NeuroQuest

Blood Biomarker for Alzheimer's Disease: Scientific Overview

NeuroQuest develops a novel blood-based diagnostic test for Alzheimer's disease based on a specific cellular immune response which reflects the failure of the immune system to cope with the disease and thereby contributes to disease escalation. The concept that created the ground for such a test was developed by Prof. Michal Schwartz of the Weizmann institute of Science, who leads NeuroQuest Scientific Advisory Board. Her research, of direct relevance for the current concept, has been published over the last two decades in top journals, including *Science*, *Nature Medicine*, *Nature Neuroscience*, *Nature Cell Biology*, *PLOS Medicine*, and others.

Alzheimer's disease (AD) is an age-related neurodegenerative condition characterized by neuronal damage, amyloid-beta (Aβ) plaque formation, and chronic inflammation within the central nervous system (CNS), leading to gradual loss of cognitive function and brain tissue destruction[1,2]. For decades, the prevailing dogma had been that the local neuroinflammatory response reflects infiltration of blood-borne immune cells that, which together with the activated microglia, indiscriminately contribute to disease escalation. Schwartz's findings, which were confirmed by numerous additional reports over the years, showed that microglia and infiltrating blood-borne macrophages are non-redundant in their functions, and that boosting recruitment of immune cells to the damaged CNS can be beneficial for resolving inflammation[3-6]. Specifically, in the context of AD, it was shown by the Schwartz group and later by others over, that in animal models of AD, recruitment of circulating myeloid cells is needed for clearance of Aβ plaques and for fighting off the disease[7-11].

The questions which emerged from these findings were how to enhance recruitment of macrophages to the CNS, and how to identify the factors that limit their recruitment. Schwartz discovered that the selective recruitment of inflammation-resolving cells to the CNS, requires systemic immune activation rather than immune suppression[12]; furthermore, she showed that these cells are not recruited through breaches in the blood brain barrier (BBB), but rather through a systemic immune response that requires interferon (IFN)-γ-dependent activation of a unique gateway - the brain's choroid plexus (CP), the epithelial layer that forms the blood-CSF-barrier (BCSFB)[4,13-16]. These findings substantiated Schwartz's original observations that autoreactive T cells are supportive for the recovery from CNS injury ("*Protective autoimmunity*") , and that they act, at least in part, by facilitating recruitment of monocyte-derived macrophages, through a mechanism that involves IFN-γ[3,17,18]. Accordingly, peripheral immunity is not a direct mirror of the local inflammatiory response in the brain.

Based on these observations, the Schwartz group suggested that recruitment of inflammation-resolving immune cells to the diseased brain is needed for arresting neuroinflammation, and is dependent on the activity of the choroid plexus, which may dysfunction under brain pathologies. This concept was supported in Schwartz's recent findings (*Science*, 2014), showing that the CP is immunologically suppressed in both mice and humans during brain aging[19]. In this work, the Schwartz group showed using a robust, non-biased transcriptional analysis, that in brain aging the CP displays an IFN type I (IFN-I) response signature, including a dramatic rise of IFN-β expression. Collaborating with the group of Tony Wyss-Coray (Stanford University, California), the Schwartz group also showed that by rejuvenating the blood composition of aged mice that CP activity is amendable for restoration, and the effect was associated to partial restoration of cognitive function in aged mice[19].

Current research of the Schwartz group (Baruch et al., 2015; *in final stage of manuscript revision*) shows that in AD transgenic mice, CP gateway activity for allowing immune cells to traffic the CNS is suppressed along disease progression. Their findings show that circulating immune cell populations, with immunosuppressive functions, are dramatically affecting CP function. Specifically, Schwartz's findings identify systemic $Foxp3^{+}CD4^{+}$ Treg-mediated immunosuppression as a negative player in AD pathology, acting at least in part by interfering with IFN-γ-dependent activation of the CP, which is needed for orchestrating recruitment of inflammation-resolving leukocytes to the CNS. Using genetic conditional ablation of $Foxp3^{+}$ Treg cells in AD-Tg mice, and various pharmacological approaches to

target these cells, Schwartz findings show that activation of the CP for supporting leukocyte trafficking to the CNS is followed by accumulation of immunoregulatory cells at cerebral sites of Aβ pathology, plaque clearance, and mitigation of cognitive decline. These findings point to immunosuppressive cell populations in AD as key players in the inability to mount a systemic immune response needed for mitigation of disease pathology[20].

Neuroquest was founded to identify the immunological signature of the cellular immune activities that might indicate dysfunction of the immune system in diseases such as AD. The test that NeuroQuest is developing could serve as an early diagnostic marker for immune cell malfunction, or may be used to follow the effect of therapy. Clinical studies performed by NeuroQuest in collaboration with the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Aging (AIBL) flag ship study in Australia, have substantiated Prof. Schwartz Theory. AIBL pilot study focused on identifying immune cell populations which are affected in AD patients, and potentially serve a critical role in mediating systemic immunosuppression (See **Figure 1**); such immune suppressor cells can be of the lymphoid origin (Tregs), or myeloid-derived suppressor cells (MDSCs). These systemic immune cell populations are crucial for maintenance of autoimmune homeostasis and protection from autoimmune diseases[21]. Nevertheless, Schwartz's group findings suggest that under neurodegenerative conditions, when a reparative immune response is needed within the brain, the ability to mount this response is curtailed by these cells. Specifically, their findings show that these cells are acting at least in part by interfering with IFN-γ-dependent activation of the CP, which is needed for orchestrating recruitment of inflammation-resolving leukocytes to the CNS[20,22]. AIBL pilot study clinical results identify specific immune cell clusters which can distinguish between amyloid negative versus positive subjects, with high level of sensitivity and specificity. Among the immune cell populations implicated are myeloid ($CD11b^{+}$) and dendritic ($CD11c^{+}$) cells which are known to regulate the adaptive immune response, and immune cells expressing the P2X7 receptor associated with ATP-mediated cell death. Taken together, the markers that comprise the suggested diagnostic panel reflect systemic immune dysfunction and, once substantiated, are expected to serve as a reliable predictive tool.

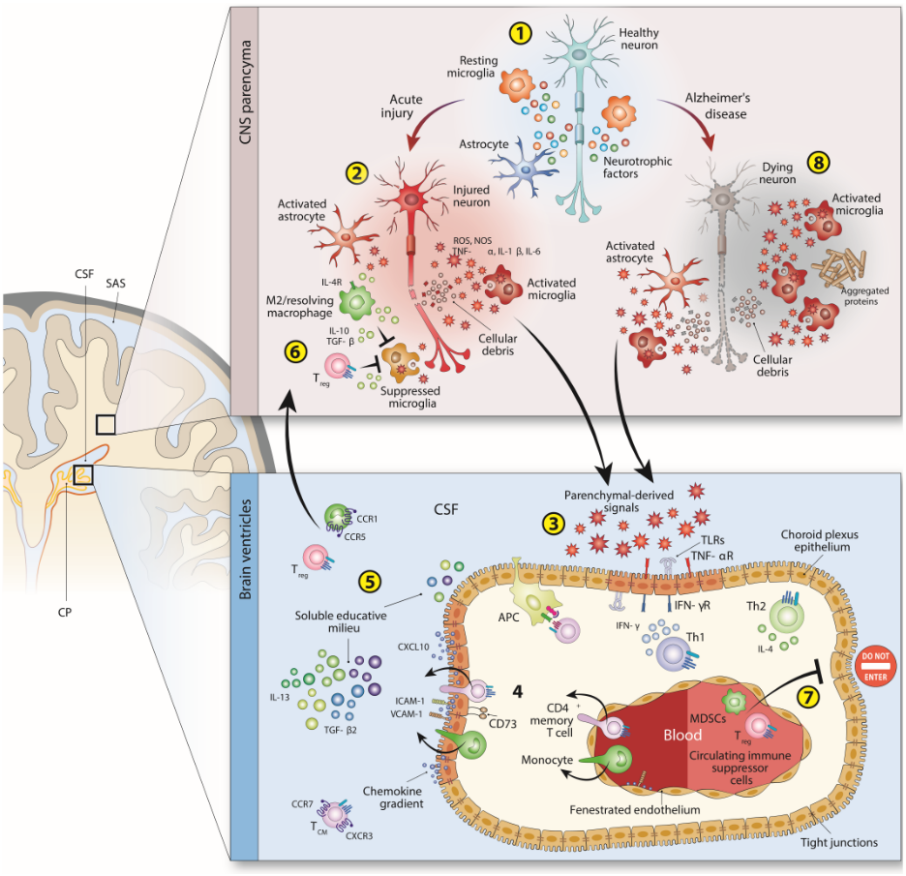


Figure 1. Suggested model:
(Adapted with permission from: Schwartz and Baruch, 2014, *EMBO Journal*)
(**1**) In the steady state, astrocytes and microglia serve as sentinels of tissue homeostasis, providing the neural parenchyma with a supportive neurotrophic environment. (**2**) Following CNS insult, dying cells and accumulation of cellular debris locally activate resting microglia and astrocytes. Activated microglia phagocytose cellular debris while concurrently secreting toxic compounds, including pro-inflammatory cytokines (such as IL-1β, TNF-α and IL-6) and reactive oxygen and nitrogen species (ROS, NOS). (**3**) Parenchymal-derived signals (e.g., TNF-α) reach the choroid plexus (CP) through the cerebrospinal fluid (CSF) and are sensed by cytokine receptors and Toll-like receptors (TLRs) expressed by the CP epithelium. (**4**) These signals, together with IFN-γ from CP stromal Th1 cells, initiate a cellular trafficking cascade for T cells and monocytes entering the CNS. This cascade includes the upregulation of integrin receptors (e.g. ICAM-1), chemokines (e.g. CXCL10) and surface enzymes (e.g. CD73) by the CP epithelium, which enables selective recruitment of leukocytes to the CNS. (**5**) Entry through the CP-CSF serves an educative role in skewing infiltrating immune cells towards an anti-inflammatory/suppressor phenotype. (**6**) Along the repair process, monocyte-derived macrophages and regulatory T cells (Tregs) are recruited to the inflamed CNS parenchyma and suppress the inflammatory response by the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. (**7**) In chronic neurodegenerative diseases in general, and AD in particular, circulating immune suppressor cells (such as Tregs and myeloid-derived suppressor cells (MDSCs)) maintain peripheral immune suppression and inhibit immune cell trafficking to the CNS. (**8**) Lacking the support of circulating inflammation-resolving leukocytes, dying cells, cellular debris and protein aggregates locally activate astrocytes and microglia in an escalating vicious cycle of local toxicity; neurons residing in this inflammatory microenvironment degenerate via apoptotic mechanisms.

Clinical Results - AIBL Pilot Study

In 2013, NeuroQuest received strong independent endorsement of its platform with the collaboration of the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Aging (AIBL). Launched on 14th November 2006, AIBL is one of the largest studies of its kind in Australia and one of the largest in the world. The trial was conducted as an exploratory case-control AIBL sub-study. The methodological approach assessed the degree to which markers measured for NeuroQuest in the AIBL study accurately identify subjects using Positron Emission Tomogrpahy (PET) who show presence (amyloid positive) or absence (amyloid negative) of amyloid in their brain, with an assumption that amyloid positive subjects have or are potentially at risk for Alzheimer's disease.

The study was a blinded, single center, exploratory case-control study in adults (minimum age 60 years). Eligible subjects, in acoordance to the AIBL inclusion criteria, were assigned NeuroQuest blood assay and PET imaging to be done within a period of 4 months (two months prior blood processing to two months post blood processing).

Models for identifying Amyloid positive subjects were developed based on the markers selected where the dependent variable is Amyloid positive or negative, out of which one model was selected as the best promising model.

The analysis included Area Under the ROC curve (AUC), along with two-sided 95% confidence interval, Sensitivity and Specificity statistics, including the statistically optimal cutoff and value of the maximal sum of Sensitivity and Specificity.

In stage I of this study, EDTA anti-coagulant blood was collected from the Melbourne site of the AIBL cohort, including patients with mild cognitive impairment (MCI) or Alzheimer's disease (AD), as well as age-matched healthy controls (HC). Whole blood was incubated with different combinations of selected cell surface biomarkers. Each combination targeted three analytes, with conjugated fluorophors (FITC, PE or APC). After erythrocyte lysis and washing, the cells were analyzed by flow cytometry.

Each sample was run blinded and a total of 69 subjects were screened in stage I, including 49 healthy controls (HC), 15 MCI and 5 AD subjects. Out of the 12 sets of carefully selected combinations, 10 of

them showed statistical significance between HC and MCI or AD, which derived over 30 promising parameters. Most of these markers remained to be significant when analyzed against PET amyloid scan data, e.g. amyloid burden negative (n=33) vs. positive (n=36), particularly between PET positive and PET negative HCs. The analysis has shown that these biomarkers are of high sensitivity and specificity, independent of age, sex, ApoE4 genotype or level of education, and have clinical utility.

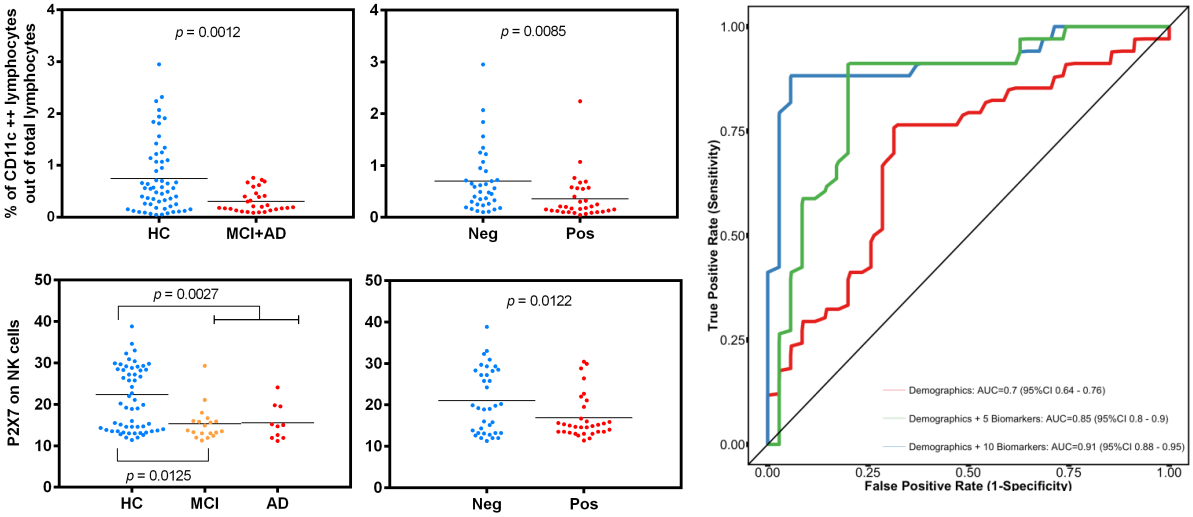
Markers retained from Stage 1 were included in various statistical models as predictors, where the dependent variable is Amyloid burden as measured by PET scans.

Two promising models were selected. The results they yield are provided below.

Results include:

Receiver Operating Curve (ROC) curve analysis that includes:

- Graph of ROC
- Area Under the ROC curve (AUC), along with two-sided 95% confidence interval
- Sensitivity and Specificity statistics, including the statistically optimal cutoff and values (those that yield the maximal sum of Sensitivity and Specificity)



(**Left**) Examples of two parameters which showed significant differences between healthy control and patients and between amyloid burden negative and positive subjects. Two-tailed T test was used for statistical analysis. (**Right**) The Receiver Operating Characteristic (ROC) curve of stage I NeuroQuest data**. Red line (Model 1)**: demographics alone (age, gender, *ApoE* and education) (area under curve 0.69, sensitivity 76.4% specificity 68.5%, accuracy 72.4%); **Green line (Model 2)**: 5 biomarkers alone (area under curve 0.84, sensitivity 91.1%, specificity 80.04%, accuracy 85.5%), **Blue line (Model 3)**: 10 biomarkers and demographics (area under curve 0.91, sensitivity 88.2%, specificity 94.2%, accuracy 91.3%).

Model 1	Model 2	Model 3
Sensitivity 76.4%	Sensitivity 91.1%	Sensitivity 88.2%
Specificity 68.5%	Specificity 80.0%	Specificity 94.2%
Accuracy 72.4%	Accuracy 85.5%	Accuracy 91.3%
AUC 0.69	AUC 0.84	AUC 0.91

Key references

1. Akiyama, H., *et al.* Inflammation and Alzheimer's disease. *Neurobiology of aging* **21**, 383-421 (2000).
2. Hardy, J. & Selkoe, D.J. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353-356 (2002).

3. Shechter, R., *et al.* Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. *PLoS medicine* **6**, e1000113 (2009).
4. Shechter, R., *et al.* Recruitment of beneficial M2 macrophages to injured spinal cord is orchestrated by remote brain choroid plexus. *Immunity* **38**, 555-569 (2013).
5. Rapalino, O., *et al.* Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. *Nature medicine* **4**, 814-821 (1998).
6. Cohen, M., *et al.* Chronic exposure to TGFbeta1 regulates myeloid cell inflammatory response in an IRF7-dependent manner. *The EMBO journal* **33**, 2906-2921 (2014).
7. Simard, A.R., Soulet, D., Gowing, G., Julien, J.P. & Rivest, S. Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron* **49**, 489-502 (2006).
8. Butovsky, O., Kunis, G., Koronyo-Hamaoui, M. & Schwartz, M. Selective ablation of bone marrow-derived dendritic cells increases amyloid plaques in a mouse Alzheimer's disease model. *The European journal of neuroscience* **26**, 413-416 (2007).
9. Town, T., *et al.* Blocking TGF-beta-Smad2/3 innate immune signaling mitigates Alzheimer-like pathology. *Nature medicine* **14**, 681-687 (2008).
10. Koronyo-Hamaoui, M., *et al.* Attenuation of AD-like neuropathology by harnessing peripheral immune cells: local elevation of IL-10 and MMP-9. *Journal of neurochemistry* **111**, 1409-1424 (2009).
11. Mildner, A., *et al.* Distinct and non-redundant roles of microglia and myeloid subsets in mouse models of Alzheimer's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**, 11159-11171 (2011).
12. Schwartz, M. & Baruch, K. Breaking peripheral immune tolerance to CNS antigens in neurodegenerative diseases: boosting autoimmunity to fight-off chronic neuroinflammation. *Journal of autoimmunity* **54**, 8-14 (2014).
13. Kunis, G., *et al.* IFN-gamma-dependent activation of the brain's choroid plexus for CNS immune surveillance and repair. *Brain : a journal of neurology* **136**, 3427-3440 (2013).
14. Shechter, R., London, A. & Schwartz, M. Orchestrated leukocyte recruitment to immune-privileged sites: absolute barriers versus educational gates. *Nature reviews. Immunology* **13**, 206-218 (2013).
15. Shechter, R. & Schwartz, M. Harnessing monocyte-derived macrophages to control central nervous system pathologies: no longer 'if' but 'how'. *The Journal of pathology* **229**, 332-346 (2013).
16. Kunis, G., Baruch, K., Miller, O. & Schwartz, M. Immunization with a Myelin-Derived Antigen Activates the Brain's Choroid Plexus for Recruitment of Immunoregulatory Cells to the CNS and Attenuates Disease Progression in a Mouse Model of ALS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **35**, 6381-6393 (2015).
17. Yoles, E., *et al.* Protective autoimmunity is a physiological response to CNS trauma. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **21**, 3740-3748 (2001).
18. Moalem, G., *et al.* Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nature medicine* **5**, 49-55 (1999).
19. Baruch, K., *et al.* Aging. Aging-induced type I interferon response at the choroid plexus negatively affects brain function. *Science* **346**, 89-93 (2014).
20. Schwartz, M. & Baruch, K. The resolution of neuroinflammation in neurodegeneration: leukocyte recruitment via the choroid plexus. *The EMBO journal* **33**, 7-22 (2014).
21. Kim, J.M., Rasmussen, J.P. & Rudensky, A.Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nature immunology* **8**, 191-197 (2007).
22. Baruch, K., Kertser, A., Porat, Z. & Schwartz, M. Cerebral nitric oxide represses choroid plexus NFκB-dependent gateway activity for leukocyte trafficking. *EMBO J (In press)* (2015).