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Article

Inhibitors of Soluble Epoxide Hydrolase and cGAS-STING Pathway Repair the Defects in Macrophage Clearance of Amyloid- β Underlying Vascular Complications of Alzheimer's Disease Patients

Milan Fiala ^{1,*}, Bruce D. Hammock ², Sung Hee Hwang ², Julian Whitelegge ³, Karolina Elzbieta Kaczor-Urbanowicz ^{4,5,6}, Andrzej Urbanowicz ^{4,5,6} and Santosh Kesari ⁷

¹ Department of Integrative Biology and Physiology, UCLA School of Medicine, Los Angeles, California 90024

² Department of Entomology and Nematology, and UC Davis Comprehensive Cancer Center University of California -Davis, Davis, California

³ Department of Psychiatry UCLA School of Medicine, Los Angeles, California 90024

⁴ Center for Oral and Head/Neck Oncology Research, Biosystems and Function, UCLA School of Dentistry, University of California at Los Angeles, Los Angeles, CA 90095

⁵ UCLA Institute for Quantitative and Computational Biosciences, University of California at Los Angeles, Los Angeles, California 90095

⁶ Institute of Control and Computation Engineering, Warsaw University of Technology, Warsaw, Poland

⁷ Providence Saint John's Health Center, Santa Monica, California

* Corresponding author: Milan Fiala mfiala@ucla.edu; Milan Fiala¹ mfiala@ucla.edu

Abstract: Alzheimer's disease (AD) and the therapies of AD with anti-amyloid₁₋₄₂ (A β) monoclonal antibodies are associated with the vascular complications of brain edema, hemorrhage, and cerebral amyloid angiopathy (CAA) termed amyloid-related imaging abnormalities (ARIAs). The neuropathology of normal and AD brains demonstrates that monocyte/macrophages (MMs) are critical in successful brain clearance in healthy brain by up-loading, transporting, and degrading A β but fail degradation and clearance in AD patients by releasing A β into vessels at the blood-brain barrier, causing vasculitis and ARIAs. The naturally-formed epoxides (EpFAs) of polyunsaturated fatty acids (PUFA's), including arachidonic, docosahexaenoic, and eicosapentaenoic fatty acids, may repair degradation of A β by regulating A β -degrading enzymes, inflammatory cytokines, and unfolded protein response (UPR) to endoplasmic reticulum stress in a homeostatic fashion. EpFAs are, however, degraded by the soluble epoxide hydrolase enzyme (sEH) but are protected by the sEH inhibitor (sEHI) TPPU. In the cultures of AD patients' macrophages, TPPU with the epoxide EEQ, and the cGAS/STING inhibitor H-151 increased uptake of A β at 2 hours and degradation of A β at 24 hours. In conclusion, the soluble epoxide hydrolase inhibitor TPPU with omega-3 fatty acids and the cGAS/STING inhibitor H-151 increase A β uptake and degradation in macrophages of AD patients, and potentially enhancing A β clearance in the AD brain.

Keywords: Alzheimer's disease; vascular complications; monoclonal antibody therapy; Aducanumab; Lecanemab; epoxide of polyunsaturated fatty acid; soluble epoxide hydrolase inhibitor EC5026; EP4 inhibitor; monocyte/macrophage; amyloid-beta degradation.

Plain language summary: Neuropathological studies revealed that the molecule amyloid- β causes inflammation in the brain of patients with Alzheimer's disease, involving the immune cells called macrophages. Current therapies of Alzheimer's disease with the monoclonal antibodies Aducanumab or Lecanemab bind to but do not clear amyloid- β from the brain and do not inhibit the inflammation. This study shows that the molecule called TPPU, which inhibits soluble epoxide

hydrolase, together with certain polyunsaturated fatty acid molecules, protect AD macrophages against inflammation and thus may clear amyloid- β from the brain. Another soluble epoxide hydrolase inhibitor called EC5026 is more suitable for human therapy than TPPU and should be tested in a clinical trial in patients with Alzheimer's disease. These experimental drugs complement other approaches for degradation of amyloid- β .

1. Introduction

Aducanumab and Lecanemab are therapeutic monoclonal antibodies to amyloid- β 1-42 ($A\beta$) that increase the clearance of soluble and insoluble $A\beta$ in the brain of Alzheimer's disease (AD) patients and may, although controversially, slow AD progression [1–3]. These anti- $A\beta$ monoclonal antibody therapies are associated with amyloid-related imaging abnormalities (ARIA), characterized by brain edema and/or hemorrhagic stroke [3–5], which are caused by $A\beta$ -induced myeloid (monocyte/macrophage (MM)) vasculitis [6] and blood-brain barrier (BBB) damage. Therefore, effective and safe therapy of AD remains an unresolved problem.

To resolve a more generally safe approach AD therapy, we have reviewed existing therapeutic studies and investigated novel approaches. We demonstrate that monocyte/macrophages (MMs) are critical in brain clearance of $A\beta$ but fail to clear $A\beta$ in AD patients. Fortunately, the defects in transcriptome, glycome and functions of AD patients' MMs are known from previous studies and illuminate four possible approaches to effective therapy of AD through biochemical modulation of sGAS, NF κ B, IRF3, and EP4 signaling, as follows:

1) cGAMP- STING synthase (cGAS) pathway is activated in AD and other diseases by cytosolic DNA, which activates STING and induces pro-inflammatory signaling by the transcription factors NF κ B and IRF3 [7–9]. cGAS signaling is important in neurological diseases (Parkinson's disease (PD), AD [10], amyotrophic lateral sclerosis (ALS) [11]), heart failure, chronic inflammatory diseases (pancreatitis), and cancer. The cGAMP-STING signaling is blocked by the STING inhibitor H-151 [7].

2) $A\beta$ induces inflammation and endoplasmic reticulum (ER) stress in macrophages through NF κ B and IRF3 signaling, which are inhibited by the bioactive signaling molecules epoxides of fatty acids (EpFAs) [12]. EpFAs are formed by cytochrome P-450 enzymes from polyunsaturated ω -3- and ω -6 fatty acids (PUFAs). EpFAs regulate macrophage inflammation but are destroyed by the soluble epoxide hydrolase (sEH) [13]. The sEH enzyme is inhibited by the sEH inhibitors (sEHI) TPPU and EC5026.

3) The signaling through prostaglandin EP2 receptor 4 (EP4) decreases energy, neuronal plasticity and memory in a mouse model of ageing and likely in AD. Blockade of peripheral myeloid EP4 signaling is sufficient to restore cognition in aged mice. [14].

Thus, three approaches are potentially therapeutic in AD: a) inhibition of cGAS by a STING inhibitor (e.g. H-151); b) blockade of sEH by an sEH inhibitor (sEHI), such as TPPU (not approved for human studies) or EC5026 (approved for human studies), c) inhibition of inflammatory signaling by an inhibitor of the prostaglandin receptor EP4 (EP2R4) [14] or, possibly, EP2 (EP2R2) [12].

In this study, we clarify the critical role of monocyte/macrophages (MMs) in the immunopathology of AD patients, including the patients on monoclonal antibody therapy (**Section 3.1**), analyze the defects in macrophage $A\beta$ clearance and transcriptome (**Sections 3.2 and 3.3**), and show possible repair of these defects using the epoxides of polyunsaturated fatty acids (EpFAs), the inhibitors of soluble epoxide hydrolase (sEHs) (e.g. TPPU) and the cGAS-STING pathway (the STING inhibitor H-151) (**Sections 3.4 and 3.5**). These therapeutic approaches increase $A\beta$ degradation in macrophages and inhibit the inflammatory vasculitis, and are potential approaches to therapy of AD.

2. Materials and Methods

2.1. Study Design

We examined the effects of epoxy fatty acids (EpFAs) on the transcriptome of peripheral blood macrophages generated from peripheral blood mononuclear cells (PBMC) of two AD patients

(patient #1, age 68 years, male gender; mini-mental state examination (MMSE) 21 points; and patient #2, age 71 years, male gender; MMSE 26 points).

2.2. Macrophage Culture and Treatments

We isolated peripheral blood mononuclear cells (PBMC) by Ficoll-Hypaque technique and cultured them in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% autologous serum for 1 to 2 weeks until CD68+ macrophages developed. We added the compounds in a DMSO solution at the final concentration 1 μ M EpFAs, 100 nM TPPU, or H151 (100 ng/ml). We confirmed macrophage type by staining with anti-CD68 antibody (monoclonal antibody against human macrophage marker in human monocytes, macrophages, and myeloid cells; clone KP1, DAKO, Inc) and macrophage phenotype as pro-inflammatory in most patients by high staining with anti-CD80 and low staining with anti-CD163.

2.3. RNA Sequencing and Analysis

Total RNA was extracted from macrophage cultures using Quick RNA Miniprep kit (Zymo Research, Inc.). Final libraries were generated from total RNA using KAPA mRNA Hyper kit, and quantified and sequenced on NovaSeq 6000 (Illumina, Inc). After removing low-quality reads, the data were aligned to the human genome GCHr38 using STAR (v2.6) and count data were normalized using DESQ2's median of ratios method. We compared the effects of EpFAs in normalized counts of macrophage samples and performed a supervised graphical analysis of individual genes of interest, according to the previous method [15].

2.4. Chemicals

The methyl esters of EpFAs and the sEH inhibitor TPPU were prepared in the laboratory of B. Hammock [16], according to the previously described synthesis methods [17,18]. Lipophilic fatty acid epoxides and the enzyme inhibitors were added in DMSO (less than 1 percent final volume, and DMSO was used as a control.

3. Results

3.1. Monocyte/Macrophages (MMs) Transport Amyloid-B To Vessels Causing Vasculitis, Which Underlies Amyloid-Related Imaging Abnormalities (ARIAs) and Cerebral Amyloid Angiopathy (CAA)

According to the A β hypothesis, the deposition of amyloid- β_{1-42} (A β) in plaques, neurons, and blood vessels in the brain is the cause of AD neuropathology [19]. AD brain contains oligomeric and soluble A β in neurons and apoptotic macrophages, and fibrillar A β in plaques and blood vessels [20]. The deposition of A β has a "biochemical phase" with A β aggregation, phosphorylation of tau, and cell-to-cell propagation of A β and p-tau, a "cellular phase" with the participation of "microglia" and astrocytes [21]. A β deposition is followed by the "inflammatory response phase" with vasculitis caused by blood-borne MMs, which transport A β from plaques to vessels [20].

3.3. Defects of Macrophage Transcriptome Underlie Macrophage Failure Of Brain Clearance

As inflammatory macrophages are pathogenic in chronic inflammatory disorders, increasing A β degradation and inhibiting inflammation are therapeutic mechanisms against AD. The anti-amyloid antibody chimeric antigen receptor epitope-expressing macrophages (CAR-Ms) have been shown to degrade A β [26]. Macrophages of AD patients are defective in uptake of fibrillar and soluble A β , and in degradation of intracellular A β [20] [20]; and they display a pro-inflammatory phenotype with increased endoplasmic reticulum (ER) stress and defective phagocytosis of A β [27].

The functional defects of AD macrophages are related to the down-regulation of the transcripts of several pathways: a) OX-PHOS energy enzymes; b) the enzymes degrading A β , i.e. membrane metalloendopeptidase (MMSE), insulin-degrading enzyme (IDE), angiotensin-converting enzyme

(ACE); and c) the enzymes in the ubiquitin-proteasome system E1, E2, and E3; and to the up-regulation of inflammatory cytokines [15].

3.2. Amyloid- β Degradation in the AD brain Fails Due to the Transcriptomic Defects in AD Patients' Monocyte/Macrophages

MMs are critical for brain clearance of A β in health but AD patients' macrophages fail in degradation. In the AD brain [15,22], MMs are attracted by chemokines to A β plaques, exit vessels (green/CD68) [22] (Figure 1a), invade A β plaques, upload but do not degrade A β (yellow) [20], return to vessels, undergo apoptosis and release A β (red) into vessels (Figure 1b), causing CAA and BBB damage resulting in a failure of brain nutrition and protection [15,20,22]. MMs are blocked from brain emigration at the blood-brain barrier (BBB) by gross distention with A β . The brain tissues of AD patients show macrophages in perivascular spaces (Figure 2a) and A β plaques (Figure 2c), in comparison, the brain tissues of a patient with epilepsy do not show perivascular macrophages (Figure 2b) [22]. MMs also attach and upload A β from neurons [20].

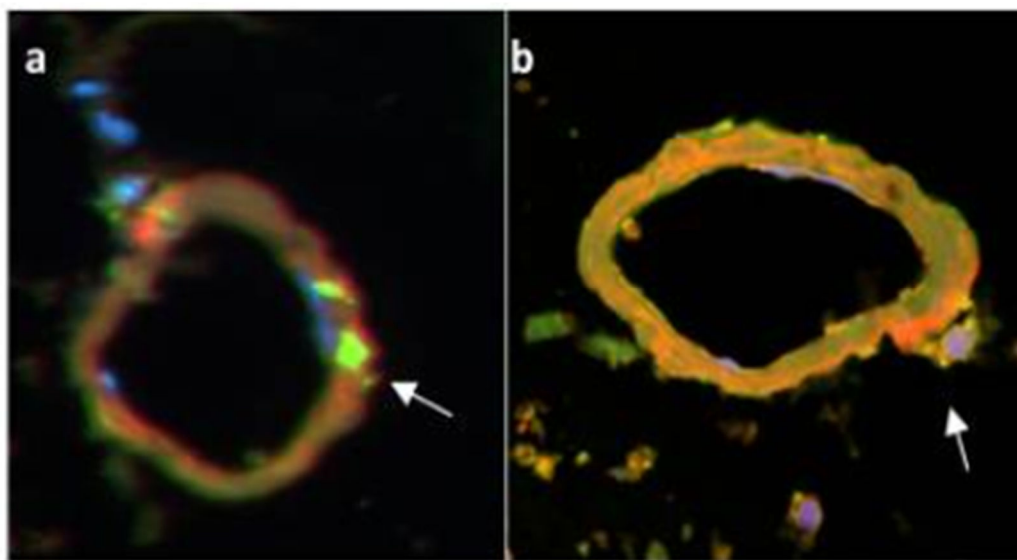


Figure 1. Monocytes exit vessels empty (green), up-load A β (red) in plaques (Figure 2c), migrate back to vessels filled with A β (yellow), suffer apoptosis, and release A β . a) The microvessel with two A β -free (green) macrophages (blue nucleus) at 3 o'clock (arrow) and a macrophage releasing A β (red) at 11 o'clock; b) abutting A β -filled (green/red, overlap yellow) macrophage at 4 o'clock releasing A β (red) into the vessel [15]. Confocal microscopy of the post-mortem AD brain tissue with macrophages stained green using CD68 antibody (clone KP1, DAKO, Inc) by immunofluorescence (by permission of the authors [23]).

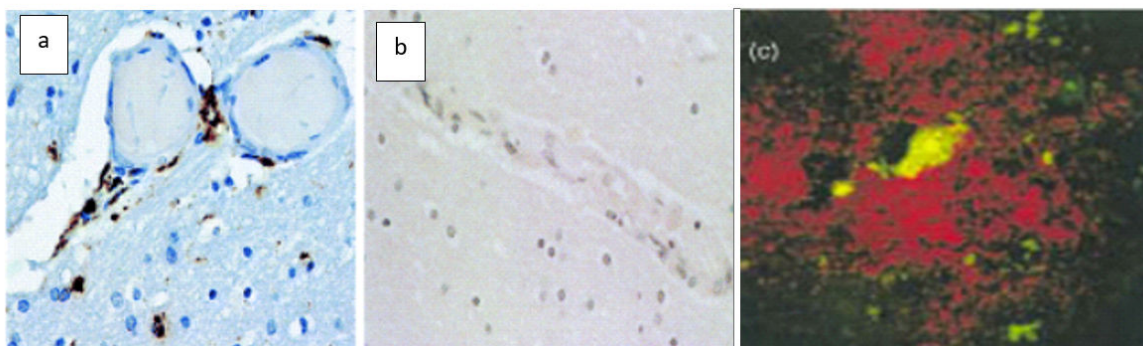


Figure 2. (a) Macrophages (brown) infiltrate perivascular spaces in the AD brain; (b) Macrophages (brown) do not infiltrate the perivascular spaces of a patient with epilepsy;

(c) macrophages (green/yellow) clear patches in A β (red) plaques and become distended [9] (by permission of authors). Note microglia around the plaque are free of A β (green).

The anti-amyloid- β monoclonal antibodies Aducanumab and Lecanemab increase macrophage clearance of A β plaques and macrophage transport of A β to vessels but do not increase A β degradation, leading to vasculitis, CAA and ARIAs. In a patient treated by Lecanemab, the neuropathology showed histiocytic (macrophage-lineage) vasculitis [6]. Anatomical studies in mice, rats, and primates show microglia engulfing A β fibrils [23]. In the human brain with AD [20,22], and in the brain of a patient treated with monoclonal A β antibody Lecanemab [6], the infiltrating cells are blood-derived CD68⁺ myelomonocytic cells. The plaques are infiltrated by CD68⁺ A β ⁺ (yellow) macrophages ingesting A β (red), whereas the microglia surrounding the plaques are negative for A β (CD68⁺ A β ⁻ (green)) (**Figure 2c**).

The AD brain contains insoluble A β in the plaques and soluble A β in vessels. Thus, both cellular and humoral mechanisms are necessary for brain clearance: soluble A β is cleared by intramural periarterial drainage (IPAD) and drainage of cerebrospinal fluid (CSF) into cervical lymph nodes; insoluble A β is cleared by macrophages. However, the intramural route is likely obstructed by A β released by apoptotic macrophages, as well as by immune complexes after monoclonal antibody immunotherapy and the complexes with anti-A β autoantibodies [24,25].

3.4. Epoxides of Polyunsaturated Fatty Acids (EpFAs) Repair the Macrophage Transcriptome in Energy Enzymes, A β degradation Enzymes, and Cytokines

Clinical use of polyunsaturated fatty acids (PUFAs) in AD therapy with preference for ω -3 over ω -6 PUFAs in seafood has a long history [28]. The clinical effects of PUFAs against cognitive decline extend beyond the benefits of cholinesterase inhibitors [29]. To clarify the beneficial mechanisms of PUFAs, we investigated whether the mechanisms arise in part through the epoxide metabolites of PUFAs (EpFAs). EpFAs are lipid mediators [30], which act *in vivo* by modulating the endoplasmic reticulum (ER) stress pathway to reduce inflammation and nociceptive pathophysiology toward homeostasis. The epoxides of ω -3 fatty acids are anti-inflammatory and more stable than those of ω -6 fatty acids. *In vivo*, ω -3 fatty acid supplementation may alone modulate ER stress [31,32]. The epoxides of a) arachidonic acid (ARA) called EETs, b) the ω -3 fatty acid eicosapentaenoic acid (EPA) called epoxyeicosatetraenoic acids (EEQs), and c) epoxydocosapentaenoic acids (EDPs) from the ω -3 fatty acid DHA are metabolized by the soluble epoxide hydrolase (sEH) to more polar and usually inactive diols [33,30] (**Figure 3**).

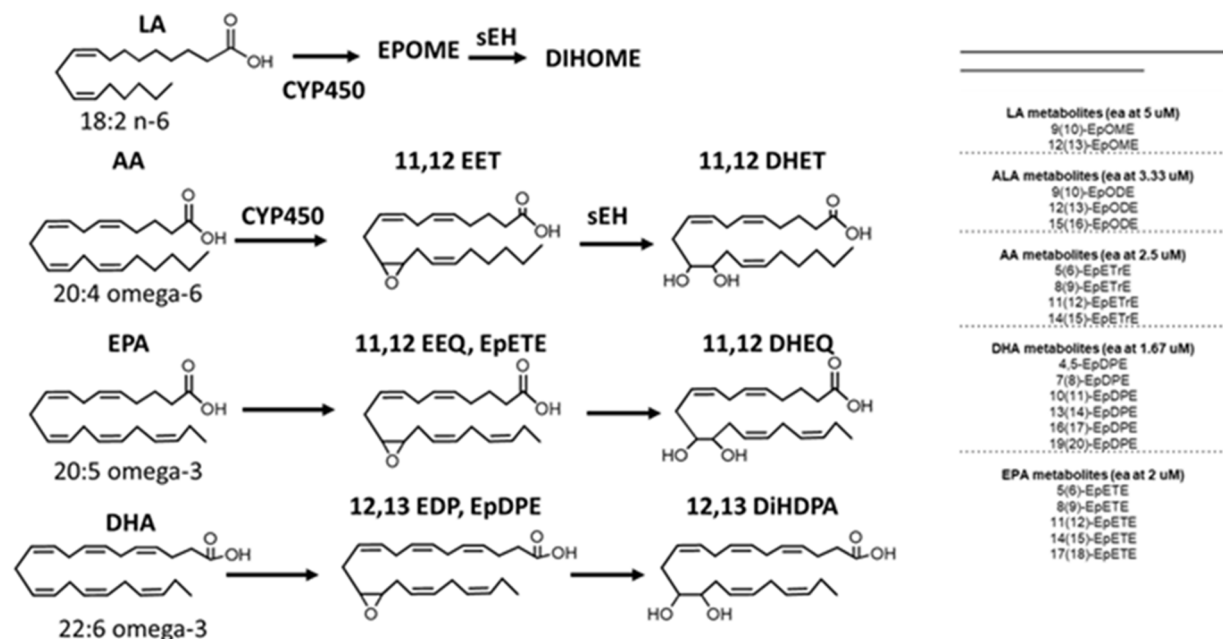


Figure 3. Epoxy fatty acids (EpFAs). EPFAs are produced from polyunsaturated fatty acids (PUFA) by cytochrome P450s. EETs are produced from ω -6 arachidonic acid (AA), EEQ from ω -3 eicosapentaenoic acid (EPA), and EDPs from ω -3 docosahexaenoic acid (DHA). Only one regioisomer of EETs, EEQs, or EDPs is shown for clarity.

We treated macrophages from two AD patients using the EpFAs and analyzed the transcriptome. The epoxides EEQ, EDP, as well as the sEH inhibitor TPPU, strongly increased the transcripts of the $A\beta$ -degrading enzyme *ACE* (**Figure 4a**). The epoxides EEQ, and EDP, regulated the transcripts of granzyme B and inflammatory cytokines *IL1B*, *IL-23A*, *IL-6*, and *TNF* in a homeostatic fashion (down-regulated high transcripts and up-regulated low transcripts) (**Figure 5**). The regioisomeric epoxides EDP up-regulated ATF4 and ATF6, which activate unfolded protein response (UPR) in folding enzymes, chaperones and endoplasmic reticulum associated degradation of denatured proteins (ERAD) (**Figure 6**).

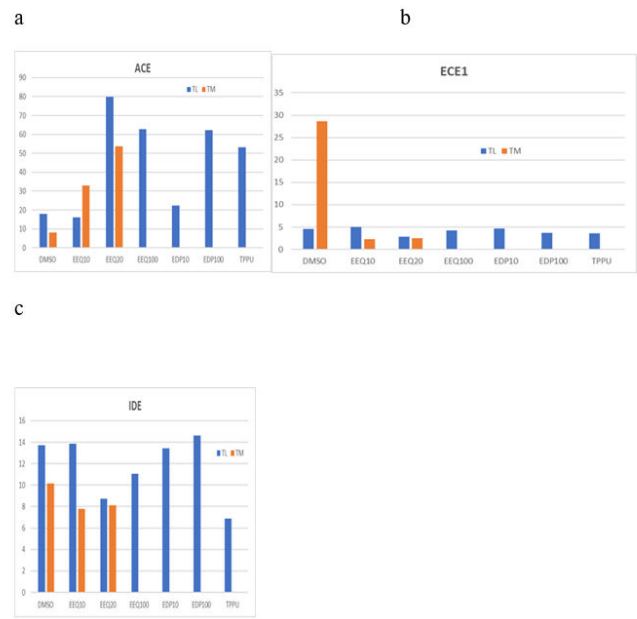


Figure 4. Epoxy fatty acids (EpFAs) up-regulate the $A\beta$ -degrading transcript ACE in macrophages of two Alzheimer's disease patients. Ω -3 EpFA effects on macrophage transcriptome of two AD patients: a) EEQ and EDP up-regulate $A\beta$ -degrading enzyme angiotensin converting enzyme (ACE), (b) EEQ down-regulates ECE1 endothelin converting enzyme (ECE1); c) EpFA effects on insulin-degrading enzyme (IDE) were divergent. When the bar is missing, no transcript was detected.

a) EpFAs regulate IL-1B, GZMB, IL-6, and TNF in a homeostatic fashion in each patient. When the bar is missing, no transcript was detected.

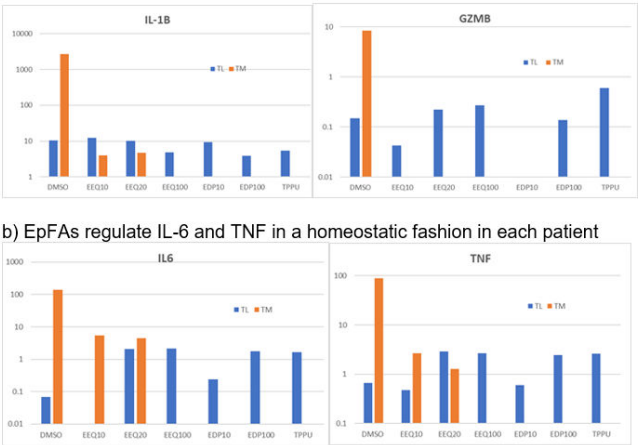


Figure 5. EpFAs regulate inflammatory cytokines and granzymes in a homeostatic fashion (down-regulate high transcripts and up-regulate low transcripts).

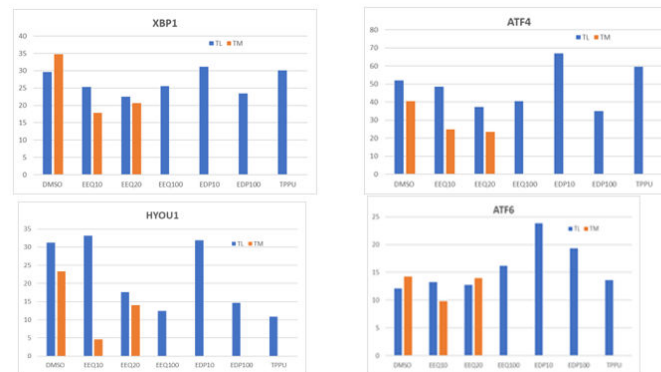


Figure 6. Effects of ω -3 EpFAs on endoplasmic reticulum (ER) stress genes and unfolded protein response (UPR) genes: a) EDP up-regulates XBP1, which activates UPR gene expression in folding enzymes, chaperones, and ERAD; b) EDP up-regulates ATF4, which activates autophagy and CHOP; c) EEQ and EDP activate HYOU1; d) EDP up-regulates ATF6, which activates chaperones.

3.5. EpFAs together With The Soluble Epoxide Hydrolase Inhibitor TPPU or the STING inhibitor H151 Increase Uptake and Degradation of FITC-Amyloid- β in AD Macrophages

We tested the effects on macrophage uptake of FITC-A β of 2-hour and 24-hour treatments with EpFAs together with the sEH inhibitor TPPU or the cGAMP/STING inhibitor H151 and showed the effects according to the mean difference at 2 hours and 24-hours. The 2-hour A β uptake in macrophages was significantly ($P < 0.0000$) increased (in descending order) by a) H-151, and b) EEQ + TPPU vs. DMSO. The 24-hr residual A β was significantly decreased by a) EEQ + TPPU, and b) H-151 vs. DMSO (**Figure 7**).

3.6. A β is Transferred From Plaques To Vessels Despite Aducanumab therapy In the Yale study [34], the Figure 2 Shows Overall Reduction of A β in the Brain Of A Patient Treated By Aducanumab vs. The Brain of An Untreated Patient, But Also The Presence Of Amyloid Angiopathy In Both The Treated And The Untreated Brain. When Adjusted for the Higher Magnification In The Figure 2a vs. Figure 2b, the Deposits Are Comparable In Both Brains. These results Are Consistent With Transfer Of A β From The Plaques To Vessels Despite Aducanumab Therapy and the Need for A β degradation.

4. Discussion

4.1. Polyunsaturated fatty Acids Protect The Brain By Increasing The Epoxides Of Polyunsaturated Fatty Acids (EpFAs), which Inhibit Macrophage Mechanisms Of Vascular Complications. A soluble epoxide Hydrolase Inhibitor Is Mandatory To Protect Against EpFA Degradation By Soluble Epoxide Hydrolase

In our previous study, a nutritional supplementation program delivering ω -3 fatty acids DHA and EPA together with anti-oxidants in the commercial Smartfish[®] drink protected the cognition of patients with mild cognitive impairment (MCI) against mental decline beyond that provided by cholinesterase inhibitors [35] but the patients continued to decline, showing that omega-3 supplementation alone is not a curative approach [36].

PUFA supplementation increases the blood EpFAs in multiple studies [37]. Here, we demonstrate that EpFAs, especially EEQ and EDP derived from ω -3 fatty acids, modulate the transcriptome and increase the clearance of A β by macrophages. EEQ upregulates in macrophages the A β degrading enzyme ACE and energy molecules [10] and regulates the inflammatory cytokines and markers of endoplasmic reticulum (ER) stress in homeostatic fashion. The 2-hour up-loading of

FITC-A β in macrophages is accelerated by EEQ with TPPU or by H-151 and the degradation of FITC-A β is strongly increased at 24 hours by these molecules (Figure 7).

(A) FITC-A β density in macrophages treated by 1uM EEQ with or without TPPU or by cGAMP inhibitor H-151 2 and 24 hours post treatment

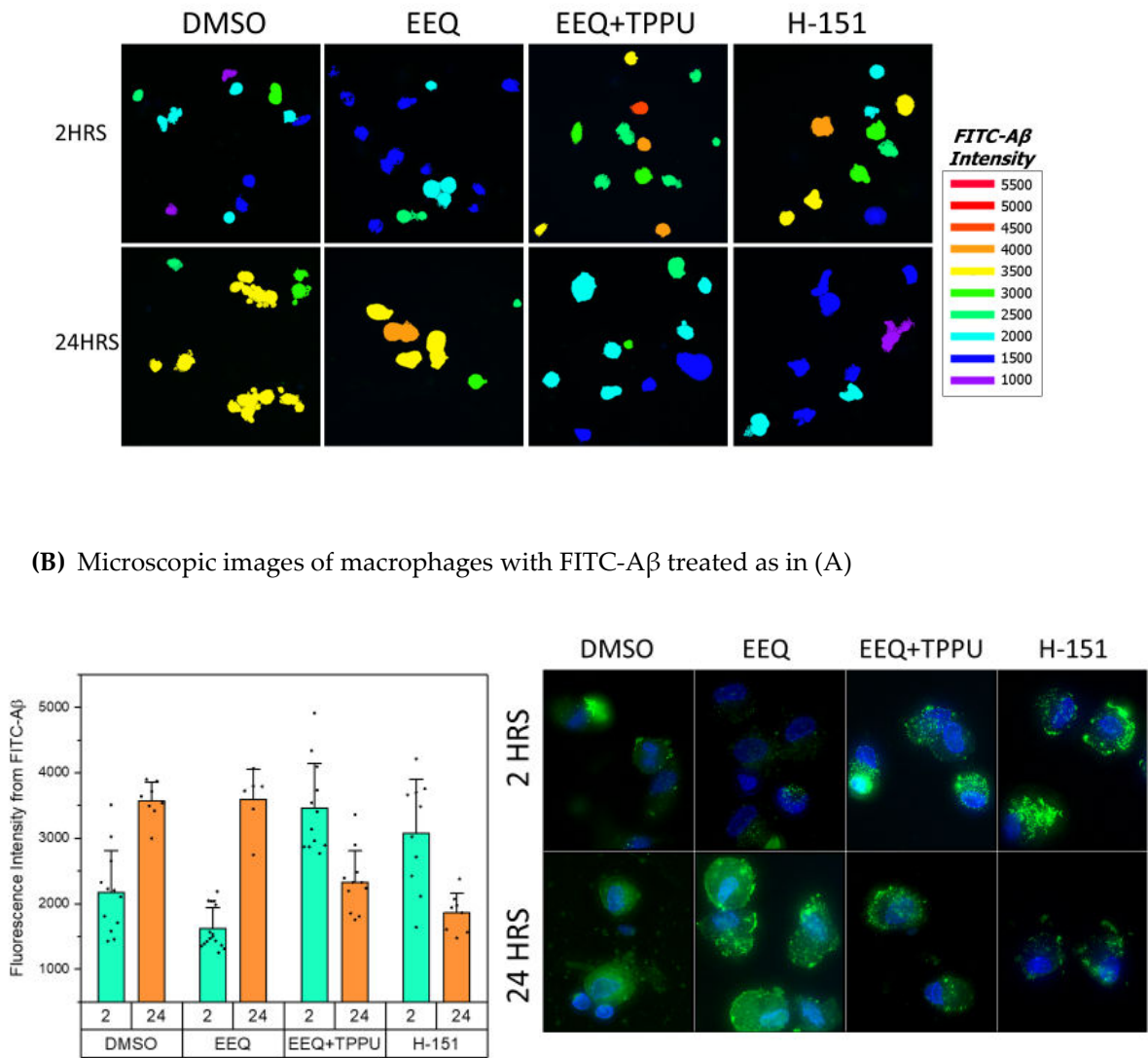


Figure 7. Microscopic imaging of EpFAs effects on a) uptake and b) degradation of amyloid- β in macrophages after addition of FITC-A β : a) at 2 hrs effects are significant a ($P<0.001$) for increased uptake by treatment with EEQ + TPPU compared to H-151; b) at 24 hrs effects are significant for increased degradation by treatment with H-151 compared to EEQ + TPPU compared to EEQ alone and DMSO. The microscopic results are shown in the panel below.

EpFAs up regulate the transcripts of enzymes involved in cellular energetics, which are necessary for phagocytosis and migration of macrophages [31]. The effects of these EpFA mechanisms are therapeutic by a) increasing A β degradation, which alleviates A β transport into vessels, CAA, and damage to the BBB, and b) decreasing inflammatory cytokines, which inhibits vasculitis. The ω -3 positions of PUFAs are more rapidly epoxidized by cytochrome P450 enzymes than other regioisomers of either ω -3 or ω -6 PUFAs and the omega 3 epoxides are the most resistant regioisomers to hydrolysis by the sEH.

4.2. Practical Use Of The Molecular Inhibitors And Polyunsaturated Fatty Acids In Therapy of AD

Several studies have indicated that sEHIs used alone offer promise in the treatment of a number of chronic neurological diseases [38,39] [40,41]. Following their discovery in the 1970's and early characterization, the first clinical targets were cardiovascular pathologies. sEHs alone, presumably by increasing EpFAs, reduce vascular inflammation, atherosclerosis, and hypertension, which may protect cerebral vasculature from AD-associated damage. Our study suggests that, in addition to these vascular effects, the sEH inhibitor TPPU in synergy with EpFAs has a direct positive AD effect on the disease through increased uptake of A β at 2 hours and completed degradation of A β at 24 hours. This is also produced by the STING inhibitor H-151. In an ageing mouse model, the restoration of cellular bioenergetics through inhibition of the prostaglandin E₂ receptor 4 (EP4) signaling in myeloid cells restored cognition [14]. Therefore, the combined inhibition of both sEH and EP4 in macrophages of AD patients should be tested in a future clinical trial, as has been examined in cancer [42].

The use of sEHs could be therapeutically valuable on their own or as a supplement to an omega-3 diet (e.g. EO3 drink). EC5026, a compound similar to TPPU, is in human safety trials on escalating oral doses from 1 mg per day [43]. In addition, the effects of the STING inhibitor H-151 were comparable to sEH effects and could be additive or synergistic. The recommendations of the best therapy will be guided further by field experience, yet these three agents (TPPU or EC5026, H-151, and the prostaglandin EP4 inhibitor ONO), offer promise of reducing the adverse effects of monoclonal antibody therapies and directly improving AD therapy.

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Data availability: The data on the EpFA effects on the transcriptome in two patients are available from the authors.

Conflict of interests: Bruce D. Hammock and Sung Hee Hwang are developing the EC5026 inhibitor of soluble epoxide hydrolase for therapy of Alzheimer's disease and other neurodegenerative diseases.

Author Contributions: Milan Fiala designed the study, performed the bulk of experiments, and wrote the manuscript; Sung Hee Hwang prepared epoxy fatty acids and sEH inhibitors in the laboratory of B. Hammock; Bruce Hammock provided intellectual content; Julian Whitelegge provided intellectual content; K. Paul provided intellectual content; Karolina Kaczor-Urbanowicz and Andrzej Urbanowicz performed RNA sequencing of macrophages treated by EpFAs by processing the final libraries and aligning the data to the human genome GCHr38 and analysis of treated and untreated samples. S. Kesari followed the patients and provided intellectual content. Haley Marks and Brian Jeong provided microscopic imaging of macrophage cultures in **Figure 7**.

Acknowledgments: We thank Jeffrey Gornbein, PhD for the statistical analysis of the immunofluorescence results in **Figure 7**.

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