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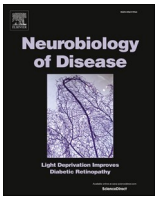

Systemic perturbations of the kynurenine pathway precede progression to dementia independently of amyloid-β

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ABSTRACT

Increasing evidence suggests that kynurenine pathway (KP) dyshomeostasis may promote disease progression in dementia. Studies in Alzheimer's disease (AD) patients confirm KP dyshomeostasis in plasma and cerebrospinal fluid (CSF) which correlates with amyloid-β and tau pathology. Herein, we performed the first comprehensive study assessing baseline levels of KP metabolites in participants enrolling in the Australian Imaging Biomarkers Flagship Study of Aging. Our purpose was to test the hypothesis that changes in KP metabolites may be biomarkers of dementia processes that are largely silent. We used a cross-sectional analytical approach to assess non-progressors ($N = 73$); cognitively normal (CN) or mild cognitive impairment (MCI) participants at baseline and throughout the study, and progressors ($N = 166$); CN or MCI at baseline but progressing to either MCI or AD during the study. Significant KP changes in progressors included increased 3-hydroxyanthranilic acid (3-HAA) and 3-hydroxyanthranilic acid/anthranilic acid (3-HAA/AA) ratio, the latter having the largest effect on the odds of an individual being a progressor (OR 35.3; 95% CI between 14 and 104). 3-HAA levels were hence surprisingly bi-phasic, high in progressors but low in non-progressors or participants who had already transitioned to MCI or dementia. This is a new, unexpected and interesting result, as most studies of the KP in neurodegenerative disease show reduced 3-HAA/AA ratio after diagnosis. The neuroprotective metabolite picolinic acid was also significantly decreased while the neurotoxic metabolite 3-hydroxykynurenine increased in progressors. These results were significant even after adjustment for confounders. Considering the magnitude of the OR to predict change in cognition, it is important that these findings are replicated in other populations. Independent validation of our findings may confirm the utility of 3-HAA/AA ratio to predict change in cognition leading to dementia in clinical settings.

Abbreviations: AA, anthranilic acid; Aβ, amyloid beta; Aβ42, 42-residue form of amyloid beta; AIBL, Australian Imaging, Biomarkers and Lifestyle flagship study of aging; 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; KMO, kynurenine 3-monooxygenase; KP, kynurenine pathway; KYN, kynurenine; KYNA, kynurenic acid; NAD^+, nicotinamide adenine dinucleotide; NMDA, *N*-methyl D-aspartate; PARP, poly ADP-ribose polymerase; p-tau, hyperphosphorylated tau; QUIN, quinolinic acid; TRP, tryptophan; t-tau, total tau; XA, xanthurenic acid.

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1. Introduction

Chronic neuroinflammation plays a known role in the progression of dementia and Alzheimer's disease (AD) by increasing amyloid (Minter et al., 2016) and tau (Didonna, 2020) toxicity. The kynurenine pathway (KP, Fig. 1) of tryptophan (TRP) metabolism is also dysregulated by chronic inflammation, which in-turn, may feedback to enhance dementia-related neuroinflammatory processes. Mechanistically, neuroinflammation-driven changes in the KP lead to excessive production of the KP metabolite quinolinic acid (QUIN) which causes excitotoxic overstimulation of the *N*-methyl D-aspartate (NMDA) receptor, inducing a cascade of molecular events that lead to neuronal death (Jacobs et al., 2017).

In addition to NMDA receptor-mediated excitotoxicity, QUIN mediates neurotoxicity by multiple other mechanisms, including the formation of redox-active metal complexes, poly ADP-ribose polymerase (PARP) activation and nicotinamide adenine dinucleotide (NAD+) depletion (Braidy et al., 2009). Importantly, in both neurons and astrocytes, QUIN at very low concentrations (<50 nM), leads to the cellular energy factor nicotinamide adenine dinucleotide (NAD+) which is essential for maintaining brain energy homeostasis and healthy brain aging. However, QUIN is cytotoxic at sub-physiological concentrations (>150 nM) in both the cell types (Braidy et al., 2009). The KP metabolite kynurenic acid (KYNA) produced by astrocytes acts as an endogenously produced neuroprotective molecule able to antagonize QUIN excitotoxity at the NDMA receptor (Jacobs et al., 2017). Also implicated in neurotoxicity is the KP metabolite 3-hydroxykynurenine (3−HK) which was recently shown to induce mitochondrial membrane destabilization and disrupt the oxygen respiratory chain (Castellano-Gonzalez et al., 2019). Other KP metabolites coordinate aspects of the immune response. Levels of 3-HK and 3-hydroxyanthranilic acid (3-HAA) modulate T-cell activity in the immune system, with higher 3-HAA concentrations reducing cytokine release from T-helper (Th) cells (Hayashi et al., 2007).

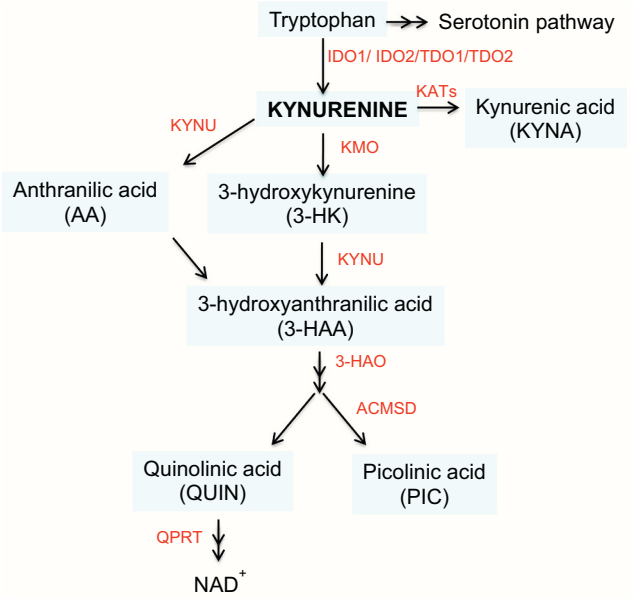

Fig. 1. Simplified schematic of the kynurenine pathway (KP). Key aspects of the KP include: Induction of the KP enzymes indolamine 2,3 deoxygenase (IDO-1 and IDO-2) or tryptophan 2,3 deogygenase (TDO-1 and TDO-2) results in the conversion of tryptophan (TRP) to kynurenine (KYN). The enzyme kynurenine 3-monooxygenase (KMO) is located at a key branch point of the KP. The activity of KMO determines if KYN is metabolised to the redox active and immunomodulatory metabolites 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HAA) which in turn leads to production of the neurotoxic metabolite quinolinic acid (QUIN) or by kynurenine aminotransferase (KAT) to the neuroprotective metabolite kynurenic acid (KYNA). Activity of the enzyme quinolinate phosphoribosyl transferase (QPRT) ultimately leads to the essential cellular energy factor nicotinamide adenine dinucleotide (NAD+). Highlighted in blue are KP metabolites measured within this study, while red text indicates a KP enzyme.

How KP dysregulation meshes with the overall clinical picture of dementia, including AD, is emerging. Activated microglia and reactive astrocytes are known to surround amyloid plaques and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau (p-tau) (Osborn et al., 2016; Masters et al., 2015). At least initially, these glial cells exert a repair by clearance function, but chronic induction of activated microglia produces pro-inflammatory cytokines linked to neuroinflammation which is then linked to the progression of dementia at both pathological and clinical levels (Masters et al., 2015; Heneka et al., 2015). Simultaneously, chronically activated microglia produce excess QUIN (Guillemin, 2012). These overlapping events may account for the clinical observations in AD patient post-mortem hippocampal tissue that show correlations between QUIN concentrations and the presence of amyloid (Guillemin et al., 2005) and tau (Wu et al., 2013), particularly when it is further appreciated that in vitro studies show that QUIN promotes hyper-phosphorylation of tau protein (Rahman et al., 2009).

Clinical evidence further confirms significant changes in KP metabolite concentrations and KP enzyme activity in the sera/plasma/CSF of AD patients compared to normal controls (Gulaj et al., 2010; Schwarz et al., 2013; Giil et al., 2017; Jacobs et al., 2019). In the Gulaj study, serum QUIN concentrations were 1.7-fold higher in AD patients compared to controls while the QUIN/3-HK ratio, increased 2-fold. In histopathologically confirmed AD patients, plasma TRP, 3-HAA, xanthurenic acid (XA) and QUIN were significantly decreased compared to cognitively normal controls (Odds ratios; 0.24–0.47; *p*-values <0.001–0.01) (Giil et al., 2017). As samples from histopathologically confirmed patients represent very late-stage disease, this observation of decreased QUIN agrees with findings of other studies showing that QUIN concentrations peak at mid-stages of AD before decreasing in late-stage disease (Guillemin et al., 2005). Interestingly, these authors also reported that increased QUIN correlated with reduced cognitive performance in elderly AD patients (Giil et al., 2017), where patients are less advanced and QUIN levels are higher. Significant positive associations between plasma Aβ42 and the KP metabolites, kynurenine (KYN; $r = 0.467$, $p = 0.007$), KYNA ($r = 0.214$, $p = 0.035$), anthranilic acid (AA; $r = 0.576$, $p = 0.0006$), QUIN ($r = 0.539$, $p = 0.001$) and the kynurenine to tryptophan (K/T) ratio ($r = 0.494$, $p = 0.004$) were also reported in individuals with high neocortical Aβ load, whereas these associations were absent in participants with low Aβ load (Chatterjee et al., 2019).

More recently, we demonstrated that in AD patients, plasma KYN and picolinic acid (PIC) were inversely correlated with CSF p-tau and total tau (t-tau), respectively. Increased 3-HK/KYN ratio also correlated with increasing t-tau and p-tau (Jacobs et al., 2019). Another study also reported KP correlations with increased KYNA and QUIN in the CSF of AD patients correlating with amyloid β1–42, tau and/or phosphorylated tau-181 (van der Velpen et al., 2019). Although clinical studies of the KP in neurodegenerative disease differ in design and often yield different results, there is general consensus that KP activation occurs in AD patients (as reflected by increased K/T ratio) with most studies also showing increased concentrations of the highly neurotoxic metabolites QUIN and 3-HK.

Considering the spectrum of clinical evidence, therapeutic modulation of the KP, aimed at rebalancing the ratio of neuroprotective/neurotoxic KP metabolites by inhibiting KP enzymes, has attracted significant research effort. Most focus has centered on the KP enzyme kynurenine 3-monooxygenase (KMO) following the demonstration that KMO inhibition improved various indicators of disease progression in murine models of AD and Huntington's disease (Zwilling et al., 2011).

Despite considerable interest in the KP in AD and neurodegenerative disease, most reports are single-point studies assessing KP metabolites after disease diagnosis. No studies have yet assessed KP metabolites in

otherwise healthy participants and followed clinical course. Hence, our purpose was to address this gap, by conducting a study using samples from the Australian Imaging, Biomarkers and Lifestyle (AIBL) flagship study of aging. Initially, we used a cross-sectional analytical design to test the hypothesis that baseline levels of metabolites in the KP would be associated with clinical disease progression, defined as a change in classification from cognitively normal (CN) to mild cognitive impairment (MCI) or from MCI to AD, henceforth denoted as progressors. The design also assessed longitudinal data in terms of how KP modulations in progressors may be related to changes in clinical classification.

2. Methodology

2.1. Participants

Initiated in 2006, AIBL is Australia's largest study of aging, aimed at understanding which blood, imaging, genetic, demographic, modifiable risk factors, and/or cognitive markers influence the development of AD. Participants were followed-up at approximately 18-month intervals, for up to 12 years (144 months). A subset of 286 AIBL participants (152 CN, 87 MCI and 47 AD at baseline) was selected at random for the conduct of a pilot study investigating baseline plasma levels of metabolites in the KP across the pre- and post- dementia diagnosis spectrum. Demographic and clinical information including age, sex, apolipoprotein E (*APOE*) ε4 allele status (major genetic risk factor for AD), the Mini-Mental State Exam (MMSE) score, Clinical Dementia Rating (CDR) global score and the AIBL preclinical Alzheimer's cognitive composite score (AIBL PACC) derived by (Donohue et al., 2014) were used to investigate the relationship of KP metabolites to cognition (Table 1). Further details of the AIBL study protocol and methods can be found elsewhere (Fowler et al., 2021).

2.2. Sample selection for progressor and non-progressor groups

Participant selection from the overall AIBL population through to those individuals included in the current analyses is shown in Fig. 2. From the subset of 286 randomly selected AIBL participants, 166 progressed from either CN at baseline to MCI ($N = 101$) or from MCI at baseline to AD ($N = 65$), denoted as progressors; while only 73 participants (51 CN and 22 MCI), denoted as non-progressors, remained at their baseline clinical state at all subsequent follow-ups. As the focus of this work is the comparison of metabolites in the KP between progressors and non-progressors, only general summaries of the 47 individuals who commenced the study with AD are provided. Plasma KP data obtained from baseline samples were analyzed with respect to change in clinical classification between 2008 and 2020. The results presented here in the Tables and Figures denote non-progressors and progressors by clinical classification at baseline as either CN or MCI, prior to any cognitive change.

2.3. Kynurenine metabolite analysis by UHPLC, HPLC and GC/MS

2.3.1. Plasma preparation

Briefly, all blinded plasma samples were deproteinized with equal volumes of 10% *w/v* trichloroacetic acid (TCA) followed by centrifugation (3600 *g* at 4 °C for 15 min). Supernatants were harvested, filtered through 0.45 μm polytetrafluoroethylene syringe filters (Merck-Millipore, CA, USA) and stored at −80 °C prior to analysis.

2.3.2. Quantification of KP metabolites

TRP, KYN, 3-HK, 3-HAA and AA were quantified by ultra-high-performance liquid chromatography (UHPLC) in accordance with published methods (Guillemin et al., 2007). KYNA analysis was performed by high performance liquid chromatography (HPLC) using an Agilent 1260 system with fluorescence detection (ex: 344 nm and em: 388 nm) in accordance with published methods (Lim et al., 2017). PIC and QUIN metabolites were quantified using gas chromatography (GC; Agilent 5975 system) coupled with mass spectrometry (MS; Agilent 7890 system) as adapted from published methods (Lim et al., 2017).

2.4. Positron emission tomography (PET) imaging

PET Aβ neuroimaging was conducted using either 11C-Pittsburgh compound-B (PiB), 18F-Florbetapir (FBP), or 18F-Flutemetamol (FLUTE) tracers with brain Aβ represented as centiloid (CL) values. Detailed information regarding the PET procedures, including derivation of PET Aβ status has been reported previously (Bourgeat et al., 2018; Rowe et al., 2010). Of the total 286 participants, only 113 had PET imaging data available (Aβ-: $N = 51$, Aβ+: $N = 61$). Complete sample size breakdown between clinical classification, progressor groups and Aβ groups is shown in Supplementary Table 1.

Table 1
Cohort demographics.

	Non-progressor (NP)				AD at baseline	Progressor (P)				NP vs P CN p-value	NP vs P MCI p-value
	All	CN	MCI	CN vs MCI p-value		All	CN	MCI	CN vs MCI p-value		
N (%)	73	51 (70)	22 (30)		47 (39)	166	101 (61)	65 (39)			
Gender Male, N (%)	35 (48)	23 (45)	12 (55)	0.6269	21 (45)	82 (49)	51 (50)	31 (48)	0.8466	0.6479	0.7573
Mean Age, years (SD)	75.7 (5.8)	75.5 (5.3)	76.2 (7)	0.6795	77.7 (7.1)	74.9 (7.2)	73.7 (7)	76.7 (7.2)	0.0084	0.0701	0.7778
APOE ε4 Carrier, N (%)	24 (33)	11 (22)	13 (59)	0.0042	33 (70)	88 (53)	45 (45)	43 (66)	0.0104	0.0094	0.7336
Mean Centiloid (SD)*	28.7 (43.2)	1.3 (4.3)	69 (42.9)	<0.0001	112.8 (35.1)	59.4 (46.9)	40.6 (42.6)	80.5 (42.8)	0.0002	<0.0001	0.3761
PET-Aβ + ve, N (%)*	13 (25)	9 (21)	4 (57)	0.0605	6 (100)	42 (75)	24 (63)	18 (100)	0.0022	0.0002	0.023
Mean MMSE (SD)	28.3 (1.6)	28.9 (1.1)	27 (1.9)	0.0001	14.6 (7.8)	27.4 (2.3)	28.4 (1.3)	25.7 (2.6)	<0.0001	0.0136	0.0171
Mean CDR global (SD)	0.2 (0.2)	0 (0.1)	0.5 (0.1)	<0.0001	1.8 (0.8)	0.2 (0.3)	0.1 (0.2)	0.5 (0)	<0.0001	0.0603	0.1621
Mean AIBL PACC (SD)	0.4 (1)	0.1 (0.5)	−1.6 (1)	<0.0001	−3.9 (1)	−1.3 (1.1)	−0.6 (0.6)	−2.3 (0.7)	<0.0001	<0.0001	0.0078
Mean months follow-up (SD)						70.5 (25)	73.4 (26.2)	65.9 (22.6)	0.0511		

Baseline demographic summaries for clinical diagnosis categories for non-progressors and progressor participants. N denotes the number of participants, with percentage (%) or standard deviation (SD) in parenthesis. Cognitively normal (CN); mild cognitive impaired (MCI); Alzheimer's disease (AD); Apolipoprotein E ε4 allele carriers (*APOE* ε4); Positron Emission Topography (PET) amyloid burden was measured by the centiloid value; Mini-Mental State Exam (MMSE); Clinical Dementia Rating (CDR); Australian Imaging, Biomarkers and Lifestyle Preclinical Alzheimer's Cognitive Composite (AIBL PACC). * denotes a reduced sample size of $N = 112$. *P*-value represents the comparison between non-progressor and progressor (All) groups. Comparisons between CN non-progressors and CN progressors MCI non-progressors and MCI progressors are not shown Comparison between CN and MCI progressor mean months follow-up is 0.0511.

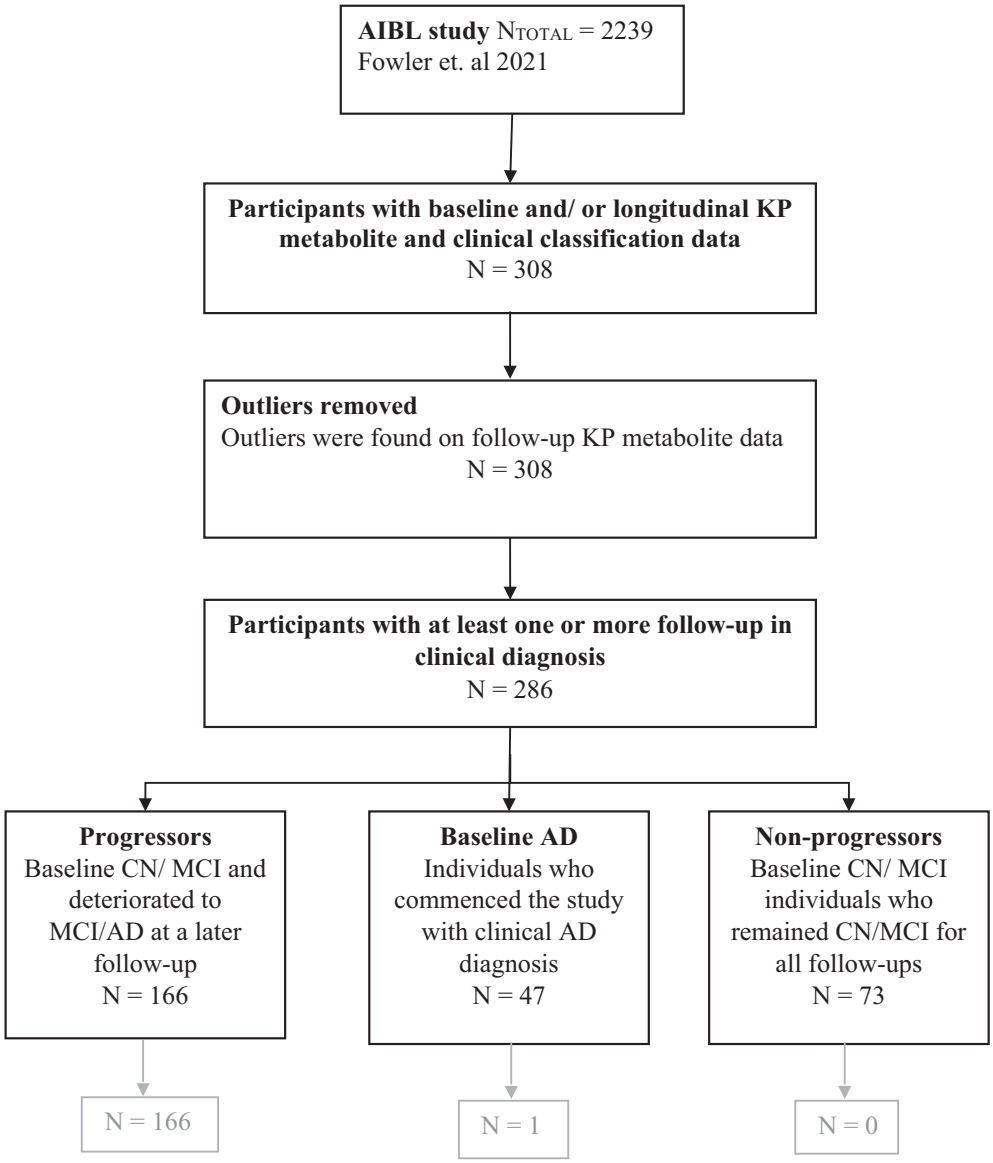

Fig. 2. Derivation of Australian Imaging, Biomarkers and Lifestyle (AIBL) participant sample size used in the current study. N_{TOTAL} denotes the total AIBL study sample size, N denotes the number of participants at each step. This study focuses on the analyses of longitudinal clinical classification (defined as progressors and non-progressors) with respect to participants baseline Kynurenine Pathway (KP) metabolite information. CN: cognitively normal, MCI: mild cognitive impairment and AD: Alzheimer's disease.

2.5. Statistical analysis

Demographic characteristics for baseline clinical classifications were compared within each progressor/non-progressor group using Chi-squared (gender) and Fisher (APOE ε4 allele status) tests. Investigation of metabolite levels between both progressor/non-progressor groups, and CN/MCI across progressor/non-progressor groups was performed using standard comparison of marginal means. Adjustment for confounders was performed using Generalised Linear Modelling, with conversion of beta coefficients to odds ratios to assess risk levels. Further details on data handling and statistical analyses can be found in the Supplementary materials section.

3. Results

3.1. Sample demographics

Comparison of demographic and clinical characteristics within both progressor and non-progressor groups showed no difference in the frequencies of males to females ($p > 0.05$), whilst participants with MCI were significantly older than CN participants in the progressor group only (Table 1). Remaining characteristics such as *APOE* ε4 carrier frequency, mean CL, MMSE, CDR global and AIBL PACC score were all significantly different between clinical classifications within both progressor and non-progressor groups ($p < 0.05$) as shown in Table 1.

Participants who were CN at baseline and did not progress over the length of the study were less likely to have an *APOE* ε4 allele as compared to those CN participants who did progress (CN non-progressor 22% vs CN progressor 45%, $p = 0.0094$). For participants classified as MCI at baseline, *APOE* ε4 allele status was not significantly different between progressor and non-progressor groups (MCI non-progressor 59% vs MCI progressor 66%, $p = 0.73$). Baseline age was not significantly different between participants who were either CN or MCI in the non-progressor group ($p = 0.6795$), however MCI participants in the progressor group were significantly older than their CN counterparts ($p = 0.0084$) as show in Table 1.

Of the total sample size ($N = 286$) 65 participants who were MCI at baseline and progressed to AD during the study were no more likely to be Aβ + as compared to the 22 participants who were MCI and did not progress (p = 0.73); however, this result should be interpreted with caution due to the small sample size meeting these clinical classification criteria coupled with available PET data.

Within the progressor group, the mean number of months of cognitive follow-up time was slightly less for participants who were MCI at baseline (65.9 months) as compared with those participants who were CN at baseline (73.4 months), even though this difference did not quite reach statistical significance ($p = 0.0511$). Correlation plots of biomarker trajectories with age, for all available samples are shown in Supplementary Figs. 1 and 2.

3.2. Metabolite biomarker mean comparisons across progressor groups

Four- and three-fold higher unadjusted baseline means for 3-HAA and 3-HAA/AA ratio respectively, were observed for progressors compared to non-progressors (p-values <0.0001 across all baseline groups), as shown in Table 2 and Fig. 3. To a lesser extent, 3-HK and the 3-HK/KYN ratio values were also higher in progressor participants (3-HK/KYN ratio: 54.71 compared to 48.25; p-value 0.0001). Compared with non-progressors, baseline mean PIC and QUIN/KYNA ratio levels were 27.4% and 26.9% lower, respectively, for progressor participants (p-values <0.0064 for both metabolites). Cognitively normal non-progressors showed lower baseline concentrations of TRP, 3-HK and KYNA metabolites compared to progressor groups (all p-values <0.014) but showed higher QUIN, K/T and PIC/QUIN ratios than progressor groups (p-values <0.0351). Refer to Table 2 and Supplementary Fig. 3 for all numeric and visual comparisons of baseline mean KP metabolites.

3.3. Metabolite biomarker mean comparisons across PET Aβ groups

Assessing differences in KP metabolites between PET Aβ groups, there were nominally significant changes in TRP, AA, KYN, QUIN and 3HK metabolites (Supplementary Table 2), although these did not meet the adjusted significance level. Both 3-HAA and its ratio with AA were significantly higher in progressors as compared with non-progressors in both Aβ- and Aβ + groups ($P < 0.0001$). Changes in all other metabolites (in either or both Aβ−/Aβ + groups), excluding AA, were seen between non-progressor and progressor groups, however only at the nominal significance level ($p < 0.05$, Supplementary Table 2, Supplementary Figs. 4 & 5).

3.4. Metabolite biomarker effect size on the odds of a progressor individual

The effect of a metabolite biomarker on the odds of a participant being a progressor compared to a non-progressor is shown in Table 3, after adjusting for confounders (age, gender and *APOE* ε4 allele status). The 3-HAA/AA ratio was found to have the largest effect on the odds of an individual being a progressor (OR 35.3; 95% CI between 14 and 104). This OR suggests that a one unit increase in 3-HAA/AA for a CN/MCI participant (for example, moving from 0.33 to 1.33) equates to a participant being approximately 35 times more likely to progress to either MCI or AD. Similarly, but to a lesser extent, other metabolites in the KP which were found to significantly increase the odds of an individual being a progressor were 3-HAA (OR 1.19; 95% CI between 1.01 and 1.25) and 3-HK/ KYN ratio (OR 1.04; 95% CI between 1.02 and 1.07). In the opposite direction, where a lower metabolite biomarker level equates to lower odds of an individual being a progressor, are QUIN/KYNA, PIC and PIC/QUIN ratios, with OR of magnitudes 0.99, 0.981 and 0.0013 respectively (all with p-values <0.01).

3.5. Changes in metabolite levels with age

Combining all participant data, we assessed metabolite biomarkers with respect to age, irrespective of clinical classification, to ensure either an increase or decrease with age occurred as expected. No interactions were found between metabolite biomarkers and clinical classification with age (data not shown) given that both CN and MCI groups contain both progressors and non-progressors. There was a large separation in

Table 2
Metabolite mean comparisons.

Metabolite	Non-progressor			AD at baseline (N = 47)	Progressor			Unadjusted p-values		
	Non-progressor (N = 73)	CN (N = 51)	MCI (N = 22)		Progressor (N = 166)	CN (N = 101)	MCI (N = 65)	All	CN	MCI
TRP	42.78 (7.61)	42.2 (6.71)	44.14 (9.42)	38.4 (6.94)	44.81 (6.89)	45.28 (6.83)	44.08 (6.96)	0.0533	0.0092	0.9813
KYN	2.21 (0.46)	2.21 (0.44)	2.19 (0.5)	2.15 (0.64)	2.14 (0.43)	2.1 (0.4)	2.21 (0.47)	0.3244	0.1233	0.8387
3-HK	104.93 (19.62)	104.66 (16.57)	105.56 (25.79)	101.92 (19.48)	115.17 (29.44)	114.91 (29.5)	115.58 (29.58)	0.0018	0.0069	0.1371
3-HAA	6.48 (7.81)	5.26 (4.75)	9.33 (11.97)	9.4 (8)	27.49 (14.57)	26.77 (14.91)	28.6 (14.07)	<0.0001	<0.0001	<0.0001
AA	24.3 (8.5)	23.99 (8.43)	25.02 (8.8)	27.71 (11.73)	26.8 (10.82)	25.89 (11.31)	28.2 (9.95)	0.0573	0.2493	0.1653
PIC	306.21 (93.36)	296.14 (84.41)	329.55 (110.0)	284.11 (113.2)	222.31 (88.18)	226.89 (97.51)	215.19 (71.46)	<0.0001	<0.0001	0.0001
QUIN	1475.7 (425.9)	1452.4 (425.8)	1529.6 (431.3)	1429.5 (565.2)	1303.6 (480.1)	1274.6 (468.4)	1348.8 (498.1)	0.0064	0.0205	0.1103
KYNA	20.24 (7.36)	20.13 (6.94)	20.51 (8.41)	17.85 (7.6)	22.83 (7.98)	23.36 (8.61)	22 (6.85)	0.0159	0.014	0.4571
K/ T	52.22 (13.16)	53.59 (13)	49.04 (13.29)	54.35 (13.2)	48.95 (12.01)	47.17 (10.19)	51.72 (14.03)	0.0718	0.0028	0.4254
3-HK/ KYN	48.25 (9.98)	47.73 (9.43)	49.45 (11.28)	49.66 (13.61)	54.71 (14.84)	55.33 (14.65)	53.74 (15.19)	0.00011	0.00016	0.167
3-HAA/ AA	0.3 (0.37)	0.25 (0.28)	0.41 (0.52)	0.38 (0.37)	1.14 (0.7)	1.15 (0.7)	1.13 (0.69)	<0.0001	<0.0001	<0.0001
PIC/ QUIN	0.22 (0.09)	0.22 (0.08)	0.22 (0.09)	0.21 (0.09)	0.18 (0.08)	0.19 (0.08)	0.17 (0.07)	0.0018	0.0351	0.0313
QUIN/ KYNA	85.59 (37.12)	83.76 (36.61)	89.81 (38.81)	81.24 (32.19)	62.58 (30.64)	62.1 (31.76)	63.34 (29.02)	<0.0001	0.0005	0.0064

Baseline mean metabolite comparisons for non-progressor and progressor participants across clinical classification groups; cognitively normal (CN), mild cognitive impaired (MCI) and Alzheimer's disease (AD) participants. Mean metabolite values and standard deviations in parenthesis. TRP (Tryptophan), KYN (kynurenine), 3-HK (3-hydroxykynurenine), 3-HAA (3-hydroxyanthranilic acid), AA (anthranilic acid), PIC (picolinic acid), QUIN (quinolinic acid), KYNA (kynurenic acid), and K/T (kynurenine/ tryptophan ratio). Pairwise comparisons were assessed across all clinical classification groups within non-progressor and progressor participants; refer to Fig. 2 for significant comparisons. P-value All refers to the unadjusted comparison between non-progressor (N = 73) and progressor (N = 166) groups, P-value CN refers to the unadjusted comparison between non-progressor CN (N = 51) and progressor CN (N = 101) groups, P-value MCI refers to the unadjusted comparison between non-progressor MCI (N = 22) and progressor MCI (N = 65) groups.

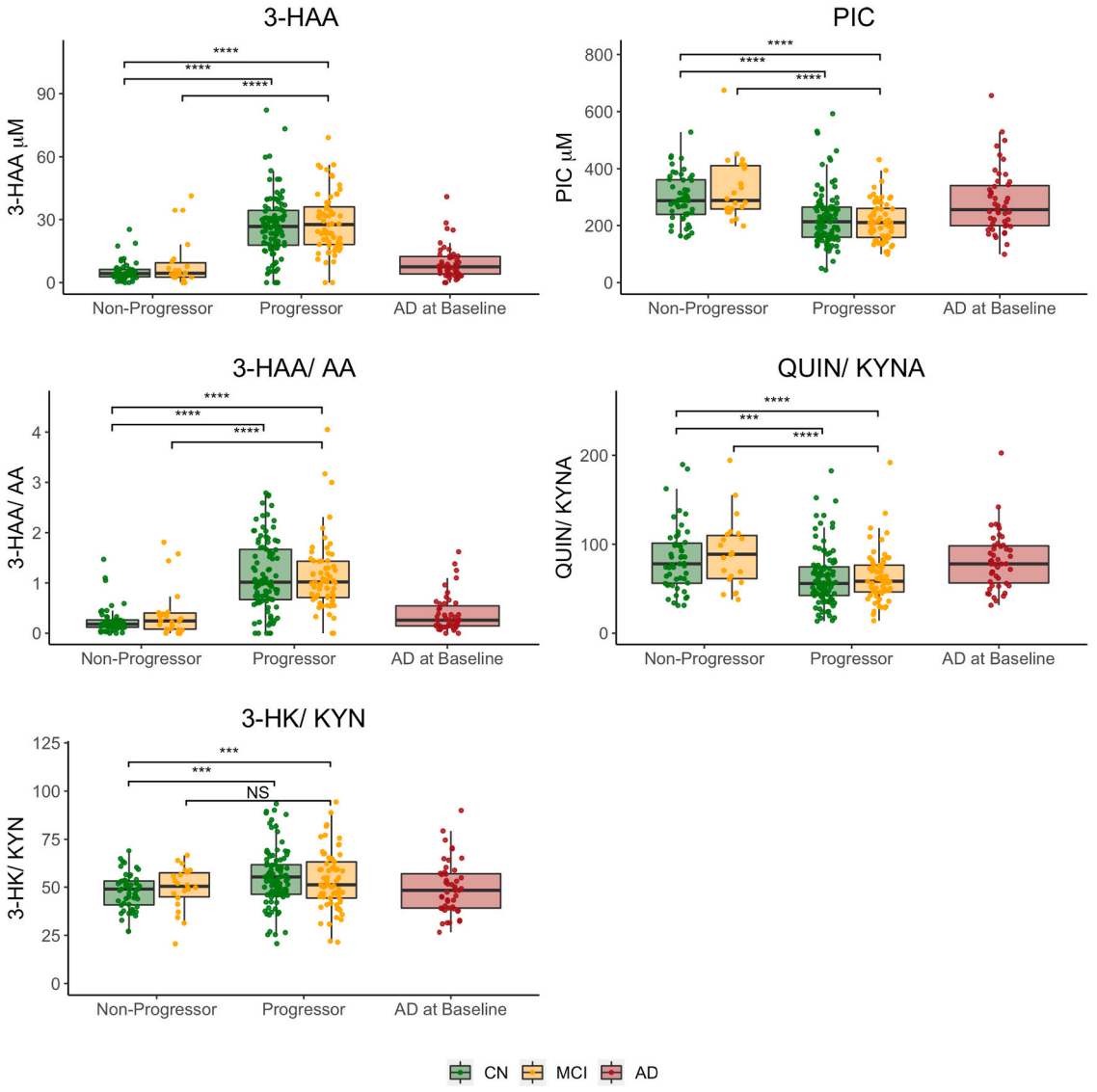

Fig. 3. Baseline metabolite value comparisons across Progressor and clinical classification groups.
Baseline mean metabolite values for progressors, non-progressors, and Alzheimer's disease (AD) at baseline. 3-HAA (3-hydroxyanthranilic acid), PIC (picolinic acid), 3-HAA/ AA (anthranilic acid) ratio, QUIN/ KYNA (quinolinic acid/ kynurenic acid) ratio, and 3-HK/ KYN (3-hydroxykynurenine/ kynurenine) ratio. Colour coded into clinical classification; cognitively normal (CN, green), mild cognitive impaired (MCI, yellow) and Alzheimer's disease (AD, red). Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

values between progressors and non-progressors for 3-HAA and the 3-HAA/AA ratio, however, slopes were similar amongst all groups (Supplementary Figs. 1 and 2). Spearman's correlation between each metabolite and age showed moderate but significant associations, with KYN, PIC, AA, QUIN the K/T ratio and the QUIN/KYNA ratio increasing with age, and TRP, 3-HK/KYN and PIC/QUIN decreasing with age.

3.6. Changes in metabolite levels by gender

Given that the metabolome has been shown to vary by gender in MCI/AD, we also assessed the influence of gender on KP metabolites in the AIBL cohort. This analysis showed that there are no significant differences by gender in all the KP metabolites (Supplementary Figs. 6 and 7). While there are some gender differences in 3HAA and 3HAA/AA ratio, these are only apparent in participants >87 yrs. of age and are likely skewed due to the small numbers of participants aged >87 yrs. (male $n = 6$; female $n = 13$).

4. Discussion

This is the first study that investigated whether baseline levels of KP metabolites were different in participants who underwent a subsequent change in their clinical disease stage during their time in the AIBL study compared to matched individuals whose disease stage remained stable. While large changes in 3-HAA occurred in progressors, this was not specific to the presence of significant amyloid pathology, as similar changes were seen in progressors whose amyloid levels remained within normal limits. It had been previously reported that higher levels of the KP metabolites KYNA, QUIN and PIC and a higher K/T ratio after AD diagnosis were associated with increased cognitive decline in patients without the *APOE* ε4 allele but not in those with *APOE* ε4 (Ervik et al., 2019). Another earlier single-point post-diagnosis study showed decreased KYNA, but stable KYN levels, in plasma and red blood cells in both *APOE* ε4 and non-*APOE* ε4 AD patients, which is in agreement with the current study (Hartai et al., 2007).

The lack of direct correlation of plasma KP parameters to Aβ load

Table 3
Logistic regression odds-ratio model results.

Metabolite	Non-progressor ($N = 73$) / Progressor ($N = 166$)	CN ($N = 51$/ 101)	MCI (N = 22/ 65)
TRP	1.05 (1, 1.09)*	1.07 (1.01, 1.13)*	1.01 (0.943, 1.08)
KYN	0.792 (0.409, 1.54)	0.5 (0.211, 1.16)	1.32 (0.407, 4.58)
3-HK	1.02 (1.01, 1.03)**	1.02 (1, 1.04)*	1.02 (0.998, 1.04)
3-HAA	1.19 (1.14, 1.25)****	1.25 (1.17, 1.36)****	1.15 (1.09, 1.24) ****
AA	1.03 (0.998, 1.06)	1.02 (0.979, 1.06)	1.05 (0.99, 1.12)
PIC	0.99 (0.986, 0.993)****	0.992 (0.988, 0.996)***	0.982 (0.972, 0.99)****
QUIN	0.999 (0.999, 1)*	0.999 (0.998, 1)	0.999 (0.998, 1)
KYNA	1.05 (1.01, 1.09)*	1.05 (1, 1.1)	1.04 (0.965, 1.12)
K/ T	0.982 (0.96, 1.01)	0.951 (0.916, 0.983)**	1.02 (0.98, 1.06)
3-HK/ KYN	1.04 (1.02, 1.07)**	1.05 (1.02, 1.09)**	1.02 (0.988, 1.07)
3-HAA/ AA	35.3 (14, 104)****	58.8 (16.1, 289) ****	18.7 (5.01, 96.9) ****
PIC/ QUIN	0.0013 (2.92e-05, 0.0488) ***	0.00471 (4.87e-05, 0.367)*	0.000161 (1.06e-07, 0.128)*
QUIN/ KYNA	0.981 (0.972, 0.99)****	0.984 (0.973, 0.995)**	0.974 (0.957, 0.99)**

Odds ratio (95% confidence interval) results on the ability of kynurenine metabolites to distinguish the difference between progressor and non-progressor participants. These models take into account the effects of baseline age, sex and apolipoprotein E (*APOE*) ε4 allele carrier/ non-carrier status. Logistic regression models on each metabolite were applied independently to three baseline groups; Progressors and non-progressors, cognitively normal (CN) and mild cognitive impaired (MCI). Top row shows the number of non-progressor and progressor individuals in parenthesis for each group, separated by a forward slash. TRP (Tryptophan), KYN (kynurenine), 3-HK (3-hydroxykynurenine), 3-HAA (3-hydroxyanthranilic acid), AA (anthranilic acid), PIC (picolinic acid), QUIN (quinolinic acid), KYNA (kynurenic acid), and K/T (kynurenine/ tryptophan ratio). Metabolite significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

may reflect the fact that KP concentrations may not be the same at the site of active disease. Indeed, we previously reported that only plasma concentrations of KYN, 3-HK, AA and PIC correlated to CSF levels (Jacobs et al., 2019). Alternatively, it may be that KP metabolites only have a weak secondary link to amyloid phenomena (as shown in Supplementary Table 3) with associations being stronger for neuroinflammation and/or tau pathology. This could also explain, in part, why Ervik found that KP metabolites only changed in non-*APOE* ε4 AD (Ervik et al., 2019), whereas in *APOE* ε4 AD, amyloid aggregation is the dominant pathological mechanism and may be largely independent of the KP.

As noted above, the most striking finding in this study is that increased plasma 3-HAA/AA ratio at baseline strongly predicted the risk that CN or MCI participants will progress to either MCI or AD. This is a novel finding and may represent a new prognostic tool to monitor risk of cognitive decline. Previous single-point studies of the KP, conducted after disease diagnosis, show that plasma concentrations of 3-HAA are typically decreased, while AA is increased, leading to lower 3-HAA/AA ratios. For example, significant decreases in plasma 3-HAA have been reported in late-stage Huntington's disease (Stoy et al., 2005) and others found a significant decrease (30%) in 3-HAA in the plasma of AD patients (Giil et al., 2017). We also previously reported a 30% decrease in plasma 3-HAA in symptomatic AD patients but found that 3-HAA was significantly increased in CSF (Jacobs et al., 2019). These findings, amongst others, prompted the suggestion that low 3HAA/AA ratios represent a marker for inflammation and its progression (Darlington et al., 2010) which may constitute a 'cleaning-up' mechanism. Hence, it is interesting to note that the current study also found that 3-HAA was decreased in symptomatic AD study participants relative to progressors. Further evidence of the interplay between 3-HAA levels, inflammation and cognition recently emerged in a study of participants with pre- and type 2 diabetes (Bakker et al., 2021). 3-HAA was associated with lower odds of cognitive decline in both groups (OR per SD 0.67 [96% CI 0.47, 0.96] in prediabetes and 0.73 [0.60, 0.87] in type 2 diabetes). It is important to note that decreased 3-HAA, rather than decreased AA, accounts for the increased plasma 3-HAA/AA ratio in progressors.

Why the plasma 3-HAA/AA ratio reverses and increases prior to progression to MCI or AD, before returning to the same level as CN non-progressors or symptomatic AD participants, is an interesting question. One possibility is that AD medications might interact directly or indirectly with the KP. However, no direct effects of galantamine, donepezil or other acetylcholinesterase (AChE) inhibitors on 3-HAA or AA have been reported to date, although one study showed approximately 20% reductions in KYNA and PIC in a limited number of patients (Koola et al., 2018). Also arguing against a potential drug effect is that AD medications tend to be taken for a relatively short period as effectiveness is limited and decreases over time. A variety of dietary supplements are also commonly used in dementia with no studies assessing the effects of *ginkgo biloba*, coconut oil or vitamin D supplementation on the KP. There is some evidence that a ginseng extract (Kang et al., 2017), resveratrol (Wirleitner et al., 2005) or curcumin (Zhang et al., 2019) can favorably remodulate the KP, although further studies are needed to confirm effects on the KP. Hence, it is possible that use of these supplements by study participants may have affected results. An alternate explanation may be that prodromal disease phase processes drive molecular changes that skew the KP, leading to increased 3-HAA, but which diminish in latter-stage disease. Disease-stage effects have been noted in AD previously where the expression of QUIN in post-mortem brain tissue is reduced in later Braak stages of the disease (Guillemin et al., 2005). As the immediate fate of 3-HAA along the major KP branch is conversion to 2-amino-3-carboxymuconate-6-semialdehyde (ACMS) by the enzyme 3-hydroxyanthranilic-acid-oxygenase (3-HAO), other mechanisms which reduce 3-HAO function, could also be potential explanations of high 3-HAA. This would also explain the significantly decreased levels of PIC we observed.

Whatever the precise mechanism, there are many potential impacts of increased 3-HAA on dementia processes. Mechanistically, 3-HAA is regarded as redox active and/or redox regulatory with early data describing a direct neurotoxic effect (Okuda et al., 1998) mediated by metal ion reduction (Goldstein et al., 2000). Other studies point to antioxidant effects (Krause et al., 2011; Oh et al., 2004) and, on balance, it seems more likely that 3-HAA would act as a reactive oxygen species (ROS) detoxifier rather than a toxic ROS generator. If indeed 3-HAA does scavenge ROS, this would likely act to reduce both amyloid and tau phenomena enhanced by the neuroinflammation-ROS axis. Despite the potential links, no reports have yet emerged that describe how 3-HAA modulations may impact formation of tau tangles.

Adaptive immunity, mediated by B- and T- cells, has attracted increasing attention in AD and has been shown to enhance amyloid clearance (Baruch et al., 2015). Studies in murine AD models featuring Aβ pathology confirm brain $CD3^+$ T-cell infiltration correlating with Aβ plaque load (Ferretti et al., 2016). Studies also show increased levels of $CD3^+$ T-cells in AD patients relative to age-matched healthy controls (Togo et al., 2002). In 3xTg AD mice, adoptive transfer of nonspecific regulatory T cells (Tregs) reduced Aβ burden and improved cognitive defects whereas depletion of Tregs worsened cognitive defects (Baek et al., 2016). Since Munn et al., showed that activation of the KP resulted in maternal-fetal tolerance mediated by the suppression of T-cells (Munn et al., 1998), interest in the immunological aspects of KP metabolites has grown. Relevant observations include that 3-HAA induced activated T-cell death by depleting intracellular glutathione but spared non-activated T-cells (Lee et al., 2013). 3-HAA also reduced expression of pro-inflammatory cytokines (interleukin (IL)-12, IL-6 and Tumour

Necrosis Factor (TNF-α) in bone marrow-derived dendritic cells (BMDCs) stimulated with lipopolysaccharide (Lee et al., 2013). Considering these observations, it is reasonable to speculate that, if indeed adaptive immunity is important in clearing amyloid, then increased 3-HAA is highly likely to interfere with this mechanism and may thus be a biomarker of impaired adaptive immunological response in pre-symptomatic dementia.

While strong results for the 3-HAA/AA ratio amongst others were found, this study also has limitations. During the years since AIBL conception, participants donated multiple longitudinal blood samples. However, at the time of sample collection for this particular project, the number of samples collected for progressor and non-progressors was unbalanced and as such we used only baseline samples for KP analyses. Future research will examine longitudinal samples from a larger selection of AIBL participants, to ensure balanced numbers of both progressor and non-progressor groups. A second limitation is the small sample size of the PET amyloid imaging group, while no direct correlations with Aβ load were observed, we cannot rule out that with increased samples our results might be different.

The current study is the first to highlight the association of KP metabolites with a significant cognitive change prior to AD diagnosis. Further work is needed to validate these findings in other populations using both cross-sectional and longitudinal designs. Notwithstanding, our current results have shown an intriguing, previously unreported finding, strongly linking increased plasma 3-HAA levels in older adult AIBL study participants to risk of progressing to MCI or AD. With further validation this finding may also have translational potential as an indicative biomarker, highlighting a possible therapeutic window for either preventative strategies or treatment.

CRediT authorship contribution statement

Marcela Cespedes: Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Kelly R. Jacobs:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Project administration. **Paul Maruff:** Methodology, Investigation, Writing – review & editing. **Alan Rembach:** Resources, Data curation, Project administration. **Christopher J. Fowler:** Methodology, Investigation, Data curation. **Brett Trounson:** Investigation. **Kelly K. Pertile:** Investigation. **Rebecca L. Rumble:** Investigation. **Stephanie R. Rainey-Smithe:** Writing – review & editing. **Christopher C. Rowe:** Methodology, Investigation, Project administration. **Victor L. Villemagne:** Methodology, Writing – review & editing, Project administration. **Pierrick Bourgeat:** Investigation, Data curation. **Chai K. Lim:** Investigation. **Pratishtha Chatterjee:** Writing – review & editing. **Ralph N. Martins:** Resources, Writing – review & editing. **Arne Ittner:** Writing – review & editing, Funding acquisition. **Colin L. Masters:** Resources, Writing – review & editing. **James D. Doecke:** Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Gilles J. Guillemin:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition. **David B. Lovejoy:** Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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