, gut-associated antigens, and nerve growth factors. The results showed equivocal to moderate reactions with BBB proteins such as S100B and aquaporin-4 (AQP4). Reactions were high with enteric nerve nitroarginine and with several proteins associated with AD. The $A\beta_{42}$ antibody was highly reactive with the nerve growth factors beta-nerve growth factor (β-NGF) and brain-derived neurotrophic factor (BDNF). This is especially significant because nerve growth factors act as fertilizer or food for brain cells; if autoantibodies attack the nerve growth factors, then the brain cells' ability to regenerate and heal themselves will be inhibited or even negated, leading to neurodegeneration, the mark of disorders such as AD. The detection of antibodies to BBB proteins also suggests damage to the BBB. It is possible, then, that should the BBB be damaged or breached, then these ubiquitous and normally harmless but potentially highly reactive autoantibodies may cross through the barriers and find their way eventually into the brain, where they may then through various mechanisms either cross-react or bind with neural tissues and with $A\beta_{42}$, contributing to the onset and/or progression of AD and perhaps other neurodegenerative disorders (see Figure 8). Not everyone will have the same autoantibodies to the same proteins or antigens.

It is therefore reasonable to measure antibodies against these neural antigens, BBB proteins and nerve growth factors in both asymptomatic individuals and patients with AD in order to suggest treatment modalities tailored to the individuals that may help delay progression or even provide remission in patients

with AD. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the specific antigens so that the appropriate treatment can be applied.

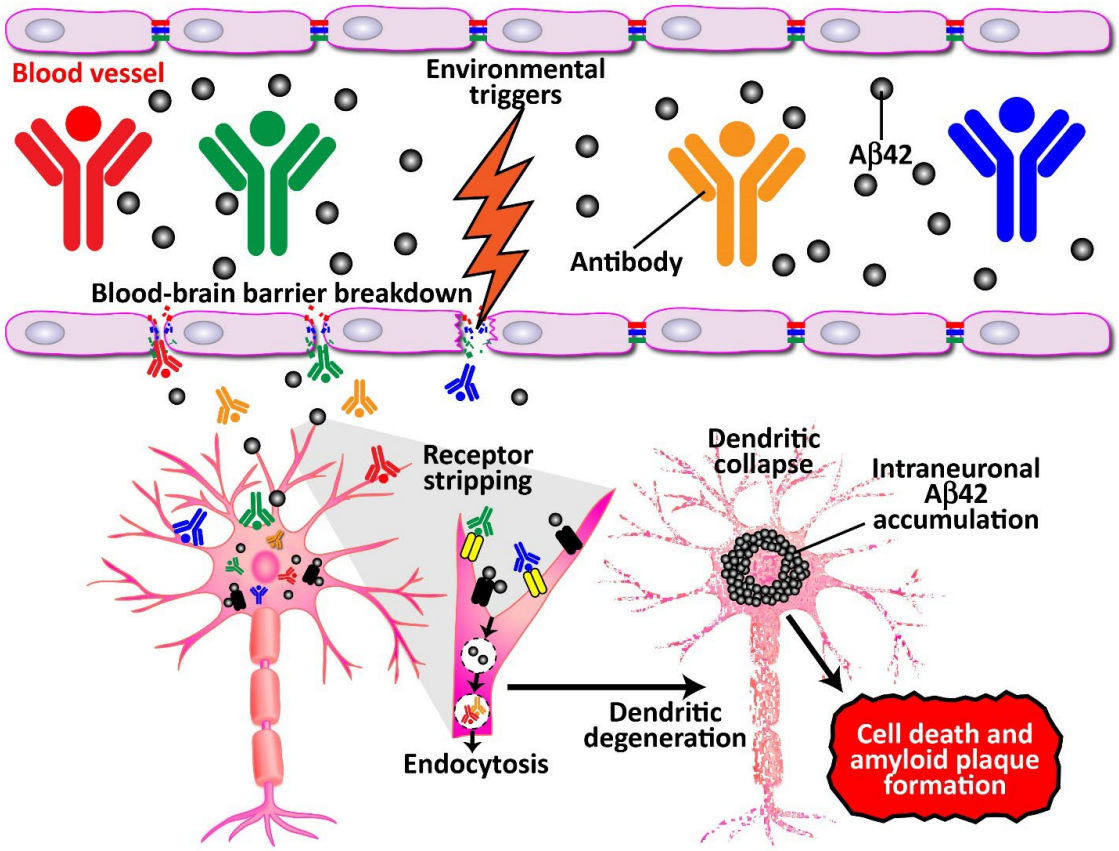


Figure 8. A Mechanism for AD Pathogenesis. *When combining blood-brain barrier breakdown with the presence of neuron-binding autoantibodies in the serum, the autoantibodies that were once prevented from entering the nervous system have direct access to target tissues. This antibody reacts with different brain antigen and may lead to neuronal cell death, neuroautoimmunity and/or amyloid plaque formation. (Modified from Nagele)*[23]

The Cyrex Difference

Alzheimer's disease is a complex disorder and as such, there is no simple test used to diagnose it. Current medicine uses a multiple step, multiple practitioner approach to diagnose AD:[4]

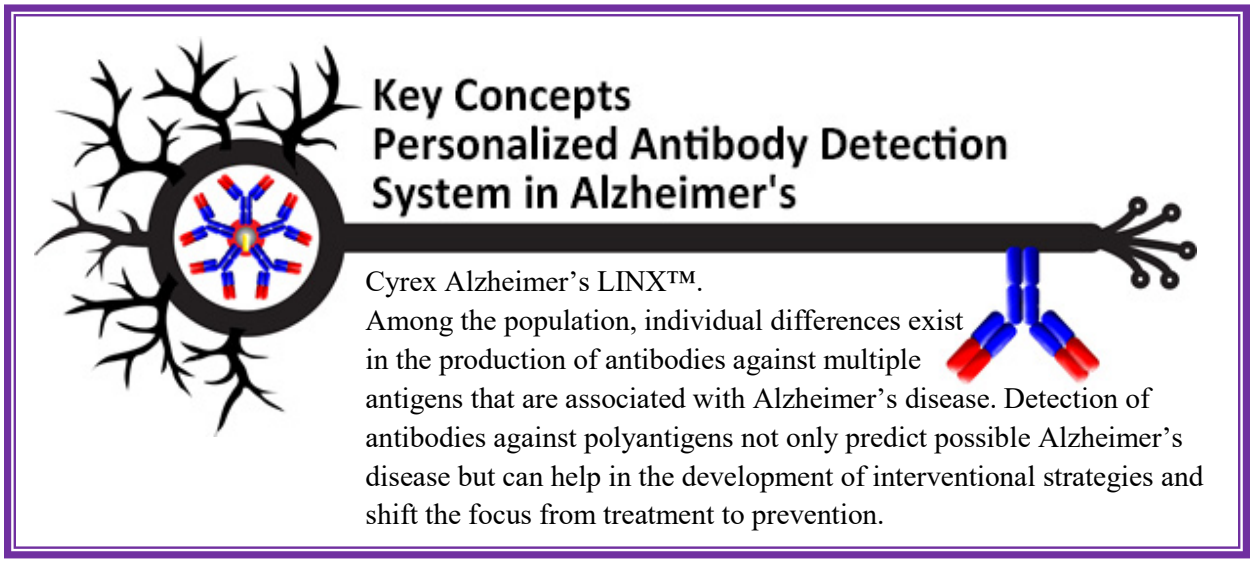
- Compile a full medical and family history from the patient, including psychiatric history and history of cognitive and behavioral changes. This step often involves a close family member to provide input about the changes observed in cognitive ability and social behavior.
- Conduct cognitive tests along with physical and neurological examinations.
- Multiple blood tests and brain imaging is done to rule out other potential causes of dementia.

Once the diagnosis of AD has been accomplished, the difficulty lies in identifying the cause of the cognitive decline. This takes many weeks of additional traditional tests and exams. Note that none of these steps involves identification of the specific environmental triggers that may have different effects due to the personalized idiosyncratic nature of the human immune system, microbiome, physique and sensitivities.

Cyrex's Alzheimer's LINX™ - Alzheimer's-Associated Immune Reactivity puts a significant emphasis on the role of triggers that affect the BBB, neural proteins and growth factors that are involved in neuronal degeneration or regeneration. Healthy individuals have been shown to have antibodies to the same tissue biomarkers used in AD analysis, such as tau protein, asialoganglioside, Aβ and myelin basic protein.[68] The difference between healthy individuals and AD patients is the presence of a broken BBB.[64] As AD is a spectrum disease with multiple possible combinations of triggers and degeneration of select neuronal tissues, finding the patient's unique signature can guide the practitioner in implementing an effective therapy to combat or prevent cognitive decline. As detailed below, a comprehensive assessment that includes

- Brain Proteins involved in Alzheimer's disease
- Growth factors involved in neuronal regeneration
- Enteric nerve, enzymes and neurological peptides
- Pathogens with Alzheimer's disease
- Chemicals with Alzheimer's disease
- Foods cross-reactive to amyloid beta
- Blood-brain barrier protein and Neurofilaments

makes it easier for the healthcare professional to see the route of pathogenesis, and devise strategies to stop it. By addressing BBB breakdown, identifying and removing triggers and enhancing neuronal regeneration, one might be able to win the war on cognitive decline.



Pathophysiology of Cognitive Decline

The pathophysiology of cognitive decline includes a variety of systems, triggers and pathways. Thus, there is no single, agreed-upon pathology for AD. Alzheimer's LINX™ is a comprehensive assessment covering many of the proposed systems, triggers and pathways leading to AD as shown in Figure 9.

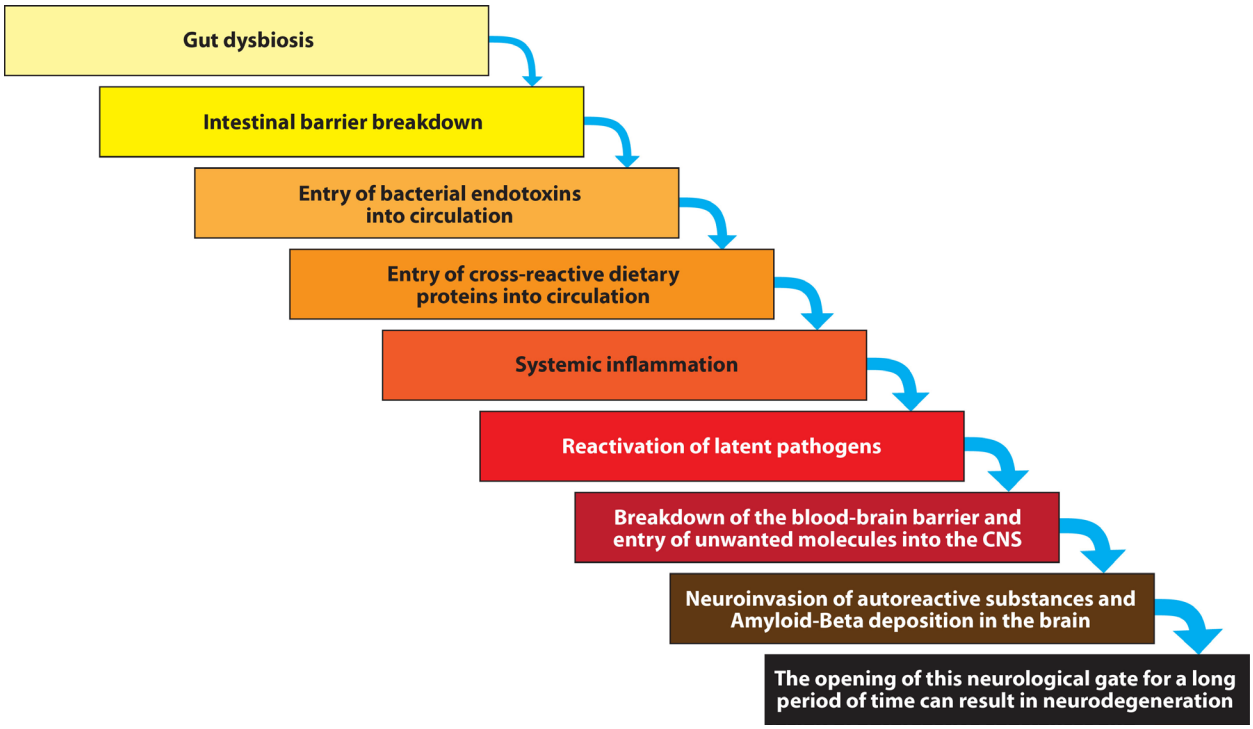


Figure 9. A Pathway to Neurodegeneration. *Although the mechanisms resulting in cognitive decline vary from person to person, the concept of 'gut-to-brain' is depicted here.*

Researchers have now determined that the gut, brain and immune system are in fact all interconnected and influence each other in what some call the gut-brain axis or the brain-gut connection.[69-70] The road to AD or other neurological disorders can include a state of gut dysbiosis in which the inflammation from the Gram negative bacterial toxins such as LPS and cytolethal distending toxin (CDT) break down the epithelial cells and/or the tight junctions of the intestinal barrier. The broken barrier allows for the easy translocation of bacterial LPS and CDT as well as dietary proteins, some of which cross-react with different tissue antigens. With all of these immunogens entering the bloodstream, systemic inflammation occurs. At this point, it is highly possible that latent pathogens of long-ago and forgotten infections may become reactivated, and contribute further to the inflammatory cascades. Systemic LPS, other bacterial toxins, inflammatory cytokines and other immunogens together target structures of the BBB, such as aquaporins and S100B, resulting in the breakdown of the BBB. When the BBB is broken, the brain is at risk for the entry of unwanted molecules including Aβ peptide originated from the gut, autoreactive antibody infiltration, and the deposition of Aβ and immune complexes into the brain. Eventually, this may manifest as AD or other neurological disorders.

These players and factors that are involved in Alzheimer's disease are summarized below. A comprehensive assessment that includes each of these categories will delineate a patient's unique pathophysiology or antibody immune print.

Brain Proteins

Researchers have pinpointed key "amyloidogenic" self-proteins involved in the pathogenesis of AD, namely Tau protein, Amyloid-beta peptide, Rabaptin-5, Presenilin and Alpha-Synuclein.[71] Each of these proteins or peptides is a potential target of the immune system which can result in the production of specific antibodies that are detectable in blood.

Autoantibodies can be protective (usually IgM), produced to clear out dead cells, or they can be pathogenic (usually IgG), which can cause tissue damage. Studies show that these autoantibodies are found in both healthy individuals and patients with AD, but in higher levels in the latter.[6,reviewed in 72]

Thus, one must consider antibody-antigen patterns along with BBB permeability when assessing patients for cognitive decline. Overall, patients with elevated circulating autoantibodies are at greater risk for the development of neurological disorders than patients who do not have circulating autoantibodies. When the BBB is broken, autoreactive antibodies may infiltrate the brain and trigger neuroautoimmunity, as shown in Figure 8. It is when the BBB is broken that neuroautoimmunity and/or cognitive decline can occur due to circulating autoantibodies or circulating immune complexes. Because circulating autoantibodies only induce amyloid plaque formation when the BBB is breached, it is imperative to protect, support and maintain the integrity of the BBB not only in patients with AD but even in healthy subjects.

It is important then to detect these autoantibodies to these different proteins and molecules that interact with $A\beta_{42}$ through various mechanisms so as to develop protocols that may stop or reverse the neurodegeneration and cognitive decline that comes with AD. In this regard Cyrex's Alzheimer's LINX™ presents a list of proteins or peptides that are major targets of the immune system and are suggested for clinical use.

Tau Protein: Tau protein (intraneuronal) is a microtubule-associated protein found in the neurons of the central nervous system. It binds to tubulin, which facilitates tubulin's ability to assemble into microtubules. This action provides stability and flexibility for distal portions of axons. To a lesser extent tau protein is found in oligodendrocytes. Anti-tau has been detected in the sera of patients with AD, with other dementia, with neuroinflammatory diseases, and in healthy controls; it was found that AD patients had significantly higher levels of autoantibodies to tau and heavy neurofilament than the other groups.[73] Fibrillar deposits of highly phosphorylated tau are a key pathological feature of several neurodegenerative tauopathies including AD and some frontotemporal dementias. Increasing evidence suggests that alterations to soluble tau proteins induce neurodegeneration.[74] In particular, aberrant tau phosphorylation is acknowledged to be a key disease process, influencing tau structure, distribution, and function in neurons. Due to cross-reactivity with amyloid beta peptide,[6] patients with circulating antibodies to tau protein may be at greater risk for AD and other neurological disorders when the blood-brain barrier is breached.

Amyloid-Beta Peptide: Amyloid precursor protein (APP) is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. It has been implicated as a regulator of synapse formation, neural plasticity and iron export. Amyloid-Beta (Aβ) (extraneuronal) is a protein fragment from an APP. Although its function has not been fully elucidated, some posited roles for Aβ have already been

explored, such as activation of kinase enzymes, protection against oxidative stress, regulation of cholesterol transport, and anti-microbial activity. There is immediate involvement of Aβ peptide in the production of antibodies when high levels of IgG against amyloid-beta are detected in the serum. Antibodies can also be produced against environmental triggers that share a similar structure or amino acid sequence such as various foods and pathogens. Exposure to toxic chemicals can form new antigens that bear a resemblance to misfolded Aβ peptide. Elevated Aβ antibody production would be expected in response to Aβ accumulation, which can be due to deficient clearance mechanisms and/or increased formation of Aβ peptides.[25]

Rabaptin-5 + Presenilin: Rabaptin (Rab) is a protein involved in cellular vesicle trafficking and in the regeneration of damaged axons and can be found in microvascular endothelial cells. It is a very promising autoantigen for AD, as it is elevated in the majority of serum samples from AD patients, but not in sera from healthy controls. On the other hand, Rab5 is not exclusive to AD, as it has also been found in patients with systemic lupus erythematosus.[reviewed in 72] Rabaptin-5 interacts with the GTP. Rab GTPases act as molecular switches cycling between "active" GTP-bound and "inactive" GDP-bound forms.[75] A lack of Rab5 strongly inhibits Rab5-dependent early endosome membrane fusion. Thus, Rab5 is a Rab effector required for membrane docking and fusion necessary for tissue regeneration. The production of antibodies against Rab5 have the ability to starve neurons from necessary growth factors such as neurocrescin that is necessary for growth and reproduction due to cross-reactivity between Rab5 and this essential growth factor. Presenilins are a family of related multi-pass transmembrane proteins which constitute the catalytic subunits of the gamma-secretase intramembrane protease complex. Presenilin proteins not only affect the γ-secretase cleavage in familial AD cases but are also required for physiological Aβ generation by direct or indirect control of beta-amyloid precursor protein, which corresponds to an exacerbation of $A\beta_{42}$ production.[76-78] Presenilins are postulated to regulate amyloid precursor protein processing. Due to cross-reactivity with amyloid beta peptide,[6] patients with circulating antibodies to Rabaptin-5 + Presenilin may be at greater risk for AD and other neurological disorders when the blood-brain barrier is breached.

Alpha-Synuclein: Physiologically, native α-synuclein promotes presynaptic soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE)-complex assembly, but its molecular mechanism of action remains unknown. α-Synuclein antibody is a strong biomarker for neurological disorders. Although present in the heart and muscles, the protein, α-synuclein, is predominantly found in the brain, specifically at the tips of neurons. It plays a role in synaptic transmission and plasticity. α-synuclein interacts with tubulin and is necessary for normal development of cognitive function. Researchers have shown that α-synuclein knockout mice have reduced learning ability in tests requiring both working and spatial memory.[79] Aggregates of fibrillated α-synuclein in the form of Lewy bodies are detected in many Parkinson's disease patients and in some individuals with Alzheimer's disease. Lewy bodies have been found in the intestinal enteric nerves, which could indicate an early site for PD. As a result, the interpretation of antibodies against Alpha-Synuclein can indicate immune reactivity to the enteric neurons and different components of the essential barriers of the brain and intestines. These α-synuclein aggregations are believed to be toxic to neurons, especially the dopaminergic neurons in substantia nigra. The aggregated form of α-synuclein induces microglial activation, the release of proinflammatory cytokines, and the degeneration of neurons. Immune reaction against dysfunctional α-synuclein results in specific antibodies that are detected in the peripheral blood of about 90% of patients with the inherited form of Parkinson's disease[7] and in some patients with AD. This aggregation may be due

to a malfunction in protein disulfide isomerase (PDI), resulting in protein misfolding. Antibodies to α-synuclein have been detected at high levels in both Parkinsonian and AD groups of patients.[reviewed in 80] Serum immunoglobulins to α-synuclein from these patients are highly (8-fold) elevated, compared to healthy controls. These antibodies are strong biomarkers of dementia with Lewy Bodies.[81] Due to cross-reactivity with amyloid beta peptide,[6] patients with circulating antibodies to α-synuclein may be at greater risk for AD and other neurological disorders when the blood-brain barrier is breached.[1]

Growth Factors

Researchers have noted that neuronal regeneration pathways may not be functioning in patients with AD. Through genetic variations or pathogen homology,[27,82] growth factors during AD may not effectively support, maintain or rebuild neuronal tissues as they are normally intended to do. Dysfunctional growth factors that may be involved, include Beta Nerve Growth Factor (β-NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophins and Somatotropin (Figure 10). Both antibodies against β-NGF and BDNF cross-react with $A\beta_{42}$ and thereby can enhance the process of amyloidogenesis and at the same time prevent the normal healing and replacement of damaged nerve cells.[6] Taken together, these mechanisms can play a role in the pathophysiology of AD and other neurodegenerative disorders.

Beta Nerve Growth Factor: Nerve growth factor (NGF) is a neurotrophic factor and neuropeptide. NGF is primarily involved in the regulation of growth, maintenance, proliferation, and survival of certain target neurons in the sympathetic and sensory nervous systems. NGF binds with tropomyosin receptor kinase A and low-affinity NGF receptor. These receptors are associated with neurodegenerative disorders. NGF circulates in the bloodstream to keep homeostasis throughout the body and contributes to cell growth and differentiation, particularly nerve cells. β-NGF specifically supports the survival and growth of neural cells, regulates neuronal cell growth, promotes differentiation of neurons, and aids in neuron migration.[83] Due to cross-reactivity with AβP,[6] patients with circulating antibodies to βNGF may be at greater risk for AD and other neurological disorders when the blood-brain barrier is breached.

Brain-Derived Neurotrophic Factor: Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family. BDNF plays a vital role in the growth, development, maintenance, and functioning of several neuronal systems, including those involved in memory such as the hippocampus, cortex, and basal forebrain.[84] It supports differentiation, maturation, and survival of neurons, and under acute conditions such as glutamatergic stimulation, cerebral ischemia, hypoglycemia, and neurotoxicity, is neuroprotective. Therefore, BDNF plays a significant role in the development and maintenance of a healthy nervous system. It plays a direct role in the early phase of synaptic plasticity by triggering the delivery of GluR1 subunits to the synapse. As BDNF levels decrease with age, and antibodies against AβP that cross-react with BDNF are produced in the blood, a breach in the BBB may lead to cross-reactive antibodies destroying what is left of BDNF levels in the brain.

Both antibodies against β-NGF and BDNF cross-react with $A\beta_{42}$ and thereby can enhance the process of amyloidogenesis and at the same time prevent the normal healing and replacement of damaged nerve cells.[6] Taken together, these mechanisms can play a role in the pathophysiology of AD and other neurodegenerative disorders.

Neurotrophins: Neurotrophins (NTs) are a family of proteins belonging to a class of growth factors. NTs induce the survival, development, and function of neurons and are capable of signaling particular cells to survive, differentiate, or grow. They work in the peripheral and central nervous systems to encourage the growth and differentiation of new neurons and synapses. Due to cross-reactivity with Aβ,[6] patients with circulating antibodies to NTs may be at greater risk for AD and other neurological disorders when the BBB is breached.

Somatotropin: Somatotropin is a growth hormone (GH) secreted by the anterior pituitary gland. It is a major participant in control of several complex physiologic processes, including growth and metabolism. It is likely that GH promotes neuronal survival through its anti-apoptotic properties. Due to cross-reactivity with Aβ,[6] patients with circulating antibodies to somatotropin may be at greater risk for AD and other neurological disorders when the BBB is breached.

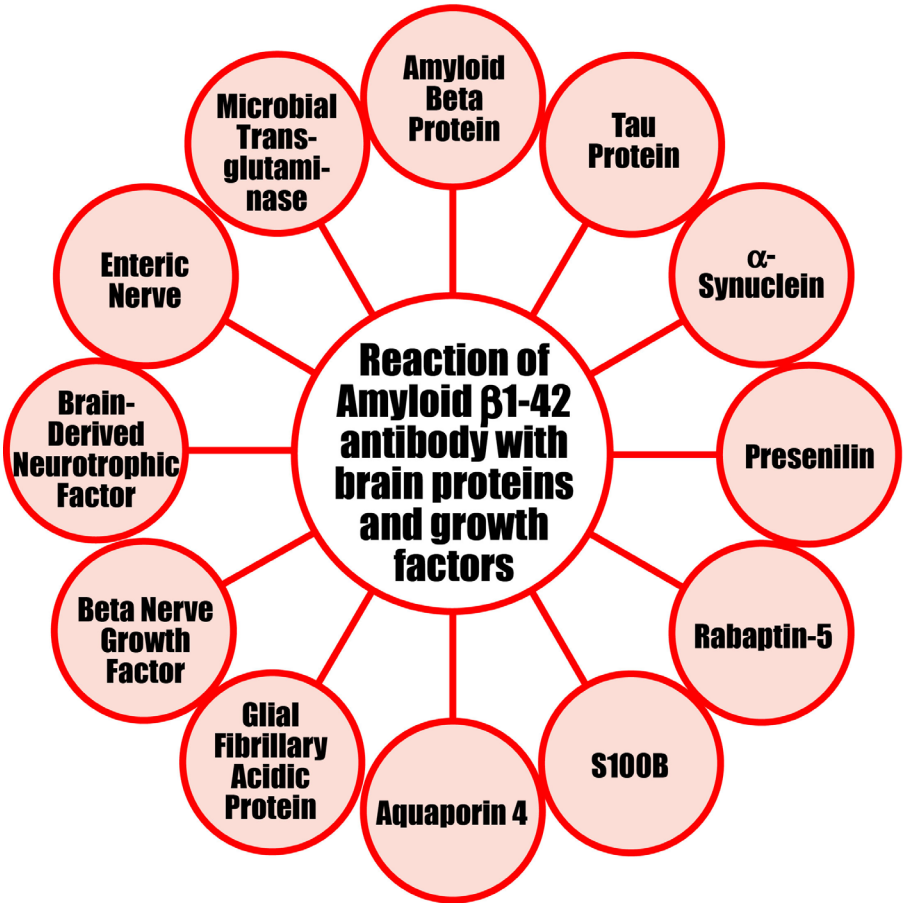


Figure 10. Neural proteins and Nerve Growth Factors with which Aβ42 may react in patients with Alzheimer's disease. *Not everyone will react to the same proteins or growth factors. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the culpable factors so that the appropriate treatment can be applied.*

Enteric Nerve, Enzymes and Neurological Peptides

The discussion about the gut-to-brain concept of AD[69-70] would not be complete without the enteric nervous system and the enzymes that are major components of the GI. In this category we include: Enteric Nerve (EN), Vasoactive Intestinal Peptide (VIP) and Transglutaminases (microbial, tissue transglutaminase-3 and tissue transglutaminase-6).

The enteric nervous system (ENS) controls gut peristalsis, sensory neurons and interneurons. Multiple sclerosis (MS) patients were noted to have digestive dysfunction in 65.6% of cases including constipation, dysphagia and fecal incontinence.[85] Antibodies to ENS tissues were found in patients with MS and thus, it is thought that autoimmunity against the ENS may be the cause of intestinal disorders seen in MS patients.[86] In the case of AD, it was shown that antibody made against $A\beta_{42}$ reacted strongly with enteric nerve.[6] This indicates that production of antibody against enteric nerve affects amyloid-β, and antibodies that are produced against Aβ can affect the enteric nerve function.[6] To compound matters, the longer a person lives, the greater the number of challenges from dietary proteins, medications, pathogens, stress and chemicals occur against the gastrointestinal system. Studies show that aging changes intestinal smooth muscle cell quality and the general morphology of enteric neurons.[reviewed in 87]

Although the intestinal immune system is in place to prevent gut dysbiosis, environmental damage to the intestinal barrier, and the upregulation of inflammation, it can get overwhelmed. Assessing ENS and gut immune tissues can identify a disruption in the system that is meant to protect the rest of the body.

Enteric Nerve + Vasoactive Intestinal Peptide: Enteric neurons (ENs) represent a vast neural network that is organized into two major ganglia, the myenteric and submucosal plexuses. This neural network is distributed throughout the entire alimentary tract and extends out to the biliary tract and pancreas.[88] ENs are involved in the sensory information sent between the enteric nervous system (ENS) and the central nervous system (CNS). There is a close structural similarity between the central and enteric nervous systems. This similarity can indicate an antibody-induced disease process that not only effects the CNS but the ENS as well. Bowel movements, transmucosal fluid exchange in the small and large intestine, and the function of the brain and immune system can be impacted by high levels of IgG antibodies against ENS components. Vasoactive Intestinal Peptide (VIP) is a widely distributed neuropeptide in both the central and peripheral nervous system. It acts as a neuromodulator in many organs/tissues including heart, lung, thyroid gland, kidney, immune system, urinary tract, and genital organs. VIP has also been shown to inhibit LPS-induced production of inflammatory cytokines. Production of antibody against VIP may dampen the anti-inflammatory and regulatory functions of VIP in the gut and its relationship to the brain.

Transglutaminases: Transglutaminases are a family of enzymes. They form protein polymers, like scaffolding, which are vital in the formation of barriers and stabilizing structures. Tissue Transglutaminase-3 (tTG3) is expressed mainly in the epidermis, and to a lesser extent in the placenta and the brain.[88] In the epidermis tTG3 plays a role in the formation of cell envelope barrier structures, and in hair follicles tTG3 helps in the hardening of the inner root sheath.[89-90] Tissue Transglutaminase-6 (tTG6) is expressed in neural tissue.[91] The tTG6 enzyme is not commonly expressed in the small intestine but can be found in mucosal antigen-presenting cells.[91] Microbial transglutaminase (mTg), also known as thrombian, is a product added to a powder used in the food manufacturing industry to adhere smaller pieces of food together for a decorative effect or to give food a pleasing texture.[92-93] It is also used to thicken some milks, yogurts and

egg whites. Vojdani[6] showed that anti-$A\beta_{42}$ antibody reacted moderately to tTG-3 and tTG-6, but strongly to microbial TG. This is highly significant because mTG is found in thousands of products that are consumed worldwide on a daily basis. If individuals react immunologically to mTG, the produced antibodies can contribute to Aβ tangle formation and the development of AD.

Again, different people will have different reactivities, and it would be a mistake to make blanket assumptions one way or another about which proteins, molecules and factors are involved in one person's pathophysiology of Alzheimer's disease. It is therefore reasonable to measure antibodies against these neural antigens, BBB proteins, nerve growth factors, enteric nerve, VIP and mTG in asymptomatic individuals and patients with AD alike in order to suggest treatment modalities tailored to the individuals that may help delay progression or even provide remission in patients with AD. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the specific antigens so that the appropriate treatment can be applied.

Pathogens

Pathogens, from chronic infections or from latent/reactivating organisms, have been identified in the pathogenesis of AD. The ones of note include, Oral Pathogens (*Streptococcus mutans* and *Streptococcus sanguinis*), *Enterococcus faecalis* (*E. faecalis*), *Escherichia coli* (*E. coli*) CDT, *Salmonella* CDT, *Campylobacter jejuni* (*C. jejuni*) CDT, and Herpes Virus Type-1.

As discussed and illustrated above, individuals with chronic or latent/reactivating infections have more Aβ upregulation. Aβ has an anti-microbial property and is thus called into action during infection. As the person ages, more build-up of Aβ in circulation and increased BBB occur. With the BBB open, cross-reactive pathogen antibodies and excess Aβ can enter the brain and nervous system.

Furthermore, due to sequence homology between pathogen antigens and amyloid proteins, the production of cross-reactive antibodies may be the cause of the deposition of Aβ and neurofibrillary tangle formation.[reviewed in 82] In this situation, the immune system makes antibodies against the pathogen antigens, which then bind to Aβ peptide. The binding inhibits the anti-microbial activity of Aβ, rendering it ineffective against the pathogen. The pathogen can evade immune attack and the bound Aβ can contribute to plaque formation (Figure 11).

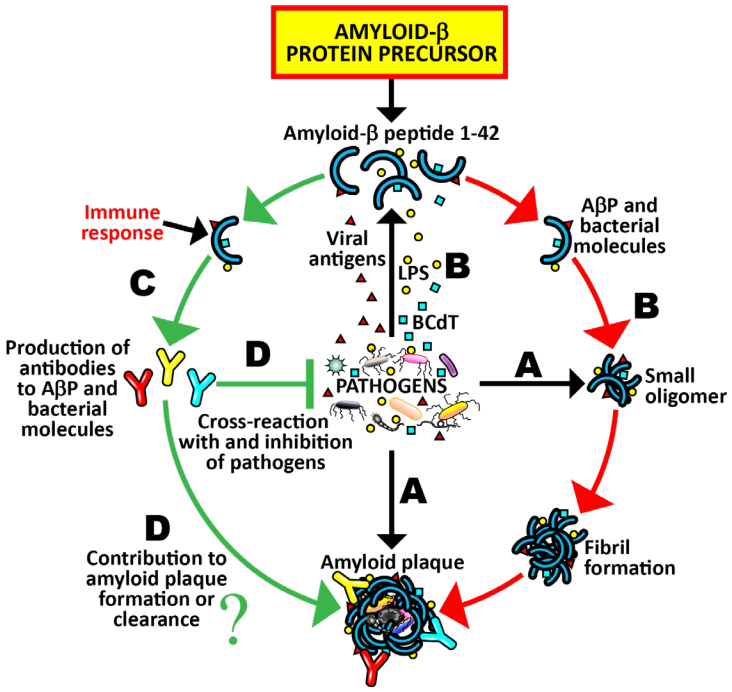


Figure 11. The Pathogenic and Protective Effects of Binding AβPs and Pathogens. *A) Pathogens can contribute directly to amyloid plaque formation. B) They can also release toxins and antigenic molecules that bind with AβPs, forming small oligomers, then fibril formation, and finally amyloid plaques. C) Antibodies produced as an immune response against these pathogens and their molecules may also bind with AβPs and enhance plaque formation, or D) possibly fulfill their original purpose by inhibiting pathogens and attacking the plaques.*

Oral Pathogens: *Streptococcus sanguinis* (*S. sanguinis*) is a commensal organism found in a healthy mouth. During dental work, *S. sanguinis* may gain entrance to the bloodstream. A gram-positive bacterium, *Streptococcus mutans* (*S. mutans*) is a known oral cavity pathogen. It may play a role in dental caries.

Enterococcus faecalis: *E. faecalis* are gram-positive anaerobic cocci. They are ubiquitous organisms present in dairy and fermented food products, natural environments (i.e. plants, soil and water bodies), and the gastrointestinal tract of humans and other mammals. It is a common hospital-acquired infection. *E. faecalis* can survive and persist in a broad range of environments, such as pH, temperature, hyper- and hypotonic conditions.

Escherichia coli CDT + Salmonella CDT: *Escherichia coli* (*E. coli*) and *Salmonella* are bacteria in the gastrointestinal tract. These bacteria release a cytotoxin called cytolethal distending toxin (CDT). CDTs can infiltrate intestinal epithelial cells, damage tissue proteins, attack the cell's nucleus and contribute to a breakdown of the intestinal barrier.

Campylobacter jejuni CDT: *C. jejuni* CDTs are endotoxins released by the gut pathogen. Non-pathogenic CdtA is utilized by pathogenic CdtB to infiltrate intestinal epithelial cells. Inside the cell, CdtB contributes to cytoskeletal damage, which may induce apoptosis (cell death). CdtB is the first bacterial toxin known to act in the nucleus of a target cell.

Herpes Type-1: Herpes Simples Virus-1 (HSV-1) is classified in the α-herpes virus group of the Herpesviridae, together with HSV-2 and varicella-zoster virus. HSV-1 has been found in brain of AD,[94] while Vojdani and Vojdani found cross-reactivity between HSV-1 and $A\beta_{42}$.[5] Herpes virus has been shown to cross-react with synuclein.[95] Indeed, Porcellini *et al*.[96] found a genetic signature for increase risk of having herpes virus in the brain. The genes involved are APP, APOE, CR-1, CLU ad PICALM. They found that virus reactivation becomes more frequent as the host ages and in these individuals, the microorganisms are more likely to induce a limited, segmental and chronic, sub-clinical pseudo-encephalitis resulting in excellerated neurodegeneration.[96]

Intestinal microflora takes part in bidirectional communication between the gut and the brain. Indeed, studies suggest that gut microbiota is associated with neuropsychiatric disorders, such as Parkinson's disease, amyotrophic lateral sclerosis, and depression; and more recent work shows the differences between the microbiota of AD patients and healthy controls.[97,98] Provasi *et al*.[97] found elevations in Phylum *Firmicutes* in AD patients compared to controls, while there was a decrease of *Bacteroidetes* in AD patients compared to healthy controls.[97] Furthermore, serum LPS levels in the AD group were much higher. Although microbial communities are generally stable, they can be altered by stress, alcohol binging, poor diet and other common occurrences. Enteric bacteria, commensal and pathogenic microorganisms may have a major impact on the immune system, brain development, and behavior, as they are able to produce several neurotransmitters and neuromodulators like serotonin, kynurenine, catecholamine, *etc*., as well as amyloids.[reviewed in 109] However, as described above, when dysbiosis occurs, a cascade of destruction can ensue. It is strongly postulated that AD may begin in the gut, and is closely related to the imbalance of gut microbiota.[97-101] This is a promising area for therapeutic intervention. Ton *et al*.[100] reviewed studies in which nutraceutical supplementations were used to improve the microbiome and reduce inflammation. Substances studied included resveratrol, vitamin D, kefir, antioxidant flavonoids, *Lactobacillus*, vitamin A and vitamin C, omega-3 PUFA.

Pathogens can have a role in the pathogenesis of AD. Figure 12 illustrates some of the pathogens that have been shown to share homology with amyloid-β. As thoroughly described above, this shared homology may lead to cognitive decline.

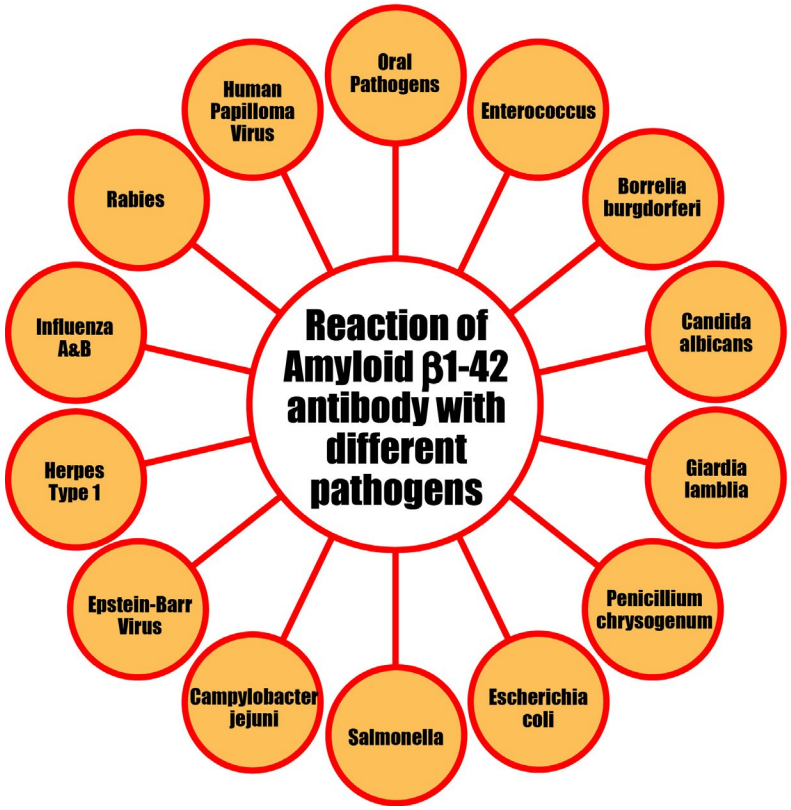


Figure 12. Pathogens associated with Alzheimer's disease with which Aβ42 shares homology or molecular mimicry. *Not everyone will harbor these pathogens, and even those that do, may not develop AD. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the culpable factors so that the appropriate treatment can be applied.*

Chemicals

Chemicals have long been considered detrimental to brain health, most notably Aluminum, which has been found in the nuclei of neurons in the brains of patients with AD.[51] Alzheimer's LINX™ expands the list to also include Dinitrophenyl, Ethyl Mercury, Methyl Mercury and Phthalates. Toxic chemicals or their metabolites can directly get into the brain and cause damage to the neurons. They can also combine with human proteins to form neoantigens and protein misfolding that mimics amyloid-beta, so that antibodies made against one will also attack the other, leading to neurodegeneration. The Aβ autoantibodies found in patients with AD may be produced due to body burden of chemicals.

Some common chemicals could bind to human tissue proteins and peptides, causing an immune reaction against the bound self-tissue.[7] Some chemicals can change the structure of human proteins, causing them to misfold into a structure similar to amyloid peptide; consequently, the antibodies made against these chemicals, especially the ones made against aluminum bound to human tissue, bind to Aβ peptide and proteins, contributing to the proteins' aggregation.[7] In their cross-reactivity study, Vojdani and Vojdani found that monoclonal anti-$A\beta_{42}$ reacted from moderately to strongly with several chemicals bound to

human serum albumin (HSA), but not to many other chemicals bound to HSA, nor to HSA alone.[7] The common culprit, aluminum, is used in multiple, everyday products, including deodorant, candy-coloring, cheese and cooking utensils, that it is ubiquitous. Over time, a build-up in the body can occur, putting the elderly at risk for neurodegenerative disorders. Aluminum accumulation in the brain affects the memory and cognition, alters synaptic activity, activates microglia, and promotes β-amyloid and neurofilament aggregation.[48]

As shown in Figure 13, a breach of the BBB allows for the entry of chemicals into the brain. The chemicals themselves, or the antibodies generated against chemicals bound to human tissue, can form neo-antigens within the brain. Direct binding of the chemical to an $A\beta_{42}$ monomer or to antibodies produced against chemicals bound to tissue antigens results in a reaction with $A\beta_{42}$ peptide, inducing amyloid fibril formation.[7]

Aluminums: Aluminum or aluminium is a chemical element with the symbol Al and atomic number 13. A ductile metal in the boron group, it is silvery-white, soft and nonmagnetic. Aluminum is the third most abundant element after oxygen and silicon and the most abundant metal in the Earth's crust. It is used in multiple, everyday products, including deodorant, candy-coloring, cheese and cooking utensils, so much so that it is practically ubiquitous. Over time, a build-up in the body can occur, putting the elderly at risk for neurodegenerative disorders. Scientists have shown that after crossing the body's protective barriers, aluminum colocalizes in the nuclei of nerve cells in the brains of patients with AD.[51] Aluminum accumulation in the brain affects the memory and cognition, alters synaptic activity, activates microglia, and promotes amyloid-β and neurofilament aggregation.[48] De Chambrun *et al*. found through oral administration in mice that aluminum enhanced inflammation and decreased mucosal healing in murine models of experimental colitis.[47] Colocalization of aluminum and iron in the nuclei of nerve cells in the brains of patients with AD might induce oxidative damage, inhibit the repair of said damage, and eventually lead to neurodegeneration and the development of AD.[51] Aluminum can also cross-link amino acids with $A\beta P\text{-}_{42}$, accelerating $A\beta P\text{-}_{42}$ monomer aggregation to form dense senile plaques.[48] Detection of antibody against aluminum bound to HSA in a patient with AD may indicate the involvement of this metal in the pathogenesis of AD.

Dinitrophenyl: Dinitrophenyl (DNP) is a chemical compound containing nitro functional groups attached to a benzene ring, which includes compounds such as phenol, toluene and more. Having biological actions, DNP is used in insecticides, ovicides, acaricides, fungicides, herbicides, some medical therapies, as haptens in some vaccine preparations, and many other products.[102,103] DNP by itself does not induce an immune response, but it can bind to body proteins and form neo-antigens which can elicit an immune response, resulting in the production of antibodies against both DNP and the human tissue proteins to which it bonded. As has been shown recently[6,7] the reaction of antibody made against $A\beta_{42}$ with DNP-HSA may indicate involvement of DNP in the pathogenesis of AD if these antibodies are detected in the blood.

Ethyl + Methyl Mercury: Mercury (Hg) is a heavy chemical element that is emitted into the air by human activities, such as manufacturing or burning coal for fuel, and from natural sources, such as volcanoes. It is deposited, via ecosystem transport, into lakes and oceans, where it bioaccumulates in fish. According to the US EPA, "nearly all methylmercury exposures in the U.S. occur through eating fish and shellfish."[104] In addition, mercury is used in thermometers, barometers, float valves, mercury switches, and other devices where exposure can occur with device breakage. It is also found in dental amalgams, energy-efficient light

bulbs, and is used in scientific research applications. Researchers have used animal models to elucidate the mechanisms by which exposure to mercury can lead to immune activation, loss of self-tolerance and autoimmune disease.[105] Reaction of anti-$A\beta_{42}$ antibody with mercury bound to HSA as shown by Vojdani may indicate the involvement of mercury in the development of autoimmunity against amyloid-β in AD.[6,7]

Phthalates: Long-chain, high molecular weight phthalates such as Diisononyl Phthalates (DINP), Diisodecyl Phthalates (DIDP) and Dipropyl Phenyl, or Di-n-propyl, Phthalates (DnPP) are commonly used to give flexibility in polyvinyl chloride (PVC) plastics. Considerable amounts of phthalates are found in consumer products such as construction materials, electrical wires and cables, automotive parts, clothing, and furniture. Many food and drink containers have phthalates in them and thus, the chemicals may leach into the food or drink product. In some individuals, phthalates may bind to human tissue, forming neoantigens and thus resulting in the formation of antibodies against both the phthalates and the human tissue. Antibody made against $A\beta_{42}$ was shown to react with phthalate bound to HSA. This indicates the possible involvement of phthalates in the pathogenesis of AD in individuals with elevated levels of phthalate antibodies.

Foods Cross-Reactive to Amyloid-Beta

No single researcher has done more work on cross-reactivity than Aristo Vojdani. In his study on the cross-reactivity of over 200 food proteins with Aβ, he found significant cross-reactivity with the following: Egg Yolk, Lentil Lectin, Pea Lectin, Canned Tuna (and to a lesser extent raw Tuna), Hazelnut Vicilin, Cashew Vicilin, Scallops, Squid, cow's milk Caseins, wheat α-Gliadin, Gliadin Toxic Peptide and Non-Gluten Wheat Proteins (globulins and amylase).[7]

Antibodies made against the above food proteins may immunologically impact Aβ protein oligomerization. Only these specific food antigens reacted with amyloid-beta antibody. This reaction can lead to a breakdown of oral tolerance which will cause toxic environmental triggers such as food peptides to cross the intestinal barrier. These toxic antigens can remain harmless in the circulation, but if the BBB becomes compromised then these food antigens can come into contact with Amyloid-Beta and contribute to amyloid fibril and amyloid plaque formation. The removal of these foods from the diet, especially for individuals with antibodies made against them, provides a therapeutic opportunity, during the early stages of cognitive decline. By eliminating these foods from the diet, a possible trigger or exacerbator can no longer fuel the AD pathogenesis. Figure 13 illustrates the effect of foods and chemicals on amyloid plaque formation.

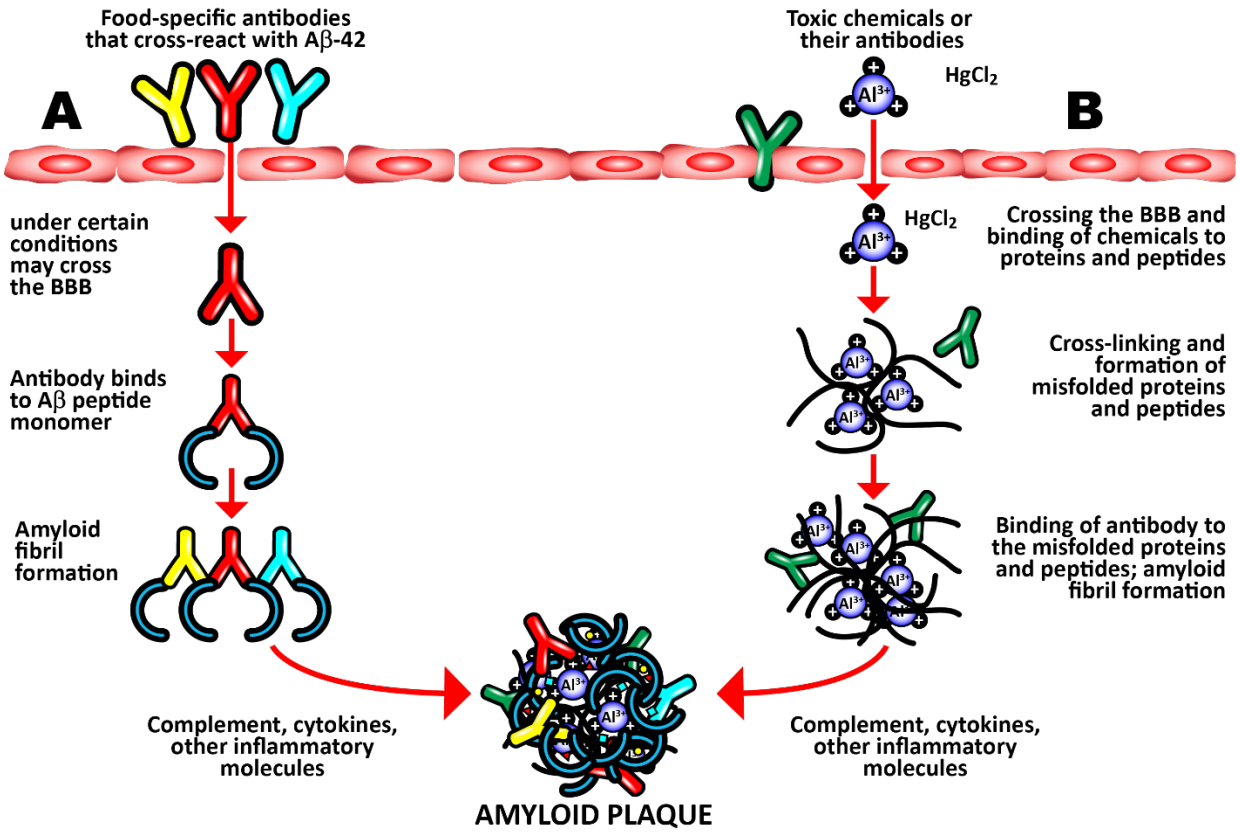


Figure 13. The Synergistic Effect of Food-Specific Antibodies and Toxic Chemicals on Amyloid Plaque Formation. *Toxic chemicals such as aluminum and mercury and food antibodies that cross-react with $A\beta_{42}$ under certain conditions may penetrate the blood-brain barrier (BBB) and contribute to neurotoxic effects. A) After crossing the BBB, $A\beta_{42}$ cross-reactive food antibodies bind to an Aβ peptide monomer and also induce amyloid fibril formation. The formation of immune complexes, release of cytokines, complement cascade activation, and other inflammatory factors further promote amyloid plaque aggregation, which contributes to AD neuropathology. B) Direct binding of toxic chemicals to different proteins and peptides results in the formation of misfolded proteins, which in conjunction with the chemical antibody also binding to the misfolded proteins contributes to protein or peptide fibril formation.*

Blood-Brain Barrier and Neurofilaments

As discussed above, researchers have identified the BBB is the protective shield against the onset of cognitive decline. Once this barrier is broken, which has been seen in patients in the early stage of cognitive decline, the neurodegeneration can occur quickly. BBB proteins, Claudin-5, Aquaporins and Neurofilament Proteins are major proteins involved in the maintenance of the BBB. This is why measurement of antibodies against these proteins is an important component of Alzheimer's LINX™.

Blood-Brain Barrier Protein: The blood-brain barrier (BBB) is a physical barrier formed by the arrangement of endothelial cells and tight junctions that line the capillaries which supply blood to the brain. It is a highly selective barrier that restricts the movement of molecules from the blood across to the brain. The BBB naturally permits the passage of essential metabolites, small hydrophobic (lipid soluble)

molecules like oxygen, carbon dioxide, hormones, *etc*. Acting like a filter, the BBB prevents the entry of infectious agents, toxins and other macromolecules into the nervous system. If antibodies against BBB proteins are detected, it may indicate a broken BBB. S100B is a specific BBB protein expressed primarily by astrocytes, acting as a neurotrophic factor and neuronal survival protein.

Claudin-5: Claudin-5 is a major cell adhesion molecule of tight junctions in brain endothelial cells. Claudin works in conjunction with other tight junction structures such as occludin, zonulin and junctional adhesion molecules to tie endothelial cells together, forming the BBB. By allowing only the entry of necessary nutrients and stopping macromolecules, tight junctions ensure the prevention of antigen invasion into the brain and nervous system. Antibodies against Claudin-5 may indicate a breakdown of BBB tight junctions.

Aquaporins: Aquaporin is the predominant water channel protein in the human brain. Aquaporin-4 (AQP4) is abundantly expressed in the end-feet of astrocytes supporting the blood vessels of the blood–brain barrier (BBB) and in astrocytic processes in contact with synapses. There are four plant aquaporins that contribute to the generation of antibodies targeting human AQP4; corn, spinach, soy, and tomato. The function of AQP4 is to ensure bi-directional cerebral water balance and removal of harmful substances, and thus, a dysfunction of AQP4 has implications in neuropathological disorders, including brain edema, stroke, and head injuries. Detection of antibody against human AQP4 may not only indicate a breakdown in the BBB and activation of the astrocytes, but may justify removal of aquaporin-containing foods from the patient's diet.

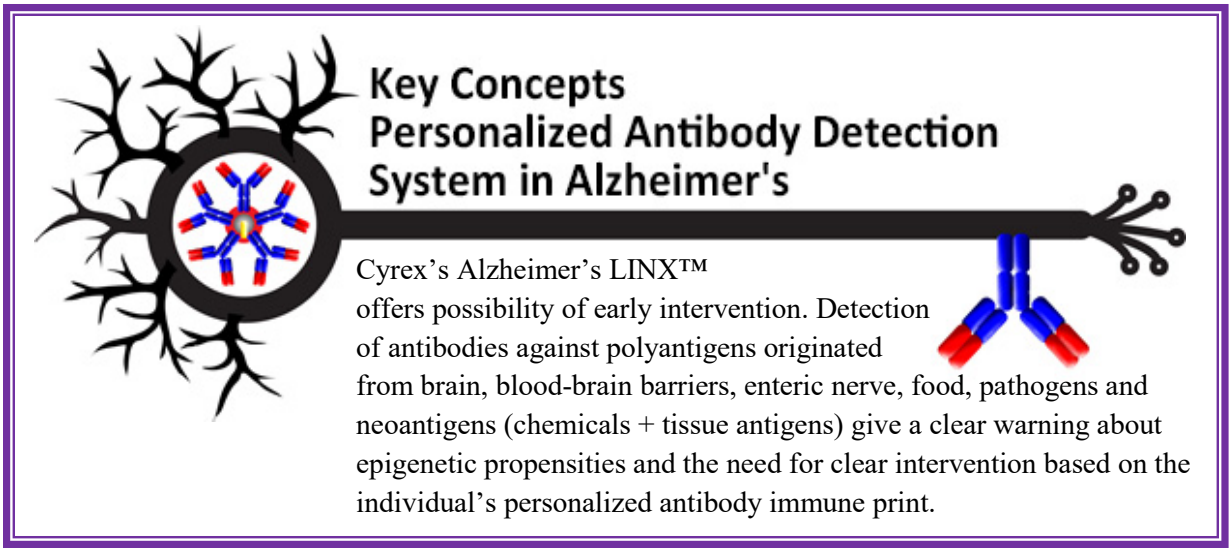
Neurofilament Proteins: Neurofilaments (NFs) are protein polymers. Along with microtubules and microfilaments, NFs form the neuronal cytoskeleton. Their primary function is to provide structural support for axons and to regulate axon diameter, which influences nerve conduction velocity.

Not everyone will react to all these proteins simultaneously. It is therefore reasonable to measure antibodies against these proteins in both asymptomatic individuals and patients with AD in order to suggest treatment modalities tailored to the individuals that may help delay progression or even provide remission in patients with AD. This is where the Alzheimer's LINX™ can be useful, since one patient may react to BDNF, another to β-NGF, and a third may react to all of these antigens. The antibody immune print can therefore guide practitioners in taking preventive measures and removing the triggers that are responsible for the production of these antibodies.

Factors that affect the BBB

- Bacterial toxins, LPS, CDT
- Pathogens such as herpes-1, *Borellia burgdorferi*
- Toxic chemicals such as heavy metals, aluminum, pesticides and more
- Some food antigens such as glutens, lectins
- Inflammatory cytokines such as TNF-α, IL-6, NF-κB, IL-1
- Traumatic brain injury
- Ischemic stroke
- Advanced glycation end products
- Drugs
- Stress

In summary, we have shown that each individual has a unique physiology, genetic makeup, microbiome composition, and immune system. Not everyone will react immunologically to the same pathogens, foods and toxic chemicals. It is therefore reasonable to measure antibodies against these environmental triggers in both asymptomatic individuals and patients with AD and classify them based on their antibody immune print. Each unique immune print can suggest treatment modalities tailored to the individuals that may help delay progression or even provide remission in patients with AD. This is where the Alzheimer's LINX™ can be useful, as it can use a patient's immune print to identify the specific antigens so that the appropriate treatment can be applied.



CLINICAL APPLICATION OF ASSESSING ALZHEIMER'S LINX™ ALZHEIMER'S-ASSOCIATED IMMUNE REACTIVITY

Alzheimer's LINX™ - Alzheimer's-Associated Immune Reactivity is all about the individual patient and customizing an intervention plan specific to the patient's immune reactivity presentation. Although genes play a role in the pathogenesis of AD, there are several environmental risk factors that are modifiable. This array helps to identify the foods, chemicals and pathogens that may be playing a role in the destruction of neuronal tissues. Autoantibodies to self-tissues and growth factors can be identified, and problems with the regeneration of neuronal tissues can be uncovered. Detailed information obtained from Alzheimer's LINX™ allows the practitioner to effectively combat the pathogenesis of AD and other cognitive impairments. Like many other spectrum disorders, there is no one disease pattern. Instead, practitioners are faced with a new puzzle with each individual patient.

Clinical Uses

Measurement of Alzheimer's LINX™ can be used to identify potential triggers or exacerbations of neurodegenerative disorders resulting in AD, MCI or dementia. For patients with a family history of neurodegenerative disorders, who wish to prevent it in themselves, Alzheimer's LINX™ can detect early warning signs, so that lifestyle modifications may stop the pathogenesis of the disorder. For patients exhibiting the first signs of cognitive decline, this array can be used to identify the individual's unique pattern of pathogenesis so that targeted therapy and lifestyle modifications can be made quickly. If implemented in time, recovery of cognitive decline is possible. For patients already diagnosed with AD, MCI or dementia, Alzheimer's LINX™ can pinpoint triggers that may be fueling the process of decline. By removing these triggers and healing the barriers, the pathogenesis of disease can be slowed down and the quality of life for the patient improved. The clinical uses of Alzheimer's LINX™ include:

- Identify asymptomatic individuals at greater risk for developing Alzheimer's disease or other neurological disorder.
- Identify reactivity to triggers of Alzheimer's disease or other neurological disorders.
- Identify the early stages of neurodegenerative processes.
- Monitor the effectiveness of lifestyle modifications for Alzheimer's disease.

Recommended For Certain Patients

The development of neuroautoimmunity requires three ingredients: genetic predisposition, environmental triggers and increased blood-brain barrier permeability. BBB integrity plays a vital role in the overall health and well-being of the brain and nervous system. Those with a family history of neurodegeneration resulting in cognitive decline should be assessed regularly. In addition to people presenting with BBB permeability, patients who tested positive to the chemicals (Aluminum, Dinitrophenyl, Mercury or Phthalates), foods (Egg Yolk, Lentil or Pea Lectins, Canned Tuna, Hazelnut, Cashew, Scallops, Squid, Casein, Gliadin, Non-Gluten Proteins, Tomato, Spinach, Corn or Soy) and/or pathogens (*Streptococcus, E. faecalis, E. coli, Salmonella, C. jejuni* or Herpes Type-1) should be assessed using the full Alzheimer's LINX™. Measurement of Alzheimer's-Associated Immune Reactivity is recommended for patients who:

- Are interested in preventing the development of Alzheimer's disease or other neurological disorder.
- Are exhibiting early signs of Alzheimer's disease or other neurological disorder.
- Have been diagnosed with Alzheimer's disease.
- Have a history of gastrointestinal disorders and/or diabetes.
- Played high-impact sports.
- Are immunoreactive against specific pathogens, chemicals and foods.

CLINICAL INTERPRETATION FOR ALZHEIMER'S LINX™ ALZHEIMER'S-ASSOCIATED IMMUNE REACTIVITY

Interpretation of elevated level of IgG antibodies against Alzheimer's LINX™ is shown in Table 1.

Table 1. General Interpretation of Alzheimer's LINX™. *Below are evaluations of IgG antibodies against Alzheimer's LINX™ Alzheimer's-Associated Immune Reactivity and related clinical correlation. Alzheimer's LINX™ test results alone are not diagnostic for any disease. For detailed interpretation, please call customer service to schedule a telephone appointment with one of our clinical consultants.*

Positive Result	Notes	Considerations
BRAIN PROTEINS		
Tau Protein	Increased risk for cognitive decline, or result of traumatic brain injury (TBI)	• Immune modulation • Protection of the BBB Management of neuroinflammation Enhancement of neuroplasticity
Amyloid-Beta Peptide	Increased risk for cognitive decline	
Rabaptin-5 + Presenilin	Possible disruption of endocytosis and increased risk for cognitive decline	
Alpha-Synuclein	Possible lack of neuroplasticity and increase risk for neurodegeneration	
GROWTH FACTORS		
Beta Nerve Growth Factor	Possible lack of neuronal regeneration and increased risk for neurodegenerative disorders	• Lifestyle modification • Immune Modulation • Protection of the BBB
Brain Derived Neurotrophic Factor	Possible lack of protection for neuronal cells and increased risk for multiple neurological disorders	
Neurotrophins	Possible inability to protect and generate growth of neuronal cells and increased risk for neurological disorders	
Somatotropin	Possible deficiency of growth hormone	
ENTERIC NERVE, ENZYMES AND NEUROLOGICAL PEPTIDES		
Enteric Nerve + Vasoactive Intestinal Peptide	Increased risk for inflammatory enteric neuropathy and bowel motility dysfunctions	• Mucosal immune modulation • Microbiome balance
Transglutaminases	Increased risk for intestinal, skin and brain disorders	

PATHOGENS		
Oral Pathogens	Increased risk for Alzheimer's disease, cardiovascular and arthritic autoimmunity	• Improvement of oral hygiene • Reducing exposure to pathogens and their antigens • Immune (Boost/modulation)
Enterococcus faecalis	Increased risk for Alzheimer's disease and inflammatory bowel disease	• Microbiome balance • Reducing exposure to pathogens and their antigens • Immune (Boost/modulation)
Escherichia coli CDT + Salmonella CDT	Increased risk for Alzheimer's disease, intestinal disorders and breakdown of the blood-brain barrier (BBB)	• Microbiome balance • Reducing exposure to pathogens and their antigens • Immune (Boost/modulation) • Protection of the BBB • Management of neuroinflammation
Campylobacter jejuni CDT	Increased risk for Alzheimer's disease, intestinal disorders and breakdown of the blood-brain barrier (BBB)	
Herpes Type-1	Increased risk for Alzheimer's disease, and cognitive decline	• Lifestyle (stress management, sleep balance) • Immune boost/modulation • Protection of the BBB
CHEMICALS		
Aluminums	Body burden of aluminum and its contribution to Alzheimer's disease	• Reduction of environmental exposure • Immune modulation • Enhancement of liver detoxification • Protection of the BBB
Dinitrophenyl	Body burden of dinitrophenyl and its contribution to Alzheimer's disease	
Ethyl + Methyl Mercury	Body burden of mercury and its contribution to Alzheimer's disease	
Phthalates	Body burden of phthalates and their contribution to Alzheimer's disease	
FOODS CROSS-REACTIVE TO AMYLOID BETA		
Egg Yolk, raw + cooked	Immune reactivity to egg yolk and its cross-reaction with $A\beta_{42}$	• Dietary management • Mucosal immune modulation • Support of digestive tract • Restore oral tolerance • Support of regulatory T cells (Tregs)
Lentil Lectin + Pea Lectin	Immune reactivity to lentil and pea lectins and their cross-reaction with $A\beta_{42}$	
Tuna, canned	Immune reactivity to canned tuna and its cross-reaction with $A\beta_{42}$	
Hazelnut Vicilin + Cashew Vicilin	Immune reactivity to hazelnut and cashew vicilin and their cross-reaction with $A\beta_{42}$	
Scallops + Squid	Immune reactivity to scallops and squid and their cross-reaction with $A\beta_{42}$	
Caseins	Immune reactivity to cow's milk caseins and their cross-reaction with $A\beta_{42}$	
Alpha-Gliadin + Gliadin Toxic Peptide	Immune reactivity to wheat gliadin, its cross-reaction with $A\beta_{42}$, and the opening/destruction of intestinal tight junctions	
Non-Gluten Wheat Proteins	Immune reactivity to non-gluten wheat proteins and their cross-reaction with $A\beta_{42}$	

BLOOD-BRAIN BARRIER AND NEUROFILAMENTS		
Blood-Brain Barrier Protein + Claudin-5	Possible blood-brain barrier (BBB) breakdown of endothelial cells and tight junctions	• Immune support • Management of Inflammation • Management of Neuroinflammation • Protection of the BBB
Aquaporins	Possible blood-brain barrier (BBB) breakdown targeting astrocytes or food immune reactivity to corn, soy, spinach, tomato aquaporin	• Dietary management • Immune Support • Management of Inflammation • Management of Neuroinflammation • Protection of the BBB
Neurofilament Proteins	Possible axonal injury and neurodegeneration	• Immune Support • Management of Inflammation • Management of Neuroinflammation • Protection of the BBB

SPECIMEN REQUIREMENT

2 mL serum
Ambient

RELATED TESTING

- **Antibody Array 2 - Intestinal Antigenic Permeability Screen (Serum)**
- **Antibody Array 3X - Wheat/Gluten Proteome Reactivity and Autoimmunity (Serum)**
- **Antibody Array 4 - Gluten-Associated Cross-Reactive Foods and Foods Sensitivity (Serum)**
- **Antibody Array 10 - Multiple Food Immune Reactivity Screen (Serum)**
- **Antibody Array 11 - Multiple Chemical Immune Reactivity Screen (Serum)**
- **Antibody Array 12 - Pathogen-Associated Immune Reactivity Screen (Serum)**
- **Antibody Array 5 - Systemic Autoimmune Reactivity Screen (Serum)**
- **Antibody Array 7/7X - Neurological Autoimmune Reactivity Screen (Serum)**
- **Antibody Array 20 - Blood-Brain Barrier Permeability (Serum)**
- **Antibody Array 22 - Irritable Bowel / SIBO Screen (Serum)**

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