Cognitive Vitality Reports® are reports written by neuroscientists at the Alzheimer's Drug Discovery Foundation (ADDF). These scientific reports include analysis of drugs, drugs-in-development, drug targets, supplements, nutraceuticals, food/drink, non-pharmacologic interventions, and risk factors. Neuroscientists evaluate the potential benefit (or harm) for brain health, as well as for age-related health concerns that can affect brain health (e.g., cardiovascular diseases, cancers, diabetes/metabolic syndrome). In addition, these reports include evaluation of safety data, from clinical trials if available, and from preclinical models.

TLR4 Inhibitors

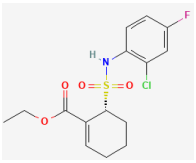
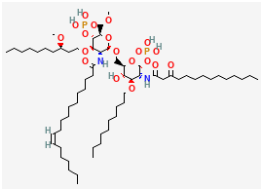
Evidence Summary

Elevated TLR4 signaling is implicated in a variety of age-related diseases, but clinical efforts to inhibit it in disease have not led to benefit, implying it may be necessary to prevent TLR4 dysregulation during aging.

Neuroprotective Benefit: The dysregulation of TLR4 signaling may drive chronic inflammation associated with cognitive aging and Alzheimer's disease, but inhibitors may be less effective in the context of an elderly immune system.

Aging and related health concerns: Dysregulated TLR4 signaling may impact risk for a variety of age-related diseases through induction of inflammation, but therapeutic attempts to inhibit it have not shown clinical efficacy for any condition, thus far.

Safety: TLR4 inhibitors may increase infection risk, but this has not been seen in clinical trials tested thus far, perhaps due to short duration of treatment and/or testing in older participants with weaker baseline TLR4-mediated immunity.

Availability: In clinical trials	**Dose**: Effective doses have not been established. All tested drugs have been administered i.v.	TAK-242 **Chemical formula:** $C_{15}H_{17}ClFNO_4S$ **MW**: 361.8 g/mol  Source: PubChem Eritoran **Chemical formula**: $C_{66}H_{126}N2O_{19}P_2$ **MW**: 1313.7 g/mol  Source: PubChem
Half-life: Eritoran 30-50 hours N1-0101 6.4 days	**BBB**: TAK-242 is penetrant	
Clinical trials: Eritoran has been tested in a Phase 3 RCT in sepsis (n=1961), TAK-242 has been tested in a Phase 3 RCT in sepsis (n=274), and NI-0101 has been tested in a Phase 2 RCT in rheumatoid arthritis (n=90)	**Observational studies**: Genetic variants in TLR4 are associated with a variety of aging-related diseases, but associations vary in different ethnic populations.	

What is it?

Toll-like receptor 4 (TLR4) is a pattern recognition receptor involved in innate immunity. It is expressed on myeloid cells, and can induce different inflammatory signaling cascades depending on the presence of adaptor proteins [1]. TLR4 is involved in the activation of the innate immune system in response to pathogens, and is particularly important for the response to gram negative bacteria. The best characterized signaling pathway is in response to the gram negative bacterial derived endotoxin, lipopolysaccharide (LPS). LPS triggers activation of TLR4 via the CD14-TLR4-MD2 receptor complex. Coupling to the MyD88-dependent pathway results in NF-κB activation and the induction of proinflammatory cytokines, while coupling to the TRIF-dependent pathway activates IRF3 and induces the production of type 1 interferons. The overall immune response downstream of TLR4 activation is influenced by the overall immune environment, such as the presence of interacting surface receptors and intracellular adaptors, as well as presence and activation status of other pattern recognition receptors, including other TLRs. Several of the TLRs have overlapping activation patterns such that loss of one may be compensated by upregulation of a related TLR. This may explain why targeting TLR4 alone has failed to show consistent clinical benefit in the studies conducted thus far.

Eritoran is an analog of the lipopolysaccharide lipid A, and acts as an antagonist of TLR4 by competitively binding to the ligand site and blocking activation of the receptor by other ligands [2]. It is being developed by Eisai Co. It was tested in a Phase 3 RCT for sepsis, but failed to meet its primary endpoint.
TAK-242, also known as resatorvid, is a small molecule inhibitor of TLR4. It disrupts the interaction of TLR4 with adaptor proteins by binding at Cys747, and thus inhibits the downstream MyD88 and TRIF-dependent signaling pathways [3]. It failed to show benefit in a Phase 3 RCT in sepsis. It was developed by Takeda Pharmaceuticals. In partnership with Takeda and Yaqrit, Akaza Bioscience is developing TAK-242 for acute alcoholic hepatitis.
NI-0101, also known as EB05, is a humanized immunoglobulin G1K monoclonal antibody that binds TLR4 and prevents signal transduction [4]. Its activity is dependent on FcyRII. It was developed by Novimmune SA. It failed to show efficacy in a Phase 2 RCT in rheumatoid arthritis. It has been licensed by Edesa Biotech in 2020, and is being tested in Covid-19.

The drugs tested thus far act by broadly inhibiting the activation of the TLR4 receptor. Selective disruption of the particular interactions (i.e. the formation of specific signaling complexes) which are known to mediate the deleterious inflammatory signaling in a given condition is expected to be a better therapeutic strategy, but to date, this type of TLR4-targeted therapeutic has not been tested.

Neuroprotective Benefit: The dysregulation of TLR4 signaling may drive chronic inflammation associated with cognitive aging and Alzheimer's disease, but inhibitors may be less effective in the context of an elderly immune system.

Types of evidence:

- 3 biomarker studies of TLR4 expression/activity
- 4 gene association studies in Alzheimer's disease
- 1 gene association study in Huntington's disease
- Numerous laboratory studies

Human research to suggest prevention of dementia, prevention of decline, or improved cognitive function:

There is no data on the use of TLR4 inhibitors in preventing dementia, however, gene association studies provide evidence that some variants of TLR4, some of which appear to impact TLR4 function, are associated with altered risk for Alzheimer's disease.
The prevalence of particular TLR4 single nucleotide polymorphisms (SNPs) varies across ethnic groups, and a variety of disease associated TLR4 SNPs have been identified in different groups. The effect of these variants appears to be modified by the environment, including both genetic (gene-gene interactions) and regional/lifestyle-related factors (gene-environment interactions). Thus, different TLR4 SNPs are differentially associated with disease risk in different populations. The ultimate functional consequences of many of these SNPs are still unclear. The best characterized TLR4 SNPs, Asp299Gly and Thr399Ile, were originally reported to impair activation in response to the bacterial-derived TLR4 ligand, LPS, and to increase susceptibility to septic shock [5; 6]. However, various other reports have found divergent results, such that they had no effect, were associated with higher constitutive activity, or were associated with an elevated inflammatory response following activation [7; 8; 9]. These discrepancies likely stem from the context dependent nature of TLR4 signaling, such that the effect of the SNPs may differ *in vivo* vs. *ex vivo*, and may differ *in vivo* in different people depending on their genetic background and environmental effects. Unless the SNP can be clearly tied to a change in TLR4 activity or downstream inflammatory signaling, the association studies do not offer compelling evidence about how the modulation of TLR4 meaningfully impacts disease risk. Another important caveat is that these genetic variants are present throughout the life of an individual, and it is not clear during which period(s) they have the most impact on disease risk. Thus, inducing a modification to TLR4 activity consistent with one of these variants with a drug later in life/during disease manifestation may not offer comparable protection.

The coding variant in TLR4 (rs4986790) is one of the best characterized. In this variant, an aspartic acid at position 299 is replaced by a glycine (Asp299Gly) due to change in base from an A to a G (+896A>G). In some studies, this missense variant has been shown to reduce the ability of TLR4 to respond to LPS, and thus attenuates the downstream pro-inflammatory signaling stemming from TLR4 activation by at least some ligands [5]. In a Northern Italian cohort (626 AD; 190 controls), the presence of the major allele (A) (Odds ratio [OR] (OR 1.4, 95% Confidence Interval [CI] 1.01 to 3.06), or being homozygous for the major allele (AA) (OR 1.9, 95% CI 1.03 to 3.52) was associated with higher risk for AD [10]. In the Québec Founder Population cohort (384 AD; 245 controls), the TLR4 rs4986790 minor (G) allele was

found to be associated with a reduced risk of developing AD (OR 0.5615), but in postmortem brain tissue (n=44 AD cases), the allele did not influence mRNA or protein levels of TLR4 [11]. mRNA levels for *IL1b*, *IL6*, and *TNF* also did not differ with genotype. In participants from the PREVENT-AD cohort in Canada (n=136), which includes pre-symptomatic individuals with parental history of AD, the mean cortical thickness was higher in the frontal and occipital lobes of minor (G) allele carriers, which was associated with better visuospatial constructional index scores on the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS). The minor (G) allele carriers also had stable CSF IL-1β levels over time, in contrast to AA individuals, in which levels increased with age. It is hypothesized that less cumulative activation of TLR4 prevents the age-related increase in IL-1β levels, which may be indicative of lower levels of inflammation-mediated neuronal damage/loss, as evidenced by higher brain volumes and cognitive performance.

The TLR4 11367G>C polymorphism is located in the 3' untranslated region (UTR) of the *TLR4* gene, and is relatively common in the Han Chinese population (minor allele frequency 14.7%) [12]. Peripheral (blood-derived) leukocytes from healthy carriers of the C variant showed reduced induction of TLR4 surface expression and lower levels of inflammatory cytokine induction (TNFα) in response to *ex vivo* LPS stimulation, in one study [12]. In a separate study, the plasma level of TLR4 was elevated in AD patient carriers of the C variant [13]. The functional effect *in vivo* is not clear. A gene association study in Han Chinese (n=137 AD; 137 controls) found the minor (C) allele was associated with higher risk for AD in both ApoE4 carriers (OR 2.03, 95% CI 1.03 to 3.98) and in non-carriers (OR 5.77, 95% CI 3.03 to 11.00) [14].
The minor alleles of the TLR4 SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs7037117, and rs7045953) were also found to be associated with lower risk for AD in a Han Chinese population (399 AD; 386 controls), with odds ratios ranging from 0.445 to 0.637 [15]. The strength of the association was modified by the haplotype. In an ethnically Chinese cohort in Taiwan (269 AD; 499 controls), homozygosity (TT) for the rs1927907 variant (A>T) was associated with increased risk for AD (adjusted OR 2.45, 95% CI 1.30 to 4.64), but the association was only significant in ApoE4 non-carriers [16]. The functional effects of these variants have not been established.

Huntington's disease: SNPs in TLR4 (rs1927911, rs1927914) were found to be associated with changes in motor progression in individuals from the European Huntington's Disease Network Registry (n=830) [17].

Human research to suggest benefits to patients with dementia:

TLR4 inhibitors have not yet been tested in dementia patients. However, data from patient-derived tissues suggest that the function and disease contribution of TLR4 signaling varies as the disease progresses.
In postmortem brain tissue (44 AD; 44 controls), the mRNA expression of TLR4 is elevated in the frontal cortex from AD patients [18]. The TLR4 levels are positively correlated with amyloid plaque density, as well as the mRNA expression levels of IL-1β, IL-6, and TNF. In the cerebellum, which is unaffected in AD, there was no difference in TLR4 expression between AD and controls. The expression of TLR4 has also been shown to be elevated in peripheral blood mononuclear cells (PBMCs) from AD patients (n=60), relative to controls (n=60) at both the mRNA and protein levels [13]. Furthermore, TLR4 expression was inversely associated with cognition, based on MMSE score ($r = -0.29$).

The expression levels alone do not capture the impact of TLR4, and how it changes over the disease course. In the context of aging alone, the expression of TLR4 on peripheral blood cells increases resulting in higher basal production of pro-inflammatory mediators, and this is inversely associated with the ability of these cells to elicit a productive inflammatory response to a TLR4 stimulus [19]. This process of immune dysfunction appears to be exacerbated in the context of AD. A study examining the activation status of PBMCs in individuals with subjective memory complaint (n=10), amnestic mild cognitive impairment (MCI) (n=14), and mild AD (n=14) found that, relative to PBMCs from healthy elderly controls (n=15, ≥ 60 years old), monocytes from those with subjective memory complaints exhibited enhanced chemotaxis, free radical production, and cytokine production in response to TLR4 stimulation [20]. The stimulus-evoked TLR4-mediated activation of monocytes peaked during the MCI stage. During the transition from MCI to AD, there was an increase in the basal activation of TLR4-mediated pro-inflammatory signaling and cytokines (IL-1β, IL-6, and TNFα) in monocytes, along with a decreased ability to respond further to a TLR4-activating stimulus. While monocytes from healthy elderly can still increase pro-inflammatory cytokine production in response to a TLR4-activiating stimulus, monocytes from patients fail to show further increases in these cytokines and/or instead upregulate anti-inflammatory cytokines, indicative of an exhausted immune response. The ability of monocytes to appropriately respond to TLR stimuli becomes increasingly anergic as the disease progresses from MCI to AD.

This then begs the question of when the optimal time would be to target TLR4. Clinical studies in other indications suggest that targeting TLR4 once innate immune cells have been reprogrammed into an

altered state where TLR4 ligands no longer elicit reliable immune-activating responses has minimal effects on modifying disease course. This may be because the overall immune environment has changed to such a significant degree that targeting TLR4 alone cannot restore immune competency. While the low level of chronic inflammation elicited by elevated basal TLR4 activity likely contributes to neurodegeneration, it is clear from these studies that this issue is not simply increased TLR4 activity, but rather that its overall response profile is dysfunctional in the context of aging, which is then further exacerbated in disease contexts.

Mechanisms of action for neuroprotection identified from laboratory and clinical research:

TLR4 is a pattern recognition receptor which is activated in response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [1]. In response to an acute insult, the activation of TLR4 triggers an innate immune activating inflammatory response designed to rapidly clear the pathogen and damaged cells. However, overactivation of this response can exacerbate inflammation-related neuronal loss. Consequently, TLR4 inhibitors (TAK-242) show neuroprotective properties in preclinical models of acute cerebral injury by mitigating neuroinflammatory damage [21; 22; 23]. Prolonged exposure to TLR4 ligands can lead to a chronic inflammatory response, and is thought to be associated with age-related 'inflammaging' [24]. Aβ acts like a PAMP to activate TLR4, thus the accumulation of Aβ can drive prolonged TLR4 activation [24].

The functional impact of TLR4 inhibition varies in preclinical models depending on the model used, the timing of the intervention relative to the disease course, and the age of the animals. Another complicating factor is that LPS, derived from gram negative bacteria, is the most commonly used TLR4 ligand in studies, and mice are the most commonly used model animal. Mice are far less sensitive to LPS than humans, which is reflected in different innate immune responses [3]. This impacts the potential translatability of these studies, and may explain why TLR4-targeted therapies have been clinically unsuccessful thus far.

Cognitive aging: TLR4 plays a role in the modulation of some cognitive processes [25]. This results in altered brain responses in TLR4 knockout mice. These developmental effects can make it difficult to translate the brain-related phenotypes in adult knockout animals with the function and potential impact of modulating TLR4 during adulthood. Similar to what is seen in humans, TLR4 responsiveness is altered with age in mice. TLR4 expression is increased in the brain in the context of aging in mice. Relative to young mice (<7 months old), old mice (19-21 months old) are less responsive to TLR4 inhibitors with

respect to effects on cognitive parameters [25]. Additionally, there appears to be a sex effect, with females being more sensitive to TLR4 inhibition. TLR4 inhibition with the small molecule inhibitor TAK-242 (3 mg/kg i.p. for 14 days) improved performance on tests of spatial learning and memory only in young female mice, likely through attenuating the stress response. In aged mice, TAK-242 did not impact cognition or globally elevated pro-inflammatory cytokines, suggesting aged animals are less responsive to TLR4 modulation.

The level of expression of TLR4 in the brain was found to be similar between APP/PS1 AD mice and age-matched wildtype mice, but the cytokine levels differed, suggesting that the altered function of TLR4 is more relevant and/or that changes in other inflammatory mediators are more relevant drivers of disease [26]. A lifetime burden of repeated bouts of infection is hypothesized to play a role in the age-related changes in TLR4 expression and function. AD model mice (5XFAD) housed in a natural pathogen-containing environment show accelerated neuronal loss relative to those housed in a specific pathogen-free environment [27]. In young (8 weeks old) wildtype mice, neuroinflammation driven by TLR4 signaling downstream of an infection (*P. gingivalis*) impaired cognitive performance on tests of learning and memory [28]. The effect on cognition could be prevented through the administration of a TLR4 inhibitor (TAK-242) at the time of infection. Repeated activation of this TLR4 pathway may result in tolerance [29]. Additionally, TLR4 and TREM2 have an antagonistic regulatory relationship, such that frequent TLR4 activation may result in reduced TREM2-mediated neuroprotective activity [30; 31].

Alzheimer's disease: TLR4 INHIBITIORS ARE UNLIKELY TO FIX TLR4 DYSREGULATION IN AD
As a TLR4 ligand, Aβ is likely the factor that exacerbates the TLR4 tolerance, reflected in the progressive declining/altered responsiveness to TLR4 ligands in AD patients [20]. This likely contributes to the polarization of microglia into an altered inflammatory state which shows tolerance to Aβ, and hampers its clearance [29]. In the APPswe/PSEN1dE9 model, microglia from 12-month-old mice showed a reduced cytokine response to a systemic LPS stimulus [29]. The effects of TLR4 and its modulation in older mouse models are more relevant to what is seen in human AD patients, which is an elderly population, than those using younger mice. Various studies have shown either that activation of TLR4 is beneficial when Aβ first starts accumulating by stimulating a productive immune response, or that starting treatment with a TLR4 inhibitor relatively early in the disease course can ameliorate cognitive deficits [32; 33; 34; 35; 36]. However, the immune-driven responses underlying these effects are present in young animals, but are often absent in aged animals, reflective of age-related changes in TLR4 function and responsiveness.

All together these studies suggest that TLR4-targeted interventions are likely to be most effective in young adults, indicating that they may not be optimally suited to the treatment of neurodegenerative diseases in elderly individuals.

APOE4 interactions: Not established

Aging and related health concerns: Dysregulated TLR4 signaling may impact risk for a variety of age-related diseases through induction of inflammation, but therapeutic attempts to inhibit it have not shown clinical efficacy for any condition, thus far.

Types of evidence:

- 1 systematic review of preclinical and clinical studies with eritoran
- 3 meta-analyses of gene association studies with cancer
- 2 meta-analyses of observational studies assessing TLR4 expression and cancer prognosis
- 3 meta-analyses of gene association studies with age-related macular degeneration
- 2 meta-analyses of gene association studies with glaucoma
- 1 meta-analysis of gene association studies with rheumatoid arthritis
- 1 meta-analysis of gene association studies with coronary artery disease
- 1 meta-analysis of gene association studies with atherosclerosis
- 1 meta-analysis of gene association studies with type 2 diabetes
- 1 clinical trial for NI-0101 in rheumatoid arthritis
- 2 clinical trials for sepsis (eritoran and TAK-242)
- 1 gene association study of TLR4 SNP with longevity
- 1 observational study of impact of antihypertensives on TLR4 expression
- Numerous laboratory studies

Lifespan: ASSOCIATION BETWEEN TLR4 AND MORTALITY IS LIKELY CONTEXT DEPENDENT
The association between the +896A>G Asp299Gly (rs4986790) polymorphism in TLR4 and longevity was examined in a small case-control study in Italian (Sicilian) men [37]. The study included 105 young men hospitalized for acute myocardial infarction (age 20-46), an age-matched control group (n=127), and a group of 55 very old men (age 96-104). Relative to the young controls (G allele frequency 15 ± 5.9%), the Asp299Gly SNP was underrepresented in men with myocardial infarction (G allele frequency 5 ± 2.4%), and overrepresented in the very old men (16 ± 15.6%). It is unclear whether a similar association would

be seen in other populations considering the large influence of genetic background and environment on TLR4 SNP associations. This SNP has been associated with increased or decreased risk for a variety of age-related diseases in different studies, largely in a cohort (region/ethnicity)-dependent manner [38]. Consequently, there is no indication that any particular variant will necessarily be linked with overall higher or lower risk for morbidity or mortality in a given individual. The direction of the association will likely be determined by the diseases of most relevance to one's region/life (i.e. infection burden, healthcare access, nutrition access, etc.). For example, the Asp299Gly variant is associated with increased mortality due to severe sepsis, but may be associated with reduced mortality due to malaria [8]. Consequently, the prevalence of haplotypes containing Asp299Gly have decreased in Eurasian populations, but have remained in Sub Saharan African populations with a high malarial burden.

Cancer: ELEVATED TLR4 IS ASSOCIATED WITH WORSE PROGNOSIS IN MANY CANCERS

Dysregulation of TLR4 signaling is a common feature of many cancers [39]. TLRs are involved in tumor growth, and their signaling can have pro- or anti-tumor effects depending on the environment [40]. The upregulation of TLR4 in cancer tends to involve the development of an immunosuppressive tumor environment, as chronic TLR4 signaling in this context impedes the cytotoxicity of anti-tumor T cells and stimulates tolerogenic T regulatory cells. Consequently, multiple meta-analyses have found that elevated tumor expression of TLR4 is associated with worse prognosis. Meta-analyses of 24 studies (n=2,812 cancer patients) and 15 studies (n=1,294 cancer patients), found that high expression of TLR4 was associated with worse overall survival (Hazard ratio [HR] 1.29, 95% CI 1.17 to 1.42; HR 2.05, 95% CI 1.49 to 2.49, respectively) [41; 42].

TLR4 polymorphisms have also been associated with altered cancer risk. A meta-analysis of 55 studies (20,107 cases; 28,244 controls) found that the TLR4 SNP Thr399Ile (rs4986791) was associated with decreased cancer risk (OR 0.764, 95% CI 0.652 to 0.894; allele model), and the association was found in both Caucasian and Asian populations [43]. A meta-analysis of gene association studies found that the TLR4 SNP Asp299Gly (rs4986790) had a nominal association with gastric cancer [44]. A meta-analysis of studies examining TLR4 SNPs in cervical cancer found that the TLR4 SNP +1196C>T Thr399Ile (rs4986791) was associated with increased risk for cervical cancer (T vs. C allele: OR 1.34, 95% CI 1.12 to 1.62) [45]. The TLR4 SNPs -2604A>G (rs10759931) (GG genotype: OR 1.48, 95% CI 1.02 to 2.17) and +7764C>T (rs1927911) (T vs. C allele: OR 1.71, 95% CI 1.31 to 2.24) were also associated with increased risk for cervical cancer. These studies highlight how different polymorphisms may have different effects in different contexts.

In terms of cancer treatment, efforts have primarily tested TLR4 agonists as an adjuvant for anti-cancer immunotherapies in order to boost the anti-cancer immune response [46]. Bacillus Calmette-Guerin

(BCG) is used for bladder cancer, and Monophosphoryl Lipid A (MPL) is an adjuvant used in anti-cancer vaccines. TLR4 agonists have generally not been associated with clear benefit when used as monotherapies.

Sepsis: NO CLEAR BENEFIT TO TLR4 INHIBITION IN SEVERE SEPSIS
Several TLR4 inhibition-based therapies have been tested for severe sepsis, and none have shown clinically meaningful benefits, thus far [3]. The most extensively tested was eritoran tetrasodium, which is a structural analog of lipid A, a TLR4 ligand, and acts by blocking access to endogenous TLR4 ligands [2]. Preclinical models indicated that effective dosing depended on the time of drug intervention as well as the route of administration for the infectious agent. Based on the heterogeneity of efficacy in models [2], it is perhaps not surprising that clinically meaningful benefits were not achieved in clinical trials. In a Phase 3 RCT (NCT00334828) in patients with severe sepsis (n=1,961), eritoran tetrasodium (105 mg i.v. over six days) did not lead to significant improvement on its primary endpoint, 28-day all-cause mortality [47]. There were also no significant effects on cytokine levels with treatment. Eritoran was administered within 12 hours of organ dysfunction, which may have been too late to benefit patients. The small molecule TLR4 inhibitor TAK-242 (1.2 or 2.4 mg/kg/day i.v.) also failed to show benefit in a double-blind placebo-controlled RCT in 274 patients with severe sepsis (NCT00143611) [48]. The trial was terminated due to lack of efficacy on its primary pharmacodynamic measure of change in serum IL-6 levels. TAK-242 also failed to significantly improve 28-day all-cause mortality. While TLR4 is strongly implicated in the dysregulated damage-inducing inflammatory response to an infection, resulting in sepsis, these trials suggest that it likely needs to be targeted earlier in the process to prevent the induction of these inflammatory cascades, such that once they are actively damaging organs, targeting TLR4 (alone) is no longer effective.

Rheumatoid arthritis: NO CLEAR BENEFIT TO TLR4 INHIBITION IN ARTHRITIS
TLR4 is implicated in the pathogenesis of rheumatoid arthritis (RA) because the autoantibodies associated with RA, anti-citrullinated protein antibodies (ACPAs), form immune complexes with citrullinated proteins and bind to TLR4 [4]. However, a meta-analysis of 14 studies found that there were no overall significant associations between the two best characterized TLR4 SNPs, Asp299Gly (rs4986790) (based on 4,554 cases and 6,449 controls) and Thr399Ile (rs4986791) (based on 1,501 cases and 2,138 controls) [49]. When stratified by ethnicity, a reduced risk for the Asp299Gly SNP (OR 0.73) was seen in a Spanish population. Modulation of TLR4 has also failed to impact clinical disease progression. In a double-blind, placebo-controlled Phase 2 RCT (NCT03241108) (n=86), the monoclonal antibody NI-0101, which blocks the activation of TLR4, failed to significantly improve disease activity

score measures, relative to placebo [4]. Biomarkers of inflammation, such as IL-6, IL-1β, and TNFα, were also not significantly impacted with treatment. Together these studies suggest that blocking the TLR4 pathway alone is not sufficient for disease modification. TLR4 may not be the optimal target in the inflammatory cascade and/or due to compensation or redundancy, multiple components would need to be targeted to significantly dampen inflammation-mediated tissue damage.

Coronary artery disease: TLR4 MAY PROMOTE VASCULAR INFLAMMATION

TLR4 is implicated in the pathogenesis of coronary artery disease (CAD) and the response to medication. The inflammation downstream of TLR4 signaling is thought to contribute to heart and vascular dysfunction. An RCT including 41 participants with CAD and 20 without CAD taking a statin (atorvastatin) with either an angiotensin II receptor blocker (telmisartan) or an angiotensin converting enzyme inhibitor (enalapril) for 12 months found that the plasma levels of TLR4 were higher in participants with CAD, and this was associated with a decrease in TLR4-responsive miRNAs (miR-31, miR-181a, miR-16 and miR-145) [50]. Treatment led to an increase in these miRNAs and a decrease in TLR4 protein levels. This effect was slightly stronger in participants taking telmisartan.

The contribution of TLR4 likely depends on a variety of factors, as the association between TLR4 SNPs and CAD has been found to be modified by ethnicity. In a meta-analysis of 14 studies (n=13,927 participants), no significant association was found for the Asp299Gly SNP (rs4986790), while a protective association was found for the Thr399Ile +1196C>T (rs4986791) (T vs C allele: OR 0.38, 95% CI 0.25 to 0.58) in Asian cohorts, but not in Caucasian cohorts [51].

Treatment with the TLR4 inhibitor, eritoran (4-hour infusion of 2, 12 or 24 mg), starting one hour before cardiac bypass surgery (n=152), did not significantly impact post-surgery organ function/injury or levels of inflammatory mediators in a double-blind, placebo-controlled Phase 2 RCT [52]. This suggests that TLR4 may not be the predominant mediator of cardiac surgery-related inflammation.

Atherosclerosis: TLR4 MAY PROMOTE IN VASCULAR INFLAMMATION

Pro-inflammatory immune cells produce high levels of reactive oxygen species (ROS), which induce oxidative stress damage on other cells. In part, this occurs through the modification (oxidation) of proteins and lipids. Oxidized LDL (oxLDL) is a pro-atherogenic form of cholesterol. The downstream impact of TLR4 activation is highly context dependent, depending on the presence of interacting receptors. OxLDL can trigger the activation of the CD36-TLR4-TLR6 complex, which drives a pro-inflammatory response that can induce damage to other cells [53; 54].

Similar to what has been seen for numerous other conditions, the associations between TLR4 SNPs and atherosclerosis appear to be ethnicity dependent, suggesting they are influenced by gene-gene and

gene-environment interactions [55]. The TLR4 Thr399Ile +1196C>T (rs4986791) polymorphism was found to be associated with susceptibility to atherosclerosis in Asian cohorts (T vs C allele: OR 2.96, 95% CI 1.58 to 5.53), but not in Caucasian or mixed cohorts [55].

Metabolic disease: TLR4 IMPLICATED IN DIET-RELATED INFLAMMATION

Metabolic syndrome is characterized by chronic low-grade inflammation, and TLR4 is thought to be one of the main mediators of obesity-related inflammation via interactions between diet and the gut microbiome [56]. Bacteria in the gut can produce short chain fatty acids as a fermentation by-product of dietary fibers, and these metabolites influence host metabolic function and immunity [57]. Short chain fatty acids act through the TLRs to regulate the immune response in a manner which protects the gut endothelium against inflammation. In contrast, diet derived saturated fatty acids can activate the CD14-TLR4-MD2 complex to trigger inflammation [53; 56]. Omega fatty acids can attenuate this effect, and trigger the protective response. Thus, the composition of dietary fatty acids consumed can influence the state of postprandial inflammation through their effects on TLRs. Saturated fatty acids also influence the gut microbiome in a manner which promotes the production of endotoxins (such as LPS), which also activate TLR4 as PAMPs [42]. Additionally, this inflammatory response in the gut damages the gut endothelium in a manner which makes the gut barrier leaky, thereby allowing these endotoxins to get out into the bloodstream, providing a trigger for systemic inflammation.

A poor diet which promotes the release of endotoxins can lead to chronic activation of TLR4, which likely lead to the same maladaptations that occur to TLR4 during aging, resulting in elevated constitutive activation, leading to chronic low-grade inflammation, coupled with an impaired immune response to an actual pathogen/infection. This may underlie the accelerated immune aging that is typical in the context of metabolic syndrome. It may also be a driver of the association between metabolic syndrome and increased risk for a variety of age-related conditions that involve chronic low-grade inflammation.

In preclinical models, TLR4 inhibition does not protect against weight gain or metabolic disturbances following a high-fat diet, however, under some conditions, it may protect against some neurological effects triggered by diet-related inflammatory responses in the brain [58].

Type 2 diabetes: ELEVATED TLR4 SEEN IN CONJUNCTION WITH INSULIN RESISTANCE

Insulin has a suppressive effect on TLR4 expression, and elevated TLR4 expression is seen in the context of obesity-related insulin resistance [56]. The surface membrane expression of TLR4 is increased on blood monocytes in individuals with type 2 diabetes, and is associated with increased serum levels of pro-inflammatory mediators in the serum (IL-1β, IL-6, IL-8, TNFα) [59]. The impact of TLR4 SNPs in type 2 diabetes is influenced by genetic background, which is read out as differences between cohorts of

different ethnic origins. A meta-analysis of 41 studies (23,250 cases; 24,760 controls) found that the Asp299Gly +896A>G TLR4 SNP (rs4986790) was associated with increased risk for type 2 diabetes in Asian cohorts (G vs A allele: OR 1.21, 95% CI 1.01 to 1.44), while the Thr399Ile (rs4986791) polymorphism was associated with an increased risk for type 2 diabetes in both Asian and Caucasian cohorts (OR 1.40, 95% CI 1.07 to 1.82) [60]. The rs11536889 TLR4 polymorphism appears to have a protective effect in Chinese cohorts (OR 0.76, 95% CI 0.59 to 0.97).

Age-related macular degeneration: NO CLEAR GENETIC ASSOCIATIONS WITH TLR4
Several meta-analyses of gene association studies have examined the impact of TLR4 SNPs on susceptibility to age-related macular degeneration (AMD). A meta-analysis of six studies (2,371 cases; 2,371 controls) and of nine studies (n=5,979) both found that the TLR4 SNP Asp299Gly (rs4986790) was associated with increased susceptibility for AMD [61; 62]. However, a meta-analysis of 12 studies (4,904 cases; 4,422 controls) found that the association for this SNP was not significant following statistical correction [63]. The TLR4 SNP Thr399Ile (rs4986791) was not associated with AMD in any of these studies.

Glaucoma: ETHNICITY-DEPENDENT GENETIC ASSOCIATIONS WITH TLR4
A meta-analysis of eight studies found that the TLR4 SNPs rs4986790 A/G and rs4986791 C/T were associated with risk for primary open angle glaucoma [64]. A separate meta-analysis of eight studies (n=1,680), found that the effect of TLR4 SNPs rs7037117, rs10759930, rs1927911, rs12377632 and rs2149356 may be modified by ethnicity [65].

Safety: TLR4 inhibitors may increase infection risk, but this has not been seen in clinical trials tested thus far, perhaps due to short duration of treatment and/or testing in older participants with weaker baseline TLR4-mediated immunity.

Types of evidence:

- 1 systematic review for preclinical and clinical studies with eritoran
- 2 clinical trials for N1-0101 (healthy volunteers and rheumatoid arthritis)
- 1 clinical trial for TAK-242 (sepsis)
- Numerous laboratory studies

Several different types of TLR4 inhibitors have been tested in clinical trials, including the ligand analog eritoran tetrasodium, which acts as a competitive inhibitor, the small molecule TAK-242, which inhibits the association of the adapters needed for downstream signaling, and the monoclonal antibody NI-0101, which blocks the activation of TLR4.

Eritoran: In Phase 1 studies, there was a dose and time dependent increase in the incidence of phlebitis, or vein inflammation, at the infusion site [2]. In Phase 2 studies for sepsis, eritoran was also associated with higher rates of phlebitis, acute renal failure, elevated creatine and transaminases, and trend toward higher incidence of atrial fibrillation [8]. In the Phase 3 RCT, incidences of these adverse events were not significantly different from placebo, and the infection rate was also similar [47].

TAK-242: In patients with severe sepsis (n=274), TAK-242 (1.2 or 2.4 mg/kg over 96 hours) was associated with a dose-dependent increase in methemoglobin levels in 30.1% of treated patients (NCT00143611) [48]. Methemoglobin is a form of oxidized hemoglobin which can no longer bind oxygen. The prevalence of anemia, hypokalemia, pyrexia, and urinary tract infections also tended to increase with drug dose, though a clear dose-response relationship was not demonstrated. Otherwise, TAK-242 was generally well-tolerated in this population.
NI-0101: In healthy young adult volunteers (n=73), N1-0101 (up to 15 mg/kg) was generally well-tolerated, and adverse events were primarily mild and resolved quickly (NCT01808469) [66]. The most common adverse event was headache (35% N1-0101 vs 23% placebo). In response to an LPS challenge, N1-0101-treated participants did not show the characteristic increase in serum IL-6 levels, or flu-like symptoms. In patients with rheumatoid arthritis (n=90), the incidence of treatment-emergent adverse events was similar between N1-0101 (5 mg/kg every 2 weeks for 12 weeks) and placebo groups (NCT03241108) [4]. In the N1-0101 group, a treatment-related severe adverse event included a severe infusion related reaction, while non-serious treatment-related adverse events included mild dermatitis, a moderate urinary tract infection and a grade 2 increase in alanine aminotransferase. Infection was the most-frequently reported adverse event, but was similar across groups (11.5% NI-0101 vs 13.8% placebo).

Due to its context-dependent effects on immune system function, the impact of TLR4 inhibition is likely to vary depending on the disease and the patient population. Increased risk for infection is the most prominent concern. However, this has not been a significant issue in the clinical trials conducted thus far. This likely stems from the age of the patient populations. Younger individuals, which have a more robust TLR4-mediated pathogen response would be expected to be more susceptible to infection,

particularly to gram negative bacteria, following TLR4 inhibition. Since the ability of TLR4 to induce a productive inflammatory immune response to a pathogen (ligand) is generally weakened with age due to changes in the balance between constitutive and ligand-mediated activation, inhibition of TLR4 may not have a further clinically meaningful impact on infection risk in an older population.

Drug interactions: Drug interactions have not been established, as TLR4 inhibitors are not currently approved, however, due to their impact on the immune system, they may increase the risk for infection when combined with other immunosuppressive drugs.

Sources and dosing:

TLR4 inhibitors have not yet been approved for any condition. All of the TLR4 inhibitors clinically tested thus far have used an i.v. route of administration. As none of the later phase trials have been successful, effective doses have not been established for any condition. Eritoran tetrasodium is under clinical development by Eisai. TAK-242 is available for research use from commercial suppliers, and is under clinical development by Akaza Bioscience in partnership with Takeda. NI-0101 is being developed by Edesa Biotech as EB05.

Research underway:

According to Clinicaltrials.gov, there are three active clinical trials for TLR4 inhibitors.
TAK-242 is being tested in a Phase 2 RCT (n=100) in patients with acute alcohol hepatitis (NCT04620148). The estimated study completion is December 2022.
Eritoran is being tested as part of the REMAP-CAP Phase 3 adaptive platform trial for community acquired pneumonia (NCT02735707).
EB05 is being tested in a Phase 2 RCT (n=396) for Covid-19 (NCT04401475).

Search terms:
Pubmed, Google: TLR4, eritoran, TAK-242, N1-0101

- Alzheimer's disease, neurodegeneration, aging, cancer, gene association, cardiovascular, metabolic disease, clinical trials, meta-analyses, safety

Websites visited for TLR4 Inhibitors:

- Clinicaltrials.gov (Eritoran), (TAK-242), (N1-0101), (EB05)

- PubChem (Eritoran), (TAK-242)
- DrugBank.ca (Eritoran), (TAK-242)
- Cafepharma (Eritoran)

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