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ORIGINAL ARTICLE

Central kynurenine pathway shift with age in women

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Abstract

Age is considered a dominant risk factor in the development of most neurodegenerative disorders. The kynurenine pathway, a major metabolic pathway of tryptophan is altered in the majority of neurodegenerative disorders. In this study, we have analysed CSF samples from 49 healthy women across a wide age range (0–90) for kynurenine pathway metabolites and the inflammatory marker neopterin. Our results show central tryptophan metabolism is increased with age in women, with an apparent shift towards the neurotoxin quinolinic acid. We also observed an increase in central levels of the inflammatory marker neopterin with age and a positive correlation between neopterin and kynurenine pathway activation. We conclude that, the changes that occur in the kynurenine pathway as a result of normal ageing are mechanistically linked to increased inflammatory signalling and have some explanatory potential with regard to age-associated degenerative diseases in the CNS. Management of health in ageing and (preventative) treatment would do well to look to the kynurenine pathway for potentially novel solutions.

Keywords: age, cerebrospinal fluid, inflammation, kynurenine pathway, quinolinic acid, tryptophan.

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Modern medicine has extended human lifespan to a point where ageing and its associated degenerative burden has become more of a medical issue than ever before. Age is now the major risk factor for the development of most neurodegenerative disorders (Evans *et al.* 1989; Moghal *et al.* 1994). Kynurenine pathway metabolism (Fig. 1) is the major pathway for tryptophan (TRYP) metabolism and is known to be altered in a number of neurodegenerative disorders, including amyotrophic lateral sclerosis (Chen and Guillemin 2009), Parkinson's disease (Zinger *et al.* 2011) and Alzheimer's disease (Guillemin and Brew 2002).

Neurodegenerative diseases are associated with chronic inflammation (Quintanilla *et al.* 2004) and it has been shown that the general inflammation marker IL-6 increases with age (Guest *et al.* 2014). Interestingly the kynurenine pathway has been shown to be up-regulated by inflammatory cytokines such as IFN-γ and tumor necrosis factor- alpha (TNFα) while some of the downstream kynurenine pathway metabolites are immunosuppressive (Mándi and Vécsei 2012).

However, not much is known about how kynurenine pathway activation and dynamics shift with age. It was only recently shown that downstream metabolite picolinic acid (PIC) increases with age (Coggan *et al.* 2009). However, other

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Abbreviations used: 3HAA, 3-hydroxy anthranilic acid; 3HAO, 3-hydroxyanthranilate; 3HK, 3-hydroxykynurenine; ACMS, aminocarboxymuconate semialdehyde; ACMSD, aminocarboxymuconate semialdehyde decarboxylase; IDO1, indolamine2,3-hydroxylase; IFN-γ, interferon gamma; KAT, kynurenine aminotransferase; KMO, kynurenine mono-oxygenase; KP, kynurenine pathway; KYNA, kynurenic acid; KYN, kynurenine; NMDA, *N*-methyl-D-aspartate receptor; PIC, picolinic acid; QPRT, quinolinate phosphoribosyltransferase; QUIN, quinolinic acid; TDO, tryptophan-2-3-dioxygenase; TRYP, tryptophan.

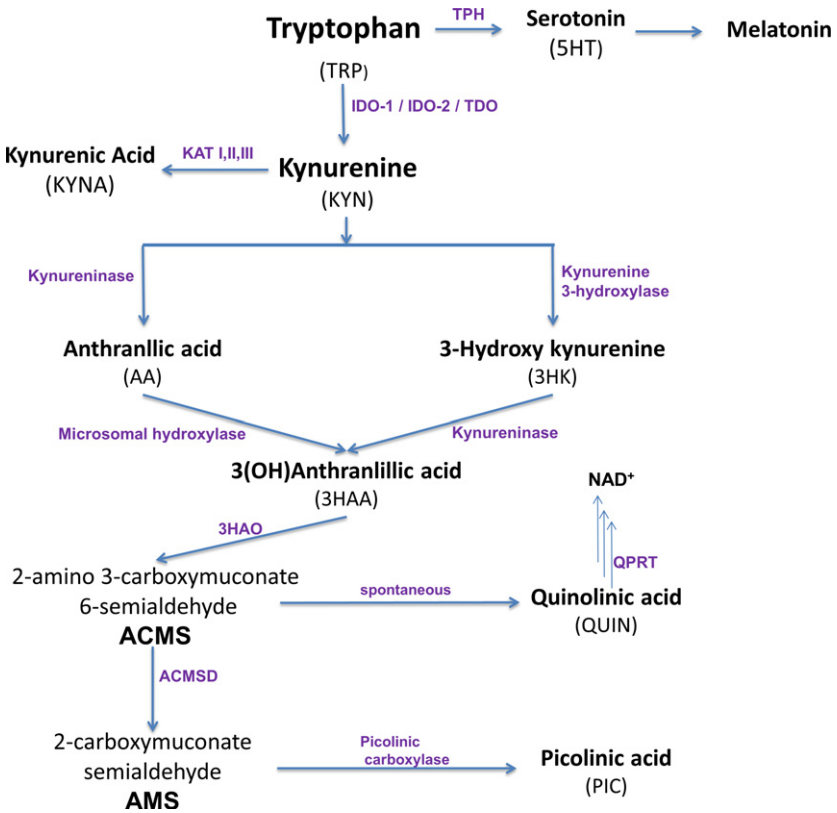

Fig. 1 The kynurenine pathway, schematic overview. Tryptophan is recruited into the pathway by activation of indolamine 2,3 dehydoxylase-1(IDO-1), indolamine 2,3 dehydoxylase-2 (IDO-2) or tryptophan 2,3 deogygenase-2 (TDO-2) and subsequent conversion into kynurenine (KYN). Kynurenine is then metabolized along the pathway into a range of neuroactive and immunomodulatory metabolites. KYN can be metabolized by kynurenine aminotransferase (KAT) −1, 2 or 3 to kynurenic acid (KYNA) or by kynureninase to anthranillic acid (AA) or kynurenine 3-hydroxylase (KMO) to 3-hydroxykynurenine (3HK). Both 3HK and AA can be metabolized to 3-hydroxy anthranillic acid (3HAA) by kynureninase and microsomal hydroxylase respectively. The latter can be metabolized by 3-Hydroxyanthranilic acid 3,4-dioxygenase (3HAO) to 2-amino 3-carboxymuconate 6-semialdehyde (ACMS) which spontaneously forms the NAD precursor quinolinic acid (QUIN). ACMS can also actively be metabolized into 2-carboxymuconate semialdehyde (AMS) which can then be metabolized into picolinic acid (PIC) by picolinic carboxylase. Factors outside of the pathway can shift the balance of metabolites produced by influencing the activity levels of the pathway enzymes.

kynurenine metabolites, while neuroactive and immunomodulatory have not been characterized in this manner.

In this study, we analysed CSF samples of 49 female subjects aged 0–98 years for kynurenine metabolites and inflammatory markers. We observed an increase in the levels of the inflammatory marker neopterin coincident with an apparent increased flux through the kynurenine pathway. This resulted in a decrease in upstream kynurenine metabolite levels accompanied by a robust elevation in the downstream metabolites quinolinic acid and PIC.

Methods

Ethics statement

This study was conducted in accordance with the Helsinki declaration. Ethical approval was obtained from the Human Research Ethics Committee, Sydney Adventist Hospital (EC00141, project number 2011-005). Written informed consent was obtained from all participants prior to commencement.

CSF samples

CSF samples (n = 49; female) were obtained with ethics approval (SAHHREC #13-02) from the Sydney Adventist Hospital, Sydney Australia. Samples were collected by standard lumbar puncture from patients assessed as clinically well after investigation for suspected meningitis, returning normal results for routine CSF pathology markers, including white cell count, protein, glucose and bacterial sterility. All samples were stored at −20 to −80°C until analysis.

Gas chromatography mass spectrometry

PIC and quinolinic acid (QUIN) in CSF samples were determined using gas chromatography mass spectrometry (GC-MS) in electron-capture negative ionization mode by a method previously described (Smythe *et al.* 2002). Briefly, 50–100 μL samples of CSF were combined with 20 μL of the internal standards deuterium labelled-

PIC (RT 4.1) and deuterium labelled QUIN ([2H3]-QUIN) RT 5.3. Samples were evaporated to dryness, and derivatized into hexafluoroisopropyl esters products. Chromatographic separations were performed in splitless mode using HP–5MS capillary columns (30 m × 0.25 mm i.d.).

High-pressure liquid chromatography

TRYP, kynurenine and kynurenic acid measurement Kynurenine pathway metabolites in CSF were detected using an Agilent 1200 high-performance liquid chromatography system. Analysis of TRYP and kynurenine were concurrently measured as previously described (Lim *et al.*, 2013). Briefly, 200 μL of samples was treated with equal volume of 10% trichloroacetic acid and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter. Treated samples were then injected into a C18 column (5 μm, 150 μm 4.6 i.d.; Agilent Zorbax, Sta. Clara, CA, USA) at an injection volume of 30 μL via an automatic liquid sampler. The assay was performed using 0.1 M ammonia acetate at pH 4.65 as mobile phase at an isocratic flow of 0.8 mL/min. TRYP and kynurenine were quantified by fluorescence (Ex 254/Em 404 RT 6.5 and UV/Vis detection at 365 nm RT 3.84 respectively). 3-hydroxykynurenine (3HK) was quantified by UV/Vis detection at 365 nm RT 1.12 and 3haa and neopterin by fluorescence (Em438/Ex320 RT 2.81, Em250/Ex438 RT 1.191 respectively).

Measurement of kynurenic acid was performed as previously described (Guillemin *et al.*, 2007). Treated samples were injected into a C18 column and kynurenic acid was eluted using a mobile phase consisting of 50 nM sodium acetate supplement with 0.25 M zinc acetate and 2.25% (v/v) acetonitrile at an isocratic flow rate of 0.75 mL/min. Quantification of kynurenic acid was carried out by fluorescence (Ex 344/Em 388) detection and expressed in nM. Intra- and inter-assay coefficient of variation (CV) was determined by standards incorporated in the sample run set to be in an acceptable range of 5–8%.

Statistical analysis

Statistical analysis was performed using IBM SPSS software. All measurements were tested for normality using the Kolmogorov–Smirnov test, non-normal distributions were log transformed to achieve normality. Correlations were calculated using Pearson coefficients. Corrections for age were done using multivariate general linear models (MVA). Statistically significant level was set at $p < 0.05$.

Results

Increased KP activation with age

Kynurenine/TRYP ratio, an accepted measure of kynurenine pathway activity, is significantly correlated with increased age (Fig. 2b). (Pearson; $r = 0.544$, $p \leq 0.000$, $n = 49$).

This remained significant after controlling for age and time of day (MVA: t (45) = 4.696, $p \leq 0.000$, $R^2 = 0.346$). See Table 1 for metabolite levels per age range.

KP metabolites 3HK, KYNA and QA interactions

Both TRYP (Fig. 2a) and 3-hydroxykynurenine (Fig. 2c) levels correlated negatively (i.e. were lower) with age (Pearson; $r = -0.647$, $p \leq 0.000$, $n = 49$), and (Pearson; $r = -0.503$, $p \leq 0.000$, $n = 49$) respectively.

Table 1 Kynurenine pathway (KP) metabolites and neopterin levels per age range

Metabolite	Age	Mean	Median	SEM
Tryptophan (μM)	20–40	2.03	2.03	0.13
	40–60	1.51	1.63	0.21
	60–80+	0.39	0.15	0.23
Kynurenine (μM)	20–40	0.51	0.25	0.29
	40–60	0.67	0.33	0.28
	60–80+	0.98	0.88	0.17
Kynurenine/tryptophan ratio	20–40	22.04	10.65	12.35
	40–60	17.98	14.33	3.02
	60–80+	41.36	40.94	4.80
Kynurenic acid (nM)	20–40	4.50	3.20	1.27
	40–60	5.23	5.29	1.22
	60–80+	3.13	1.45	1.12
3HK (nM)	20–40	3.71	3.00	0.74
	40–60	4.92	5.36	0.86
	60–80+	0.28	0.25	0.05
3HAA (nM)	20–40	1.29	1.30	0.13
	40–60	1.03	1.06	0.16
	60–80+	2.16	1.79	0.32
Picolinic acid (nM)	20–40	13.73	12.88	1.59
	40–60	12.77	9.61	2.64
	60–80+	20.32	18.69	2.85
Quinolinic acid (nM)	20–40	6.93	6.35	1.24
	40–60	9.33	7.77	2.47
	60–80+	24.87	23.44	4.71
Quinaldic acid (nM)	20–40	0.32	0.36	0.06
	40–60	0.32	0.17	0.09
	60–80+	0.49	0.57	0.08
Neopterin (nM)	20–40	0.28	0.29	0.03
	40–60	0.41	0.28	0.10
	60–80+	0.88	0.77	0.16

3HK, 3-hydroxykynurenine; 3HAA, 3-hydroxy anthranilic acid.

There was no significant correlation between kynurenic acid (KYNA) and age (Pearson; $r = -0.231$, $p = 0.132$, $n = 44$). CSF levels of KYNA also did not correlate with 3HK levels (Pearson; $r = 0.167$, $p = 0.278$, $n = 44$). There was also no correlation between 3HK and the dehydroxylated form of KYNA; quinaldic acid (QA) (Pearson; $r = 0.052$, $p = 0.772$, $n = 34$). Surprisingly KYNA levels also did not correlate with QA (Pearson; $r = -0.106$, $p = 0.565$, $n = 32$).

However, QA levels were significantly positively correlated with age (Pearson; $r = 0.445$, $p = 0.006$, $n = 37$).

Downstream metabolites

Concentrations of the downstream metabolites QUIN (Fig. 3a) and PIC (Fig. 3b) were increased with age (Pearson; $r = 0.553$, $p \leq 0.000$, $n = 48$), (Pearson; $r = 0.560$, $p \leq 0.000$, $n = 49$) respectively.

A positive correlation was observed between age and neopterin levels (Pearson; $r = 0.547$, $p \leq 0.000$, $n = 44$)

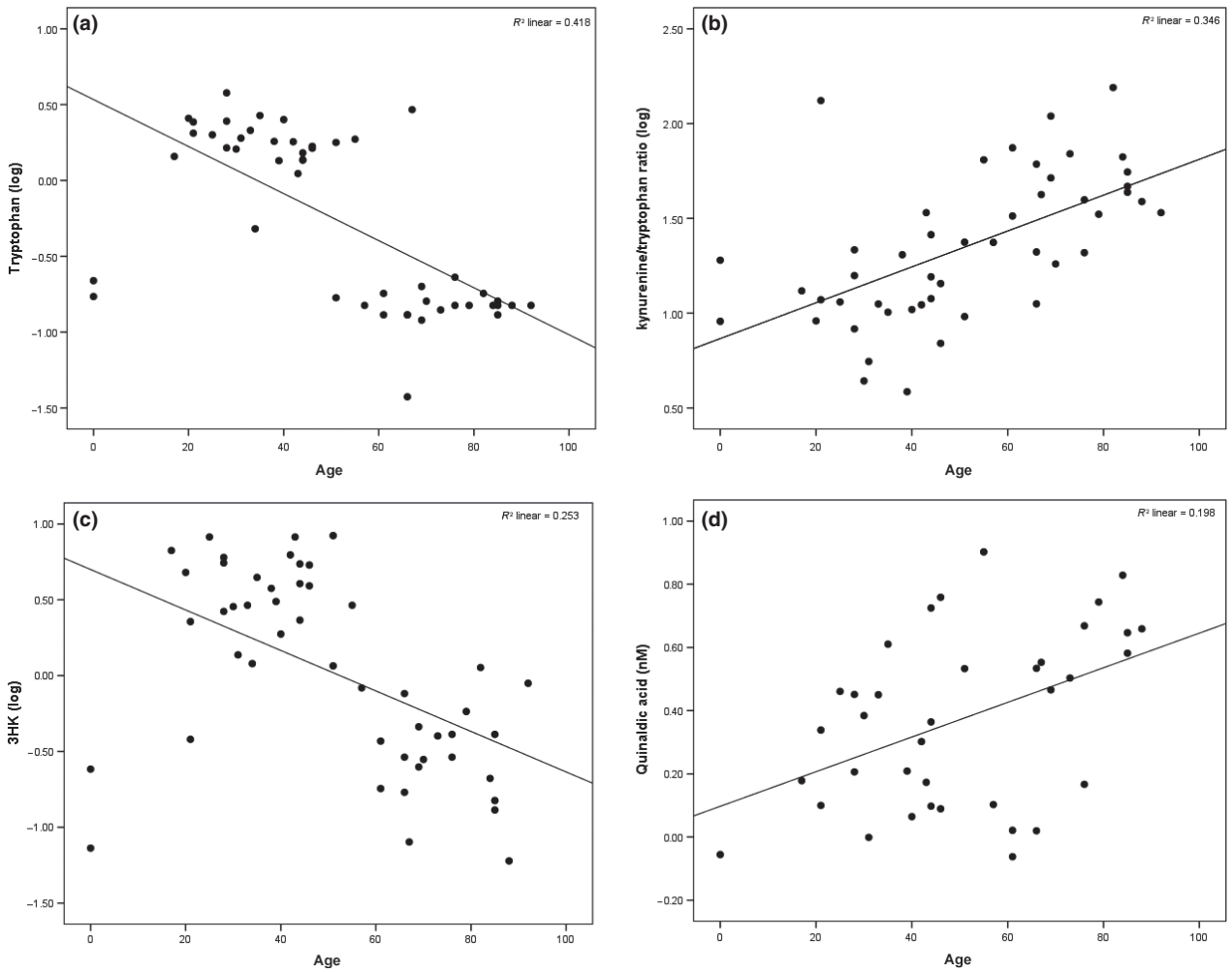

Fig. 2 Correlations between age and CSF upstream kynurenine pathway metabolites tryptophan (a), kynurenine/tryptophan ratio (b), 3-hydroxykynurenine (c) and quinaldic acid (d). (a)Tryptophan levels in CSF were inversely correlated with age (Pearson; $r = -0.647$, $p \leq 0.000$, $n = 49$, $R^2 = 0.418$), (b) Kynurenine/tryptophan ratio is considered a measure of kynurenine pathway activation. There was a positive correlation between age and kynurenine/tryptophan ratio in CSF (Pearson; $r = 0.544$, $p \leq 0.000$, $n = 49$, $R^2 = 0.316$). This was mostly because of a decrease in tryptophan as there was no correlation between age and kynurenine (Pearson; $r = 0.243$, $p = 0.085$, $n = 51$, $R^2 = 0.059$). (c) An inverse correlation between age and 3-hydroxykynurenine was observed (Pearson; $r = -0.503$, $p \leq 0.000$, $n = 49$, $R^2 = 0.253$). (d) A significant positive correlation between age and quinaldic acid levels in CSF was observed (Pearson; $r = 0.052$, $p = 0.772$, $n = 34$, $R^2 = 0.196$). Comparisons were made using the Pearson correlation coefficient and multiple linear regression.

(Fig. 4), neopterin and kynurenine/TRYP ratio (Pearson; $r = 0.427$, $p = 0.004$, $n = 43$) (Fig. 5a).

PIC levels did not correlate with neopterin levels (Pearson; $r = 0.180$, $p = 0.249$, $n = 43$) (Fig. 5b) whereas QUIN did (Pearson; $r = 0.713$, $p \leq 0.000$, $n = 44$) (Fig. 5c). PIC/Quinolinic acid ratio showed no statistically significant association with age (Pearson; $r = -0.223$, $p = 0.128$, $n = 48$).

Discussion

It has been recognized for many decades that age is the most significant risk factor for neurodegeneration, yet the mechanisms are largely unclear. Evidence for links between neurodegenerative disease, neuroinflammation and alterations in the kynurenine pathway is building. Most of the studies supplying support for these links often correct for age, yet there has been no study to directly correlate age with kynurenine pathway alterations.

Kynurenine pathway activity increases with age

In our cohort of healthy women, kynurenine pathway activity as evidenced by the kynurenine TRYP ratio increased with age in CSF (Fig. 2b). The increase in kynurenine/TRYP ratio was mostly because of a significant decrease in CSF TRYP levels, as there was no significant increase in kynurenine levels. However, the absence of a rise in kynurenine is likely

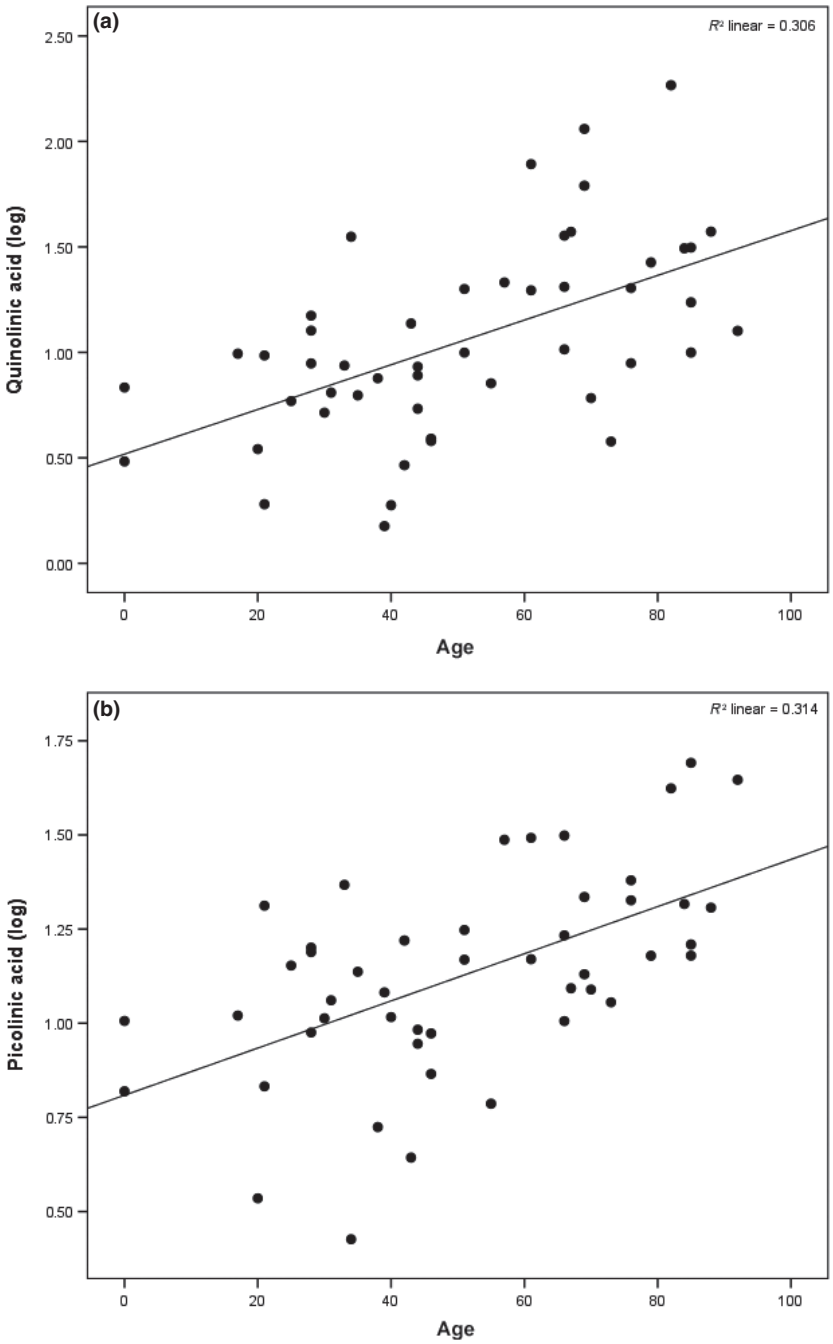

Fig. 3 Correlations between age and CSF downstream kynurenine pathway metabolites picolinic acid (PIC) (a) and quinolinic acid (b). (a) A significant positive correlation was observed between age and CSF quinolinic acid levels (Pearson; $r = 0.553$, $p \leq 0.000$, $n = 48$, $R^2 = 0.305$). (b) A significant positive correlation was observed between age and CSF PIC levels (Pearson; $r = 0.560$, $p \leq 0.000$, $n = 49$, $R^2 = 0.314$). Comparisons were made using the Pearson correlation coefficient and multiple linear regression.

because of elevated catabolism of kynurenine to downstream metabolites. Both quinolinic acid and PIC were significantly increased with age (Fig. 3a and b). This is consistent with a PIC increase found in human CSF with age (Coggan *et al.* 2009) and of increases in QUIN with age in rats (Moroni *et al.* 1988). While most studies in human subjects correct for age when reporting on QUIN levels, we are the first to report a significant generalized central increase in QUIN levels with age.

Quinaldic acid was also significantly increased with age (Fig. 2b). While an apparent increase in kynurenic acid was observed it was not statistically significant, this may be because of a sample size effect. CSF KYNA levels have been reported to significantly increase with advancing age in healthy volunteers (Heyes *et al.* 1992) and in patients without detectable neurological disease (Kepplinger *et al.* 2005). However, the observed concomitant rise in QA with age suggests any increase in kynurenic acid may be modulated by its dehydroxylation to quinaldic acid. Surprisingly, however, we did not find a significant correlation between the two parameters suggesting their final pathways of degradation and excretion remain somewhat uncoupled.

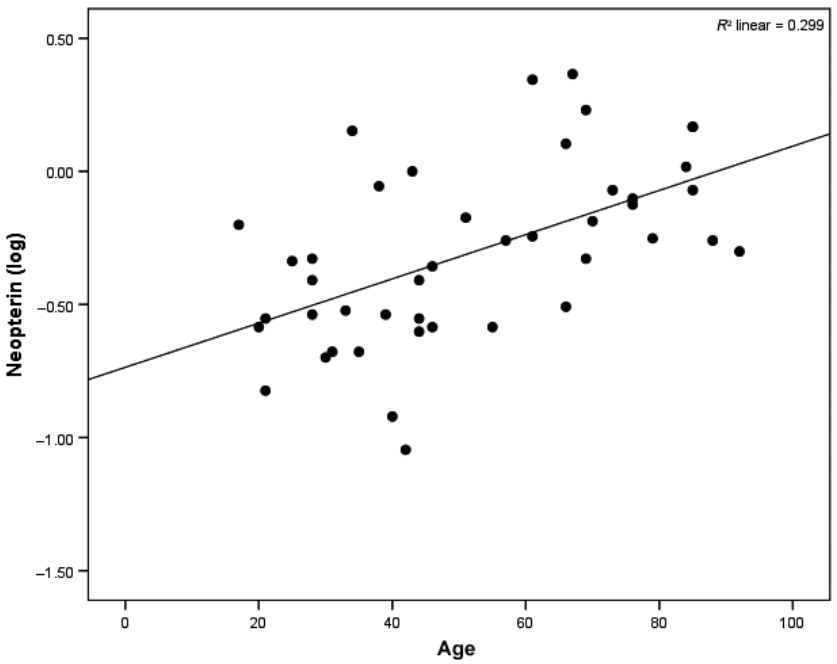

Fig. 4 Correlations of inflammatory marker neopterin with age. A positive correlation was observed between age and neopterin levels (Pearson; $r = 0.547$, $p \leq 0.000$, $n = 44$, $R^2 = 0.299$). Comparisons were made using the Pearson correlation coefficient and multiple linear regression.

Consequences: quinolinic acid

Age is still the best predictive risk factor for neurodegenerative disease such as Alzheimer's and Parkinson's disease. However, the aetiology of neurodegenerative disease in the ageing context is still not well characterized but has been shown to involve factors such as neuroinflammation and oxidative stress.

It is therefore poignant that our results show an increase in neopterin, a parameter that is associated with inflammation as well as with oxidative stress (Murr *et al.* 2002). The significant elevation in neopterin with age observed in these CSF samples (Fig. 4), strongly suggests there is a generalized state of increased inflammation of the brain with an accompanying increased level of general oxidative stress.

The latter is very likely to be caused by the former. Inflammation can drive the kynurenine pathway, especially in the production of quinolinic acid (Guillemin *et al.* 2003). Quinolinic acid is neurotoxic in a variety of ways including induction of tau phosphorylation, oxidative stress and excitotoxicity (Guillemin 2012; Pérez-De La Cruz *et al.* 2012). It has already been suggested that the kynurenine pathway is part of the underlying causative mechanism of Alzheimer's disease (Guillemin and Brew 2002; Guillemin *et al.* 2005) and Parkinson's disease (Zinger *et al.* 2011). The parallel trajectories of increased neurodegenerative disease risk with age and elevated inflammation-driven kynurenine pathway, skewed towards the production of quinolinic acid, is consistent with a causal relationship.

Our results reflect this with a strong positive correlation between neopterin and kynurenine pathway activity (Fig. 5a) as well as a positive correlation between neopterin and quinolinic acid (Fig. 5c), though not PIC. PIC was increased with age (Fig. 3b), consistent with a previous report which found an elevation in human CSF PIC with age (Coggan *et al.* 2009). However, PIC levels did not correlate with neopterin levels. PIC/Quinolinic acid ratio showed a negative trend consistent with a preferential production of quinolinic acid in the presence of inflammation.

The increased inflammation with age appears to drive kynurenine pathway activity, with a preferential production of quinolinic acid which may contribute to an increased risk of neurodegenerative disease (Kalonia *et al.* 2010; Pérez-De La Cruz *et al.* 2012).

Consequences: quinaldic acid

In addition to the elevation in downstream metabolites we also observed an increase in the upstream kynurenic acid metabolite, quinaldic acid, with age. While this did not directly correlate with kynurenine/TRYP ratios (Pearson; $r = 0.300$, $p = 0.071$, $n = 37$. $R^2 = 0.090$) it is still likely that the increase in the metabolite is because of kynurenine pathway activation. Quinaldic acid has been reported to be diabetogenic through its inhibition of gluconeogenesis (Hanson *et al.* 1969; Okamoto 1975) and interference with insulin release (Okamoto 1975). An increase in this compound may contribute to the increased risk of diabetes mellitus with age. Interestingly, while the risk of diabetes mellitus is generally described as increasing with age (2010), no definite mechanisms have been put forward apart from generalized suggestions about reduced exercise and increased food intake. The kynurenine pathway may provide a more tangible handhold for this phenomenon, though our observed

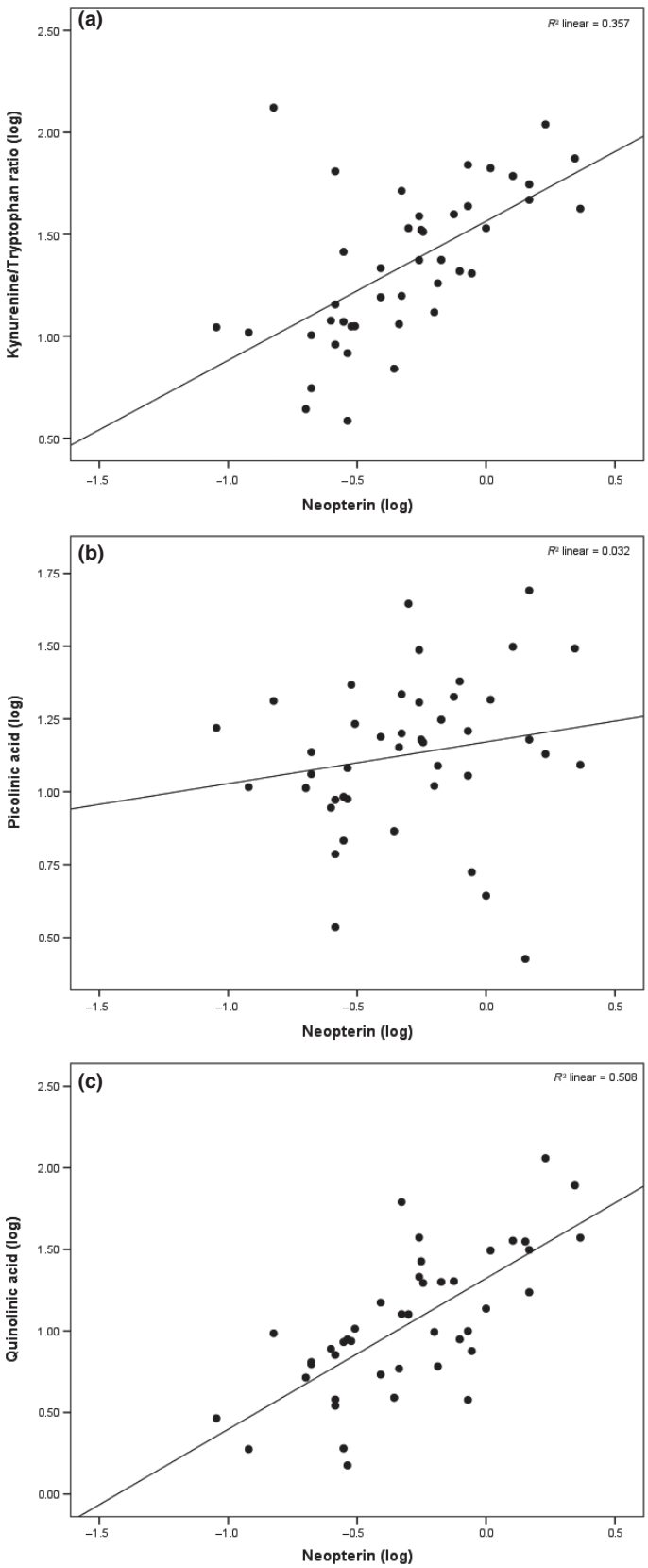

Fig. 5 Correlations of inflammatory marker neopterin with kynurenine pathway metabolites. Neopterin versus kynurenine pathway: k/t quin pic. (a) A significant positive correlation was observed between CSF kynurenine/tryptophan ratio and CSF neopterin levels (Pearson; $r = 0.427$, $p = 0.004$, $n = 43$, $R^2 = 0.357$). This remained significant after controlling for age and time of day (t (45) = 3.087, $p = 0.004$, $R^2 = 0.411$). (b) No significant interaction was observed between CSF picolinic acid levels and neopterin levels (Pearson; $r = 0.180$, $p = 0.249$, $n = 43$, $R^2 = 0.032$). (c) A significant positive correlation was observed between CSF quinolinic acid and CSF neopterin levels (Pearson; $r = 0.713$, $p \leq 0.000$, $n = 44$, $R^2 = 0.508$).This remained significant after controlling for age and time of day (t (45) = 5.209, $p \leq 0.000$, $R^2 = 0.496$). Comparisons were made using the Pearson correlation coefficient and multiple linear regression and univariate general linear models.

increase in central QA would have to translate to a systemic (i.e. serum) increase in QA. These data were not available in this cohort.

Conclusion

This study shows central kynurenine pathway activation increases with age in healthy women, which is likely to be driven by increased inflammation as indicated by elevated neopterin levels with age. This activation of the kynurenine pathway seems to favour the neurotoxic branch of the pathway represented by increased quinolinic acid levels. While the neuroprotective metabolite PIC is also elevated with age, we observed a downward trend in the PIC/QUIN ratio with age in combination with raised neopterin levels (an independent marker of inflammation). Thus, there appears to be a skewing of KP metabolism towards the neurotoxic quinolinic acid, rather than the protective PIC with age. Since the subjects in this study were assessed as clinically healthy, the increase in PIC appears to be an effective compensation for the neurotoxic potential of increasing quinolinic acid. However, the observed mismatch in favour of QUIN suggests a potential for ongoing subclinical damage.

We also reported an increase in quinaldic acid with age, which, if it translates systemically, may have consequences for glucose management and provide some explanatory power with regard to late life diabetes and mood disorders.

In summary, the changes that occur in the kynurenine pathway as a result of normal ageing have some explanatory potential with regard to age-associated degenerative diseases in the CNS. Management of health in ageing and (preventative) treatment would do well to look to the kynurenine pathway for potentially novel solutions.

Acknowledgments and conflict of interest disclosure

We wish to thank the staff from the emergency and Clinical pathology departments of the Sydney Adventist Hospital for their assistance in collecting samples for this study. This study was co-funded by the Australasian Research institute and a Research Excellence Award from Macquarie University. Authors declare no conflict of interest.

All experiments were conducted in compliance with the ARRIVE guidelines.

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