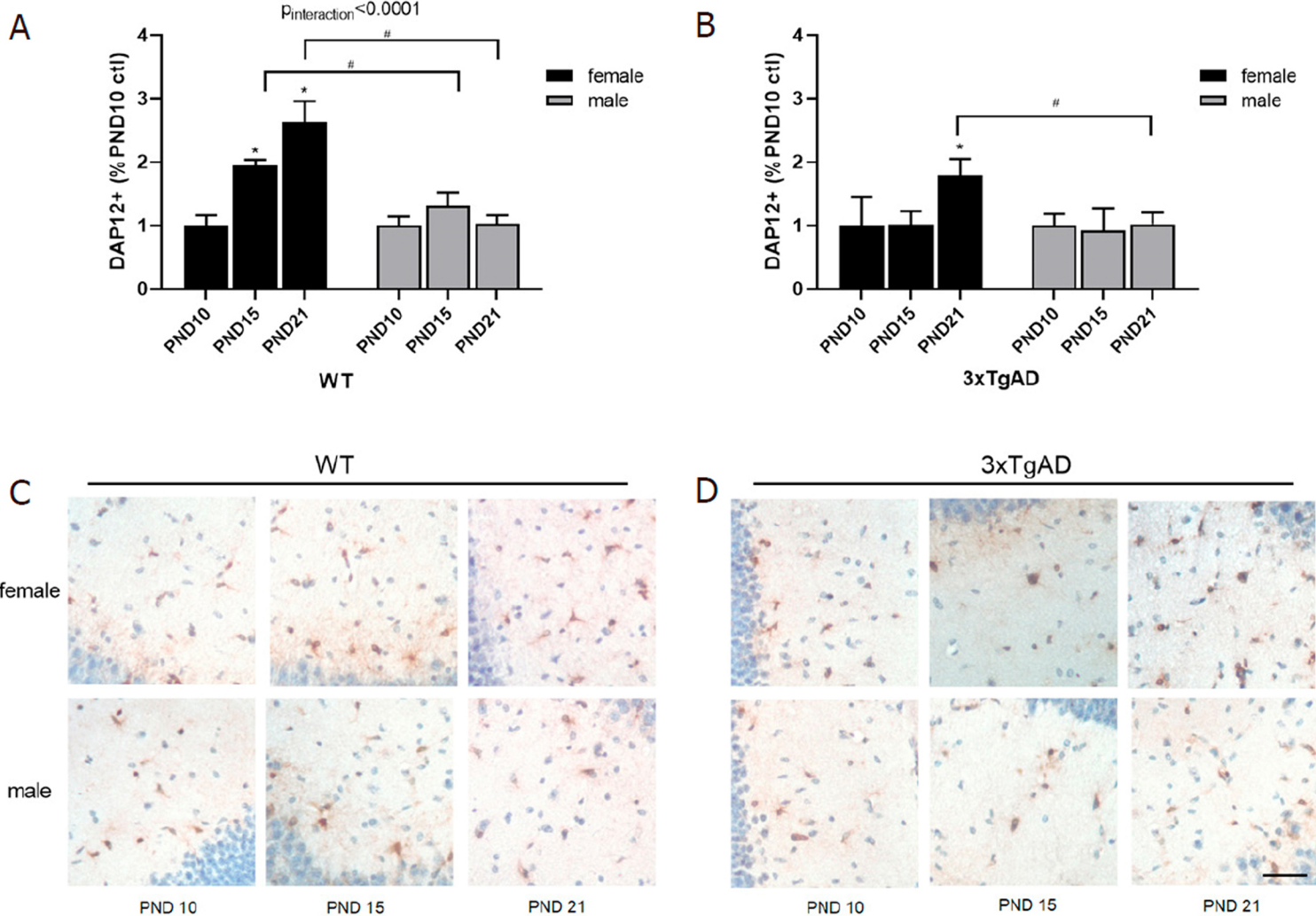


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Original Article

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β -funaltrexamine differentially modulates chemokine and cytokine expression in normal human astrocytes and C20 human microglial cells

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Abstract

Aim: Emerging evidence implicates astrocyte/microglia dysregulation in a range of brain disorders, thereby making glial cells potential therapeutic targets. The novel anti-inflammatory actions of beta-funaltrexamine (β -FNA) are of particular interest. β -FNA is a derivative of naltrexone, and recognized as a selective, irreversible antagonist at the μ -opioid receptor (MOR). However, we discovered that β -FNA has novel anti-inflammatory actions that seem to be mediated through a MOR-independent mechanism. Thus far, we have focused on the acute effects of β -FNA on inflammatory signaling.

Methods: The effect of β -FNA treatment on interleukin-1 β (IL-1 β)-induced inflammatory signaling in normal human astrocytes (NHA) and C20 human microglial cells. Cytokine/chemokine expression was measured using ELISA, and nuclear factor-kappaB (NF- κ B) p65 activation was evaluated by immunoblot.

Results: IL-1 β -induced interferon- γ inducible protein-10 (CXCL10) production in NHA was more sensitive to chronic (3 day) β -FNA as indicated by an approximately 3-fold lower EC₅₀ compared to that observed in acutely treated cells. Chronic β -FNA did not affect IL-1 β -induced monocyte chemoattractant protein-1 (CCL2) or IL-6 production in NHA. β -FNA inhibited phosphorylation of NF- κ B p65, suggesting that the inhibitory effects may be due in part to reduced NF- κ B activation. We showed for the first time that C20 human microglial cells were insensitive to the anti-inflammatory actions of acute β -FNA.



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Conclusion: β -FNA differentially affects inflammatory cytokine/chemokine expression in human astrocytes and microglia. These findings warrant further investigation into the novel anti-inflammatory actions of β -FNA, with a particular focus on astrocytes. These insights should contribute to the development of strategies to treat brain disorders that involve neuroinflammation.

Keywords: Neuroinflammation, brain, opioid, nuclear factor-kappaB, pro-inflammatory

INTRODUCTION

Neuroinflammation is present in brain infection, trauma, neurodegenerative diseases, and psychiatric disorders as well^[1-5]. Glial cells, including astrocytes and microglia are instrumental in neuroinflammation. The therapeutic effectiveness of certain antidepressant and antipsychotic drugs reportedly results, in part, from anti-inflammatory actions^[6-9]. Astrocytes and microglia have integral roles in metabolic/neurotrophic support, ion and neurotransmitter homeostasis, synaptic plasticity, and modulation of neuronal excitability; and they respond to infection and cellular insults associated with neuropathology^[10-13]. Astrocytes have a fundamental role in neuropathogenesis, in part, through the release of neurotoxic/neuroinflammatory factors including cytokines [i.e., interleukin (IL)-1 β and IL-6] and chemokines [interferon- γ inducible protein-10 (CXCL10) and monocyte chemoattractant protein-1 (CCL2)]. These secreted factors can be directly neurotoxic or can induce further glial activation/dysregulation^[14-17]. Microglia are resident CNS macrophages and thus play an important role in neuroinflammation, in part, through phagocytosis of cellular debris and release of inflammatory mediators including cytokines/chemokines. Importantly, emerging evidence highlights the importance of astrocyte/microglia dysregulation in a wide range of CNS disorders, thus implicating glial cells as potential therapeutic targets^[18-21].

We, and others, are therefore interested in identifying novel, anti-inflammatory agents that are therapeutically effective in the treatment of neurological disorders. We are interested in the previously identified, novel anti-inflammatory actions of beta-funaltrexamine (β -FNA). As a fumaramate methyl ester derivative of naltrexone, β -FNA is most notably recognized as a selective, irreversible antagonist at the mu-opioid receptor (MOR)^[22,23]. In both behavioral and *in vitro* assays, β -FNA acts initially as a reversible kappa-opioid receptor agonist, and then later results in MOR antagonism^[24,25]. As an alkylating agent, β -FNA irreversibly antagonizes MOR by covalently binding at Lys233 on the receptor^[23]. However, we discovered that β -FNA also has novel anti-inflammatory actions which seem to be mediated through MOR-independent actions^[26-28]. For instance, neither naltrexone (a nonselective opioid receptor antagonist) nor D-Phe-Cys-Tyr-D-Trp-D-Arg-Pen-Thr-NH₂ (CTAP) inhibits pro-inflammatory-induced CXCL10 expression in human astroglial cells^[27,28]. Additionally, we predicted that if the anti-inflammatory actions of β -FNA were due to alkylation, this covalent modification should then remain after washout. Indeed, pre-treatment of astroglial cells with β -FNA for 60 min, followed by drug washout prior to stimulating with IL-1 β (or tumor necrosis factor α), resulted in inhibition similar to 24 h co-exposure (cytokine stimulus + β -FNA). These findings suggested that β -FNA-induced modifications (i.e., alkylation) are *persistent* and lead to the disruption of signal transduction. Notably, our *in vitro* findings also showed that β -FNA reduces inflammatory signaling in astroglia, regardless of whether the stimulus is tumor necrosis factor α , IL-1 β or bacterial lipopolysaccharide (LPS). We also determined that β -FNA inhibits LPS-induced pro-inflammatory cytokine expression in mouse brain (but not in plasma)^[29]. Furthermore, treatment with β -FNA reduced LPS-induced sickness behavior in mice suggesting important translational implications^[29].

The primary goal of the present study was to determine the effect of chronic β -FNA treatment on inflammatory signaling in NHA. Additionally, we assessed for the first time the effects of β -FNA on inflammatory signaling in human microglial cells.

METHODS

Cells

Normal human astrocytes (NHA, cat# HA1800; ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured as previously reported^[28]. Briefly, cultures were maintained in growth medium containing Astrocyte Medium (cat# 1801), 1% Astrocyte Growth Supplement (cat# 1852), 1% penicillin/streptomycin solution (cat# 0503), and 2% fetal bovine serum (FBS). Culture medium was replaced every 48 h. Experimental cultures were seeded in either 24-well plates (1.15×10^4 cells/well) or 100-mm dishes (2.5×10^5 cells/dish) and grown until 90% confluent (15 days).

C20 human microglial cells were obtained from David Alvarez-Carbonell, PhD (Case Western Reserve University), and the details regarding the generation of this cell line have been reported^[30]. The C20 cells that we received were evaluated by the Human Identity Testing Laboratory at Oklahoma State University Center for Health Sciences and confirmed to be of human origin as previously described (Davis *et al.*^[31], 2018). Cultures were maintained in growth medium [Dulbecco's Modified Eagle Medium/Ham's F-12 50/50 mix supplemented with 2.5 mmol/L L-glutamine (Corning 10-090-CV), 10% FBS (Atlanta Biologicals S11550), and 1% penicillin/streptomycin (Lonza 17603E)] as previously reported^[31].

Treatment of cells

To determine the dose-dependent effect of chronic (72 h) β -FNA (NIDA reagent supply program; Bethesda, MD, USA) on CXCL10 expression in NHA, cells were initially cultured in growth medium containing 0.04–10 μ mol/L β -FNA for 24 h. The medium was then replaced with serum-free medium (SFM) containing β -FNA for an additional 48 h; IL-1 β (3 ng/mL; Peprotech, Rocky Hill, NJ, USA) was added to cultures for the final 24 h of the 72 h exposure period. To determine the differential effects of β -FNA on chemokine/cytokine expression, NHA were treated as described above; however, only a single concentration of β -FNA was used (3 μ mol/L; EC₅₀ for inhibition of CXCL10 expression). To assess the effects of β -FNA on IL-1 β -induced activation of (NF)- κ B p65, cells were chronically exposed to β -FNA (3 μ mol/L) as described above, then stimulated for 30 min with IL-1 β (3 ng/mL). IL-1 β -induced NF- κ B p65 activation was assessed at 30 min after stimulation (when peak activation is observed).

This was our initial investigation into the effects of β -FNA on chemokine/cytokine expression in C20 microglial cells. Thus, we used our acute exposure model. Consistent with the acute model previously used with astrocytes, we used a higher concentration range of β -FNA (3–30 μ mol/L). Briefly, C20 microglial cells were serum deprived for 24 h and then treated with β -FNA (3–30 μ mol/L) alone or in combination with IL-1 β (20 ng/mL) for 24 h in SFM.

Cell viability

Cell viability was measured using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously reported^[27]. Briefly, cells were exposed to MTT (0.55 mg/mL) for 45 min; next, the medium was removed, and cells were dissolved in 1 mL DMSO. The absorbance was then measured at 492 nm using a BIO-TEK HT spectrophotometer.

Chemokine/cytokine expression

Standard dual-antibody solid-phase immunoassays (ELISA Development Kit, Peprotech) were used for quantitation of cytokines/chemokines in culture medium as previously described^[27]. Values were normalized to total protein content, which was determined using the bicinchoninic acid protein assay as previously described^[32].

NF- κ B activation

Phosphorylated NF- κ B p65 in nuclear fractions was measured as an indicator of NF- κ B p65 activation. Following experimental treatments, NHA (in 100-mm dishes) were washed two times with PBS; nuclear

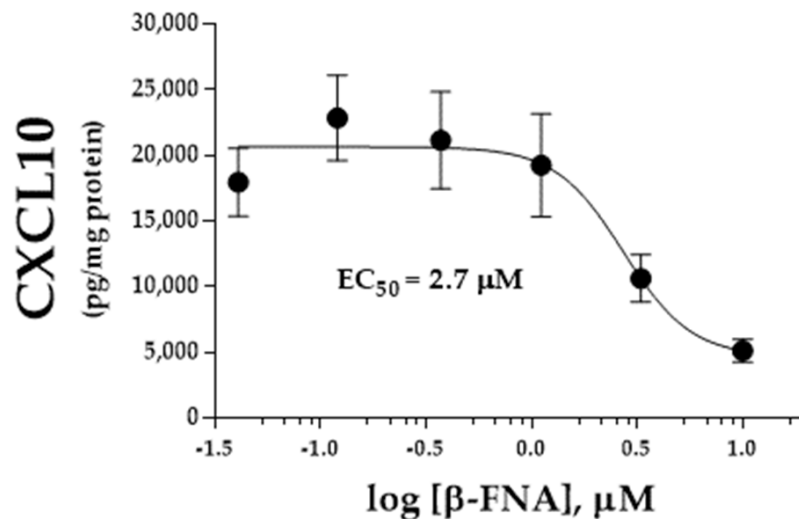


Figure 1. Chronic β -FNA dose-dependently inhibits CXCL10 expression in NHA. Cells were initially cultured in growth medium containing 0.04-10 μ mol/L β -FNA for 24 h; the medium was then replaced with serum-free medium containing β -FNA for an additional 48 h. IL-1 β (3 ng/mL) was added to cultures for the final 24 h. CXCL10 in the medium was measured by ELISA. Data are presented as mean \pm SEM (n = 8-9). CXCL10: interferon- γ inducible protein-10; NHA: normal human astrocytes; ELISA: enzyme-linked immunoabsorbant assay; SEM: standard error of the mean; β -FNA: beta-funaltrexamine; IL-1 β : interleukin-1 β

protein extracts were then collected as previously reported^[33], except the lysis buffers contained 1 mmol/L sodium orthovanadate (Sigma, #450243). Total protein (30 μ g) was loaded on 7.5% polyacrylamide gels (BioRad TGX FastCast Acrylamide kit, #161-0171), electrophoresed and then transferred to PVDF membranes. Membranes were incubated (with rocking) for 15-18 h at 4 $^{\circ}$ C. Primary antibodies included anti-phospho-NF- κ B p65 (1:1000, Cell Signaling #3033S), anti-NF- κ B p65 (1:1000, Cell Signaling #4764S), and anti- β -tubulin (1:1000, Cell Signaling #2146S). Restore Western blot stripping buffer (Thermo Scientific #21059) was used to remove antibodies and allow for re-labelling of membranes. Anti-rabbit IgG, AP-linked (1:1000, Cell Signaling #7054S) was used as the secondary antibody. The blots were scanned in a Typhoon 9410 phosphorimager (GE Healthcare, Uppsala, Sweden) using enhanced chemifluorescence reagent (GE Healthcare, UK). Densitometric analysis was performed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analyses and figure presentations were performed using PrismTM version 7.04 (GraphPad Inc., San Diego, CA). Dependent measures were analyzed by either one-way or two-way analysis of variance (ANOVA). In those instances where two-way ANOVA was used, stimulus and drug dose were the grouping variables. Data that were > 2 SD from the mean were considered outliers and removed from the analyses. When ANOVA revealed a statistically significant interaction, data were further assessed using a Fisher's LSD test. The data are all presented as mean \pm SEM.

RESULTS

Effects of chronic β -FNA on IL-1 β -induced CXCL10 expression in NHA

Exposure of NHA to β -FNA for 72 h resulted in a concentration-dependent inhibition of IL-1 β -stimulated CXCL10 expression, with an EC_{50} = 2.7 μ mol/L [Figure 1]. In a subsequent experiment, chronic exposure to a single concentration of β -FNA (3 μ mol/L) significantly ($P < 0.01$) inhibited IL-1 β -induced CXCL10 expression as indicated by ANOVA and pairwise comparison by a Fisher's LSD test. However, neither IL-1 β -induced CCL2 nor IL-6 expression was significantly ($P \geq 0.22$) affected by β -FNA [Figure 2].

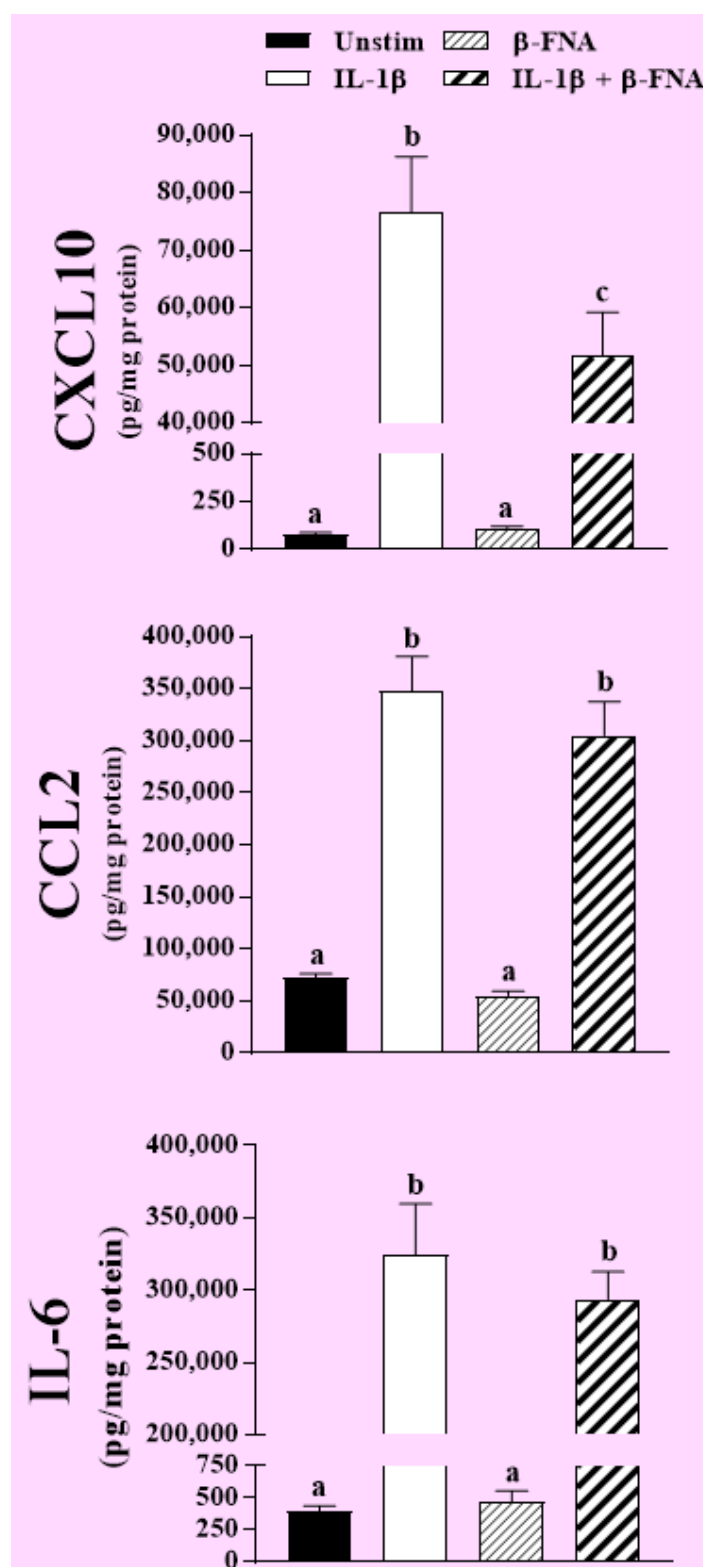


Figure 2. Chronic β -FNA differentially affects chemokine/cytokine expression in NHA. Cells were cultured in growth medium containing 3 μ mol/L β -FNA for 24 h; the medium was then replaced with serum-free medium containing β -FNA for an additional 48 h. IL-1 β (3 ng/mL) was added to cultures for the final 24 h. Chemokine/cytokine levels in the medium were measured by ELISA. Data are presented as mean \pm SEM ($n = 11-16$). Differing letters above the bars indicate the means are significantly ($P < 0.01$) different as determined by ANOVA and subsequent Fisher's LSD. NHA: normal human astrocytes; ELISA: enzyme-linked immunoabsorbant assay; ANOVA: analysis of variance; LSD: least significant difference; SEM: standard error of the mean; β -FNA: beta-funaltrexamine; IL-1 β : interleukin-1 β

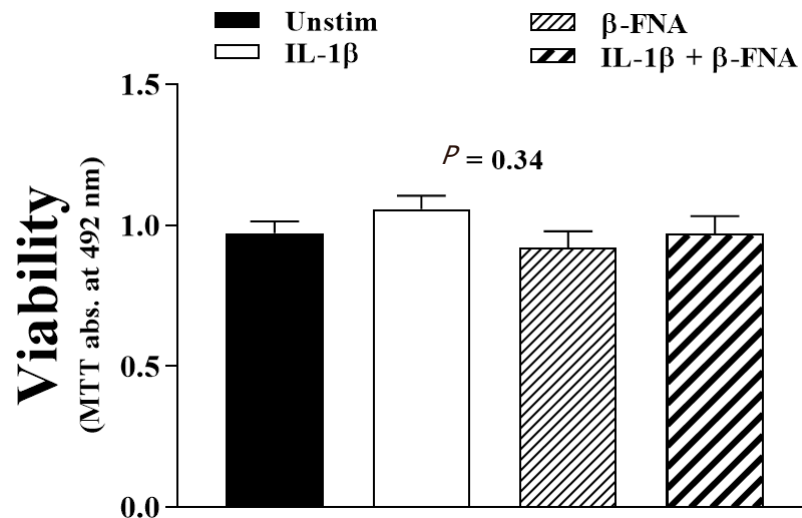


Figure 3. Effects of chronic β -FNA on viability of NHA. Cells were cultured in growth medium containing 3 μ mol/L β -FNA for 24 h; the medium was then replaced with serum-free medium containing β -FNA for an additional 48 h. IL-1 β (3 ng/mL) was added to cultures for the final 24 h. Cell viability was assessed using the MTT assay. Data are presented as mean \pm SEM ($n = 8$). ANOVA did not reveal any significant differences. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM: standard error of the mean; β -FNA: beta-funaltrexamine; NHA: normal human astrocytes; IL-1 β : interleukin-1 β

Effects of chronic β -FNA on NHA viability

Cell viability was not significantly ($P = 0.34$) affected by chronic exposure to β -FNA alone or in combination with IL-1 β , as revealed by ANOVA [Figure 3].

Effects of chronic β -FNA on NF- κ B activation in NHA

One-way ANOVA and pairwise comparison by a Fisher's LSD test revealed that chronic exposure to β -FNA significantly ($P < 0.05$) inhibited IL-1 β -induced phosphorylation of nuclear NF- κ B p65 in NHA [Figure 4]. The expression of total nuclear NF- κ B p65 in NHA was not significantly ($P = 0.2$) affected by IL-1 β or β -FNA alone, or the combination of chronic β -FNA plus stimulation with IL-1 β [Figure 4].

Effects of acute β -FNA on IL-1 β -induced chemokine/cytokine expression in C20 microglial cells

Two-way ANOVA revealed that IL-1 β significantly increased CXCL10 ($F_{1,79} = 51.4$; $P < 0.0001$), CCL2 ($F_{1,66} = 0.71$; $P < 0.0001$), and IL-6 ($F_{1,51} = 55.6$; $P < 0.0001$) levels in C20 microglial cells [Figure 5]. Acute (24 h) exposure to β -FNA did not significantly affect expression of CXCL10 ($F_{3,79} = 0.19$; $P = 0.9$), CCL2 ($F_{3,66} = 0.71$; $P = 0.54$), or IL-6 ($F_{3,51} = 0.065$; $P = 0.98$) in C20 microglial cells. Furthermore, there was no significant interaction between stimulation and β -FNA dose for CXCL10, CCL2, or IL-6 ($F_{3,79} = 0.24$; $P = 0.86$; $F_{3,66} = 0.60$; $P = 0.62$; $F_{3,51} = 0.07$; $P = 0.98$, respectively).

Effects of acute β -FNA on C20 microglial cell viability

Two-way ANOVA indicated that the viability of C20 microglial cells was not significantly affected by IL-1 β ($F_{1,86} = 0.025$; $P = 0.87$), yet there was a significant main effect of β -FNA ($F_{3,86} = 4.99$; $P < 0.005$) [Figure 5]. Additionally, there was no significant interaction between stimulation and β -FNA ($F_{3,86} = 0.26$; $P = 0.85$).

DISCUSSION

Astrocytes and microglia are instrumental in neuroinflammation and both cytokines and chemokines are among the inflammatory molecules released by these cells during neuroinflammation^[34-36]. Hence, there is substantial interest in targeting neuroinflammation as a therapeutic strategy for selected brain disorders. The therapeutic potential of β -FNA is of particular interest to our group, and the results of this study are in

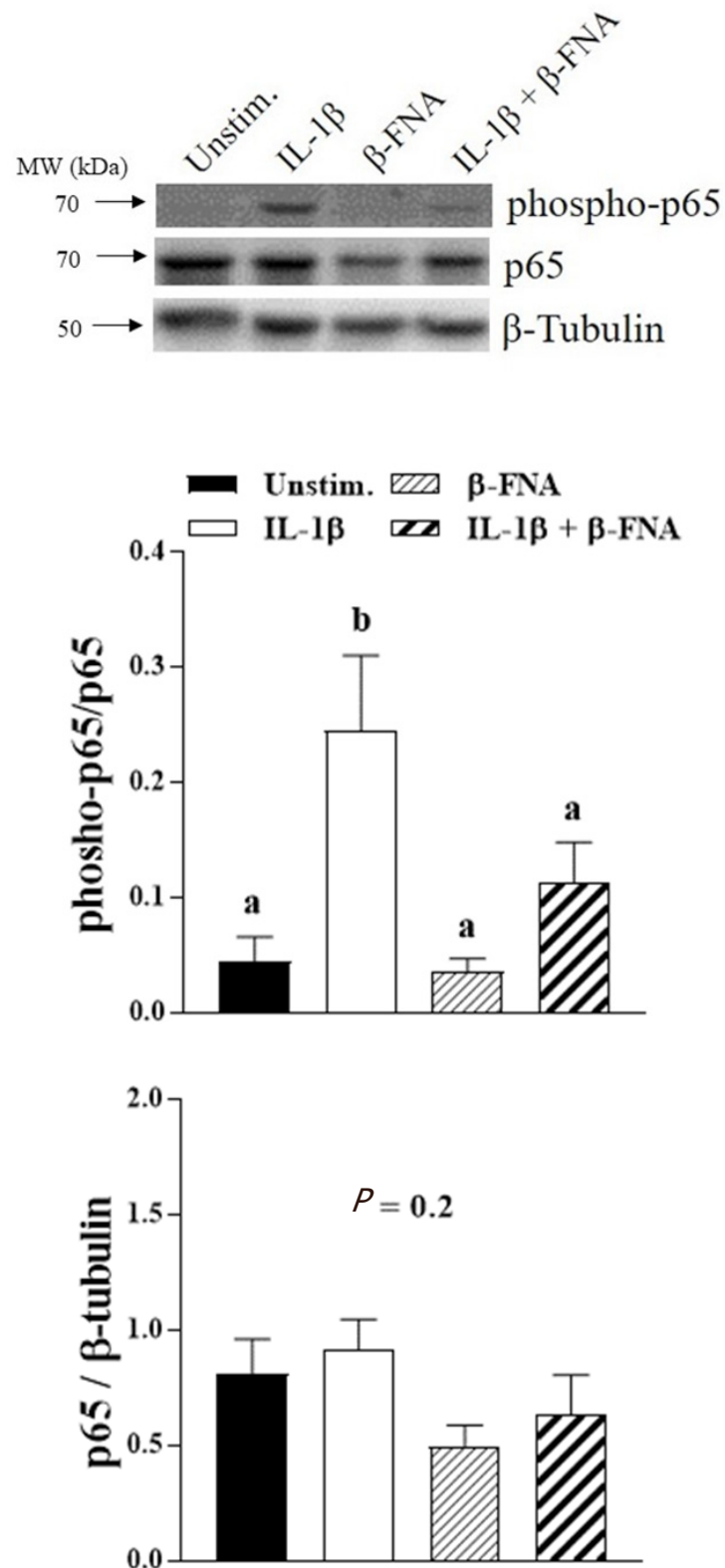


Figure 4. Chronic β -FNA inhibits NF- κ B activation in NHA. Cells were cultured in growth medium containing 3 μ mo/L β -FNA for 24 h; the medium was then replaced with serum-free medium containing β -FNA for an additional 48 h. IL-1 β (3 ng/mL) was added to cultures for the final 30 min. Western blot was used to measure levels of phospho-NF- κ B p65, NF- κ B p65, and β -tubulin in nuclear extracts (a representative blot is presented at the top of the figure). Data are presented as mean \pm SEM ($n = 5$). Differing letters above the bars indicate the means are significantly ($P < 0.05$) different as determined by ANOVA and subsequent Fisher's LSD. LSD: least significant difference; ANOVA: analysis of variance; SEM: standard error of the mean; IL-1 β : interleukin-1 β ; β -FNA: beta-funaltrexamine; NF- κ B: nuclear factor-kappaB; NHA: normal human astrocytes

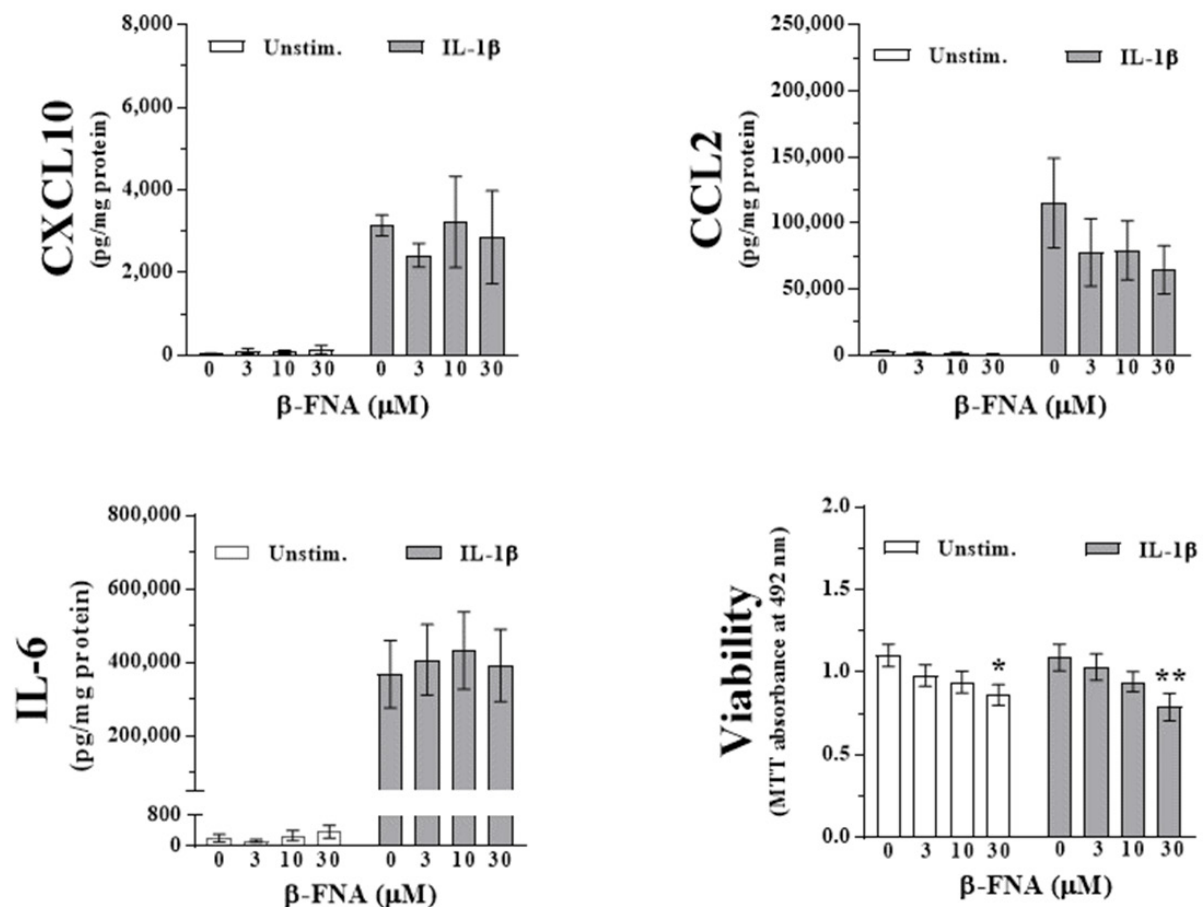


Figure 5. β -FNA differentially affects chemokine/cytokine expression in C20 human microglial cells. Cells were serum deprived for 24 h and then exposed to β -FNA (3-30 μ mol/L) alone or in combination with IL-1 β (20 ng/mL) for 24 h. Chemokine/cytokine levels in the medium were measured by ELISA; and viability was determined using the MTT assay. Data are presented as mean \pm SEM ($n = 8-12$) and were analyzed by two-way ANOVA and subsequent Fisher's LSD. * $P < 0.05$ vs. unstimulated, 0 μ mol/L β -FNA; ** $P < 0.005$ vs. IL-1 β , 0 μ mol/L β -FNA. ELISA: enzyme-linked immunoabsorbant assay; LSD: least significant difference; IL-1 β : interleukin-1 β ; β -FNA: beta-funaltrexamine; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM: standard error of the mean

line with our previous findings and advance our understanding of the anti-inflammatory effects of β -FNA. In our earlier studies, we found that β -FNA inhibition of IL-1 β -stimulated CXCL10 expression was at least in part transcriptional, given that both protein and mRNA levels were significantly reduced by β -FNA^[37]. We now show that CXCL10 inhibition in human astrocytes is even more sensitive to chronic (3 day) β -FNA as indicated by an approximately 3-fold lower EC_{50} compared to that observed in acutely treated cells. Importantly, and consistent with our previous findings^[27], the inhibitory effects of β -FNA are not due to cytotoxicity, as we have shown that even extended exposure to β -FNA does not reduce the viability of human astrocytes. These preclinical findings that astrocyte viability is not compromised are quite important as future therapeutic potential will likely involve extended exposure to this drug.

Interestingly, β -FNA did not inhibit IL-1 β -induced expression of either CCL2 or IL-6 in NHA, suggesting a level of selectivity for CXCL10. However, it may be that higher concentrations would inhibit the expression of CCL2 and IL-6. Therefore, further investigation, including a dose-response, is necessary.

Mechanistically, there is still more to learn about the anti-inflammatory effects of β -FNA. Importantly, as stated above and discussed previously in detail, we have determined that the anti-inflammatory effects in astrocytes do not seem to be due to actions at MOR (or other opioid receptors)^[26-28]. Overall, our previous

findings on the acute effects of β -FNA suggest that the anti-inflammatory actions are likely mediated, at least in part, by disrupting the NF- κ B signaling pathway^[26-28,37]. In this study, we found that NF- κ B signaling is also inhibited by chronic exposure to a lower concentration of β -FNA. Interestingly, the overall level of NF- κ B p65 in the nucleus was not impacted by β -FNA; rather, phosphorylation of NF- κ B p65 was reduced in the presence of β -FNA. Together, these findings suggest that β -FNA acts at a common factor in the signaling pathways activated by these diverse stimuli, in turn implicating the NF- κ B signaling pathway. We hypothesize that β -FNA exerts these anti-inflammatory effects via alkylation of one or more lysines of the signaling proteins in the NF- κ B pathway, and we are currently testing this hypothesis using *in vitro* approaches. Further studies are warranted to determine the mechanism by which β -FNA inhibits the phosphorylation of NF- κ B p65.

We have also determined that β -FNA exerts anti-inflammatory actions *in vivo*^[29,37]. For instance, LPS-induced neuroinflammation and sickness behavior in mice were attenuated by peripherally administered β -FNA^[29]. More specifically, β -FNA inhibited LPS-induced expression of both CXCL10 and CCL2 in the brain, but had no effect on IL-6 levels. Furthermore, β -FNA did not impact plasma levels of CXCL10, CCL2, or IL-6. These *in vivo* findings are largely in line with our *in vitro* findings in human astrocytes, except that CCL2 expression in NHA was inhibited by β -FNA. Certainly, the differential sensitivity of CCL2 to β -FNA could be related to species differences or model systems (*in vitro* vs. *in vivo*). However, it may also reflect cell type-specific differences in sensitivity to β -FNA. For example, microglial cells (at least IL-1 β -stimulated human microglial cells) are not sensitive to the anti-inflammatory actions of β -FNA. Because relatively high concentrations of β -FNA had no effect on chemokine/cytokine expression in C20 microglial cells and because of the observed cytotoxicity, we did not pursue the chronic effects of β -FNA on these cells at this point. However, in future experiments, we expect to assess chronic exposure to lower concentrations of β -FNA. Together, it is conceivable that the protective effects of β -FNA *in vivo* are largely due to modulatory effects on astrocytes; however, further investigation is needed to clearly establish the cell types affected and mechanisms involved.

In summary, we advanced our understanding of the anti-inflammatory effects of β -FNA by demonstrating that chronic exposure inhibits NF- κ B p65 activation and CXCL10 expression in astrocytes more effectively than does acute treatment. We also found that expression of neither CCL2 nor IL-6 in astrocytes is affected by chronic β -FNA. Lastly, we provided evidence of cell type-specific effects of β -FNA, as indicated by the relative resistance of C20 human microglial cells to the anti-inflammatory effects of β -FNA. Further study is warranted and expected to advance the therapeutic potential of β -FNA, or related compounds, in the treatment of brain disorders that involve neuroinflammation.

DECLARATIONS

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Authors' contributions

Concept, experimental design, literature review, statistical analysis, manuscript preparation: Davis RL
Performed experiments, data acquisition and analysis, and manuscript editing: McCracken K, Buck DJ

Availability of data and materials

Data can be made available upon valid request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Original Article

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Clinical efficacy and safety of single cycle rituximab as induction therapy for aggressive neuromyelitis optica spectrum disorder in a resource limited center: a preliminary study

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Abstract

Aim: To analyse the efficacy of single dose rituximab (RTX) as induction therapy followed by conventional oral steroid-sparing agents (azathioprine, mycophenolate mofetil or methotrexate) in a cohort of patients with aggressive neuromyelitis optica spectrum disorder (NMOSD) without CD19, 20 and 27 biomarker testing.

Methods: A retrospective analysis of clinical outcomes in eight patients with aggressive NMOSD treated with one course of RTX induction therapy in the Neurology Department at Kuala Lumpur Hospital from 2005 to 2018 was performed. The effectiveness of the treatment was determined by the number of relapses, expanded disability status scale, annualized relapsed rates, and modified Rankin Scale both before and after treatment. B cell enumeration testing was done instead of CD19, 20 and 27 biomarker testing.

Results: There was a reduction in the mean annualized relapse rate from 4.7 to 0.5 attacks per year after treatment ($P = 0.011$). Mean expanded disability status scale and modified Rankin Scale values improved from 5.4 to 3.6 ($P = 0.018$) and 3.6 to 2.6 ($P = 0.023$), respectively. No patient developed any adverse effect.

Conclusion: Single-course RTX induction therapy regime may be an alternative therapeutic option in resource limited hospitals to suppress NMOSD disease activity in the short term as pulse induction therapy whilst awaiting the effectiveness of conventional steroid-sparing agents. Further prospectively designed studies are required to prove efficacy.



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Keywords: Neuromyelitis optica spectrum disorder, single-course rituximab, aggressive neuromyelitis optica spectrum disorder

INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune inflammatory demyelinating disorder of the central nervous system with preferential involvement of the optic nerve and spinal cord^[1]. The disease has been reported worldwide with a prevalence ranging from 0.5-4.4/100,000^[2]. In Malaysia, the crude prevalence rate of NMOSD is 1.9 per 100,000 population with a crude annual incidence of 0.3 per 100,000 population^[3]. In this study, NMOSD patients notably experienced more relapses resulting in attack related disability, had higher expanded disability status scale (EDSS) scores and longer disease duration than MS patients^[3]. If untreated, 80% of NMOSD patients experience a second attack within 2 years and the 5-year mortality rate can be as high as 68% with 50% of the surviving patients developing permanent disability^[4]. The mainstay of treatment for NMOSD consists of immunosuppressant therapy. In the acute management of relapses, corticosteroid and plasmapheresis are frequently used. However, long term use of corticosteroid often leads to multiple side effects^[5]. Thus, conventional steroid-sparing immunosuppressive agents such as azathioprine (AZA), methotrexate and mycophenolate mofetil (MMF) are widely utilised as maintenance therapy to achieve remission and prevent relapses^[4].

Recent evidence points to B cell-mediated humoral immunity in the pathogenesis of NMOSD. Rituximab (RTX), a monoclonal antibody targeting the CD20 antigen on B cells, has been found to be effective in several modest quality studies in terms of reducing relapse rates and improving patients' outcomes^[6-9]. RTX offers the prospect of an alternative steroid-sparing regime in the treatment of NMOSD, particularly when rapid disease control is required. Kim *et al.*^[10] demonstrated the benefits of induction RTX followed by repeated doses to control disease activity when CD27 and memory B cells in the blood increased to more than 0.05%. Nevertheless, treatment with RTX is expensive and can cause profound B cell depletion, leading to the risk of severe immunosuppression and infection. Additionally, the monitoring of B cells and CD27 or CD19 are costly and multiple courses of RTX are a burden in regions with limited resources. At our institution, RTX is used as short course induction pulse therapy in patients with aggressive NMOSD followed by de-escalation to steroid-sparing agents such as AZA or MMF. Recent studies also suggest clustering of attacks occurring within 12 months of disease diagnoses, suggesting the hypothesis of "hitting hard" with early therapy to dampen the early aggressive inflammatory activity and later, down regulating to maintenance therapy may help in these fulminant situations^[11].

METHODS

In each patient, a diagnosis of NMOSD was made based on criteria described by the International Panel for Neuromyelitis optica (NMO) Diagnosis of 2015^[1]. Relapses in this study were defined as objective worsening or new neurological symptoms and signs lasting for at least 24 h. Aggressive NMOSD in our cohort of patients was defined as one or more disabling relapses in the preceding 12 months in patients with or without maintenance immunosuppressant therapy. Disabling relapses were defined as worsening EDSS at the time of relapse to 6.5 or more, Medical Research Council power of 3/5 or worse in the lower limbs, or visual acuity worse than 6/36 in one or both eyes at the time of relapse over the preceding one year. The patients in our observational study were on stable doses of oral prednisolone for at least three to six months prior to commencement of RTX.

This study was registered under the National Medical Research Register, Ministry of Health Malaysia (NMRR-19-291-46157 S1).

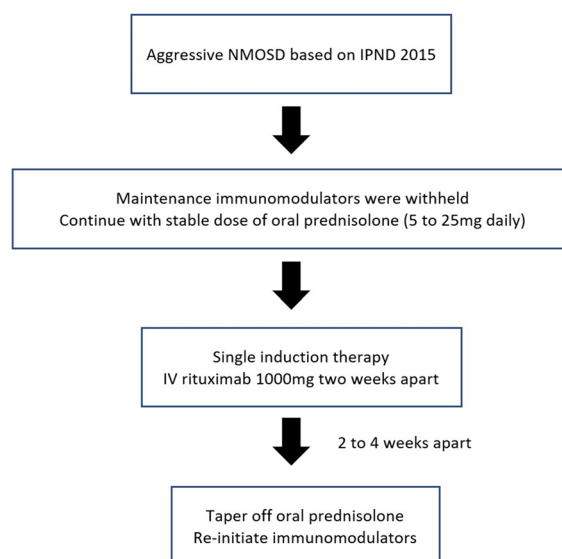


Figure 1. Protocol of single dose rituximab as induction therapy for aggressive NMOSD patients in Kuala Lumpur Hospital, Malaysia. NMOSD: neuromyelitis optica spectrum disorder; IPND: international panel for neuromyelitis optica diagnosis

This clinical outcome study is a retrospective analysis of patients with aggressive NMOSD treated with RTX in the Neurology Department at Kuala Lumpur Hospital from 2005 to 2018. The study included eight NMOSD patients who had received one course of RTX induction therapy. Clinical, laboratory and neuroimaging data from both in-patient admission and outpatient records of all patients were collected and analyzed. The primary outcomes included were the number of relapses, EDSS, annualized relapse rates (ARR), and modified Rankin Scale (mRS) before and after treatment. The EDSS was used to quantify the disability of our patients. It was assessed during six-monthly follow-up. ARR was defined as the number of clinical attacks per year. The secondary objective was to analyse the side effects of a single course rituximab treatment.

Treatment protocol

During the acute attack, high dose intravenous methylprednisolone at 1000 mg daily was given for 5 days. Patients who continued to deteriorate or did not show significant improvement in clinical signs and symptoms after 2 weeks treatment were given plasma exchange (consisting of five cycles each). RTX was used thereafter for aggressive NMOSD patients who continued to have frequent relapses in a year while on oral immunosuppressant therapy.

All patients received RTX infusion as per our Department's protocol [Figure 1]. At the initiation of treatment, the medication was given as a slow intravenous (IV) infusion of two doses 2 weeks apart at 1000 mg each. A similar regime was applied by Cree *et al.*^[6] and other RTX studies^[5,12-14]. Patients were given premedication which consisted of IV promethazine 12.5 mg, IV hydrocortisone 100 mg and oral acetaminophen 1000 mg before the start of RTX infusion. All patients were then followed-up regularly at the Neurology outpatient clinic and assessed for further relapses, EDSS, mRS and side effects. Patients were monitored with full blood counts including lymphocyte counts and B cells (in selected patients), renal profile, liver function tests and C-reactive protein. Screening for chest infections were done with imaging and serum immunoglobulin levels were checked annually, if necessary.

While on RTX therapy, all patients had their pre-existing conventional steroid-sparing agents withdrawn and they were only re-initiated as per protocol 2 to 4 weeks after RTX induction therapy to prevent cumulative effects of infection or hematological and liver dysfunction amongst the patients.

Table 1. Characteristics of 8 neuromyelitis optica spectrum disorder patients treated with rituximab

Pt	Age	Sex	Dis dur (yrs)	AQAP4 ab	Number of relapses					Prior ISS received before			Dis dur before RTX (yrs)	Dur of ISS		EDSS		EDSS		ARR (yrs)		mRS		Post RTX f/up (yrs)	TBNK B cell %	Maintenance post RTX
					before RTX			RTX		alli	Pre RTX	Post RTX (6mo)		Post RTX (1yr)	Pre RTX	Post RTX	Pre RTX	Post RTX								
					ON	TM	AZA	MMF	MTX																	
1	56	F	3	+	3	1	+	-	+	-	0.5	0.3	5.0	2.0	1.0	4.0	0	4	2	2	NA	MTX 5 mg weekly Pred 5 mg od				
2	31	F	10	+	7	1	+	+	-	IVIg Cyclo	3	0.6	4.0	2.5	2.5	1.7	0.1	3	2	6	NA	MMF 1 g bd				
3	53	F	11	+	3	4	+	-	-	INF beta-1a	4	2.2	6.5	2.0	1.0	1.4	0	4	2	6	1%	AZA 50 mg od				
4	61	M	8	+	-	2	+	-	-	-	3	0.7	9.0	9.0	9.0	0.6	0	5	5	4	NA	Pred 10 mg od				
5	30	F	17	+	9	2	+	-	-	-	10	3	3.5	2.0	1.0	1.0	0	3	2	7	NA	AZA 50 mg od Pred 5 mg od				
6	41	M	6	+	3	1	+	-	+	-	3	3.7	2.0	1.0	1.0	1.3	0	2	1	2	NA	AZA 125 mg od				
7	43	F	10	+	1	3	+	-	-	-	3	1	7.0	6.5	6.5	1.3	0.1	4	4	6	3%	Pred 5 mg od AZA 100 mg od				
8	41	M	13	+	10	7	+	+	-	IVIg	7	5	6.5	4.5	4.0	3.0	0	4	3	5	NA	AZA 100 mg od				

Pt: patient; Dis: disease; dur: duration; yrs: years; mo: month; ali: others; AQAP4 ab: aquaporin 4 antibody; RTX: rituximab; ON: optic neuritis; TM: transverse myelitis; ISS: immunosuppressants; AZA: azathioprine; MMF: mycophenolate mofetil; MTX: methotrexate; IVIg: intravenous immunoglobulin; ARR: annualized relapsed rate; mRS: modified rankin scale; EDSS: expanded disability status scale; TBNK: T-cell, B-cell and NK cell; bd: twice a day; od: once daily; pred: prednisolone; -: negative; Nil: nothing; +: positive

Statistical analysis

Data collected was analyzed using SPSS version 24 software, to look at descriptive data; means, medians, percentages and Wilcoxon signed rank test for differences between pre and post treatment in patients with NMOSD. *P* value ≤ 0.05 was taken as significant.

RESULTS

From January 2008 to May 2018, 8 out of 194 patients (4.1%) with NMOSD in our hospital database received a single course of RTX infusion. The mean age of disease onset was 44.5 years (*SD* = 11). Six (75%) of them were female, with a female to male ratio of 3:1. The patient's demographic and clinical characteristics are listed in [Table 1](#). The mean duration of disease prior to RTX was 4.1 years (*SD* = 2.9). Our cohort of patients mostly had optic neuritis or transverse myelitis manifestations and all of them were AQAP4 antibody positive.

All patients had previously received immunosuppressants for a mean duration of 2 years (*SD* = 1.7) prior to RTX; they were either on AZA (eight patients), MMF (two patients) or methotrexate (two patients) [[Table 1](#)]. The patients had an average of 5 optic neuritis and 2 transverse myelitis relapses prior to RTX therapy.

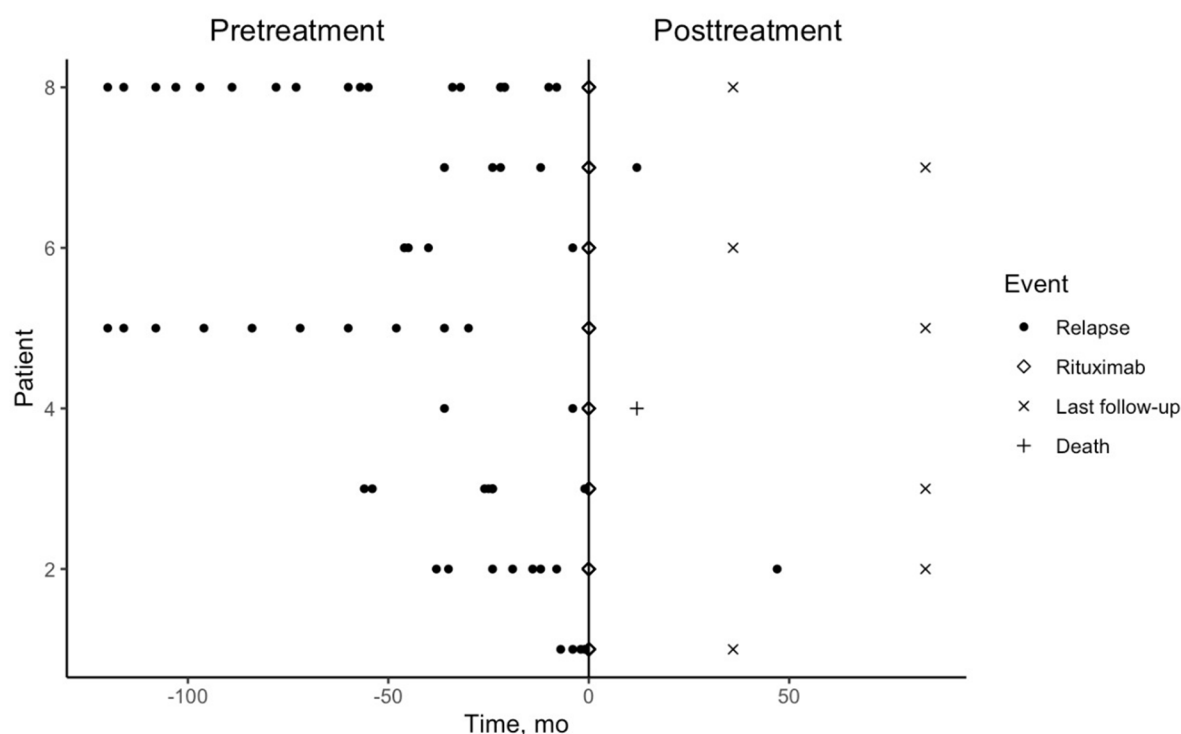


Figure 2. Relapses in patients with neuromyelitis optica spectrum disorder before and after treatment with rituximab. The mean follow-up duration after RTX therapy was 4.7 years. The use of rituximab was associated with significant reduction in the mean annualized relapse rate from 4.7 to 0.5 attacks per year after treatment ($P = 0.011$). Six patients (75%) remained relapse-free during this period of follow up. Patient 2 suffered an attack of transverse myelitis, which occurred 4 years after the last dose of treatment due to poor compliance with maintenance oral immunosuppressants [Figure 1]. Patient 7 had a relapse five years after the last dose of rituximab therapy. This patient was on maintenance azathioprine from 2010 to 2016 when she developed transaminitis. Azathioprine was withheld, and she was on prednisolone 10 mg daily when the disease relapsed

Treatment efficacy

The mean EDSS value prior to RTX treatment was 5.4 and after treatment, it improved to 3.6 ($P = 0.018$). Seven patients had reduction in EDSS scores whereas one patient (patient 4) did not show improvement following treatment. The same patient passed away due to myocardial infarction 1 year later [Figure 2]. The mean mRS prior to treatment was 3.6 and the mean mRS score post RTX improved to 2.6 ($P = 0.023$). On follow up, most of the patients following treatment were able to achieve independence in performing their activities of daily living.

Safety profile

Rituximab was well tolerated and none of our patients who received the therapy developed any short-term adverse effects such as infusion related allergic reaction, fever, abdominal symptoms or hematological disorders. There was no reported case of progressive multifocal encephalopathy, malignancies, hypogammaglobulinemia or septicemia during the mean follow up period of 4.7 years (2 to 7 years) following RTX treatment.

DISCUSSION

In patients with NMOSD, recurrent clinical relapses can lead to rapid accumulation of disability and clustering of attacks. Aggressive immunosuppressive therapy remains the mainstay of NMOSD treatment to reduce the number of destructive demyelinating events. NMOSD is thought to be an aquaporinopathy that is predominantly humorally mediated, with complex interplay between B cells, T cells, complement and cytokines. Since the first open-label study evaluating RTX in NMO patients by Cree *et al.*^[6] in 2005,

there have been several studies assessing the effectiveness of monoclonal antibody therapy, targeting CD20 epitope of the B cell lineage that is important in the pathogenesis of the disease^[8,9,13,15-17]. In these studies, there was reduction in annualized relapse rates and improvement in disability as measured by the EDSS.

However, in our resource limited setting, RTX is expensive and obtaining the medication for our patients remains a huge challenge. Thus, we had to use a dosing schedule of lower frequency and a single course of RTX was applied followed by oral immunosuppression to treat these patients. Data collected retrospectively, showed a significant reduction in the frequency of relapses, ARR in NMOSD patients with aggressive disease especially in those failing conventional immunomodulators. Remission was also maintained in 75% of patients for 4 years. Similar to the study conducted by Bedi *et al.*^[5], we avoided abrupt withdrawal of oral prednisolone to prevent early relapses in those patients. Other immunomodulators were switched off while RTX was given. All our patients were on stable doses of oral prednisolone and they were either tapered off or reduced to the lowest possible maintenance dose of 5 to 20 mg after subsequent doses of RTX were given, or when reasonable doses of conventional steroid-sparing agents was on board for an appropriate time limit without further disease relapse. Such an approach may have a positive effect on relapse control and contributed to the stability of disease activity after single dose RTX therapy.

To date, there has been no standard guideline or consensus on RTX treatment for patients with NMOSD. Existing protocols used to induce and maintain remission are characterized by heterogeneity in terms of infusion and monitoring schedules and methods. Previous studies practiced either prescheduled RTX induction regimen every 6 months, or retreatment based on B cell depletion monitoring^[6-8,16-18]. CD19, CD20 and CD27 are among the commonly used treatment-related biomarkers^[13,14,16,17]. However, our center lacks the facilities to monitor treatment with these tests though we are aware of the need to objectively test this for treatment response. We utilized lymphocyte enumeration tests by flow cytometry to estimate the B cell population as a guide to treatment. Nevertheless, the test is expensive and was only done in 50% of our patients who underwent RTX therapy. Thus, the data was too scarce to make a definite conclusion on retreatment decisions. Therefore, retreatment was guided mainly by the severity and frequency of ongoing attacks. In addition, our data demonstrated that eighty seven percent of our patients showed improvement in EDSS score and none of them required retreatment with RTX.

In resource-limited hospitals, a single induction course of RTX treatment may be a therapeutic and economic option to suppress disease activity. We recognized that oral prednisolone during RTX treatment may potentially confound the analysis, however the overlapping benefit of steroids with RTX may be crucial to avoid sudden withdrawal of immunosuppressants that can potentially cause an early relapse. The doses of steroids were kept stable during this period and other immunosuppressants were withdrawn to prevent possible adverse events.

Several studies have shown the benefits of low dose RTX in terms of improvement in ARR, disability scores and time to next relapse^[17,19,20]. Kim *et al.*^[17] reported a less frequent RTX retreatment approach which could maintain remission in a subset of NMOSD patients. It is postulated that the initial high induction dose of RTX (IV 1000 mg \times 2 doses given 2 weeks apart) may be adequate to prevent early B cell repopulation, which is seen in some studies with lower initial doses of RTX^[14,15]. To our knowledge, there has been no study to address the efficacy of a single course of RTX therapy followed by oral immunosuppressants maintenance in the management of aggressive NMOSD. In our small cohort of patients, regardless of the initial number of relapses and EDSS scores, majority responded very well to this treatment strategy and remained relapse-free.

Unlike the meta-analysis published by Damato *et al.*^[21], which recorded 26% of patients treated with RTX had adverse reactions, none of our patients exhibited any side effects. Specifically, Damato *et al.*^[21] reported

that 10% of the patients experienced infusion-related adverse effects, but we managed to reduce this risk by premedicating our patients with IV promethazine, IV hydrocortisone and oral acetaminophen as stated in the treatment protocol. In addition, the absence of infection and leukopenia in our cohort of patients may be related to the withdrawal of immunomodulators prior to RTX and a delay of reinitiating the therapy for two to four weeks after treatment. We acknowledge that our small sample size might not reflect the actual safety profile of such an approach, however the absence of major side effects in our cohort of patients is promising for our therapeutic plan. We postulate that the lack of adverse events may be due to the absence of the cumulative effect of immunomodulators with RTX and the short frequency dosing as reported by others^[22].

Nevertheless, there are several limitations to our study. Firstly, being a retrospective study, the analysis of data from medical records was subjected to recording bias. In addition, the patient group at baseline was heterogenous with regard to their pre-RTX status and have variable disease duration, number of relapses and EDSS severity. Oral immunosuppressant maintenance therapies and steroid doses following RTX treatment were also variable. Although our results were derived from a tertiary care institution, our sample size was small, which further highlights the challenges of access to RTX at tertiary establishments in a resource-limited setting.

In conclusion, pulse induction therapy with a single course of RTX followed by subsequent de-escalation to oral immunosuppressants may be a convenient and economical approach in managing NMOSD patients. In resource-limited hospitals with restricted access to RTX, such an approach can potentially be effective to reduce relapses and improve EDSS scores with minimal side effects. This treatment plan allowed adequate time for optimization of other oral medications to achieve their therapeutic benefits. Moreover, the ability to achieve and maintain remission suggests that RTX has long-term effects extending beyond treatment discontinuation. Nevertheless, we concede that a further, larger prospective cohort study is required to demonstrate the efficacy of such a treatment approach.

DECLARATIONS

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Authors' contributions

Performed data acquisition, analysis and interpretation, prepared the manuscript, Tables and Figures: Ong TL

Made substantial contributions to the conception and design of the study and performed data analysis and interpretation: Viswanathan S

Performed data acquisition, analysis and interpretation. Contributed in manuscript preparation: Ong S, Hiew FL

Availability of data and materials

All data used for this study were collected as part of ongoing work under the auspices of the Demyelinating Disease Database, established at the Neurology Department of Kuala Lumpur Hospital.

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None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Informed consent, either verbal or written, was obtained from patients for the use of retrospective data with longitudinal follow-up for this study.

Consent for publication

Not applicable.

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Case Report

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Biomarker-based diagnosis of cognitive disorders in a case series

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Abstract

The classical cerebrospinal fluid biomarkers of Alzheimer's Disease (namely total tau, phospho-tau and amyloid beta peptide) have received much attention, since they can detect the biochemical fingerprint of Alzheimer's disease and serve as a diagnostic aid for correct diagnosis of cognitive disorders during life. In this case series, we present 6 examples of patients with cognitive impairment of various types and severities and how biomarker data were helpful in every day diagnostic approach, combined with clinical, neuropsychological and imaging data and based on the most recent guidelines and recommendations.

Keywords: Cerebrospinal fluid, tau, phospho-tau, amyloid-beta, Alzheimer's disease, frontotemporal dementia, vascular cognitive impairment

INTRODUCTION

Until relatively recently, Alzheimer's disease (AD) was diagnosed according to clinical criteria proposed more than 30 years ago, requiring the patient to be demented^[1]. With time it became evident that



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AD patients may present with mild cognitive impairment^[2], or may even be asymptomatic^[3]. When symptomatic, amnesic dementia of the hippocampal type is the typical presentation^[4,5]. However, atypical presentations are not infrequent, especially in presenile patients, including frontal-predominant, language-predominant, “posterior” or mixed presentations^[4,5]. Such presentations may lead to diagnostic confusion, whilst even the typical hippocampal amnesic presentation may occur in non-AD disorders^[6]. Thus, clinical presentations or phenotypes are rather viewed as syndromes, and they are by no means synonymous with a specific disease. Various types of biomarkers may be helpful in the diagnostic approach of such typical or atypical presentations, and they have been incorporated in various diagnostic criteria for AD^[2,4,5].

Recently, the National Institute of Ageing and Alzheimer Association (NIA-AA) Research Framework group recommended a system for classifying subjects/patients on the basis of their biomarker profile, since it may result from different biomarker categories, especially neurochemical and imaging^[7]. The objective was to update a scheme for defining and staging AD mainly across its entire spectrum, to be used for research purposes, either observational or interventional. A further shift in thinking is the separation of the syndrome from the disease, as symptoms are considered part of the disease continuum and not its definition. Looking towards a biological definition of AD, as it is identified post mortem by accumulation of amyloid- β and tau and reflected in vivo by biomarkers, the group discriminates them according to their molecular specificity [i.e., amyloid- β (A β) and pathological tau (phospho-tau)]. For this scope, they propose the AT(N) system^[7], where A stands for amyloid- β plaques or associated pathological state, T for aggregated hyperphosphorylated tau or associated pathological state and (N) for neurodegeneration. The parentheses are to indicate that it represents cumulative brain injury/neurodegeneration from all etiologies and is not specific for any certain etiology. A (C) component is used to define mental decline and staging, from cognitively unimpaired to mild cognitive impairment and finally dementia, according to cognitive symptoms and neuropsychological testing. Thus, each biomarker category can be dichotomized as positive (+) or negative (-), resulting in eight different biomarker profiles and 3 “biomarker categories”: normal [A-T-(N)-], Alzheimer’s continuum [A+T-(N)-, A+T+(N)-, A+T+(N)+, A+T-(N)+] and suspected non-AD pathological change [A-T+(N)-, A-T-(N)+, A-T+(N)+]^[7].

Here, we present a case series of six patients with different types of cognitive disorders using this system. Cases were selected with the only criterion being that they were educationally useful and interesting for clinicians and medical students, and all co-authors helped in the selection of cases, in an unblinded manner. We describe their clinical, imaging and cerebrospinal fluid (CSF) biomarker data and how these could be suggestive of diagnosis, according to the AT(N) system. All patients were analyzed routinely in our department as part of the everyday diagnostic approach and they were not included in any particular study.

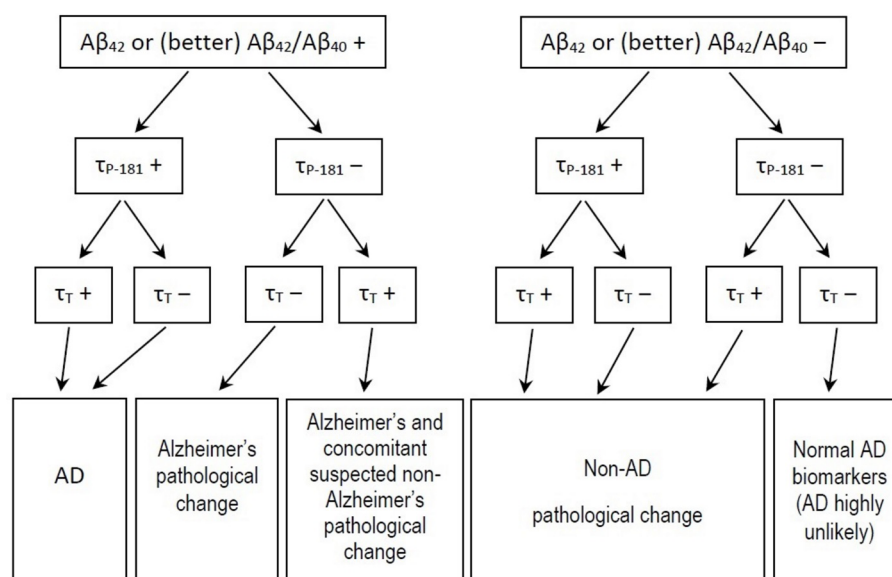
Lumbar puncture was performed at 10-11 am, after overnight fasting, at the L4-L5 interspace, according to recently proposed recommendations on standardized operating procedures (SOPs) for CSF biomarkers^[8], as described elsewhere^[9]. In brief, 4 polypropylene tubes were used for CSF collection. The initial tube (2 mL) was used for routine cytology and biochemistry and the 2nd tube (2 mL) was used for determination of IgG index, oligoclonal bands and for syphilis serology. The last 2 tubes (5 mL each) were immediately centrifuged, aliquoted in polypropylene tubes (750 μ L each) and, finally, stored at -80 °C until analysis. Aliquots were thawed only once, just before analysis, which was performed within 6 months of storage.

CSF levels of total tau protein (τ_T), amyloid- β peptide and tau phosphorylated at threonine-181 (τ_{P-181}) were measured blindly, in duplicate by double-sandwich, enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Fujirebio, Gent, Belgium) according to the manufacturer’s instructions, as previously described^[9]. In-house standards were used during every to ensure minimal measurement error

Table 1. Normal (cut-off) values of our laboratory^[9]

CSF biomarker	Normal value
total tau protein (τ_T)	< 376 pg/mL
tau phosphorylated at threonine-181 (τ_{P-181})	< 57 pg/mL
amyloid- β peptide with 42 amino acids ($A\beta_{42}$)	> 682 pg/mL
$A\beta_{42}/A\beta_{40}$	> 0.09

CSF: cerebrospinal fluid

**Figure 1.** Flow chart of the use of cerebrospinal fluid biomarkers in clinical practice, according to the AT(N) system^[7]. AD: Alzheimer's disease

($\leq 3.3\%$), and inter-assay and intra-assay variations were $\leq 6.6\%$ for all biomarker assays^[10]. Cut-off values have been previously calculated by receiver operating curve (ROC) analysis^[9,11]. Table 1 shows the CSF biomarker categories used in our clinic/laboratory and their most recently used normal (cut-off) values^[9]. Figure 1 presents a proposed simplified scheme for the diagnostic use of CSF biomarkers, according to the “philosophy” and nomenclature of the AT(N) system^[7].

CASE REPORT

Case 1

A 63-year-old female patient with no significant past medical history neither family history was admitted to the neurology department for gradually developed memory complaints over the last year with no impact on activities of daily living. Neuropsychological assessment revealed mild cognitive impairment with mini mental state examination (MMSE)^[12] score 27/30 and frontal assessment battery (FAB)^[13] score 16/18. On magnetic resonance imaging (MRI) some degree of cortical atrophy in the parietal lobes was observed with relative preservation of the hippocampus [Figure 2]. Functional imaging study using single photon emission computerized tomography (SPECT) with ^{99m}Tc-HMPAO was normal. CSF biomarker analysis revealed increased $\tau_T = 545$ pg/mL and $\tau_{P-181} = 81.8$ pg/mL and decreased $A\beta_{42} = 480$ pg/mL and $A\beta_{42}/A\beta_{40} = 0.059$. With all 3 biomarkers abnormal, the CSF profile was compatible with AD pathology and the patient was classified as $A^+ T^+ (N)^+$, suggesting “Alzheimer's disease with mild cognitive impairment”^[7]. During follow-up, she underwent two more neuropsychological assessments 4 and 8 years later, revealing progressive deterioration of cognition [Figure 3].

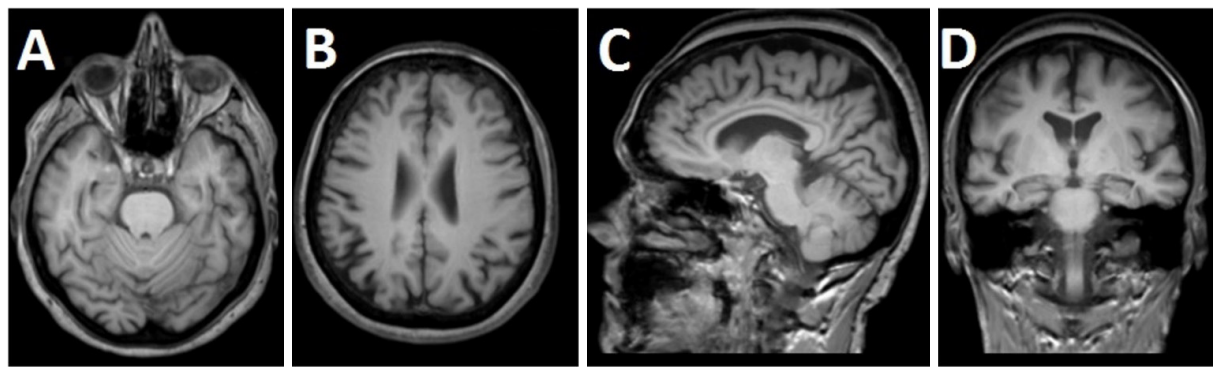


Figure 2. Brain magnetic resonance imaging of case 1 (T1 sequence), showing relative preservation of the hippocampus (A,D) and some degree of parietal atrophy (B,C)

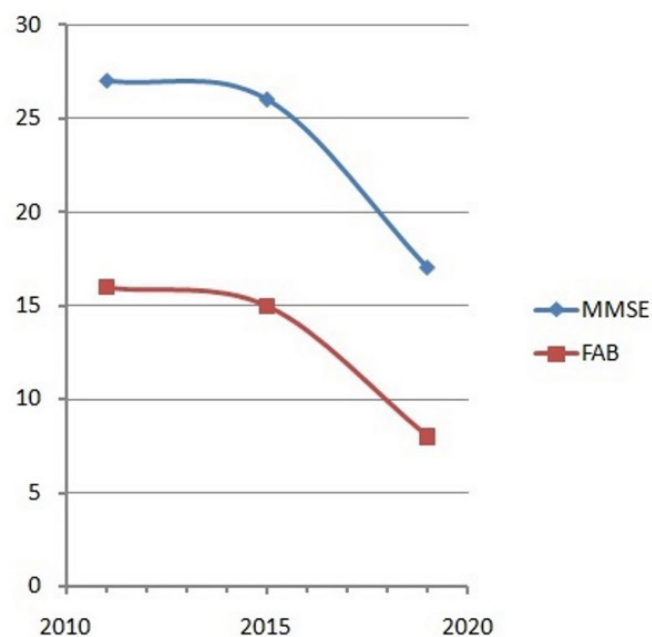


Figure 3. Progressive cognitive deterioration on mini mental state examination (MMSE) and frontal assessment battery (FAB) neuropsychological testing during the 8-year follow-up of case 1

Case 2

A 54-year-old female patient was referred for neurological evaluation due to progressive amnesic type dementia (MMSE: 21/30), with frontal and visuospatial components, evolving for approximately 5 years. MRI showed absence of atrophy. Biomarker analysis of CSF revealed normal τ_T (261 pg/mL), increased τ_{P-181} (75 pg/mL), decreased $A\beta_{42}$ (168 pg/mL) and decreased $A\beta_{42}/A\beta_{40}$ (0.04). The CSF profile was compatible with AD, and according to the most recent recommendations, the patient was classified as $A^+ T^+ (N)^-$, suggesting “Alzheimer’s disease with dementia”^[7]. In follow-up MRI, 3 and 4 years later, a progressive hippocampal and frontal-parietal atrophy was observed [Figure 4].

Case 3

A 71-year-old male patient was admitted to the neurology department due to dementia of mixed amnesic and frontal type. His brain MRI revealed ischemic lesion load but also frontal, perisylvian and frontoparietal atrophy more evident in the left hemisphere on axial fluid attenuated inversion recovery images, whereas according to T1 coronal images, the hippocampus was preserved [Figure 5]. Levels of

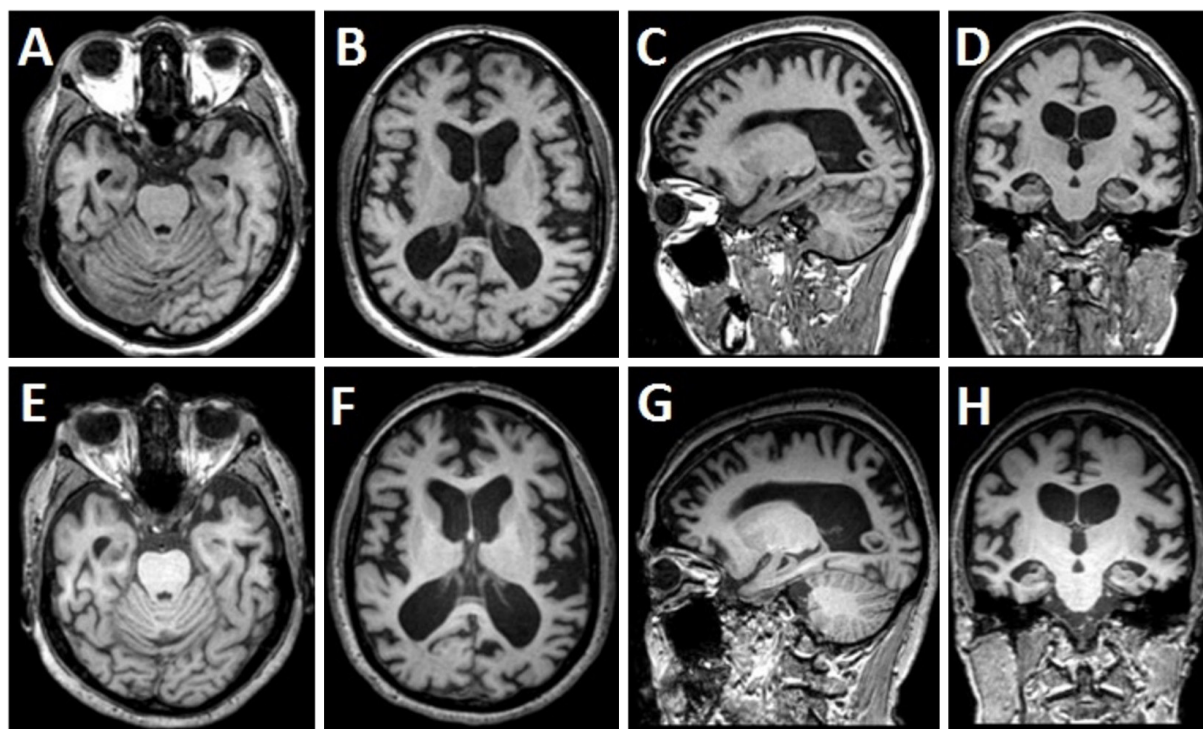


Figure 4. Brain magnetic resonance imaging of case 2 (T1 sequence) at age 57 (A-D) and 58 (E-H), showing progressive hippocampal atrophy (A, D and E, H), and frontal, perisylvian and parietal atrophy (B, C and F, G)

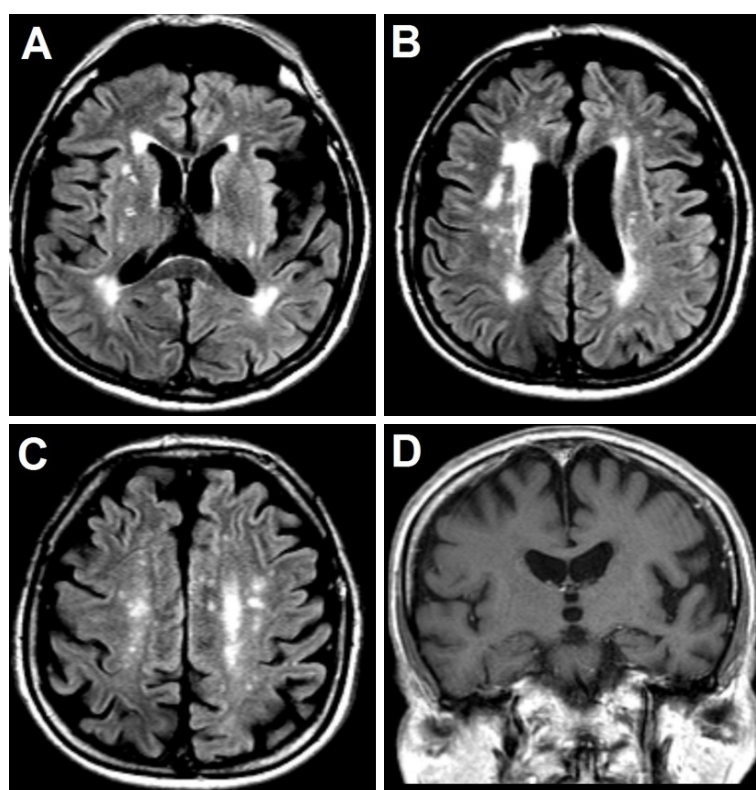


Figure 5. Brain magnetic resonance imaging of case 3. Fluid-attenuated inversion recovery images (A, B, C) show ischemic lesion load and frontal, perisylvian and frontoparietal atrophy more evident in the left hemisphere. In coronal T1 section (D), the hippocampus is preserved

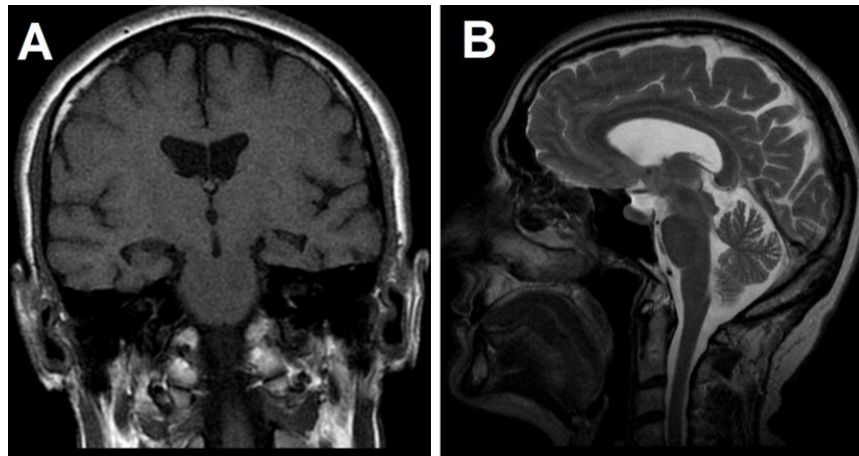


Figure 6. Brain magnetic resonance imaging of case 4. Coronal T1 section (A) reveals hippocampal atrophy. In sagittal T2 section (B), some degree of posterior frontal and parietal atrophy is observed

CSF biomarkers were: $\tau_T = 963$ pg/mL, $A\beta_{42} = 495$ pg/mL, $\tau_{P-181} = 87$ pg/mL and $A\beta_{42}/A\beta_{40} = 0.061$. With all 3 biomarkers abnormal, the CSF profile was compatible with AD pathology^[7] (in addition to subcortical small vessel disease).

Case 4

A 59-year-old female patient with typical amnesic dementia, fulfilling the clinical diagnostic criteria for probable AD^[1], was referred to the neurology department for evaluation. Hippocampal atrophy was observed on coronal T1 sequences and, additionally, some degree of posterior frontal and parietal atrophy on sagittal T2 sequences [Figure 6]. The CSF biomarker levels were: $\tau_T = 308$ pg/mL, $A\beta_{42} = 921$ pg/mL, $\tau_{P-181} = 36$ pg/mL and $A\beta_{42}/A\beta_{40} = 0.11$. Clinically, this “suspected non-Alzheimer disease pathophysiology” (SNAP)^[14] was otherwise compatible with an AD phenotype. However, with all 3 biomarker levels well within normal limits, the CSF profile was not compatible with AD^[15] and, according to the most recent recommendations, the patient was classified as A⁻ T⁻ (N)⁺ suggesting “non-Alzheimer’s pathological change”^[7].

Case 5

A 54-year-old female patient presented to our department with frontal-behavioral dementia, language disorder (mixed non-fluent and semantic components) and clinical and electrophysiological evidence of upper and lower motor neuron involvement. Her family history was positive for autosomal dominant dementia and/or ALS. On MRI T1 sequences, frontal and frontoparietal atrophy more evident to the left were present with relative preservation of the hippocampus [Figure 7]. Levels of CSF biomarkers were: $\tau_T = 268$ pg/mL, $A\beta_{42} = 513$ pg/mL, $\tau_{P-181} = 20.4$ pg/mL and $A\beta_{42}/A\beta_{40} = 0.125$. Although the clinical presentation was suggestive of frontotemporal dementia (FTD)-amyotrophic lateral sclerosis (ALS), reduction of $A\beta_{42}$ was unexpected. However, correction for the total amyloid status revealed a normal $A\beta_{42}/A\beta_{40}$ ratio, excluding amyloid reduction^[16,17] and suggesting non-AD pathology. Given the clinical presentation, a TDP-43 proteinopathy was considered the most probable disorder. Indeed, genetic testing was positive for *C9orf72* repeat expansion.

Case 6

A 40-year-old female with no past medical history was referred to the neurology department for presenile dementia. Neuropsychiatric symptoms began at the age of 34 and cognitive symptoms began three years later at the age of 37 and gradually deteriorated, fulfilling the clinical criteria for probable behavioral variant frontotemporal dementia^[18]. MRI showed atrophy in the frontal and parietal lobes [Figure 8]. Levels

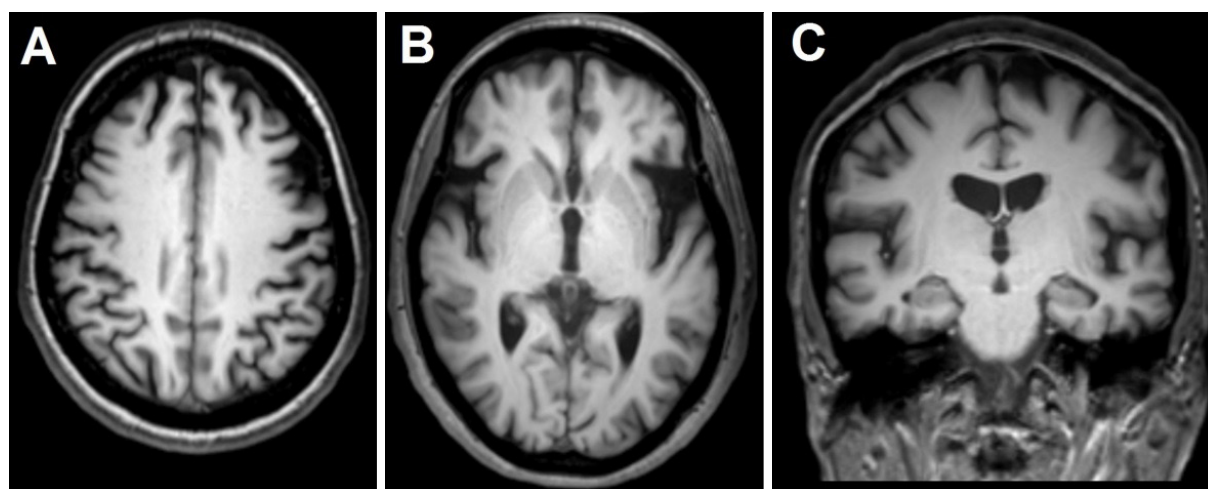


Figure 7. Brain magnetic resonance imaging (T1 sequence) of case 5, showing frontal frontoparietal and sylvian atrophy more evident to the left (A,B) with preservation of the hippocampus (C)

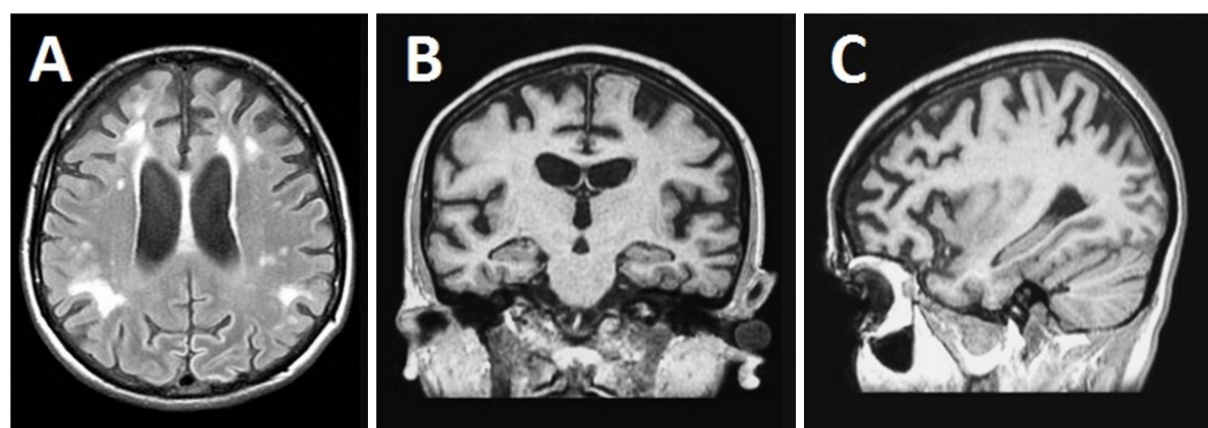


Figure 8. Brain magnetic resonance imaging of case 6. Axial fluid-attenuated inversion recovery image (A) showing white matter hyperintensities; coronal (B) and sagittal (C) T1 images reveal atrophy in frontal and parietal lobes (with some degree of left predominance) and preservation of the hippocampus

of CSF biomarkers were: $\tau_T = 1813$ pg/mL, $A\beta_{42} = 706$ pg/mL, $\tau_{P-181} = 67$ pg/mL and $A\beta_{42}/A\beta_{40} = 0.12$. With 2 biomarkers abnormal, the patient was classified as $A^-T^+(N)^+$, suggesting “non-Alzheimer’s pathological change with dementia”. Given the increased levels of τ_{P-181} , it was tempting to assume that frontotemporal dementia with tau pathology would be the most probable diagnosis^[19]. Cerebral biopsy revealed severe tauopathy without accumulation of amyloid- β or the presence of astrocytic plaques or tufted astrocytes. Genetic testing was negative for mutations in the *MAPT* and *GRN* genes.

DISCUSSION

We presented 6 patients as examples of a combined diagnostic approach based on clinical, imaging and CSF biomarkers, according to the AT(N) system^[7]. In case 1, the diagnosis of AD was made in a symptomatic yet predementia stage (MCI). Case 2 was an amnesic dementia patient, and atypical features included presenile onset and absence of atrophy at presentation; however, CSF biomarkers revealed AD biochemistry and clinical-imaging progression was typical.

In case 3, a moderate ischemic lesion load could have contributed to the patient’s symptoms, but AD was additionally present. This is a frequent scenario^[20], and CSF biomarkers are helpful in the discrimination between cases with pure vascular cognitive impairment and mixed cases (with additional AD)^[21,22].

In case 4, a 59-year-old-female, clinically fulfilled the clinical criteria of an amnesic dementia of the AD type^[1]. However, clinical presentation does not always predict brain pathology. For example, AD can present with common amnesic dementia but also with a frontal behavioral-dysexecutive syndrome, the so-called “frontal variant of AD”^[4,5]. Likewise, patients with FTLN pathologies may also present with an amnesic, AD-like syndrome. The term limbic-predominant age-related TDP-43 encephalopathy (LATE) has been recently introduced for at least some of these cases, and consensus-based recommendations and guidelines for diagnosis and staging have been formulated^[6]. Thus, clinical, biochemical, neuropsychological and imaging data, all should be considered. Of course, there is always the possibility of false-negative or false-positive results. Since all biomarkers become abnormal during prodromal stages of AD, all would be expected to be abnormal in a well-established AD dementia^[23]. However, in this patient, all biomarkers were normal, dramatically reducing the possibility of false-negative results and pointing to a non-AD pathology. Indeed, with all CSF biomarkers normal, AD is considered highly unlikely according to recent recommendations^[15]. Clinically the patient is not compatible with dementia with Lewy bodies (DLB), and to our knowledge, there are no robust, evidence-based data to support the use of standard AD treatments in non-AD, non-DLB patients. Thus, correct diagnosis would also avoid possibly unnecessary treatment(s) suitable for other diseases.

In case 5, TDP-43 proteinopathy was strongly considered from clinical presentation of combined phenotype FTD-ALS in the family, which is known to be related to a TDP-43 histopathology^[24,25]. The CSF biomarker profile was compatible with non-AD pathology. In case 6, an AT(N) profile suggestive of non-AD pathology was also observed. However, in this patient, τ_{P-181} was increased. Recently, it has been suggested that in an FTD-like patient, with no AD biomarker profile, increased τ_{P-181} is more compatible with tau-pathology, while low τ_{P-181} may be compatible with TDP-43 pathology^[19]. Thus, the tauopathy observed in brain biopsy was in accordance with this notion.

Soon after its publication, the AT(N) system triggered a lot of discussion and criticism. The concept of a disease viewed as a pathological/pathophysiological/biochemical entity unrelated to symptoms may not be easily accepted by some clinicians or the community^[26]. However, given that the same disease may present with different clinical syndromes and that the same clinical presentation may be caused by different diseases, this new view is really a step forward, and this holds true not only for AD but also for many other neurodegenerative disorders. Furthermore, since the AD pathological process starts even decades prior to symptomatic onset, whilst CSF or imaging biomarkers become abnormal in the preclinical stage^[23], the need for adopting such a view/concept is further strengthened. However, many questions seek answers. For example, what about an A⁺T⁺N⁺ patient with a clinical presentation suggestive of DLB. Is this due to mixed pathology (synucleinopathy and AD)^[5,27] or due to AD with atypical presentation^[28]? Another related question is a DLB-like patient with only amyloid biomarkers being positive. This is very common in DLB^[29]. But, is this due to the synucleinopathy alone somehow triggering amyloid deposition unrelated to AD mechanisms, or are such patients “destined” to develop full-blown AD pathology if they live long enough? Furthermore, reduced A β_{42} levels have been observed in some patients with pure vascular dementia^[20], including patients with inherited subcortical small vessel disease^[30], who do not have additional AD pathology, raising questions as to whether reduced A β_{42} always suggests Alzheimer’s pathological change.

Other CSF biomarkers may be of further help and improve the AT(N) system. Other forms of phospho-tau such as τ_{P-217} may perform better, compared to τ_{P-181} ^[31]. TDP-43 combined with τ_T and τ_{P-181} could enhance the diagnostic accuracy in the FTD spectrum^[32,33]. CSF α -synuclein levels could be useful in discriminating patients with AD from cognitively unimpaired subjects, patients with DLB and patients with Parkinson’s disease dementia^[34,35]. Blood-based biomarkers are quite promising as well, since classical AD biomarkers may also be measured in plasma. Plasma τ_{P-181} could differentiate AD dementia from non-AD neurodegenerative diseases with accuracy similar to that of CSF τ_{P-181} and tau-PET^[36], while plasma A β_{42} /

A β_{40} ratio has been associated with amyloid PET status in cognitively normal subjects^[37].

Inflammation biomarkers in CSF and blood have received much attention; however, whether they offer any added diagnostic value remains a matter of investigation. CSF α 1-antichymotrypsin levels are increased both in vascular cognitive impairment (VCI) and clinically evident AD, while elevated peripheral CRP levels may be associated with increased risk for VCI, but not AD^[38]. Serum interleukin-15 levels have been found to be significantly lower in patients with AD in comparison to healthy subjects and patients with VCI^[39]. On the other hand, CSF interleukin-15 levels are increased in AD and FTD, compared to patients with non-inflammatory neurological disorders^[40], while CSF interleukin-12 is reduced in AD, indicating altered inflammatory reactions^[41].

In neurodegenerative disorders, diagnosis should be established as soon as possible and preferably in a prodromal phase, before the onset of clinically significant dementia. Additionally, new emerging treatments or medications under investigation may be more effective when given in early stages. Therefore, timely and accurate diagnosis is mandatory to obtain potential benefits of novel treatments, but also for accurate inclusion of patients in clinical trials and for determining prognosis. As noted above in case 4, clinical phenotypes are not always tightly linked to the underlying pathology^[5,6,9] in contrast to biomarkers, some of which may have high molecular specificity. Nonetheless, CSF biomarkers are not a panacea, and their value should not be over-rated. They have disadvantages mainly due to the heterogeneity of research to date, but they still offer a very useful tool in early etiological diagnosis of neurodegenerative diseases, especially when combined with clinical and neuroimaging data^[17].

DECLARATIONS

Authors' contributions

Concept and definition of intellectual content: Kapaki E, Paraskevas GP

Clinical data acquisition and interpretation: Kapaki E, Constantinides VC, Pyrgelis ES, Paraskevas PG, Papatriantafyllou JD, Paraskevas GP

Biomarker determinations and interpretation of results: Kapaki E, Paraskevas GP

Manuscript preparation, editing and review: Kapaki E, Pyrgelis ES, Paraskevas PG, Papatriantafyllou JD, Paraskevas GP

Availability of data and materials

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None.

Conflicts of interest

The authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

All patients and/or relatives gave informed consent for publication of their clinical, biochemical and imaging data.

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Letter to Editor

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Anti-CASPR2 antibodies clinical significance and its main phenotypes

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Contactin associated protein 2 (CASPR2) is cellular adhesion molecule (CAMs) part of the neuroxins family. It is a transmembrane protein with its C-terminal portion interacting with an ankyrin protein called 4.1B and also a PDZ binding motif^[1].

It is localized along the axon, especially in the synaptic terminal and in the juxta-paranodal portion in the node of Ranvier. It is found in neurons on the basal ganglia and other motor areas, limbic system, sensitive pathways, and appears richly expressed in the temporal lobe, especially in inhibitory GAD65 positive neurons^[1-3]. CASPR2 forms with Contactin-2 part of the VGKC complex, where it has the function of clustering Kv1 channels at the juxta-paranodal^[1-3] [Figure 1].

Antibodies against CASPR2 have been described in various forms of clinical presentation^[4] [Figure 2], part of this great variation is believed to be associated with great variation in epitopes, although antibodies against the discoidin domain and laminin G1 are found in every patient^[1,5,6]. As well the overlapping found between peptides of CASPR2 and LGI1 could explain the variation of phenotype when exposure to the same VGKC complex antigens^[7,8]. Additionally the HLA DRB1*11:01 implicated in CASPR2 is not associated with LGI1^[7].

The pathogenesis in anti-CASPR2 disease is believed to occur due to a block on the interaction between CASPR2 and Contactin-2^[6,9] [Figure 3], and the disrupting the Kv1 channels expression. In some cases, there is reduced expression such as in the dorsal root ganglia^[10] and in others inducing an increase



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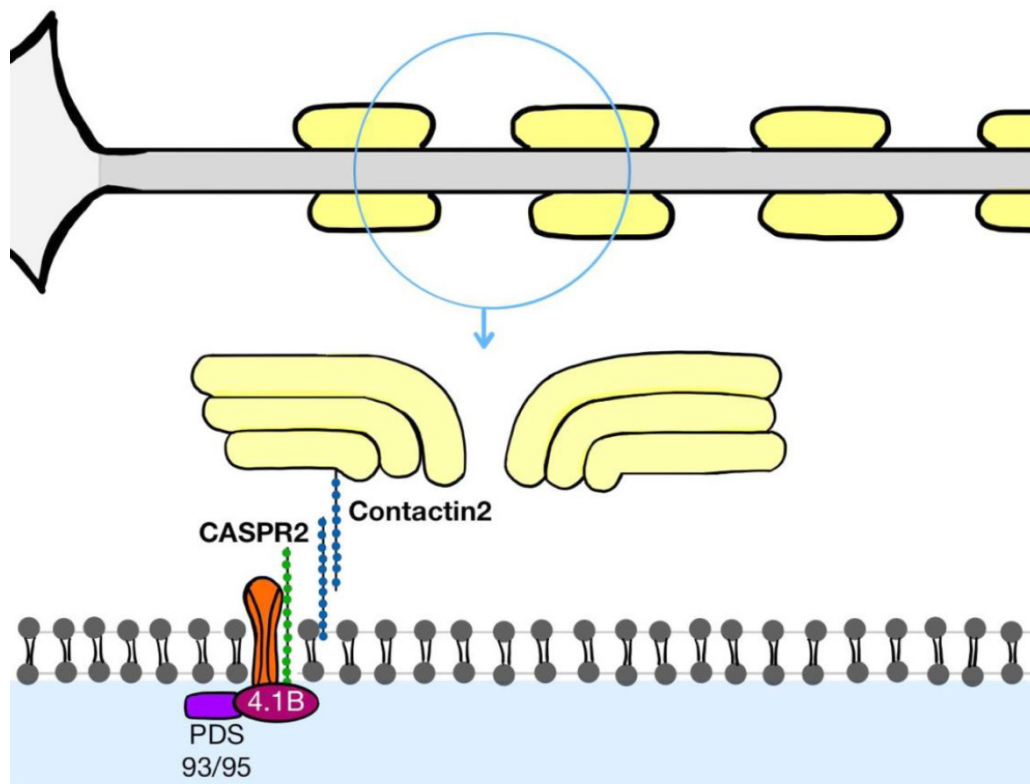


Figure 1. It represents CASPR2 localization at the juxta-paranodal portion in the node of Ranvier, showing its interaction with Contactin-2 forming the voltage gated potassium channel complex (VGKC complex)

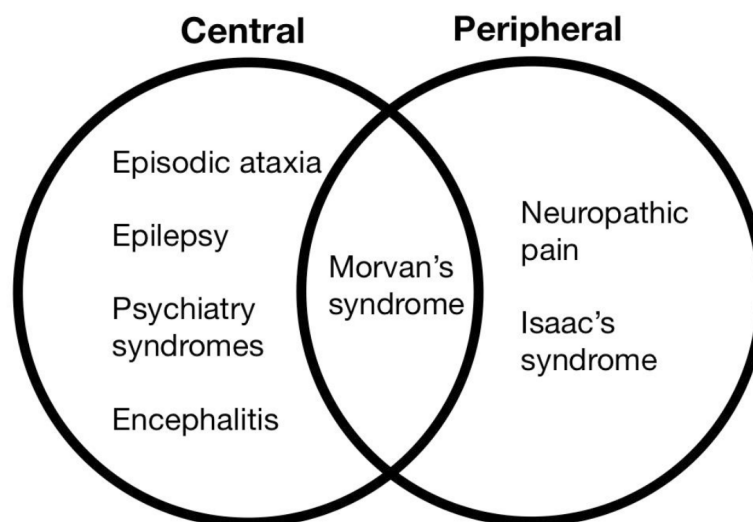


Figure 2. Shows the variety of clinical phenotypes involving the anti-CASPR2 antibodies and its overlapping between central and peripheral nervous system syndromes

in the expression, especially in the inhibitory interneurons in the hippocampus^[6,8,9]. This may cause hyperexcitability that could cause epilepsy^[8] and network imbalance supported by neuroimaging studies^[11].

Morvan's syndrome is an autoimmune disease that affects CNS, PNS and autonomic system^[12]. It consists of neuromyotonia (cramps, stiffness, fasciculation)^[13], encephalopathy, insomnia, dysautonomic signs

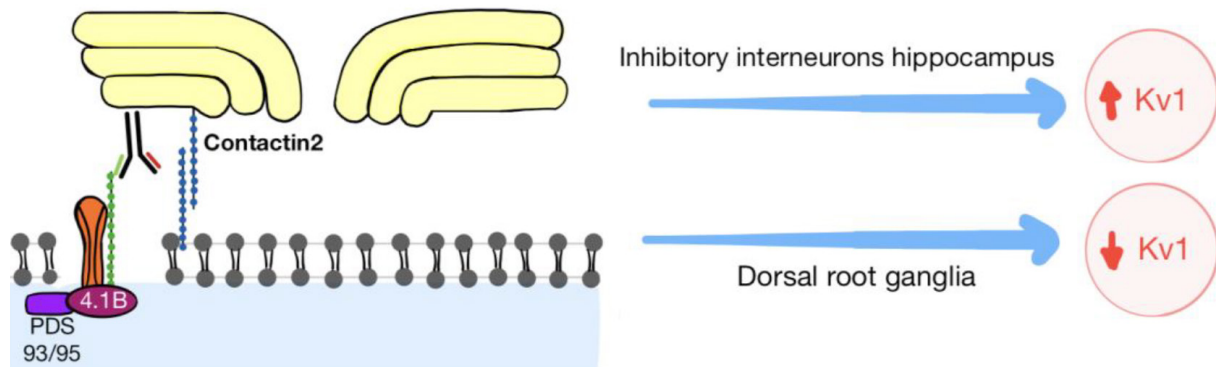


Figure 3. The IgG4 anti-CASPR2 blocking the interaction between CASPR2 and Contactin 2 protein, without activating complement, but disturbing the expression of the voltage gated potassium channel

specially hyperhidrosis and cardiovascular instability, and neuropathic pain^[4,13-15]. Weight loss, skin lesions or itch, which may result from skin dysautonomia, seizures, fever and SIADH may also be present^[12,14].

The largest study investigating Morvan's Syndrome included 29 patients^[14]. 93% of them were male and the median age was 57, varying from 19 to 80. VGKC-complex antibodies were positive in 79%. Tumor was found in 41% of the patients, and the most prevalent tumor was thymoma in 11 patients, 9 of them had history of acetylcholine receptor antibodies^[14].

Most of the patients have both CASPR2 and LGI1 antibodies^[4,15], but CASPR2 antibodies titers are usually higher^[14]. Antibodies against Contactin-2, a protein expressed in heart conduction tissue, may also be found, associated with cardiovascular instability^[14]. There are also some patients whose sera are not consistent with CASPR2, LGI1 or contactin-2, increasing suspicion about an unknown antibody^[14,16].

Isaac's syndrome is an acquired Peripheral Nerve Hyperexcitability that can include myokymia, muscle cramps, fasciculation, twitching, stiffness, and pseudomyotonia (slow relaxation of the muscles after contraction that happens especially in hands, eyes and jaw)^[16-18]. Muscle activity persists even when the patient sleeps^[18] and it may result in muscle hypertrophy^[17], mainly calf muscles^[16,17], but also forearm and hand muscles. Cramps are often worsened by voluntary muscle contraction^[17]. Dysautonomia (hyperhidrosis, sialorrhea), Trousseau and Chvostek sign may also be present; sensory manifestations are infrequent^[16], strength and reflexes are usually normal^[17]. The symptoms are usually insidious, presenting over years^[16].

Antibodies against VGKC complex are found in 38%-50% of the patients. VGKC is associated with repolarization of synaptic membranes^[12] and its blockage consequently causes nerve excitability^[17]. Other autoimmune disorders including myasthenia gravis, Addison disease, Hashimoto thyroiditis, are associated, such as neoplasms, mainly thymoma^[17]. Encephalopathy is not present in Isaac's syndrome^[17].

Limbic encephalitis, or limbic encephalopathy, is a reversible cause of cognitive impairment when correctly diagnosed and early treated^[19]. The main symptoms suggest infectious encephalitis^[19], but there are many differential diagnosis besides infection: neurodegenerative dementias, Wernicke-Korsakoff syndrome, Hashimoto's encephalopathy, overdose, schizophrenia and others^[4,19]. It affects mostly men over 50 years old and has poor association with tumors^[4].

The symptoms include amnesia, confusion, psychiatric features, seizures, sleep disturbance and dysautonomia^[4,19-21]. The presentation vary from acute to insidious^[4] and may be preceded by neuromyotonia^[22]. Focal seizures are the most common type, frequently assuming medial temporal-lobe topography;

and generalized seizures are less frequent^[4]. Chandra *et al.*^[19] described 12 patients with anti-VGKC encephalitis; all of them had cognitive decline, sleep disturbance and symptoms of panic, 9 had agitation and 3 had faciobrachial dystonic seizures. Catatonia was observed in anti-NMDA related encephalitis, but not in anti-VGKC^[19].

Neuropathic pain is frequently found in patients with CASPR 2 antibodies, likely due to CASPR 2 large expression in peripheral nervous system^[13].

CASPR2 antibodies were positive in 10% of patients with idiopathic ataxia^[13]. Orthostatism and emotions are triggers to episodic ataxia related to anti-CASPR2 antibodies^[23].

First-line therapy consists in steroids, IV immunoglobulin and plasma exchange; while second line consists in rituximab, cyclosporine, mycophenolate mofetil and cyclophosphamide. Immunotherapy leads to better outcomes than no immune therapy^[24].

In conclusion, the great variability of syndromes that can be caused by anti-CASPR2 antibodies, despite its rarity, should be remembered in clinical practice as a differential diagnosis of diseases like limbic encephalitis, epilepsy especially in older patients, new onset psychiatry symptoms, neuropathic pain, idiopathic ataxia, because the immunotherapy has been associated with improved outcomes in patients.

DECLARATIONS

Authors' contributions

Performed data acquisition and data analysis as well helping with the writing in the text: Breis LC, Schlindwein MAM

Made substantial contributions to conception and design of the study, as well as provided technical support: Gonçalves MVM

Availability of data and material

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Review

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Management strategies in acute traumatic spinal cord injury: a narrative review

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Abstract

Worldwide, spinal cord injury (SCI) affects around 500,000 people each year and results in significant morbidity. The primary insult to the spinal cord occurs at the time of the initial injury, which may result from a contusion, laceration or more rarely a transection. Secondary damage in SCI is more insidious and subacute; it is the result of a combination of an inflammatory response, vascular changes and ionic dysregulation. Early clinical intervention is vital after the acute, primary insult to ensure the best possible outcomes for these patients. Current evidence on the demographics and mechanisms, underlying basic science and management strategies of spinal cord injuries are outlined.

Keywords: Spinal cord, trauma, orthopaedics, neurosurgery

INTRODUCTION

Worldwide, spinal cord injury (SCI) is a significant pathology that affects around 500,000 people each year. The injury itself results in significant morbidity.

These injuries are typically of a traumatic aetiology and carry significant impairment to function and quality of life. 40.4% are involved in road traffic accidents, 27.9% in falls, 8% are sports injuries, 15% related to violence and 8.5% are due to tumours or other causes. The burden of these injuries to both the patient



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and society as a whole is significant. The financial cost for each individual with such an injury is US\$3 million with an estimated overall annual cost of \$10 billion per year^[1].

Primary SCI occurs at the time of initial insult to the cord, which may result from a contusion, laceration or more rarely a transection. Maximal neurological deficit is observed immediately after a SCI and this results from the loss of effective axonal transmission, which is hampered by neuronal damage, damage to endothelial cells, ongoing haemorrhage and shifts in ionic concentrations.

Secondary damage in SCI is more insidious and subacute^[1]. It results from the combination of an inflammatory response, vascular changes and ionic dysregulation. As such, timely intervention after the acute primary insult is vital to ensure the best possible outcomes for these patients.

The often-permanent functional impairment in the injured spinal cord is due to poor healing potential. This contrasts to the repair and functional recovery of other tissues such as skin and muscles. The reason for this is not entirely understood; however, as described below, inflammation likely plays a key role^[2].

Obviously, this adds additional necessity for timely intervention and appropriate treatment, not least for the patients themselves, but for society at large.

Current evidence on the demographics and mechanisms of SCIs, the basic science of SCIs, and management strategies are outlined.

The authors aim to perform a narrative review of SCI. Basic science and pathophysiology, mechanisms, management strategies and current best evidence will all be presented to offer a rounded and thorough review of SCIs and their management, for both scientific and clinical reference.

BASIC SCIENCE OF SPINAL CORD INJURY

The spinal cord consists of many multitudes of neurons, which are the component active cell in the central and peripheral nervous systems. Neurons, while there is a certain variety in morphology, contain the following components: cell body, dendrites, axon and axon terminals. The cell body contains the nucleus and contains neuronal proteins and membranes. Axons, coupled with axon terminals, function to relay electrical impulses known as action potentials to stimulate responses in the central nervous system. Axons are layered with myelin sheaths, which enable swifter transmission of action potentials. Dendrites extend out from the cell body, which act to receive impulses from other neuron axons. These are extremely long, particularly in the central nervous system, and have many complex interactions with other neurons^[3].

Neuron progenitor cells are progenitor cells within the CNS that result in the glial and neuronal cell types that populate the CNS. NPCs have no role in generating the non-neural cells that are also present in the CNS, such as immune system cells. NPCs are present in the developing embryo's CNS but are also found in the neonatal and mature adult brain^[4].

Astrocytes are among the number of glial cells. They are ectodermal neural cells that maintain homeostasis and help defend the central nervous system. They are heterogeneous in form and function and have adaptive plasticity that defines the functional maintenance of the CNS with growth and age. They transport major ions and protons, remove and catabolize neurotransmitters, and release neurotransmitter precursors and scavengers of reactive oxygen species^[5].

A SCI can be divided into (1) a primary injury which has occurred as a direct result of the initial insult; and (2) an ensuing secondary injury which is more insidious and subacute^[1]. It results from the combination of

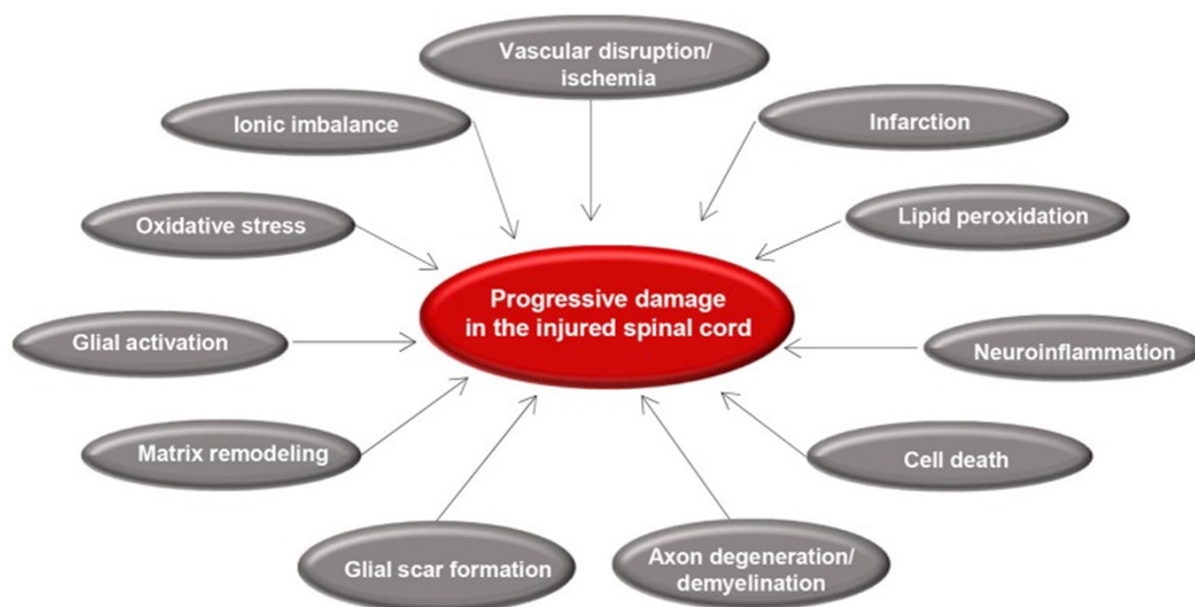


Figure 1. Primary and secondary mechanisms of spinal cord injury. Adapted from Alizadeh *et al.*^[41]

an inflammatory response, vascular changes and ionic dysregulation (all explored in Figure 1). Primary SCI occurs from a series of direct insults. Transection, whereby the spinal cord is transected by a blunt or sharp force, can be complete or partial. Any ascending or descending neuronal tissue will be damaged by such an injury. Contusion of the spinal cord occurs after a transient physical impact. This can result in compression or impact related damage to the cord. The fundamental factor in contusion is that the impact is transient and brief. Compression is a contusion-type injury following by prolonged force application over time^[6].

All primary SCIs will result in secondary damage

Inflammatory response

The spinal cord is not exposed to inflammatory processes in a healthy individual and as a result, when inflammation does occur, it can have devastating consequences. The spinal cord is separated from ongoing inflammatory cells within the body by endothelial cells, which form a physical barrier^[7]. The inflammatory response is initiated by damage to these endothelial cells, which leads to an increase in permeability and intracellular oedema, both of which are key factors in recruiting pro-inflammatory cells, leading to ongoing secondary injury^[8].

The initiation of this inflammatory response is an almost immediate consequence of SCI. The inflammatory response is mediated by pro-inflammatory cytokines including IL1B, IL6 and TNF-alpha, which are released from damaged endothelial cells^[9]. TNF-alpha induces damage in acute inflammation through stimulating apoptosis and necrosis. It is produced by activated macrophages. IL1B increases the levels of TNF-α. This results in reduced neuronal survival, exacerbating lesion size and astrogliosis, and dampening axonal plasticity^[10]. IL6 promotes the infiltration and activation of mononuclear leucocytes while suppressing neutrophil infiltration^[11].

These inflammatory cytokines are very much involved in the acute phase of the injury. It has been found in histochemical analysis of human patients with SCIs, IL1B, IL6 and TNF-alpha were detected in neurons within thirty minutes of an acute SCI. These levels declined within two days after the injury^[12].

Other studies have shown that these cells increase in the first four days following the injury^[13]. This acute inflammatory phase is a transient process lasting approximately ten days following SCI, and end at 3 weeks

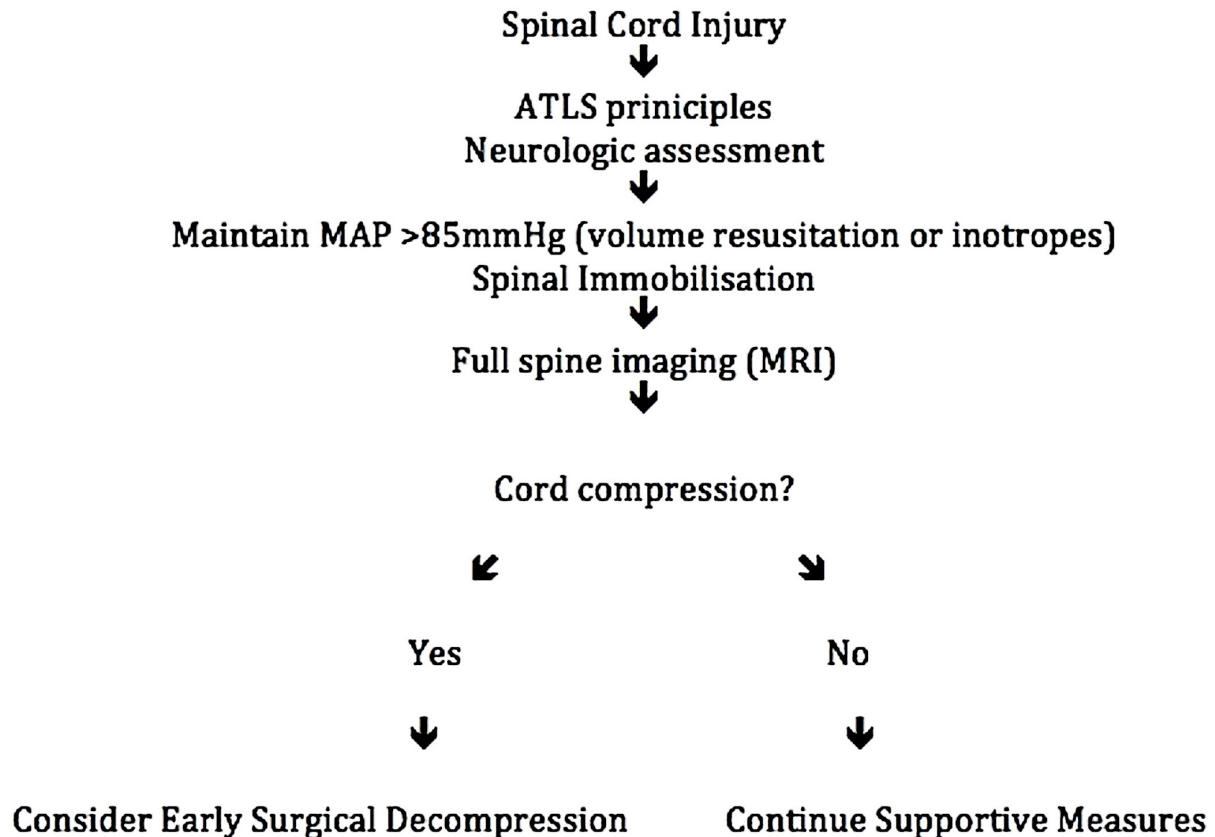


Figure 2. Treatment algorithm for acute traumatic spinal cord injury. Adapted from Vale *et al.*^[35]

following injury with inflammatory cells becoming absent from CSF then to reflect the end of ongoing secondary damage^[9].

Cellular role

Remyelination plays an important role in the recovery of axons after SCI. While pro-inflammatory cytokines mediate inflammation and cell damage in SCI, there is a parallel action of proliferation-orientated cells.

Microglia are a key cellular component of the scar that develops after SCI to protect neural tissue. They are dynamic and proliferate extensively during the first two weeks, accumulating around the lesion. There, microglia move to the interface between infiltrating leukocytes and astrocytes, where they proliferate and form a scar^[14].

Oligodendrocyte precursor cells (OPCs) regulate the inflammatory reaction after SCI. After injury, OPCs migrate to the injury site and rapidly proliferate. From the day of injury to day 7, the number of OPCs persistently increases.

Activated astrocytes influence proliferation, differentiation, and maturation of inflammatory reactions after injury. Astrocytes are also involved in synaptogenesis and control the immune response. It is these factors that play a fundamental role in remyelination after acute SCI^[15]. After trauma, astrocytes surrounding the lesion become reactive and become hypertrophic. They migrate centripetally to the epicentre of the lesion and help with the tissue repair process. These reactive astrocytes do eventually become scar-forming

astrocytes and form a glial scar. Within and around the glial scar, cells deposit extracellular matrix proteins that affect axon growth^[16]. This can result in axonal growth inhibitors and then prevent axonal regeneration. Glial scar formation is one of the main causes of the limited regenerative capability of the CNS^[17].

Macrophage activation also plays an important cellular role in regulating neuronal damage in the injured spinal cord. Macrophages have the ability to promote the repair of injured tissue by regulating transitions through the different phases of the healing response. In the injured spinal cord, pro-inflammatory macrophages potentiate a prolonged inflammatory phase and remodelling is not properly initiated^[18].

Vascular changes

Mechanical damage to the spinal cord results in immediate vasospasm of superficial vessels and intraparenchymal haemorrhage. This damage initially occurs in the highly vascularised, yet most vulnerable grey matter^[19]. This leads to immediate mechanical damage to the grey matter microvasculature, which further impairs the microcirculation to the cord and impedes perfusion^[19]. The impaired blood flow of the damaged spinal cord may then become further damaged by systemic responses to the injury such as hypotension, bradycardia and a decreased CO₂, leading to further ischemic damage^[13].

Free radical damage

Cells under stress, in pro-inflammatory states such as the acutely injured spinal cord, generate large quantities of free radicals. These reactive species lead to ionic dysregulation when generated in excess. They can overload and block normal cellular signalling pathways. Impaired electron pumps such as Na⁺/K⁺/ATPase causes increased intracellular calcium. This leads to apoptosis, as well as mitochondrial dysfunction, contributing to ongoing spinal cord damage^[20,21]. Redox potentials within the cells then plummet and result in oxidative damage. Such oxidative damage can continue for up to five days following the initial injury, contributing to the pathogenesis of secondary injury. Proteins and nucleic acids are damaged by the free radicals from red-ox reactions, leading to further ongoing damage to the spinal cord^[10].

MANAGEMENT STRATEGIES IN SPINAL CORD INJURY

Management strategies for acute SCIs are typically focused on negating any secondary insult, mediated by the vascular, inflammatory and free radical changes after the primary injury. A thorough grounding in the mechanisms described above is therefore essential for guiding appropriate management.

Cardiovascular support

Cardiovascular support for acute SCIs is essential in maintaining spinal cord perfusion after a traumatic injury. As described, physical damage to the cord results in immediate vasospasm of the microvasculature of the cord. Maintaining an adequate mean arterial pressure optimises cord perfusion. In particular, patients with complete high cervical SCIs are likely to develop spinal shock with loss of sympathetic drive. This results in hypotension due to the loss of peripheral vascular tone and concomitant bradycardia^[22]. These patients are more likely to require vasopressor support to maintain their mean arterial pressure at the required levels, compared to incomplete injuries and those with thoracic or lumbar levels of injury ($P = 0.001$)^[23].

An observational study of 91 patients demonstrated that spinal cord perfusion pressure is an independent predictor of neurologic recovery in acute SCI [odds ratio (OR) = 1.039, $P = 0.002$]^[24]. These study findings support the need for vasopressor support in acute SCI.

High levels of evidence are not available but cohort studies have demonstrated improvement in neurologic outcomes in patients with high average mean arterial pressure values. A mean arterial pressure of 85–90

mmHg is appropriate^[25-27]. The duration of vasopressor support however, does not appear to be absolutely supported in the literature. A systematic review of vasopressor support did mention that a duration of five to seven days should be considered. The choice of vasopressor though does appear to be important in some cohorts. A retrospective cohort analysis of 34 patients in California showed that in a subgroup of patients over 55 years of age, dopamine produced statistically significant increases in the complication rates when compared with phenylephrine [83% vs. 50% for dopamine and phenylephrine, respectively; OR with dopamine 5.0 (95%CI: 0.99-25.34), $P = 0.044$]^[28]. This subgroup of 34 patients also demonstrated a median improvement of one ASIA grade from admission to discharge, with no difference between vasopressor agents.

Steroid administration

Steroids have traditionally been given in acute SCI. The hypothesis is that steroids reduce inflammation and prevent secondary cord injury. However, a recent meta-analysis has debunked the evidence for their routine use. A Cochrane review of three randomised controlled trials has shown no difference in neurology between treatment and placebo groups at six and twelve months post-injury^[29]. Nevertheless, despite the lack of evidence for routine administration of steroids in acute SCI, they appear to be routinely administered in many institutions still. The rationale for this is varied, but the fear of medico-legal consequences is one such reason. In the same study, surgeons also felt that there was little risk associated with the routine administration of these treatments regardless, despite minimal demonstrated clinical benefit^[30].

Surgical treatment

Evidence suggests that decompression within 24 h of injury carries the greatest potential improvement in neurologic function for patients with incomplete SCI after trauma.

The timing of surgical decompression is a factor that plays a role in neurologic recovery. There has been some debate and certainly, this is a factor in the trauma patient with SCI and multiple concomitant injuries, particularly chest injuries. Indeed, as discussed later, there is some evidence to suggest conservative management will result in neurologic recovery also.

In a recent meta-analysis of nine studies, patients with traumatic SCIs who were decompressed within 24 hours had a significant neurologic improvement rate (OR = 1.66, 95%CI: 1.19-2.31, $P < 0.01$), a shorter length of hospital stay by almost five days ($P = 0.04$) as well as fewer post-operative complications (OR = 0.61, 95%CI: 0.40-0.91, $P = 0.02$)^[31]. Surgery within 24 h for acute traumatic SCI is thus superior to delaying surgery for neurologic outcomes.

In another meta-analysis performed by Ter-Wengel *et al.*^[32], 422 patients with complete cervical traumatic SCI showed that improvement was more likely after early surgery [respectively, 22.6%, 95%CI: 16.6%-28.7% and 10.4%, 95%CI: 5.6%-15.8%; OR = 2.6 (95%CI: 1.4-5.1)]. The same meta-analysis showed that in 636 patients with incomplete cervical traumatic SCI, there were no differences between early or late surgery. The authors thus suggest a shift in the treatment of patients with complete cervical traumatic SCI. The authors' previous understanding of the literature was that there was equivocal evidence for recovery in complete traumatic cervical SCI. The findings from this study changed that previously held position, in favour of early surgical decompression. In incomplete cervical traumatic SCI, neurological outcome is similar between early and late surgery^[32].

In traumatic central cord syndrome, a retrospective cohort analysis of 50 patients treated acutely (within 24 h) was noted to have shorter intensive care stay, overall length of stay and greater motor improvement ($P = 0.04$) compared with those decompressed later. This was only noted to be the case in acute fractures or disc

herniations. There were no statistically significant improvements in patients who underwent surgery for cervical stenosis or spondylosis^[33].

Another retrospective cohort study of traumatic central cord syndrome, consisting of 126 patients, did not demonstrate any statistically significant difference in neurological recovery based on the timing of surgery. Patients in this cohort treated with surgery did have a shortened length of hospital stay^[34].

A prospective cohort analysis of 98 patients with traumatic cervical SCI has showed that early surgical decompression (within 24 h) demonstrated higher rates of ASIA grade recovery post-operatively. At 6 months post-operatively, 23% of the early surgical group had an ASIA grade improvement of at least two grades, compared with 8.7% of the later surgical group^[35].

Acute traumatic conus medullaris injury in spinal trauma between levels T12 and L1 has not been shown to have any correlation between neurologic recovery and timing of surgery^[7]. The same author performed another retrospective cohort analysis of patients with complete traumatic thoracic SCIs. A cohort of 12 patients showed that in complete thoracic SCIs, two patients demonstrated some sensori-motor improvement, and one patient had motor functional improvement. The median time to surgery in these patients was 11 days, ranging from one to 36 days. In all patients with a documented mechanism of injury, they were all high-energy road traffic accidents, either in vehicles or on motorcycles^[6].

However, surgical treatment in traumatic SCI, whether complete or incomplete, is controversial. Conservative management of traumatic SCI has been described in the literature and was utilised to a greater extent in the past. A retrospective cohort analysis published in 1987 reviewed the outcomes of 207 patients with traumatic SCI; 56% of this cohort underwent spinal surgery. There was however, no statistical difference between the patients who underwent surgical treatment versus those managed conservatively in terms of length of stay or neurological recovery^[36]. More recently, El Masri *et al.*^[37] discussed the evidence for surgical management compared with Active Physiological Conservative Management. He concluded that conservative, non-surgical management in patients with incomplete SCIs will often recovery enough power to ambulate and suggested the need for review of the current standards of care in relation to the acute management of traumatic SCIs.

The findings of these studies are certainly at odds with the approach to acute traumatic SCI in a study published in 2010. Fehlings *et al.*^[38] assessed the professional opinions and clinical approaches to traumatic SCIs in 972 spinal surgeons. 80% of the surveyed participants would prefer to decompress within 24 h.

Early and aggressive medical management of these patients, followed by appropriate surgical decompression has demonstrated, in prospective studies, improvement of at least one ASIA grade in complete SCIs at one year follow up in 60% of patients. 92% of patients with incomplete cervical spine injuries demonstrated improvement at one-year follow-up in the same study.

CONCLUSION

SCI carries significant morbidity for affected patients and has a serious economic burden on society. As illustrated by evidence from both the pathophysiology of SCI and clinical outcomes, timing is the key variable that determines treatment outcomes. The acute inflammatory response responsible for demyelination and neuronal damage occurs within minutes of the injury, but peaks at four days after. The vascular and cellular sequelae of these acute inflammatory events, a direct response to trauma and injury, exacerbate the damage and the degree of injury. If clinicians can intervene with the appropriate support to delay, offset or reverse this catastrophic cascade of pro-inflammatory cytokines and ischaemia, patient outcomes will obviously be greatly improved. These interventions must be timely, given how rapidly these

cellular responses to injury occur.

Appropriate supportive therapy to maintain mean arterial pressure as described is essential. These patients are often the victims of serious trauma events. As illustrated in the introduction, 40% of these patients are involved in serious road traffic accidents, and may not be suitable for surgical intervention due to concomitant injuries. Coagulopathy, acidosis or renal failure may all render a prolonged surgical intervention dangerous^[39-40]. It is in these circumstances that timely and appropriate management from intensivists is paramount. This should, of course, be carried out in a multi-disciplinary manner, with input from local neurosurgical or orthopaedic services.

Wherever possible, the prevailing standard of practice appears to guide surgeons to decompress the injured spinal cord within 24 h of injury, particularly in incomplete injuries. This is illustrated by adapted treatment algorithm in [Figure 2](#). This has been shown to offer favourable outcomes in terms of neurologic recovery, compared with delaying surgical intervention. Results are less favourable for patients with complete cord injury. While some studies have offered equivocal results for early decompression, even in incomplete injuries, such as central cord syndromes, the prevailing clinician preference is to decompress as soon as possible.

While there has been a vogue for administering steroids acutely in these patients prior to decompression, they appear to add little in terms of long-term recovery and should be discouraged.

LIMITATIONS

Conclusions drawn from this narrative review are derived from a combination of retrospective and prospective cohort analyses, as well as questionnaire publications documenting the treatment preferences of spine surgeons in their treatment protocols. While some evidence cited in this text does reach Level 1, further investigations including randomised controlled trials and meta-analyses would benefit the evidence base in terms of blood pressure management, steroids and surgical decompression.

SUMMARY OF RECOMMENDATIONS

- (1) Patients with acute SCIs should have their mean arterial pressure maintained above 85-90 mmHg after injury for a period of several days;
- (2) Where possible, early surgical decompression within 24 h should be undertaken, particularly in incomplete spinal cord injury;
- (3) While the timing of establishing musculoskeletal stability of traumatic spinal injuries with surgical decompression and fixation may not determine neurologic return, it certainly allows for earlier mobilisation and easier nursing. This certainly explains the shorter lengths of stay and intensive care stays noted in patients who undergo earlier surgery;
- (4) Steroids appear to have no therapeutic value.

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Authors' contributions

All authors contributed equally to the concepts, research, writing and overall academic workload in writing this paper.

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Original Article

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Postnatal toxicant exposure in 3xTgAD mice promotes gene x environment-related early alterations to neuroimmune epigenetic profiles

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Abstract

Aim: The purpose of this study was to evaluate sex-biased, maladaptive changes to epigenetic regulation critical for development of neuroimmune crosstalk resulting from an early-life toxicant exposure previously associated with increased susceptibility to later-life neurodegeneration.

Methods: An evaluation of early-life gene x environment (GxE) interactions was performed in a mouse model of Alzheimer's disease (Tg) orally exposed to lead acetate (Pb) from postnatal day (PND) 5-9. Following exposure, immunohistochemical analysis was used to evaluate hippocampal expression of DAPI12, a marker for perinatal microglia related to microglial-mediated postnatal synaptic pruning of neurons. Altered profiles of three microRNAs critical to homeostatic microglia: neuron signaling (miR-34a, miR-124, miR-132) were measured by qRT-PCR.

Results: Atypical and deleterious expression patterns in Pb-exposed Tg mice were detected with significant female bias by PND 10. Early exposure to Pb resulted in the upregulation of miR-124, a microRNA involved in microglial quiescence, as well as miR-34a, involved in p53-dependent apoptosis and decreased phagocytosis, by PND 21 and during a period of microglial-mediated synaptic pruning specific to females. In addition, we observed a sustained, imbalanced upregulation of miR-132 in Pb-exposed Tg females as well as decreased expression of DAPI12.



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Conclusion: This study demonstrates the exacerbating effects and early manifestation of GxE interactions in this model. Furthermore, these findings underscore a period of female-specific vulnerability to epigenetic maladaptation during postnatal development, with implications on the faulty later-life adaptability of neuroimmune signaling. Further investigation is warranted to evaluate the persistence and relative contribution of these early influences on the etiopathology of Alzheimer's disease.

Keywords: MicroRNA, microglia, Alzheimer's disease, developmental origins of adult disease

INTRODUCTION

Numerous studies examining the developmental origins of adult disease (DOAD) hypothesis have shown that environmental perturbations to neuroimmune development may contribute to later-life neurodegenerative diseases, like Alzheimer's disease (AD)^[1-3]. The consequence of gene x environment (GxE) interactions is much more profound during development due to critical windows in which phenotypic plasticity is instructed by, and extremely sensitive to, exogenous signaling. In our previous studies using a DOAD model for AD, we reported that the interaction of GxE exacerbated AD susceptibility later in life in a transgenic AD mouse model postnatally exposed to lead acetate (Pb)^[4,5]. This GxE-related vulnerability was concurrent with dysfunctional microglial phenotypes and synaptic defects indicative of atypical microglia-neuron interactions, and significantly biased towards females. In agreement with the literature, microglia in aging brains can become senescent, dystrophic, and mount ineffective or inappropriate responses to neuronal signaling^[6-8]. Importantly, the spatially- and temporally-synchronized development of microglia and neurons during the perinatal period fine-tunes this crosstalk necessary for homeostatic signaling in the adult^[9]. Thus, imbalances in neuroimmune signaling and function may be developmentally promoted.

Healthy homeostatic interactions between microglia and neurons in adulthood depend on the tightly-controlled developmental programming of microglia in response to cues from neurons^[9], clearly demonstrated in studies of early immune insults resulting in cognitive decline, impaired memory, and even neurodegeneration in adulthood^[2,10-13]. Mutations to the transmembrane adaptor protein DAP12 or its ligand TREM2 are associated with a form of presenile dementia called Nasu-Hakola disease in humans^[14], and patients with heterozygous variants of TREM2 have a significantly higher susceptibility to AD^[15-17]. Furthermore, the typical expression of DAP12 is limited to perinatal microglia and is critical for healthy developmental synaptic pruning by microglia^[18,19]. DAP12-deficient rodent models exhibit persistent synaptic defects to glutamatergic synapses in adulthood^[20,21]. Therefore, DAP12 may highlight a particular junction of the neuroimmune interface during development by which microglia and neurons establish and promote healthy synapse dynamics throughout life, with implications for the promotion of early AD-like phenotypes in its absence. However, little is known about the molecular mechanisms through which early-life toxicant exposure alters this junction to promote atypical signaling between neurons and microglia that then persist beyond development.

As environmentally sensitive genomic regulators, microRNAs represent a form of long-term epigenetic regulation relevant to GxE studies to fine-tune cellular phenotypes. Nearly 60% of all protein-encoding genes are thought to be regulated by miRNAs^[22], and, with half-lives nearly 10x longer than mRNA^[23], numerous studies have already begun exploring the potential of these molecules to act as biomarkers of disease^[24]. In the healthy adult brain, transient changes to miRNAs are often the result of the immediate microenvironment, promoting functional changes to parenchymal targets as a cellular adaptation to exogenous influences - temporarily shifting the requirements for homeostasis. However, altered miRNAs during critical windows of neuroimmune development have direct consequences on the promotion

of homeostatic baselines, immune maturation and learning, and even patterns of functional response phenotypes. MicroRNA expression patterns are both evidentiary of past exogenous influences and predictive of future cellular response, such that altered miRNAs promoting the disjointed development of microglia and neurons early in life may impact the intertwined functionality of neuroimmune cells throughout life. MicroRNAs are highly involved in central nervous system development^[25] and, more pertinently, the promotion of varied microglial states of activation and quiescence and the dynamic transitions therein^[26]. Notably, a cluster of microRNAs referred to as “NeurimmiRs” have been described in modulating both neuronal and immune processes, such as miR-124 and miR-132, that act as negotiators at the neuroimmune interface^[27]. The most abundant microRNA expressed in the adult brain, miR-124 exhibits highly conserved expression patterns consistent with a critical role in neurodevelopment and neurogenesis^[25,28]. The expression of miR-124 has also been shown to promote microglial quiescence transitioning from an activated amoeboid state through the downregulation of M1-associated markers^[26,29]. Likewise, miR-132 is involved in the regulation of neurotransmission and synaptogenesis and is upregulated during postnatal development^[27]. Correspondingly, both miR-124 and miR-132 are downregulated in the brains of patients with AD^[30,31]. Although a recent report by Gillet *et al.*^[32] underscored how certain neurodevelopmental disorders were correlated with altered microRNA expression from toxicant exposures, there is still a disconnect with etiopathological relevance in DOAD models for aging-related neurodegenerative diseases like AD. Furthermore, while microRNAs are persistently modified due to early-life Pb exposure^[33], there is a little characterization of the sexually dimorphic effects in Pb-altered epigenetic profiles related to neuroimmune function.

Both rodent and human brains undergo sexually dimorphic neuroimmune development, specifically within the postnatal period, during which PGE2 secreted by microglia critically regulates the masculinization of the male brain^[34]. Blocking PGE2 with the NSAID indomethacin (indo) resulted in a “feminization” of male microglia, significantly reducing the number of amoeboid, but not total, microglia in the preoptic area of 2-day-old male mice^[35]. Importantly, the number and phenotype of microglia at varying time points and brain regions varies dramatically by sex throughout development; a spike in the number of amoeboid microglia in males parallels a testosterone surge at PND 4, whereas amoeboid microglia numbers within specific brain regions don’t peak in female brains until PND 30^[36]. Thus, the transition from DAPI12-positive immature amoeboid phenotypes to fully mature, ramified microglia occurs earlier in males than females. Previously, the first two postnatal weeks were generally considered a period of male-specific microglial vulnerability to later-life immune-related priming^[37], but, here, we report a comparable postnatal window of female-specific microglial and neuronal vulnerability to epigenetic regulation in a GxE model for AD. Nearly 2/3rds of the 250 miRNAs surveyed in neonatal mouse brains by Morgan and Bale^[38] were shown to be differentially expressed in males and females, implicating miRNAs as major epigenetic regulators of sex differences in the developing brain^[39,40]. In the current study, miR-34a was also evaluated alongside the neurimmiRs miR-124 and miR-132, given reports that the upregulation of miR-34a decreased TREM2 expression by targeting parts of its mRNA^[41]. Furthermore, expression of miR-34a both strongly promotes and is itself promoted by, p53 as a cellular stress response leading to apoptosis and senescence^[42], and modifications to this microRNA may suggest the presence of cellular stress signals that are readily detectable by microglia. Interestingly, mutations to presenilin2, one of the genes implicated in familial AD, has been shown to trigger neuronal apoptosis via the miR-34a/p53 axis^[43]. Likewise, TP53, the “apoptosis gene” that encodes for p53, is mutated in some cases of AD^[42].

The present study aimed to delineate the early epigenetic regulation of neuroimmune phenotypes related to the promotion of lifelong homeostatic microglia: neuron signaling as a consequence of toxicant exposure. The adaptive response of immune cells to fine-tune signaling in homeostatic pathways is not only critical in adulthood but defined during development. Thus, we hypothesized that the combination of a genetic proclivity to AD and postnatal exposure to Pb would result in persistent, differential changes in interrelated

neurimmiRs known to regulate microglial quiescence and neuronal maturation and sprouting, thereby promoting lifelong neuroimmune imbalances associated with increased vulnerability to AD. By including in utero exposure, we aimed to discriminate sex-biased effects derived from sexually dimorphic microglial signaling during this postnatal period related to PGE₂. Here, we confirm the hypothesis that postnatal Pb exposure in a genetic mouse model of AD significantly alters early epigenetic regulation related to the neuroimmune interface in a sexually dimorphic manner, highlighting the exacerbating effect of the GxE model in the formation of a developmental phenotype for later-life female-biased susceptibility to neurodegeneration.

METHODS

Animal handling

All handling and experimental manipulations were carried out in accordance with procedures approved by the East Carolina University Institutional Animal Care and Use Committee (IACUC). Pregnant wildtype (WT) and transgenic dams [3xTgAD; B6; 129*Psen1*^{tm1Mpm}Tg (APP^{Swe}, tauP301L) 1Lfa/Mmjax] were obtained from the seed colony in the ECU Department of Comparative Medicine and kept on a 12:12 hour light/dark cycle, with access to food and water *ad libitum*. Litters were culled to eight after birth (postnatal day, PND 1), if needed, and monitored for overt signs of toxicity.

Dosing and tissue preparation

Exposure to lead acetate was based on our previous findings that an identical exposure concentration and timing in the 3xTgAD mouse model resulted in significantly altered microglia and exacerbated AD pathology in adult females^[4,5]. Indomethacin concentration (1 mg/kg/day) was chosen to recapitulate previous reports of efficacious concentrations for PGE₂ inhibition in rodent microglia^[35,44,45]. Dosing solutions were dissolved in sterile water and prepared fresh weekly for lead acetate (100 ppm) and indomethacin (1 mg/kg/day) (Sigma-Aldrich, Milwaukee, WI, USA). From PND 5-9 neonates were dosed once per day (10 µL/g body weight/day) with a vehicle, indomethacin (1 mg/kg), lead acetate (100 ppm), or indomethacin followed by lead acetate 30 min later using a modified gavage technique^[46]. One mouse per sex, litter, and treatment group were randomly assigned and euthanized at PND 10, 15, or 21, with *n* = 3 mice/sex/age/treatment/strain for each assay. As per ethical use protocol, animals were euthanized with inhaled isoflurane followed by immediate decapitation. The brain was then carefully removed and placed in ice-cold PBS. For histochemical analysis, the left hippocampus was dissected and fixed for 24 h in 10% neutral buffered formalin followed by 70% ethyl alcohol before paraffin fixation. The right hemisphere sans cerebellum was flash-frozen whole and stored at -80 °C.

Immunohistochemistry

Formalin-fixed, paraffin-embedded hippocampi were sliced on a rotary microtome at 10 µm and mounted on Superfrost Plus slides (Azer Scientific, Germany). Briefly, slides were dewaxed in Histo-Clear II (Electron Microscopy Sciences, Hatfield, PA, USA), followed by washes in 100% and 95% ethyl alcohol and phosphate-buffered saline (PBS). Antigen unmasking was accomplished using a heat-mediated citrate buffer, followed by incubation in 0.3% hydrogen peroxide for 30 min. All subsequent staining was performed using Sequenza-Coverplate racks (Thermo Scientific, Waldorf, Germany). Sections were permeabilized with PBS with Tween-20 and blocked with diluted normal serum (ABC Vectastain; Vector Laboratories, Burlingame, CA, USA). Slides were then incubated with anti-DAP12 primary antibody (4 µg/mL; unconjugated rabbit polyclonal IgG, Cat#orb156537, Biorbyt, Cambridge, UK), for 60 min at room temperature or overnight at 4 °C. Indirect labeling was then performed using biotinylated anti-rabbit IgG secondary antibodies and reagents from a high-sensitivity avidin-biotin kit with peroxidase-based detection (Vectastain Elite ABC-HRP kit, Peroxidase (Rabbit IgG), Cat# PK-6101; Vector Laboratories). Slides were visualized with diaminobenzidine (DAB) (DAB Substrate Kit, Peroxidase (HRP), Cat# SK-4100; Vector Laboratories), with a consistent DAB development time of 60 s for all slides, and counterstained

with Harris' Alum Hematoxylin. After sequential washing in ethyl alcohol and Histo-Clear, slides were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA) and cured overnight before visualization.

Slides were visualized using a Leica DM1000 light microscope at 20× magnification (image resolution at 1.35 $\mu\text{m}/\text{pixel}$) with a SPOTTM Idea camera attachment and Advanced Imaging software. Regions of interest (ROI) were chosen at random along the dentate gyrus with the viewing frame containing as much tissue as possible and averaged for each of the 3-4 serially-sliced tissue sections per sample for a total of $n = 6-8$ ROIs/animal. Negative and positive controls were routinely employed to determine immunopositive DAP12 reactivity and to instruct background thresholding to minimize batch effects. Immunopositive DAP12 was determined over a manually predetermined background threshold via FIJI^[47] analysis, blinded to sample grouping. Use of a Color Deconvolution plugin using predetermined vectors for DAB and hematoxylin [Supplementary Figure 1], and the % area DAP12 positive/ROI was determined along the dentate gyrus hippocampal subregion. Mean % area DAP12+ was averaged from $n = 6-8$ ROIs (technical replicates) per animal, for each of the $n = 3$ animals/exposure/sex/age/strain. All data are represented as the mean % over control at PND10 \pm SEM. Raw, untransformed values for % DAP12 immunopositive staining at each age, and exposure are listed in Supplementary Figure 2, stratified by sex, strain, and age.

Quantitative real-time polymerase chain reaction

RNA Isolation and cDNA synthesis

Total RNA was purified from no more than 20 mg frozen brain tissue taken from the cortex of the right hemisphere of 3xTgAD or WT mice using the miRCURYTM RNA Isolation Kit - Cell & Plant (Exiqon Inc., Woburn, MA, USA) and associated Lysis Additive (Exiqon) specific for fatty tissue. Following homogenization and cell lysis, RNA was purified against a proprietary resin spin column separation matrix, washed with the associated buffers, and eluted at 50 μL , as per manufacturer's instructions. Purified RNA was measured on a NanoDropTM One/One^C Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Madison, WI, USA) for purity and concentration, which was then adjusted to 5 ng/ μL for subsequent reverse transcription.

For each sample, cDNA was synthesized from 10 ng purified RNA, in duplicate, using the Universal cDNA synthesis kit (Exiqon) and the following protocol: 60 min at 42 °C, followed by heat-inactivation of the reverse transcriptase for 5 min at 95 °C. Newly synthesized cDNA was then stored at -20 °C, and thawed and diluted to 80× in RNase-free water before RT-PCR.

Real-time PCR

MicroRNA relative quantification was performed on an iQ5 Multicolor Real-Time PCR Detection System thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using 96-well PCR plates (VWR, Radnor, PA, USA). A master mix was prepared for each pre-designed LNATM PCR Primer set (Table 1, Exiqon), in conjunction with ExiLent SYBR[®] Green master mix (Exiqon), for the target primers (miR-124, miR-132, miR-34a), endogenous reference primer (SNORD110), and UniSp6 RNA Spike-in control primer included in the kit. Each well consisted of 6 μL master primer mix and 4 μL cDNA template per sample, for a final well volume of 10 μL per sample, performed in duplicate. Reaction conditions included polymerase activation/denaturation at 95 °C for 10 min, 40 amplification cycles at 95 °C for 10 sec and 60 °C for 1 min (1.6 °C/s ramp rate), and a melt curve analysis consisting of a setpoint at 60 °C and endpoint at 95 °C, incrementally increasing by 0.5 °C with a 10 s dwell time.

Automatically generated threshold cycle values (C_t) were evaluated using LinRegPCR^[48] quality assessment to account for amplicon and assay efficiency. Fold change in relative miRNA expression compared to sex-, strain-, and age-matched controls was determined by the Pfaffl method, accounting for differential amplicon efficiencies calculated by LinRegPCR.

Table 1. Primer sequences of miRCURY LNA® PCR Primer sets (Exiqon)

MicroRNA	RT primer sequence 5'-3'	
hsa-miR-124-3p	UAAGGCACGCGGUGAAUGCC	target
mmu-miR-132-5p	AACCGUGGCUUUCGAUUGUUAC	target
hsa-miR-34a-5p	UGGCAGUGUCUAGCUGGUUGU	target
SNORD110	[UGACUUUAUAUCUGUCAAUCCCCUGAGAGAUACUGACGACUCCAUGUGUCUGAGCAA]	reference
UniSp6	CUAGUCCGAUCUAAGUCUUCGA	control

RT: reverse transcriptase

Statistical analysis

All statistics were carried out using the Statistical Analysis System (SAS Institute, Cary, NC, USA) or GraphPad PRISM (GraphPad, La Jolla, CA, USA) software. Initially, exploratory analyses were carried out using mixed modeling (PROC MIXED, SAS) with random intercepts to evaluate possible relationships between GxE variables with sex and age. Immunohistochemical (IHC) analyses were conducted in untreated mice stratified by strain modeling the fixed effects of age and sex. The fixed effects of treatment and age were then modeled, stratified by strain and sex. Possible interactions between these variables were also evaluated. Two-way ANOVA (PROC GLM, SAS) for age and sex was performed for each strain and treatment group for quantitative real-time polymerase chain reaction (qRT-PCR). Individual pairwise comparisons were made with a *t*-test corrected for multiple comparisons using the Holm-Sidak method or a Tukey's studentized range distribution method. IHC data are represented as % mean DAP12 immunopositive staining/ROI over strain- and sex-matched controls at PND10 ± SEM, and qRT-PCR data are represented as mean fold change over sex-, strain-, and age-matched controls, with respect to endogenous reference gene levels, ± SEM. Statistical significance was determined at **P* < 0.05 and **P* < 0.01 for interactions.

RESULTS

Sexually dimorphic hippocampal DAP12 expression during postnatal development is altered by early-life exposures

DAP12 expression on perinatal microglia is critical for phagocytosis of apoptotic neurons, and thus the development of healthy neuroimmune interactions^[19,49]. To determine baseline expression of DAP12 during the postnatal period in this GxE mouse model immunohistochemical analysis was quantified in the hippocampus of untreated control mice, stratified by sex and genetic strain. In both WT and Tg males DAP12+ expression along the dentate gyrus was not significantly altered by age [Figure 1]. In WT females, DAP12 expression was significantly increased at both PND 15 and PND21 compared to expression levels at PND 10 [Figure 1A and C]. Similarly, untreated Tg females had increased DAP12 by PND 21 compared to PND 10 but was not significantly altered at PND 15 [Figure 1B and D]. Notably, while significant sex differences were detectable in WT mice at both PND 15 and 21 [Figure 1A and C], only PND 21 differed significantly by sex for DAP12 expression in the Tg mouse strain [Figure 1B and D]. These data suggest that DAP12 expression in the hippocampus during the postnatal period is sexually dimorphic regardless of genetic background and that this sexual dimorphism is dependent on age in WT mice (**P*_{interaction} < 0.0001).

Postnatal Pb exposure significantly increased DAP12 expression in both WT and Tg female mice at PND 10 that then decreased over time [Figure 2A and C], with an inverse age-related trend compared to controls, suggesting a profound and persistent effect on female hippocampal DAP12 expression due to Pb. Interestingly, Tg males exposed to Pb also had significantly increased DAP12 at PND 10 that persisted with age [Figure 2D], whereas WT male expression was unaffected by Pb at all ages [Figure 2B].

Importantly, the addition of indomethacin before Pb exposure was able to moderate the long-term depression of DAP12 by Pb in Tg females [Figure 2C]. This is striking when contrasted with the nearly

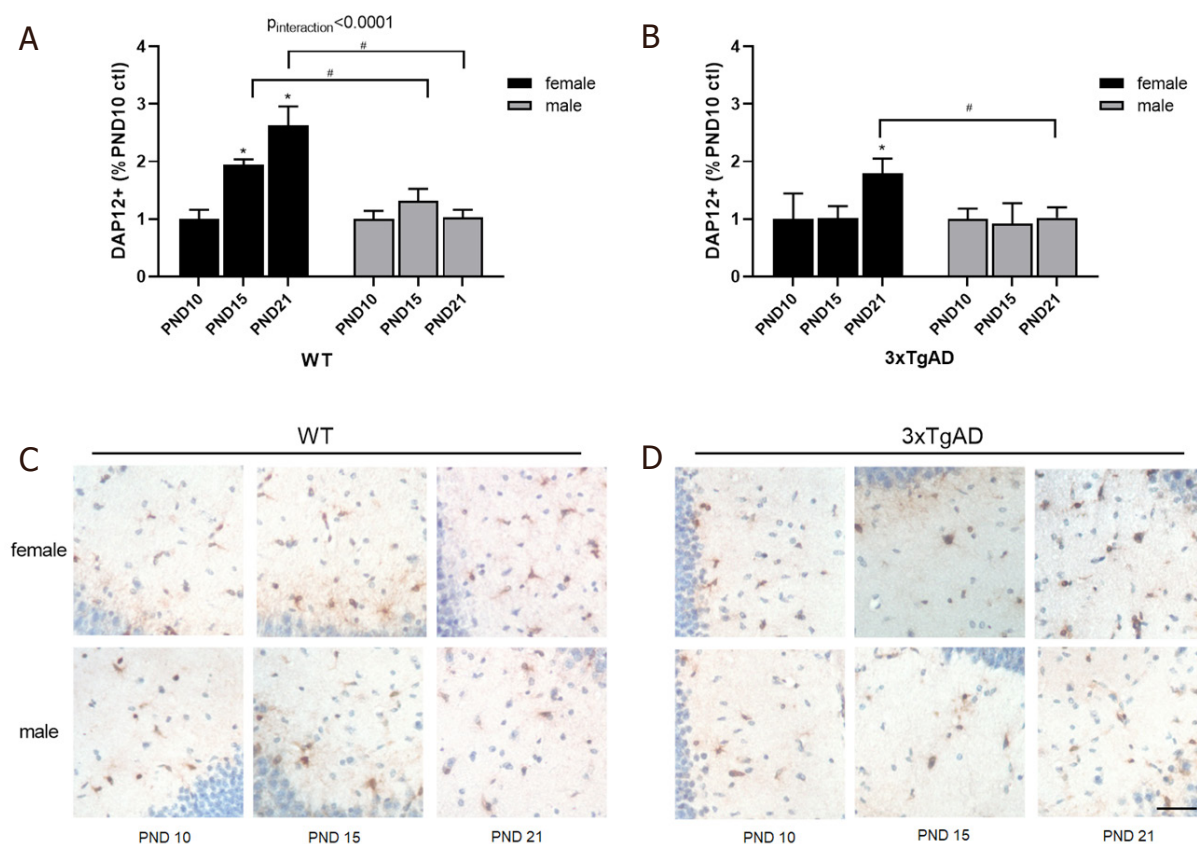


Figure 1. Quantification of hippocampal DAPI2+ expression by sex over time in untreated WT (A) and 3xTgAD (B) mice. Representative images of hippocampal DAPI2+ expression at PND10, 15, and 21 for females and males in WT (C) and 3xTgAD (D) hippocampus. Data expressed as mean % of sex- and strain-matched PND10 controls \pm SEM. $n = 3$ mice/sex/age/strain. scale bar = 26 μ m. Statistically significant at $P < 0.05$ (*-by age) (#-by sex), $*P < 0.01$ interaction (age*sex). PND: postnatal day; WT: wildtype; ctl: control

identical pattern of long-term depression by either Pb or indo alone in this group, suggesting that the combination of Pb and indo was able to rectify individual detriments over time. Although this indo+Pb rescue was similarly beneficial over time compared to Pb only exposure in WT females [Figure 2A], the individual effect of indo did not mimic that of Pb, suggesting the genetic proclivity to AD altered the female DAPI2 response to postnatal indo exposure.

Due to the absence of temporal change in DAPI2 expression in male mice regardless of strain [Figure 1], the significant effect of indo+Pb in differentially altering DAPI2 expression over time in WT male mice [Figure 2B] would suggest that the inundation of both agents acted to modify typical developmental expression more so than either agent alone. However, male DAPI2 expression at PND 15 was particularly sensitive to the effects of indo alone or in conjunction with Pb, with directionality dependent on genetic strain as expression decreased and increased in WT and Tg mice, respectively [Figure 2B and D]. This effect was not seen at PND 10 or 21, indicating a temporal vulnerability in male DAPI2 expression patterning at PND 15 in response to the NSAID indo.

Postnatal toxicant exposure induced aberrant mouse brain miRNA expression profiles by PND 10

To assess early regulatory changes to neuroimmune signaling following postnatal toxicant exposure, as well as any sex bias and effect of genetic strain, we performed qRT-PCR for three distinct, yet interrelated microRNAs at the neuroimmune interface at PND 10 [Figure 3] and PND 21 [Figure 4]. At PND 10, one day after cessation of exposure, there was a significant interaction between microRNA expression levels

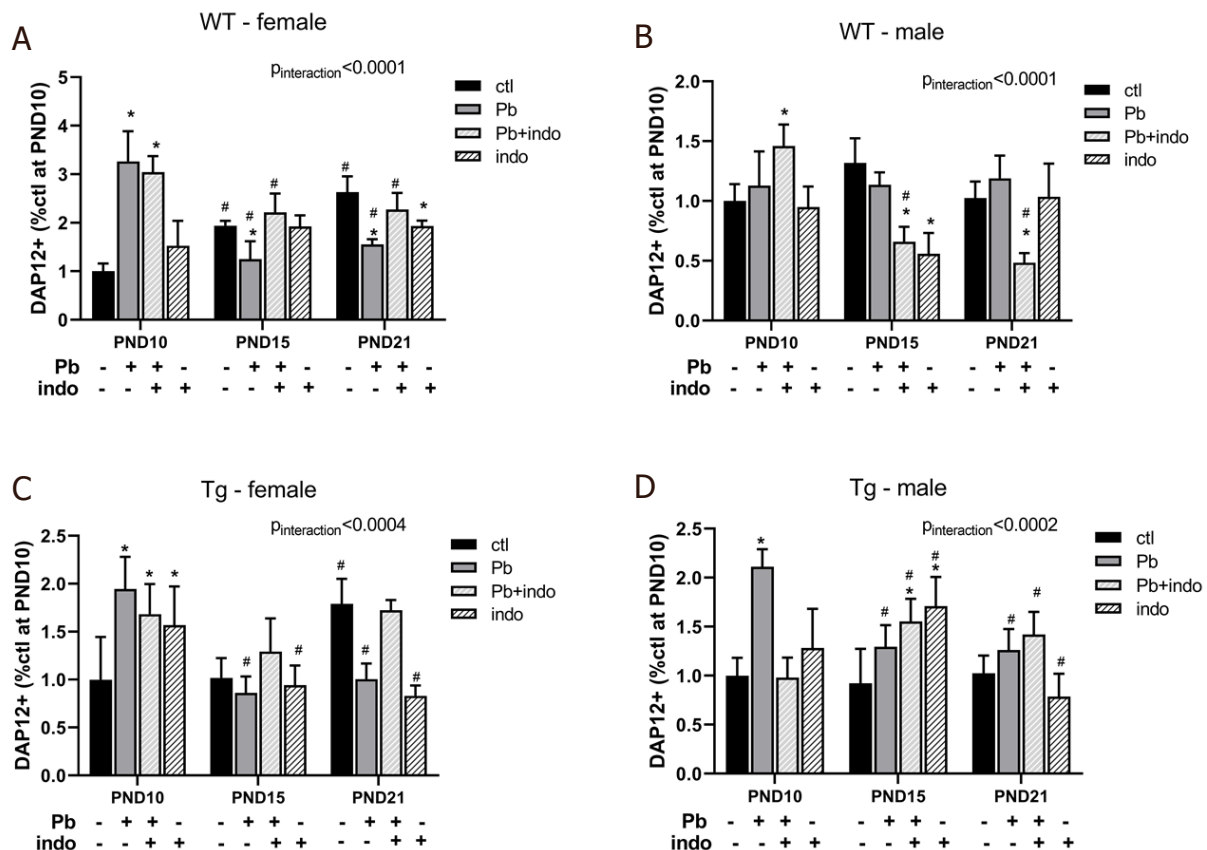


Figure 2. Quantification of hippocampal DAPI2+ expression by treatment over time in WT females (A), WT males (B), 3xTgAD females (C), and 3xTgAD males (D). Data expressed as mean % of sex- and strain-matched PND10 controls \pm SEM. $n = 3$ mice/sex/age/treatment/strain. $P < 0.05$ (*-by treatment, #-by age) and $*P < 0.01$ interaction (treatment*age) was considered statistically significant. PND: postnatal day; ROI: region of interest; WT: wildtype; Tg: 3xTgAD; Pb: lead; indo: indomethacin; ctl: control

in WT mice and treatment but not a significant effect of treatment itself [Figure 3A and B]. These data suggest, first, that atypical environmental cues due to exposure to either Pb, indo, or both affect miR-124 and miR-132 in a divergent manner than miR-34a very early during the postnatal period. Second, the inverse trends in these profile dynamics with sex would suggest that neuroimmune-related epigenetic regulation parallels the sexually dimorphic postnatal development of the neuroimmune system itself.

While transgenic mice at PND 10 also exhibited significant interactions between treatment and microRNA expression, the effect of treatment varied significantly [Figure 3C and D]. Surprisingly, miR-124 expression levels were dramatically upregulated in both Tg females and Tg males only exposed to indo, whereas Pb and indo+Pb did not affect, suggesting that genetic proclivity to AD impacted the immediate miR-124 response to indo. On the other hand, miR-132 expression levels in Tg mice were similar in directionality by sex to that of the WT; specifically, increased in females and decreased in males by either Pb or indo. This would suggest that the directionality and altered expression of miR-132 at PND 10 consequent to either Pb or indo was a consequence of sex-related vulnerabilities to this particular epigenetic reprogramming.

The expression of miR-34a in Tg males was significantly decreased by the combination of Pb and indo but not by the agents individually [Figure 3D], in contrast to the overall trend of postnatal exposures increasing miR-34a in WT males [Figure 3B].

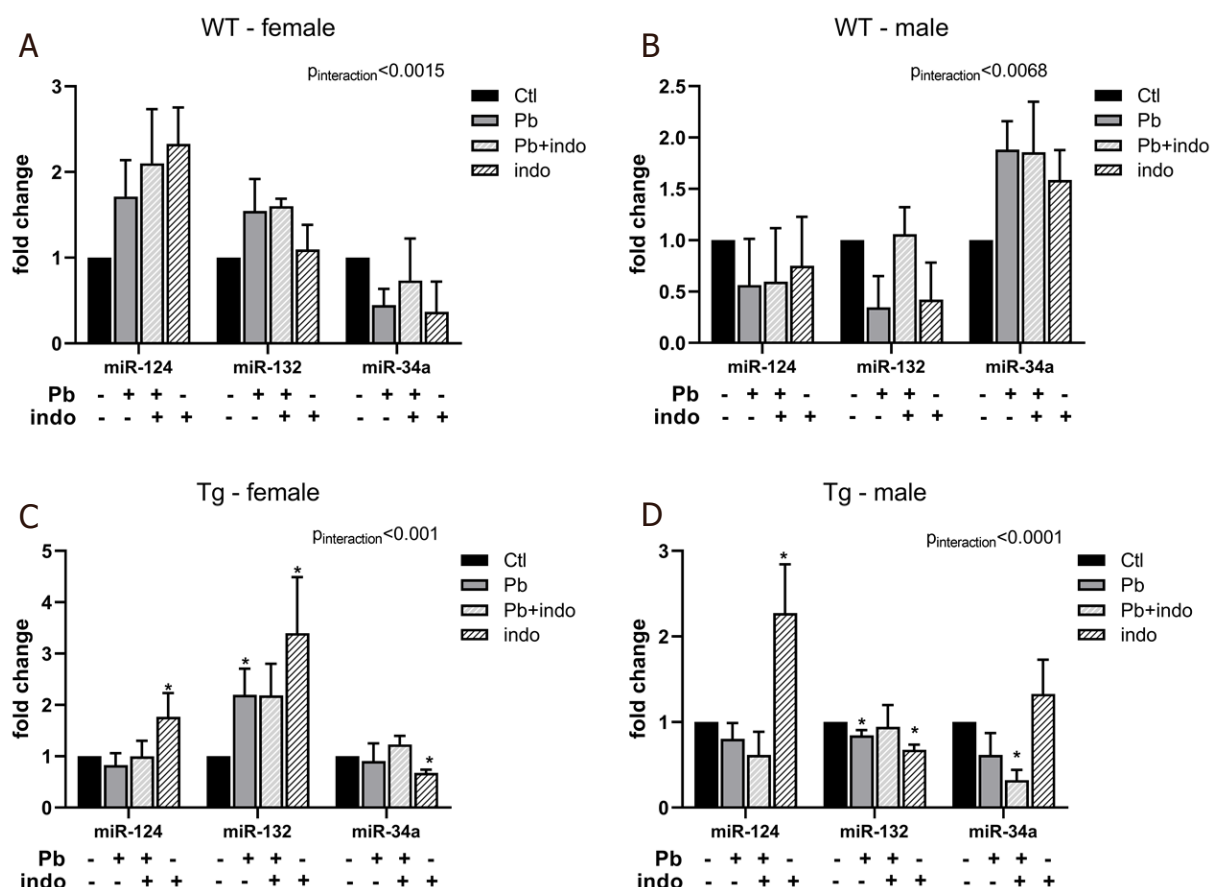


Figure 3. MicroRNA expression at PND 10 related to postnatal exposures in WT female (A), WT male (B), Tg female (C), and Tg male (D) mice. Quantification of microRNAs relative expression expressed as fold change \pm SEM over sex-, strain-, and age-matched controls in WT (A, B) and Tg (C, D) mice. $n = 3$ mice/sex/age/treatment/strain. $P < 0.05$ (*-by treatment) and $*P < 0.01$ interaction (treatment*microRNA) was considered statistically significant. PND: postnatal day; Tg: 3xTgAD; indo: indomethacin; ctl: control; ns: not significant

Postnatal toxicant exposure induced aberrant mouse brain miRNA expression profiles that persisted until PND 21

qRT-PCR analysis of microRNA expression at PND 21 revealed expression profiles in WT mice generally devoid of any carryover or long-term upregulation from PND 10, with the notable exception of dramatically increased miR-132 in WT males in response to postnatal Pb exposure [Figure 4B]. No other microRNAs were significantly affected by postnatal exposures in WT males at this time, nor was miR-132 upregulated at PND 10 in this group [Figure 3B], suggesting that Pb induced persistent epigenetic remodeling in WT males in the form of long term changes in miR-132 expression. In contrast, WT females exposed to indo exhibited decreased expression of both miR-124 and miR-34a, with miR-124 also decreased by Pb at PND 21 [Figure 4A].

Although no significant interaction between treatment and microRNA expression was detected in Tg males ($P_{\text{interaction}} = 0.1532$), indo exposure increased the expression of both miR-124 and miR-34a at PND 21 [Figure 4D], with the increase in miR-124 persisting from PND 10 [Figure 3D]. This indo-related increase in miR-124 at both PND 10 and PND 21 was also detectable in Tg females [Figure 3C, Figure 4C], suggesting that in the transgenic strain indo exposure alone was enough to result in persistently elevated miR-124 regardless of sex. Importantly, Tg females exposed to Pb alone or in combination with indo also exhibited significantly increased miR-124 at PND 21 [Figure 4C], which was not seen in the earlier time point [Figure 3C]. These data suggest that increased miR-124 may act as a long-term response to postnatal

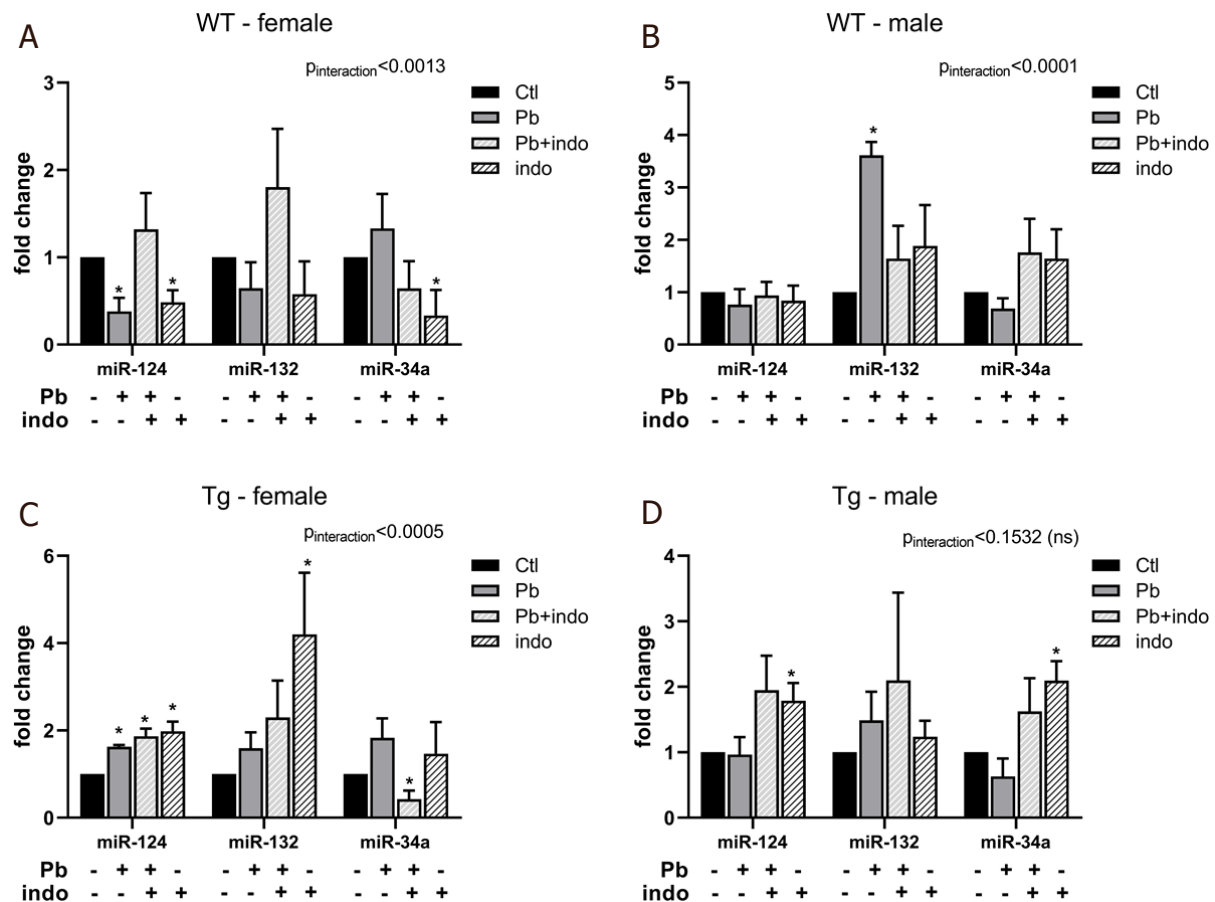


Figure 4. MicroRNA expression at PND 21 related to postnatal exposures in WT female (A), WT male (B), Tg female (C), and Tg male (D) mice. Quantification of microRNAs relative expression expressed as fold change \pm SEM over sex-, strain-, and age-matched controls in WT (A, B) and Tg (C, D) mice. $n = 3$ mice/sex/age/treatment/strain. $P < 0.05$ (*-by treatment) and $*P < 0.01$ interaction (treatment*microRNA) was considered statistically significant. PND: postnatal day; Tg: 3xTgAD; indo: indomethacin; ctl: control; ns: not significant

exposures in Tg females, implicating this microRNA in the regulation of dysfunctional neuroimmune phenotypes observed in postnatal Pb-exposed Tg females with exacerbated AD pathologies^[4]. Likewise, the decreased expression of miR-34a in Tg females with indo+Pb exposure at PND 21 [Figure 4C] mimicked that of Tg males at PND 10 [Figure 3D], suggesting similar epigenetic remediation of neuronal injury-related events, but more delayed.

DISCUSSION

In the current study, we demonstrated a critical and previously unrecognized early alteration to epigenetic patterning related to the long-term regulation of neuroimmune phenotypes consequent to postnatal toxicant exposure in a GxE model of AD. Our results indicate that these exposure-related epigenetic changes parallel the established sexual dimorphism in microglial and neuronal postnatal development, thereby highlighting a critical period of neuroimmune vulnerability for sex bias not only in neurodevelopmental disorders but also neurodegenerative diseases like female-biased AD. Although previous studies have shown the critical role of miRNAs in sexually dimorphic brain development and AD^[25,32], this study is the first to our knowledge to directly address immediate changes to the epigenetic landscape during the postnatal environment for GxE interactions with implications for chronic aging-related neurodegenerative diseases. The data presented here support our global hypothesis that disruption of neuroimmune development initiates sex-biased susceptibility to later-life neurodegeneration through GxE interactions and epigenetic regulation for a susceptible phenotype.

This GxE interaction, with a double hit from environmental toxicant exposure and genetic proclivity to AD, exacerbated and prolonged maladaptive microRNA expression profiles in response to an atypical developing environment for each sex. Contrary to the observed upregulation of DAP12 expression over time in females regardless of strain [Figure 1], postnatal Pb exposure significantly increased DAP12 immediately following cessation of exposure at PND 10, that then declined over time in both females [Figure 2A and C] as well as Tg males [Figure 2D]. These results not only corroborate reports of sexually dimorphic postnatal microglial development^[36] using DAP12 as a marker for immature, phagocytic perinatal microglia^[20], but also the heightened vulnerability of immature female microglia during this temporally critical maturation. Also, these data further corroborate our model of GxE vulnerability in a DOAD model of AD, as Pb-exposed Tg males also exhibited increased DAP12 expression [Figure 2D] during a postnatal window in which expression does not temporally fluctuate in healthy male mice [Figure 1]. Interpretation of these results is limited due to a lack of quantification of microglial number and phagocytosis or neuronal apoptosis, and future studies are warranted to delineate early physiological alterations to microglial-directed synaptic pruning resulting from atypical DAP12 expression, as well as the ramifications for total microglia numbers in the adult brain. Likewise, without the addition of colocalized staining with microglia-specific markers, cell-type-specific inferences cannot justifiably be made regarding DAP12 expression. However, reports of its expression are limited to perinatal microglia and its critical role in developmental phagocytosis of apoptotic neurons by microglia^[19,49] lend credence to the interpretation that DAP12-related neuroimmune development was significantly altered in a sex-biased manner in this GxE model.

Importantly, this striking GxE effect on temporal DAP12 expression in Pb-exposed Tg females was accompanied by equally striking effects on epigenetic changes related to neuroimmune function. Namely, latent upregulation of miR-124 by Pb, indo, or both indo+Pb was observed in Tg females at PND 21 [Figure 4C]. The microRNA miR-124 critically regulates both microglial and neuronal development, maturation, and function^[27]. Upregulation of miR-124 has been shown to promote microglial quiescence and restrict proliferation through inhibition of CEBP α , the transcription factor PU.1, and its downstream target CSF1R^[29], while also promoting neuronal differentiation via repression of Sox9^[28]. The female-specific expansion of amoeboid microglia populations around PND 30, preceded by a highly proliferative period to populate various brain regions^[36], would suggest that a premature increase in miR-124, such as that observed in postnatally exposed Tg females at PND 21 [Figure 4C], may not only prematurely limit temporally-critical microglial maturation but also the proliferation and subsequent region-specific populations in the adult brain. While the additional investigation is needed to confirm any long-term changes to microglia and neuron population numbers, there is considerable evidence for a central role of miR-124 in our experimental DOAD model of AD. Long-term neuronal changes have been reported consequent to upregulation of miR-124, in which inhibition of *REST* by miR-124 delayed the maturation of NMDA receptors by retaining the prevalence of highly excitable GluN2B subunits, the ectopic form of which is associated with amyloid- β production^[50,51]. Also, defects in glutamatergic synaptic function along with reduced TrkB expression have been reported in DAP12-deficient mice^[21]. Given our previous research demonstrating a compensatory increase in hippocampal TrkB expression in Pb-exposed Tg female mice compared to WT at PND 120 that decreased with age and correlated with dysfunctional microglial phenotypes^[4], the decrease in hippocampal DAP12 expression [Figure 2] and upregulation of miR-124 [Figure 4] reported here substantiate the hypothesis that perturbation to neuroimmune development promotes epigenetic changes for maladaptive microglia-neuron interactions involved in early AD pathologies. Furthermore, increased miR-124 at PND 21 in Tg females regardless of exposure type [Figure 4C] would suggest that this postnatal window represents a previously unseen window of vulnerability for female microglia development to any type of immune-altering exogenous insult, be it proinflammatory (Pb) or anti-inflammatory (indo).

Notably, primary cultures of immature microglia from female, but not male, whole mouse brain at PND 3 have been shown to exhibit increased expression of proinflammatory cytokines, such as IL-6 and IL-1 β ^[52]. While indo was originally administered in the present study to inhibit the masculinizing effect of microglia-secreted PGE2 to elucidate its role in the sex-biased epigenetic modulation in developing males, the anti-inflammatory mechanism of action also inhibited pro-inflammatory cytokines like IL-1 β . Thus, indo-related suppression of these pro-inflammatory signals critical for postnatal female microglial development also underscored the role of not only PGE2, but pro-inflammatory cytokines as well, in the epigenetic regulation of microglia in males and females, respectively. While suppression of PGE2 via indo exposure did not detectably “feminize” the vulnerability of male microglia to heightened neuroimmune epigenetic modification, indo exposure in females did reveal how timely modulation of microglia phenotype during a critical window of postnatal development significantly altered certain epigenetic cascades in neuroimmune development. For example, the significant and persistent upregulation of miR-132 in indo-exposed Tg females at PND 10 [Figure 3C] and PND 21 [Figure 4C] implies the presence of heightened sensitivity to cytokine-mediated miR-132 epigenetic reprogramming associated with neurite sprouting, synaptogenesis, and neurotransmission. Although miR-132 is upregulated for the first 2-4 weeks of postnatal development during a period of NMDA-dependent synaptic pruning^[27], additional upregulation may disrupt the miR-132-promoted positive feedback loop in NMDA depolarization, while interfering in GABAergic interneuron maturation^[53]. These data further corroborate the heightened GxE-related vulnerability and female bias for the epigenetic reprogramming of microglia during this postnatal window.

Further, given reports that the majority of microRNAs only persist for about five days without external stimulus^[23], this upregulation of microRNA profiles persisting until or occurring at PND 21 would suggest the continuation of an atypical epigenetic stimulus despite the cessation of exposure. In further validation of the GxE female-specific vulnerability, postnatal exposures increased miR-124 at PND 10 in WT females [Figure 3A] but PND 21 was rectified via significant downregulation with Pb or indo exposure [Figure 4A]. This was in contrast to Tg females exposed to Pb, in which upregulation of miR-124 did not occur until PND 21 [Figure 4C], indicating that a rectifying epigenetic signal for Tg miR-124 was not present for a similarly-vulnerable population of immature female microglia as with WT females despite a parallel trend for significant reductions in hippocampal DAP12 expression [Figure 2]. The current study is limited by a hypothesis-driven bias in the neurimirs selected for analysis in our GxE model for AD, and future analyses might benefit from wider microarrays to further characterize this window of heightened sensitivity to epigenetic reprogramming for female neuroimmune development. While little is known regarding this subtle and life-long maladaptive neuroimmune phenotype, the data presented here demonstrate that changes to the epigenetic landscape are detectable early in postnatal development and are significantly biased by sexual dimorphism for neuroimmune development during this time.

As hypothesized, our study demonstrated a clear female bias in the exacerbating effect of GxE interactions in early epigenetic regulation promoting later life maladaptability and neuroimmune dysfunction, which we have previously correlated with heightened susceptibility to AD^[4,5]. We have also shown that, although the most detrimental phenotype occurred in Pb-exposed Tg females, there was evidence to suggest that this DOAD model may be useful in investigating toxicant- and timing-specific windows of vulnerabilities for other sex-biased adult diseases. Ultimately, investigation of these early perturbations to epigenetic regulation of cellular phenotype could reveal thresholds of adaptability consequent to atypical developmental conditions, and potential identification of biomarkers for susceptibility.

DECLARATIONS

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Authors' contributions

Made substantial contributions to the conception and design of the study: vonderEmbse AN, DeWitt JC

Assisted with technical expertise and dissections: vonderEmbse AN, Hu Q

Performed immunohistochemistry: vonderEmbse AN

Performed data analysis and interpretation: vonderEmbse AN

Provided administrative, technical, and material support: DeWitt JC

Involved in the writing and editing of the manuscript: vonderEmbse AN, DeWitt JC

Discussed the results and commented on the manuscript: vonderEmbse AN, Hu Q, DeWitt JC

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All handling and experimental manipulations were carried out in accordance with procedures approved by the East Carolina University Institutional Animal Care and Use Committee (IACUC), and were conducted in accordance with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of laboratory animals (NIH publication No. 8023, revised 1978).

Consent for publication

Not applicable.

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Author Instructions

Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
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The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

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Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

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Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. <i>BJOG</i> 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
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Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. <i>The genetic basis of human cancer</i> . New York: McGraw-Hill; 2002. pp. 93-113.
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Tables should be cited in numeric order and placed after the paragraph where it is first cited;

The table caption should be placed above the table and labeled sequentially (e.g., Table 1, Table 2);

Tables should be provided in editable form like DOC or DOCX format (picture is not allowed);

Abbreviations and symbols used in table should be explained in footnote;

Explanatory matter should also be placed in footnotes;

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2.4.7 Abbreviations

Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, *etc.*, can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

2.4.8 Italics

General italic words like *vs.*, *et al.*, *etc.*, *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

2.4.9 Units

SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

2.4.10 Numbers

Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

2.4.11 Equations

Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

2.5 Submission Link

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