

## Age and circadian influences on picolinic acid concentrations in human cerebrospinal fluid.

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*Abbreviations used:* ACMSD, 2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase; CRP, C-reactive protein; F-PIC, fluoro-picolinic acid; GC-MS, gas chromatography mass spectrometry; HIV, human immunodeficiency virus; HSV, herpes simplex virus; ICD-10-AM, International statistical classification of diseases 10<sup>th</sup> revision Australian modification; INF- $\gamma$ , interferon gamma; KP, kynurenine pathway; KYNA, kynurenic acid; MIP, Macrophage inflammatory protein; PIC, picolinic acid; QUIN, quinolinic acid; SV, simian virus; TRP, L-tryptophan; WCC, white cell count.

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### Abstract

It has been suggested that picolinic acid, an endogenous metabolite of L-tryptophan, possesses neuro-protective and anti-proliferative effects within the CNS. However, the literature surrounding picolinic acid is limited, and its exact endogenous function is not known. Picolinic acid is produced via the kynurenine pathway which has been implicated in the pathogenesis of a range of neuro-inflammatory diseases. Although not extensively studied, there have been reports of altered picolinic acid production alongside other kynurenine metabolites in inflammatory disorders. In order to investigate whether picolinic acid concentrations are altered with disease in the CNS, we analysed picolinic acid levels in the CSF of 241 patients who underwent lumbar puncture as part of their standard clinical evaluation. In patients with no apparent CNS disease, CSF picolinic acid levels were ten fold higher in samples taken between 8pm and 4am compared to those collected between 4am and 12pm. This result suggests a diurnal variation in picolinic acid synthesis within the CNS. In addition, we observed a direct correlation between a patient's age and their picolinic acid concentration. No significant correlations were observed between CSF picolinic acid levels and any specific disease state.

**Keywords:** Picolinic acid, quinolinic acid, kynurenine pathway, CNS, cerebrospinal fluid, diurnal.

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## Introduction

Picolinic acid (PIC) is a small six-membered ring structure compound that is an isomer of nicotinic acid (Fig 1). It has been detected in cell-free supernatants, blood serum (Dazzi *et al.* 2001), CSF (Smythe *et al.* 2002), human milk, pancreatic juice and intestine homogenates (Rebello *et al.* 1982).

Little is known about the endogenous action of this molecule, and its significance in the CNS remains unclear. PIC is synthesised through a side branch of the kynurenine pathway (KP) from L-tryptophan (TRP) (Fig 2). While the primary role of this pathway within the CNS has not been completely established, maintenance of cellular NAD concentrations may be a critical function in brain cells (Grant *et al.* 1999; Grant and Kapoor. 2003).

Experimentally PIC seems to have a number of potential effects within the body, particularly involving the immune system and macrophage function. *In vitro* studies suggest that PIC can enhance macrophage interferon gamma (INF- $\gamma$ ) dependant gene expression (Varesio *et al.* 1990; Melillo *et al.* 1994), and selectively induce expression of macrophage inflammatory proteins (MIP)1 $\alpha$  and 1 $\beta$  (Bosco *et al.* 2000). High PIC concentrations have also been shown to selectively inhibit a number of viruses in culture including Human Immunodeficiency virus (HIV), Herpes Simplex virus (HSV), and Simian virus (SV) infected cell lines (Fernandez-Pol and Johnson. 1977; Fernandez-Pol *et al.* 2001). Further, antimicrobial effects of PIC have been observed against *Mycobacterium avium* complex infection, with additional potentiation of the antibiotics, clarithromycin, rifampin and various fluoroquinolones (Cai *et al.* 2006; Shimizu and Tomioka. 2006).

PIC has also been shown to affect tumour growth. *In vivo* studies on mice inoculated with tumour cells showed that those treated with injections of PIC in combination with activated macrophages, had significant increases in lifespan compared to control (Ruffman *et al.* 1987). Additionally, micromolar concentrations of PIC have been recently observed to

significantly decrease growth in human neuroblastoma cell lines (Guillemin *et al.* 2007).

Very few studies have reported PIC concentrations in disease states (Medana *et al.* 2003). However, altered TRP catabolism via the KP has been consistently observed in many inflammatory disorders of the CNS resulting in changes in concentrations of the KP metabolites kynurenic acid (KYNA) and quinolinic acid (QUIN) (Jhamandas *et al.* 2000) (for summary see Table 1). While QUIN is neurotoxic through activation of the N-methyl-D-aspartate (NMDA) receptor, KYNA offers neuroprotection by down-regulating NMDA receptor function (Jhamandas *et al.* 2000). Emphasis has, therefore, been placed upon maintaining metabolic balance within the KP.

A study of African children suffering from cerebral malaria found that elevated PIC and QUIN concentrations predicted a fatal outcome to the disease (Medana *et al.* 2003). Similar results were also observed in an animal model of malaria (*Plasmodium berghei*) using centrally infected mice (Clark *et al.* 2005).

The report by Medana and colleagues remains the only publication to date reporting changes of PIC concentrations in human disease. This lack of research into endogenous PIC levels highlights an important gap in the knowledge of this metabolite, despite the growing body of literature surrounding conditions that affect other members of the KP, such as QUIN and KYNA.

In this study we investigated possible associations between PIC concentrations in the CNS and selected physiological, clinical and demographic variables including; age, sex, disease states, time of sample collection (diurnal rhythm), QUIN, white cell count (WCC) and serum C-reactive Protein (CRP).

## Methods

### Sample collection

CSF samples (n=241; male=99, female=142) were obtained from the Sydney

Adventist Hospital Pathology Department (SAH HREC Project ID No 13-02). Samples were collected by standard lumbar puncture from suspected meningitis patients. All samples were stored at -20 to -80°C until analysis. A broad spectrum of co-morbidities and disease severities were included in the sample population, ranging from no apparent disease to fulminant CNS inflammation.

### **Gas chromatography mass spectrometry**

PIC and QUIN in CSF samples were determined using gas chromatography mass spectrometry (GC-MS) in electron-capture negative ionisation mode by a method previously described (Smythe *et al.* 2003). Briefly, 50-100µl samples of CSF were combined with 20µl of the internal standards fluoro-picolinic acid (F-PIC) and deuterium labelled QUIN ((<sup>2</sup>H<sub>3</sub>)-QUIN). Samples were evaporated to dryness, and derivatised into hexafluoroisopropyl esters products. Chromatographic separations were performed in splitless mode using HP-5MS capillary columns (30 m x 0.25 mm i.d.).

### **Medical record information**

The recent medical history (surrounding the lumbar puncture procedure) of each patient was de-identified and entered into a database modified for the study. CSF total and differential WCC, glucose and protein levels were noted, in addition to any other pathology test performed. General clinical conditions as recorded in the medical history were noted. Medications taken up to 12 hours prior to CSF sample collection were also recorded.

### **Diagnosis categorisation:**

Information in casemix summary reports from the hospital's medical database was used to categorise each patient's principal diagnosis. A diagnostic code was assigned to each patient's in-hospital admission according to the International Statistical Classification of Diseases, 10th Revision, Australian Modification (ICD-10-AM) (Roberts *et al.* 1998).

The casemix summary codes were grouped into diagnostic categories based on ICD-10-AM coding practices. Diagnoses

according to this classification system were divided into seven major categories of disease: a) Infectious and parasitic diseases: including central and systemic bacterial, viral, fungal or infections of unknown origin; b) Neoplasms: all origins; c) Mental and behavioural disorders: neurological disorders, delirium, optic neuritis, migraines, headaches, and head injuries; d) Nervous system disorders: demyelinating disease, polyneuropathy, epilepsy and convulsions; e) Diseases of the circulatory system: cerebrovascular disease, cardiovascular disease and arteritis; f) Respiratory system disorders: nose, mouth, throat and lung disorders; and g) Other: digestive system, metabolic, genitourinary, musculoskeletal and other peripheral diseases and causes.

### **Statistical analysis**

Sex, age, glucose, protein level, Total WCC, differential WCC, RCC, medications taken, principle diagnosis, time of sample taken, serum CRP levels, and PIC and QUIN concentrations were all investigated.

Normal distributions of PIC data were obtained by taking the natural logarithm of the quantitated values, and confirmed using the One-sample Kolmogorov-Smirnov test. From the logarithmic values, mean PIC concentrations of the whole sample, CNS control and CNS disease populations were calculated. Average PIC concentrations of diagnostic categories within the CNS disease population were compared with patients not having the specific diagnosis (e.g. patients with infectious diseases versus patients without infectious diseases). Analysis of variance (ANOVA) compared CNS diagnosis categories and the CNS control population. (Table 3). The means and 95% confidence intervals (95% C.I.) of PIC values were presented for each disease category (Table 3). Dunnet's *t*-test and ANOVA were used to investigate diurnal fluctuation within the CNS control group and other CNS disease categories.

### **'CNS control' and 'CNS disease' populations**

A 'CNS control' population from the sample cohort was determined using the following criteria:

1. CSF pathology reports on WCC, protein

and glucose levels were within normal range.

2. The patient has no clinical or biological evidence of diseases affecting the CNS.
3. The patient had not taken any antimicrobial, steroidal, or cytotoxic medications 12 hours prior to the lumbar puncture
4. QUIN concentrations in the tested sample were less than concentrations that have been previously associated with the neuropathology of infection and immune stimulation (i.e. <200 nM) (Heyes *et al.* 1991).

Use of the above criteria ensured that the 'CNS control population' were free of apparent CNS disease. The 'CNS disease' population was determined from the remaining patients that displayed CNS disease (i.e. Whole population minus the 'CNS control' group).

From the selected 'CNS control' and 'CNS disease' populations, reference ranges were calculated using the values falling within the central 95% of the distribution. Diurnal rhythm within the above populations was investigated using the Student's *t*-test.

## Results

The mean PIC concentration for the CNS disease population (0.45  $\mu$ M) was not statistically different to the CNS control group (0.31  $\mu$ M;  $p=0.15$ ; Table 2).

CSF PIC levels did not vary with any category of medication taken by the patient, and a significant gender difference in PIC levels was not found.

We also did not observe any significant association between PIC concentrations and markers of CSF inflammation or infection, including glucose, total protein level or white cell count (WCC). CSF PIC also showed no associated variation with serum CRP levels.

However, CSF PIC did show a statistically significant positive correlation ( $p<0.01$ ; Fig. 3) with the advancing age of the patient. This association remained true when calculated for both the control and disease populations (Fig. 3) In addition, a clear diurnal pattern was observed with PIC levels in the CSF of

the control group but not the disease group. Samples collected between 20:00 and 04:00 had significantly higher mean PIC concentrations than those taken between 04:00 and 12:00 midday ( $p=0.001$ ); or between 12:00 to 20:00 ( $p=0.011$ ) (Fig 4a).. This diurnal pattern was not observed in the CNS disease population.

Concurrent analysis of QUIN concentrations in the CNS disease population were significantly lower in the time period of 20:00-04:00 compared to those taken between 12:00-20:00 ( $p=0.006$ ). No significant differences in mean QUIN levels were observed in the CNS control population between the 3 time periods.

## Discussion

This study investigated possible associations between PIC concentrations in human CSF and relevant clinical and demographic variables. As outlined in the method section, a control population was identified as those patients having no evidence of CNS disease or other centrally acting disorders.

The mean PIC concentration in the CNS control group was not statistically different to that calculated for the CNS disease group. However, a linear correlation between CSF PIC concentration and the age of the patient in the whole sample was observed. Importantly, CSF concentrations were also observed to fluctuate markedly with the time of sampling in the control population. These results suggest that PIC synthesis and/or catabolism is influenced by daily biological rhythms.

### *PIC concentration and disease*

While previous studies suggest that PIC concentrations may correlate with inflammatory disease states (Medana *et al.* 2003), our results indicate that other factors may also significantly contribute to PIC synthesis and catabolism.

In this study, no clear association was found between CSF PIC levels and any disease category, including the general group of CNS infections (Table 2 and 3). One possible explanation for this is that PIC, being synthesized from a side branch in KP

metabolism may not be as directly connected to pathophysiological changes in KP metabolism as other kynurenines such as QUIN and KYNA (Table 1). However the diurnal fluctuation of PIC, identified in this study, may also have blunted the potential influence of inflammation mediated KP activation on PIC levels.

Importantly, direct analysis of variance (ANOVA) of the data set did show evidence of a significant difference between some diagnostic disease groups, including higher PIC levels in the neoplasm group. However, after multiple comparisons adjustment (Tukey HSD, Bonferroni), significant differences between disease state(s) were no longer evident. Weak associations with particular diseases may therefore require larger numbers to gain valid statistical power.

#### *Age related associations*

While an association between PIC and disease was not observed, a significant relationship was seen between PIC levels and the age of the patient (Fig. 3). This observation has not been previously reported, although other KP metabolites have found similar associations. 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). QUIN levels have also been shown to increase with age when measured in the rat cortex (Moroni *et al.* 1988).

Consistent with our result, the activity of the PIC producing enzyme 2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase; (ACMSD; EC.4.1.1.45.) has been shown to increase with age in animal models of rat kidney, liver and small intestine (Comai *et al.* 2005). However, the effect of age on ACMSD activity in the CNS has not been previously investigated. Pucci *et al.* (2007), have reported that ACMSD expression within the CNS is constitutively low suggesting that an age associated increase in ACMSD activity would significantly influence the Pic:Quin synthetic ratio. A postulated increase in ACMSD activity with age would also be consistent with our additional

observation that CSF Pic levels and age still showed a significant association even in the disease population.

#### *Diurnal Fluctuation*

Another novel finding in this study was that average PIC concentrations varied significantly throughout the day depending on the time of sample collection of the CSF (Fig 4). This suggests that a diurnal rhythm does exist and may be disrupted by immune activation or other factors. Importantly, we did not observe any similar diurnal pattern for QUIN levels in this same population, thus making this observation unique to PIC alone.

This diurnal variation in PIC levels is remarkably consistent with a previously observed fluctuation in CSF TRP where a series of CSF samples were taken and analysed for TRP over 24 hours in a healthy patient population (Kennedy *et al.* 2002). Low TRP levels occurred at near noon with maximum levels reached between 11pm and midnight. From this, it was proposed that the peak to trough availability of TRP as a precursor molecule may be of sufficient magnitude to influence other metabolite processes, such as melatonin secretion, which also follows a diurnal cycle (Kennedy *et al.* 2002).

Our observed diurnal fluctuation in PIC is closely mirrored by these previously reported variations in TRP levels (Fig. 4). It therefore seems likely that the availability of TRP in the CSF significantly impacts PIC concentrations in the absence of CNS pathology. Future investigations into the function of CNS PIC should consider this potential diurnal rhythm when formulating experimental design.

As research involving the study of KP metabolites in disease is commonly based upon observations of altered levels of CSF kynurenines, it appears prudent to establish whether diurnal rhythms in other KP metabolites also occur.

#### **Conclusion**

In summary, we have reported a significant association between CSF PIC levels and advancing age and a marked diurnal fluctuation in PIC concentrations. While this study did not show a significant

association between CSF PIC concentration and inflammatory disease states as previously reported for other KP metabolites, further research using larger sample cohorts may yet identify disease correlations with this enigmatic KP metabolite.

### Acknowledgments

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**Table 1**

Altered kynurenine levels in selected disease states affecting the CNS

Disease state	Alteration of kynurenines in the CNS (CSF/ brain tissue <sup>ψ</sup> )			Reference
	QUIN	KYNA	PIC	
	Alzheimer's	-	-	
	-	↓	-	Heyes <i>et al.</i> (1992)
	↑	-	-	Guillemin <i>et al.</i> (2005)
Parkinson's	-	↓	-	(2005)
Multiple sclerosis	-	↓	-	Hartai <i>et al.</i> (2007)
Huntington's	↑ <sup>ψ</sup>	-	-	Rejdak <i>et al.</i> (2002)
Cerebral Malaria	↑	↑	↑	Guidetti <i>et al.</i> (2004)
HIV infection	↑	↑	-	Medana <i>et al.</i> (2003)
Down Syndrome	-	↑ <sup>ψ</sup>	-	Heyes <i>et al.</i> (1991)
Amyotrophic lateral sclerosis	-	↑	-	Baran <i>et al.</i> (1996)
	↑	-	-	Ilzecka <i>et al.</i> (2003)
				Guillemin <i>et al.</i> (2005)

↑=elevated levels compared to control, ↓=decreased levels compared to control, - =not tested, n/s=not significant.

**Table 2**

Mean CSF PIC levels per sample group

CSF sample group	PIC concentration (μM) (95% confidence interval)	No of patients
Whole sample	0.42 (0.36-0.50)	241
CNS disease	0.45 (0.37-0.54)	207
CNS control	0.31 (0.21-0.46)	34

The CNS pathology population was not significantly different from the CNS control population (p<0.15).

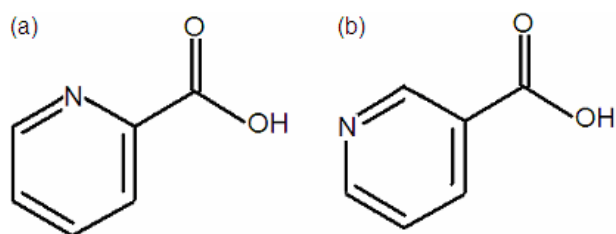


**Table 3**

Mean CSF PIC levels of the various diagnostic categories making up the CNS disease group

Diagnostic categories	PIC concentration ( $\mu\text{M}$ ) (95% confidence interval)	No of patients
Infectious and parasitic disease	0.38 (0.27-0.51)	76
Neoplasms	0.86 (0.36-2.10)	12
Mental and behavioural disorders	0.62 (0.41-0.96)	39
Nervous system disorders	0.25 (0.13-0.48)	24
Circulatory system disorders	0.36 (0.18-0.71)	14
Respiratory system disorders	0.45 (0.15-1.32)	10
Other diseases and disorders	0.61 (0.38-0.97)	32
No disease (CNS control)	0.31 (0.21-0.46)	34

Analysis of variance (ANOVA) showed a significant difference between the diagnostic categories ( $p=0.034$ ). However, after adjusting for multiple comparisons (Tukey HSD, Bonferroni) due to high variability and small sample size of some of the categories the significant differences were not identifiable.



**Fig. 1**  
Chemical structures of the isomers (a) picolinic acid and (b) nicotinic acid.

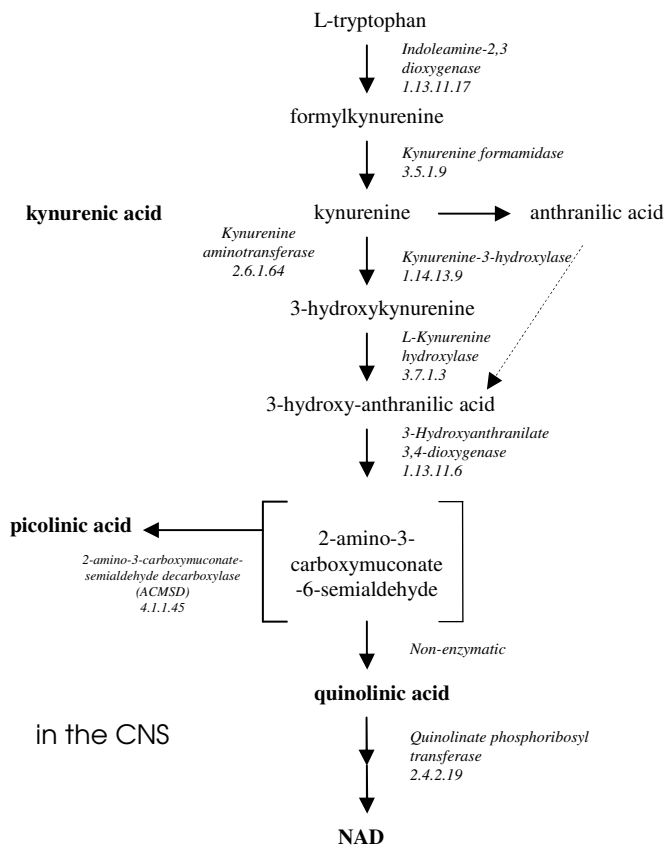


Fig. 2 The kynurenine pathway

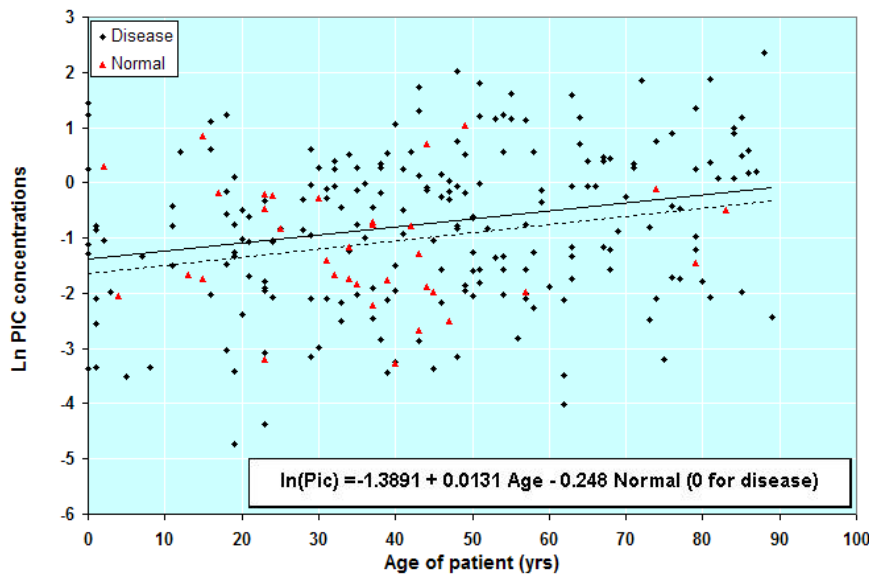
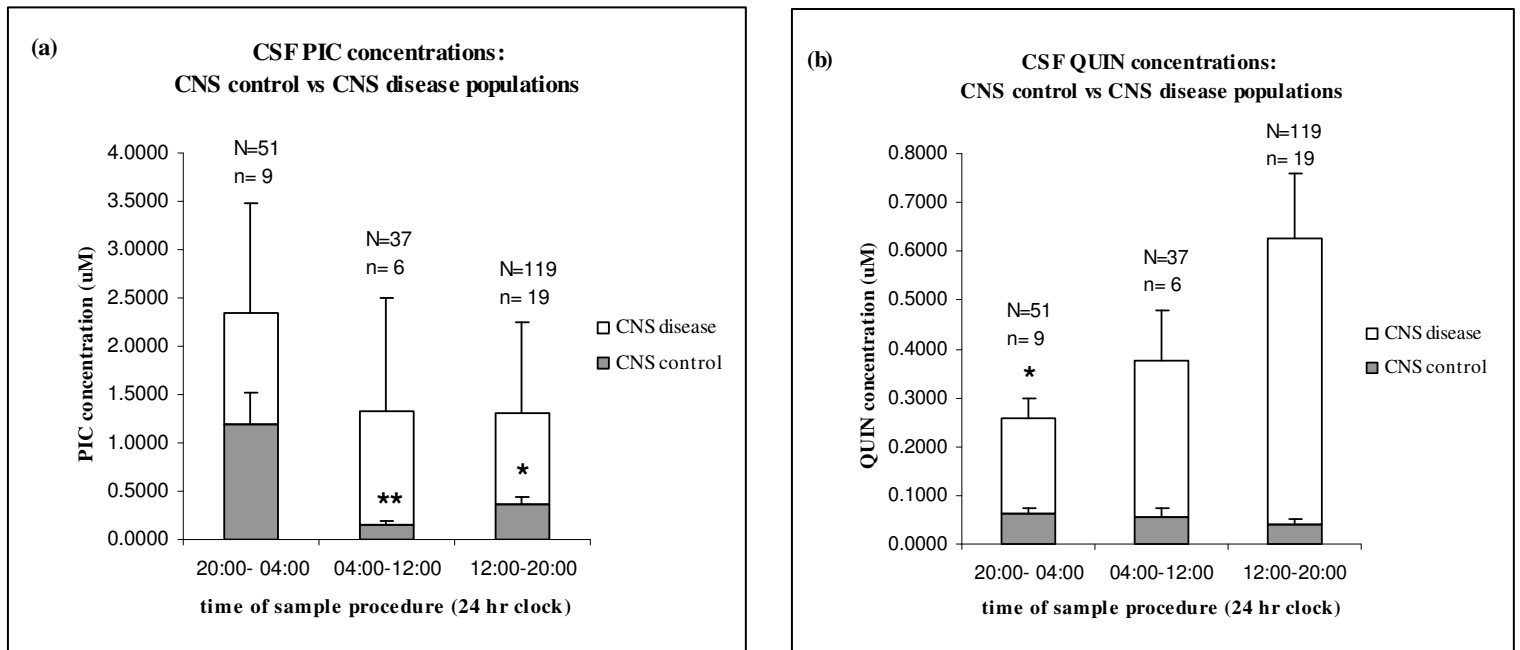


Fig. 3 Scatterplot of picolinic acid concentrations Vs subjects age. Each data point represents the natural logarithm of the picolinic acid concentration for each subject plotted against the age of the subject (in years) at the time of sample collection..



**Fig. 4** Effect of diurnal rhythm on mean CSF (a) PIC and (b) QUIN levels in the CNS disease and CNS control populations. (a) CNS disease and CNS control populations are separated according to the time of the CSF sample collection. For control group; mean picolinic acid levels between each of the 3 time periods were significantly different from each (ANOVA,  $p=0.001$ ). After correcting for multiple comparisons by Tukey's HSD, samples collected between 20:00 and 4:00 had significantly higher mean PIC concentrations than those taken between 04:00 and 12:00 (\*\* $p=0.001$ ); and those taken between 12:00 and 20:00 (\* $p=0.011$ ). There were no significant differences observed for mean CSF Pic levels between the three time periods for the CNS disease group. (b) There were no significant differences observed for mean CSF Quin levels between the three time periods for the CNS control group. Mean CNS disease QUIN concentrations taken between 20:00 and 04:00 were significantly lower than those taken between 12:00 and 20:00 (\* $p=0.006$ ). Error bars indicate standard error of the mean. N= number in CNS disease population, n= number in CNS control population.