INVESTIGATING A SUSTAINABLE MEANS OF DETECTING AND MONITORING ALZHEIMER'S DISEASE RISK VIA NON-INVASIVE BIOMARKERS

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution. Chapter 4 of this thesis includes the analysis of archival data. I warrant that I have obtained, where necessary, permission from the biobank custodians to use and report these data.

Najwa-Joelle Metri

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Something I learnt early on in my science journey is that things never quite go to plan. I've been so lucky to be supported by the most generous and patient academics and family.

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List of Abbreviations

3-HAA	3-Hydroxyanthranilic acid
3-HK	3-Hydroxykynurenine
AA	Anthranilic acid
ACN	Acetonitrile
AD	Alzheimer's disease
AF	Ammonium formate
ANZCTR	Australia New Zealand Clinical Trial Registry
APOE-ε4	Apolipoprotein E-ε4
APP	Amyloid Precursor Protein
Aβ	Amyloid beta
BBB	Blood-brain barrier
CNS	Central nervous system
CRE	Creatinine
CSF	Cerebrospinal fluid
DASS	Depression, Anxiety, and Stress Scale
ECD	Electron-capture dissociation
ECL	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assay
Ex/Em	Excitation/Emission
FA	Formic acid
FBB	^{18}F-Florbetaben
FDG	F^{18} fluorodeoxyglucose
FL	Fluorescence detection
HFBA	Heptafluorobutyric acid
IDO	Indoleamine dioxygenase
IFN-γ	Interferon-gamma
KA	Kynurenic acid
KARVIAH	Kerr Anglican Retirement Village Initiative in Ageing Health
KP	Kynurenine pathway
KYN	Kynurenine
LC	Liquid chromatography
LOD	Limit of detection

LOQ	Limit of quantification
m/z	Mass/charge ratio
MCI	Mild cognitive impairment
MeOH	Methanol
MeSH	Medical Subject Headings
MMSE	Mini-Mental State Examination
MoCA	Montreal Cognitive Assessment
MOD	Mode of detection
MRI	Magnetic resonance imaging
MRM	Multiple-reaction monitoring
MS	Mass spectrometry
NAL	Neocortical amyloid load
NDDs	Neurodegenerative disease
NFL	Neurofilament light chain
NMDA	N-methyl-D-aspartate
PA	Picolinic acid
PDA	Photo diode array
PET	Positron emission tomography
PiB	Pittsburgh Compound B
PICOS	Population, Intervention, Comparison, Outcomes, Study Design
POCT	Point-of-care testing
PP	Potassium phosphate
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PSEN1	Presenilin 1
PSEN2	Presenilin 2
QA	Quinolinic acid
SA	Sodium acetate
SUVR	Standard uptake value ratio
TFA	Trifluoroacetic acid
TRP	Tryptophan
UV-Vis	Ultraviolet-visible spectroscopy
XA	Xanthurenic acid
ZA	Zinc acetate

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TRP and KYN are presented in μM, while KA, 3-HK, 3-HAA, AA, PA, and QA are reported in nM.

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Abstract

Compelling evidence has identified the kynurenine pathway (KP) as an avenue to predict, diagnose, and measure the progression of neurodegenerative diseases, such as Alzheimer's disease (AD). Because of this, the KP may offer promising directions for the development of novel and precise treatment options for individuals experiencing metabolic dysregulation and cognitive challenges. The overarching aim of this thesis was to characterise the relationship between the KP and AD risk by profiling a variety of minimally invasive biofluids from healthy adults across the lifespan, as well as older adults at risk of AD. This aim was interrogated across three studies that are presented in Chapters 2, 3, and 4, and bookended by a general introduction and general discussion (Chapters 1 and 5, respectively). Chapter 2 systematically mapped the state of the targeted metabolomics literature and meta-analysed normative data for tryptophan (TRP) and kynurenine (KYN) quantified from the serum and plasma; Chapter 3 applied a validated analytical method to quantify the major biologically active KP metabolites of healthy adults in a range of human biofluids; and Chapter 4 analysed the full suite of KP metabolites in the serum and urine of older adults with and without risk of AD to assess their responsivity to treatment from interventional data. A major outcome from this thesis was to generate normative data on the KP, as achieved in Chapters 2 and 3. The normative metabolite concentrations reported in these studies may serve as an essential component of developing routine diagnostic tests for inflammation-based diseases as we move towards incorporating KP into point-of-care testing. Chapter 2 also sought to contextualise the KP research landscape by mapping study characteristics and evaluating studies in terms of analytical rigour and methodological validation; and determine the relationship between metabolite, sample matrix, biological sex, participant age, and study age. The findings reported in Chapter 2 improve the availability and quality of the data summarising the normative KP profile. Chapter 3 utilised correlation analyses to calculate the strength of association between all major biologically active KP metabolites across serum, plasma, saliva, and urine. A total of three significant and novel relationships were found involving saliva and urine, including a negative correlation between anthranilic acid (AA) in serum and urine. Chapter 4 sought to expand on the findings of Chapter 3 by examining the theragnostic potential of KP markers in relation to cognitively healthy older adults who were considered at risk of AD and undergoing curcumin therapy. Findings indicate that KYN levels within the serum were found to increase as neocortical amyloid load increased in the brain in females, and cognitive testing scores were found to improve as concentrations of TRP in serum and AA in urine increased. The findings regarding

AA are in line with those reported in Chapter 3, which showed that AA levels in the serum decrease as urine levels increase, suggesting that excreting AA via the urine may be protective in terms of cognitive outcomes. Within-group analyses showed that these correlations occurred within the curcumin treatment group only, and not the placebo group, regardless of AD risk. Taken together, this suggests that serum TRP and urine AA may be useful proxy markers of cognitive function and may have utility in monitoring response to treatment in future interventional studies. Collectively, the findings reported in this thesis make an important contribution to the development of point-of-care testing for diagnostic, prognostic, and theragnostic purposes in an AD-risk setting. The outcomes seed new directions for pragmatic biomarker discovery in the fields of AD prevention, risk reduction, and the monitoring of responsivity to treatment.

Chapter 1: General Introduction

Foreword:

In this chapter, an introduction to detecting and monitoring Alzheimer's disease risk via non-invasive or minimally invasive biomarkers is provided. A general overview of Alzheimer's disease aetiology and pathophysiology is described, as well as population incidence in an Australian setting. The role of the kynurenine pathway and its major metabolites in Alzheimer's disease pathophysiology is outlined. Diagnosis of Alzheimer's disease is described in terms of possible biological markers for disease, and the potential prognostic, diagnostic, and theragnostic role of the kynurenine pathway biomarkers is argued.

1.1 Dementia and Alzheimer's Disease

Dementia is a clinical syndrome involving impairments in cognition, such as executive function, perception, memory, and language, and reduced functional independence [1], resulting in significant individual, social, healthcare, and economic burden of disease [2, 3]. In 2021, it was estimated that between 386,200 and 472,000 Australians were living with dementia, and this number is projected to increase to 849,000 by 2058 [4]. Due to the increasing prevalence of dementia, the Australian economy will incur a cost of over one trillion dollars in the next four decades [1]. Of the projected dementia diagnoses in Australia over the next forty years, it is estimated that between 50–70 % of cases will be attributed to Alzheimer's disease (AD) [5, 6]. The dementia syndrome for AD is hallmarked by deficits in episodic memory (event-based memory), and the pathophysiological process underlying this initiates long before these clinical symptoms emerge forming what is known as the preclinical asymptomatic and prodromal symptomatic phases of AD, the latter of which is referred to as mild cognitive impairment (MCI) [7].

1.2 Alzheimer's Disease Aetiology and Pathophysiology

AD may be described as either familial or sporadic. Familial AD has been attributed to approximately 1 % of all AD cases, and is typically associated with the relatively younger onset of the disease [8]. Familial AD is caused by the inheritance of autosomal dominant mutations of *PSEN1*, *PSEN2*, or *APP* genes, that encode proteins presenilin 1, presenilin 2, and amyloid precursor protein, respectively [9]. Over 95 % of AD cases are attributed to sporadic AD, which typically occurs after 65 years of age [9]. Sporadic AD has been linked to non-modifiable and modifiable risk factors. Age and the high-risk genetic variant apolipoprotein E-ε4 (*APOE-ε4*) are non-modifiable risk factors of concern [10]. Relative risk of AD with *APOE-ε4* is associated with a dose-response, where an increasing number of *APOE-ε4* alleles can increase the relative risk of AD onset from 20 % to 90 %, and reduce mean age at onset from 84 to 68 [11]. Recent genome-wide association studies have also identified nine other genetic loci associated with sporadic AD, including *ABCA7*, *EPHA1*, *CD33*, *CD2AP*, *MS4A4* and *MS4A6E*, *PICALM*, *CLU*, *BIN1*, and *CR1* [12]. Despite identifying these loci, the genetic effect attributable to individual loci is approximately 50 %, indicating that there may be additional risk genes linked to sporadic AD that have not yet been identified [12].

While familial and sporadic AD differ in disease aetiology, they are thought to share some overlapping pathophysiology. The assumption of shared pathophysiology is particularly in relation to amyloid-beta [Aβ] in the context of the amyloid cascade hypothesis [13].

1.2.1 Major Proteinopathies in AD

The amyloid cascade hypothesis is concerned with Aβ peptides, which are metabolic products consisting of 35 to 43 amino acids [14]. Aβ plaques are primarily composed of the extracellular aggregation of Aβ peptides, which are produced as a by-product of the enzymatic cleavage of amyloid precursor protein (APP) [14-16] via the amyloidogenic or non-amyloidogenic pathway [14] (Figure 1.1). While the Aβ40 isoform is the most common, the Aβ42 isoform exhibits greater neurotoxicity and a greater tendency to aggregate and form Aβ plaques [14, 17]. Although Aβ is present in healthy ageing brains, it is thought that the elevation of the Aβ load, coupled with a greater ratio of neurotoxic Aβ42 to Aβ40, is linked to cognitive decline, neurodegeneration, and the onset of AD [18].

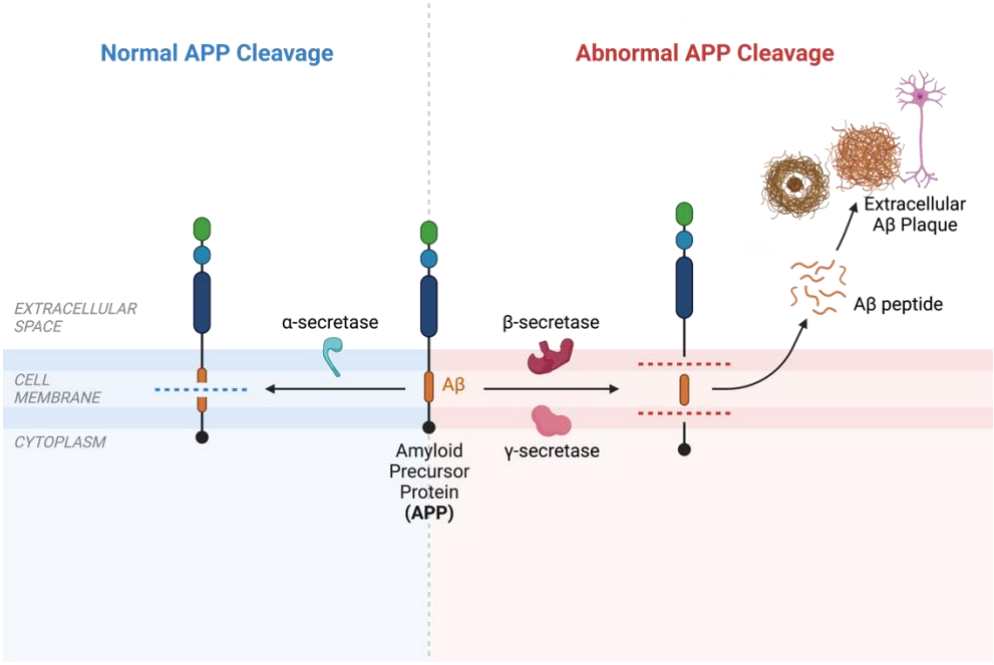

Figure 1.1. *Schematic depiction of APP cleavage, adapted from Querfurth & LaFerla (2010). In the amyloidogenic pathway, APP is cleaved at the catalytic core by β-secretase BACE1 to produce C99 and sAPPβ. C99 is then digested by γ-secretase to form Aβ peptides and amyloid intracellular domain [14]. In the non-amyloidogenic pathway, APP is cleaved by α-secretase to generate sAPPα and C83. C83 is then cleaved by γ-secretase to release amyloid intracellular domain and extracellular p3 [14].*

The amyloid cascade hypothesis for AD was officially proposed by Hardy and Higgins in 1992 [13]. According to this hypothesis, neurofibrillary tangles would then follow as a direct result of the deposition of Aβ plaques [13]. Neurofibrillary tangles are largely composed of hyperphosphorylated filaments of the tau protein, associated with assembling microtubules [13, 19]. Hyperphosphorylation of insoluble tau results in the proteins polymerising into paired helical filaments and forming cytotoxic intracellular neurofibrillary tangles [13, 14, 19, 20]. Neurofibrillary changes exhibit a well-defined distribution pattern throughout the AD brain [21]. Together with Aβ plaques, neurofibrillary tangles may initiate gliosis, a process where astrocytes and microglia in close proximity to the plaques proliferate in efforts to mediate neurotoxicity [22].

The amyloid cascade hypothesis has dominated therapeutic developments for AD for the past three decades [23-25]. However, the role of the amyloid cascade hypothesis in AD clinical outcomes has been hotly contested. Recent findings have brought into question the diagnostic (and therapeutic) value of Aβ, as anti-Aβ drugs and drugs which seek to reduce Aβ production only show modest clinical benefits in people with AD [26, 27]. Additionally, anti-Aβ drug semagacestat was linked to significant adverse events, including skin cancer and infections [28]. It has been hypothesised that Aβ may interact with various other pathological factors, as reviewed (for example) in the neuroinflammation hypothesis [29], the tau hypothesis [30], the oxidative stress hypothesis [31], the cholinergic hypothesis [32], and the metabolic dysfunction hypotheses [33, 34] (Figure 1.2). It is reasonable to suggest that Aβ may be acting in parallel with other pathophysiologies that commonly co-occur such as alpha-synuclein [27, 35], and may not be an accurate indicator of clinical relevance.

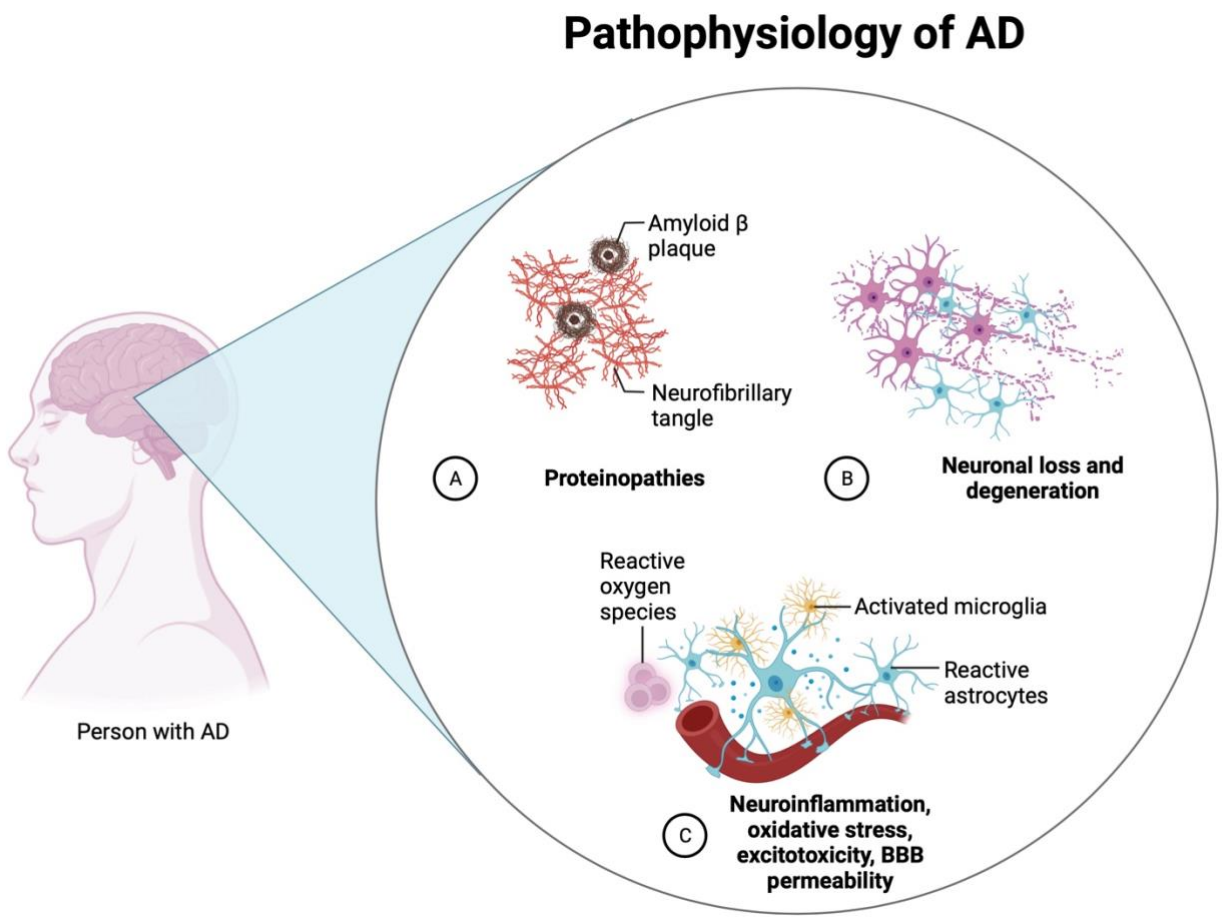

Figure 1.2. *Schematic depiction of AD pathophysiology, adapted from on Ji et al., (2013). Stage A describes the formation of Aβ plaques and neurofibrillary tangles, where abnormal levels can collect between and within neurons and impair cell function (Stage B). Stage C describes a series of immunological mechanisms observed in AD, including neuroinflammation, oxidative stress, and excitotoxicity. Microglia are the main immunocompetent cells of the central nervous system and play a key role in these processes. It is well known that the motile processes of microglia act to survey the local brain parenchyma. Recent evidence suggests that the communication between non-activated microglia may regulate the number of functional synapses in the central nervous system and modulate synaptic activity in this way [36, 37].*

1.2.2 Neuroinflammation, Oxidative Stress, and Excitotoxicity in AD

Neuroinflammation is a key element of AD pathophysiology where immunological mechanisms interact with proteinopathies such as Aβ and neurofibrillary tangles [38, 39]. Once activated by pathologies such as Aβ plaques and neurofibrillary tangles, microglia migrate to the site of the trigger to initiate an innate immune response as directed by pathogen-associated

molecular patterns or danger-associated molecular patterns [40]. The initial pro-inflammatory response is helpful in initiating phagocytosis and microglia to clear the proteinopathy debris [41, 42]. However, the excessive deposition of the Aβ plaques and neurofibrillary tangles ultimately compromises the microglial plaque clearance mechanisms, therefore contributing to the progression of neuroinflammation and AD pathophysiology.

The chronic activation of glial cells results in the release of toxic by-products such as reactive oxygen species and nitric oxide [43]. The high concentrations of nitric oxide have been linked to neuronal toxicity and this upregulation has been observed in the AD brain [44, 45]. Oxidative stress has been linked to synaptic loss and neurodegeneration in AD through the formation of non-nitrated Aβ [46-48]. This oxidative stress is linked to the accumulation of Aβ plaques and neurofibrillary tangles [49, 50]. Chronic oxidative stress promotes neuroinflammation, leading to the increased deposition of Aβ plaques and neurofibrillary tangles [51]. In turn, inflammatory mediators may stimulate the upregulation of β-secretase BACE1 and subsequent APP processing, creating a vicious pathophysiological cycle [52].

Moreover, many of the previously mentioned pathophysiologies and disease-risk genes may contribute to excitotoxicity, which is defined as the loss of neurons due to decreased energy production and subsequent overexcitation of the neuron [53]. These alterations lead to the neurotoxicity of glutamate and the subsequent overexcitation of the N-methyl-D-aspartate (NMDA) receptors in a tonic manner [53]. The excitability that occurs as a result of excessive NMDA activation may enhance the vulnerability of local neurons in AD, leading to neuronal loss and degradation [54, 55]. Together, the neuroinflammation, oxidative stress, and excitotoxicity accounts for AD provide strong evidence for the role of immunological perturbations in disease pathophysiology [39].

1.3 The Kynurenine Pathway

Recent findings have demonstrated that various metabolic pathways may contain promising diagnostic, prognostic, and therapeutic targets for major neurodegenerative diseases [34]. The metabolism of the essential amino acid tryptophan is one such pathway. Tryptophan, an essential amino acid obtained through dietary sources, is responsible for the de novo synthesis of the neurotransmitter melatonin and its precursor serotonin. These neurotransmitters play a key role in the sleep-wake cycle and mood control. Specifically, the role of melatonin is well-characterised in modulating dopamine release and reuptake in the sleep-wake cycle, while serotonin plays an integral role in emotional regulation [56]. The production of serotonin and melatonin is highly dependent on the levels of available tryptophan

and the functionality of the kynurenine pathway (KP), which plays a key role in metabolising the amino acid. As part of the KP, tryptophan is catabolised into the metabolite kynurenine. The neuroactive substances produced by this pathway may be neuromodulatory, immunomodulatory, or vasoactive in effect (Figure 1.3).

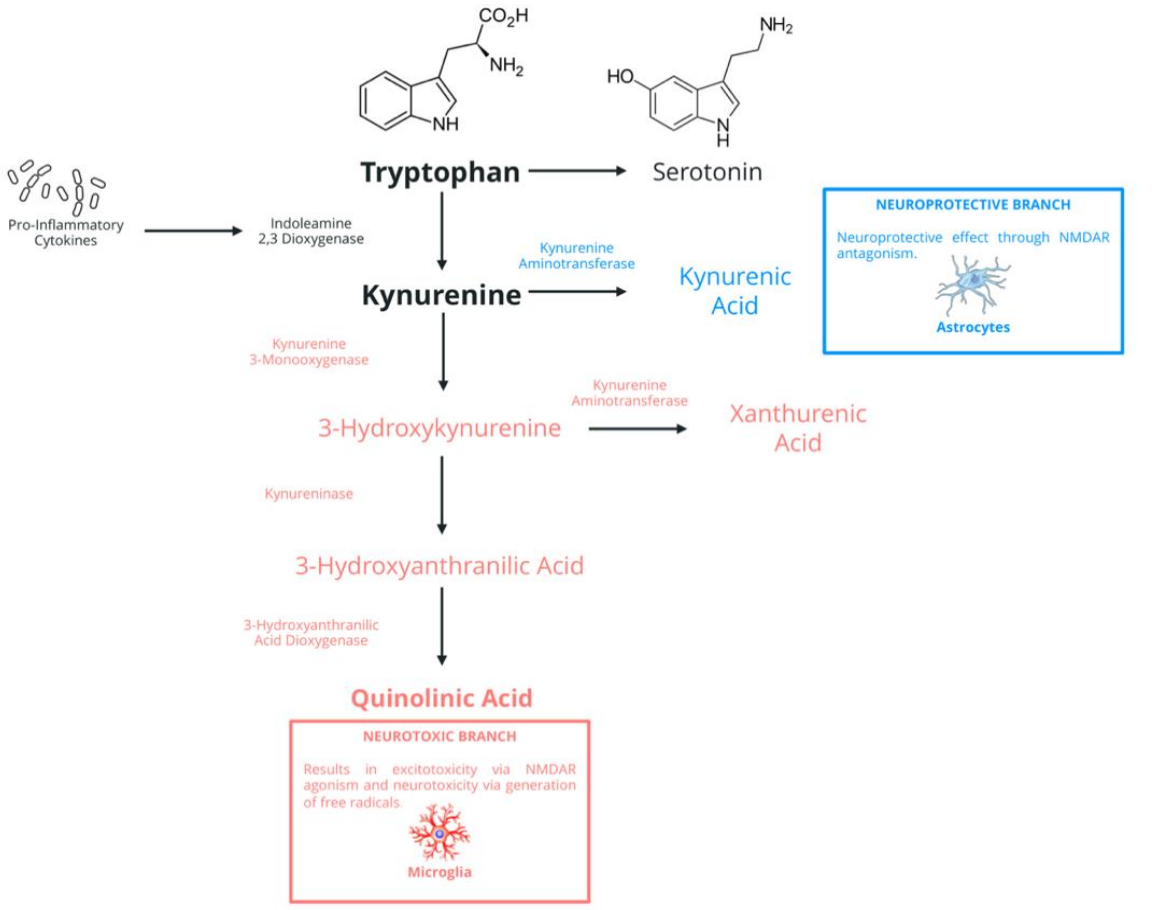

Figure 1.3. *Abbreviated schematic depicting the neurotoxic and neuroprotective branches of the KP, adapted from Tan et al. (2012). The KP produces many biologically active molecules including 3-hydroxykynurenine (3-HK), quinolinic acid (QA) and kynurenic acid (KA). These molecules act on N-methyl-D-aspartate receptors (NMDAr) to provide neuroprotective or neurotoxic effects, and are regulated by rate-limiting enzymes indoleamine 2,3-dioxygenase (IDO) and Trp 2,3-dioxygenase [37, 57]. An abbreviated depiction of the neuroprotective branch is illustrated in blue, while the neurotoxic branch is illustrated in red.*

Tryptophan metabolism and the KP have been proposed as the link between neurological dysfunction, inflammation, and neurotoxicity [58]. Neuroinflammation has been directly linked to tryptophan metabolism [58]. The presence of pro-inflammatory cytokines,

including interferon-gamma (IFN-γ), have been observed to mobilise the metabolism of tryptophan toward the KP as opposed to neurotransmitter synthesis via the activation of rate-limiting enzyme IDO [58, 59]. The upregulation of this pathway results in the increased production of neurotoxic metabolites, including QA, an agonist of the NMDA receptor, and the decreased production of neuroprotective metabolites, including KA (Figure 1.3). The KP-mediated changes in neurotransmitter levels observed in pro-inflammatory environments indicates that there is a link between the KP, excitotoxicity, inflammation, and neurotransmission that is yet to be fully investigated in the context of biomarker discovery.

1.3.1 The Kynurenine Pathway in AD

The KP is the subject of increasing efforts to understand the role of neuromodulatory metabolites in AD pathophysiology [59]. Specifically, mounting evidence has linked QA-associated neuroinflammation to the development and onset of AD [59] (Figure 1.4). KA also exhibits strong immunomodulatory functions. While largely considered neuroprotective, the actions of KA vary depending on whether inflammatory or homeostatic conditions are present [60]. While QA and KA are considered the most relevant KP metabolite in terms of biological neurotoxicity [61], there are various other neuroactive metabolites in the KP that have been implication in neuropsychiatric and neurodegenerative diseases. For example, 3-HK is considered a free-radical generator and neurotoxic compound as it plays a key role in oxidising interacting molecules [62]. 3-hydroxyanthranilic acid (3-HAA) exhibits various immunomodulatory effects and generates free-radicals. This metabolite readily auto-oxidises molecules to generate highly reactive species [63]. Alongside 3-HK and 3-HAA, anthranilic acid (AA) also possesses redox-modulating activity [64]. It is important to note that various KP metabolites may act to produce an additive immunomodulatory effect [65], which supports the case for using these neuromodulatory metabolites as measures of AD risk and progression.

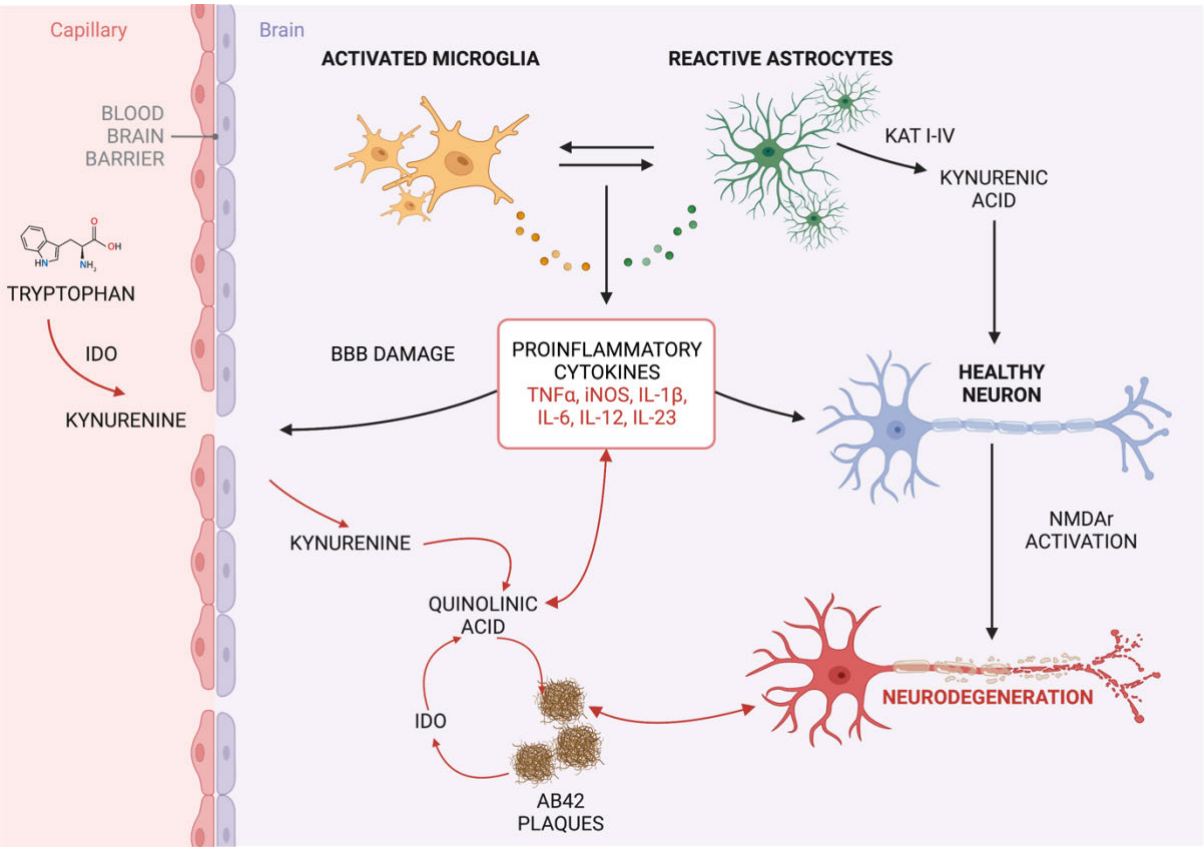

Figure 1.4. *Schematic depiction of the role of the KP in AD pathophysiology, adapted from Guillemin et al. (2003). The accumulation of neurotoxic QA, as well as the increased expression of rate-limiting enzymes such as IDO, has been well-documented in the AD brain [66-69]. Transgenic mouse studies and human studies have shown that rate limiting KP enzymes are often co-localised with these proteinopathies [67, 68]. Further, Aβ42 has been shown to induce IDO, therefore resulting in a significant increase in the production of QA by microglia and macrophages [70, 71]. The formation of tau and neurofibrillary tangles have been observed to increase in a dose-dependent manner following exposure to QA [72]. QA also has a role in inducing the expression of several chemokines and pro-inflammatory cytokines, therefore amplifying the pro-inflammatory cascade [71].*

Furthermore, the link between KP metabolites, neuroinflammation, and neurodegeneration is observable within the peripheral metabolome. These KP metabolite changes have been directly linked with biomarker evidence for neurodegeneration [73]. For instance, a recent study implicated an interaction between KP, neurofilament light chain (NFL; a biomarker for neurodegeneration), and traditional AD hallmarks. Specifically, NFL and Aβ42 were positively correlated with the kynurenine to tryptophan ratio, reflecting significant IDO upregulation [73]. When participants in this research were stratified by their neocortical Aβ load (NAL), which is considered one indicator for AD risk, associations between KP metabolites and Aβ in the plasma were pronounced in individuals with high NAL [73]. This growing body of evidence has inspired drug development initiatives to inhibit rate-limiting enzymes within the KP for multiple diseases including AD [59]. In turn, this also raises the prospect for further biomarker discovery initiatives for the KP in AD.

1.4 Biomarker Discovery and AD Diagnosis

There are many potential targets within AD's pathology for developing biological markers (biomarkers). The term biomarker refers to biological characteristics that may be objectively evaluated and used as an indicator of normal and pathogenic biological process [74]. In cases of AD, pathophysiological abnormalities precede clinical symptoms and are detectable by *in vivo* biomarkers. Currently, five AD biomarkers have been validated for inclusion in diagnostic criteria, largely concerned with Aβ plaques and tau-related neurodegeneration; three of which are imaging measures and two are CSF fluid analytes (Table 1.1) [75].

Table 1.1. Validated AD biomarkers that may be used modern in diagnostic criteria.

Biofluid or Tissue	**Biomarker**	**Method of Detection**	**Evidence for Use**
CSF	Aβ42	Spinal Tap	→ AD diagnosis has had a focus on disease fluid biomarkers and Aβ due to the predominance of the amyloid cascade hypothesis [76]. → CSF Aβ42 has an inverse relationship with amyloid plaque load [77, 78].
Brain	Aβ42	Positron Emission Tomography (PET)	→ Pittsburgh Compound B (PiB), a thioflavin T, has been used in PET scans to image Aβ in neuronal tissues. → People with AD show a marked retention of PiB in areas containing large amounts of amyloid deposits, such as the association cortex. → PiB retention is typically increased within the frontal, parietal, temporal, and occipital cortices, as well as the striatum [79]. → In cortical areas, PiB retention is correlated inversely with F^{18} fluorodeoxyglucose (FDG) [79]. → PET studies are considered the gold standard and are increasingly being used to aid early detection of dementia, or where a clinical diagnosis is challenging [80, 81].
Brain	Glucose Hypometabolism	PET	→ Metabolism, including glucose uptake, is reduced in AD [81]. → Multiple PET studies which have used the glucose metabolic tracer, FDG [81]. These studies have identified distinctive patterns and metabolic abnormalities within the AD brain.

			→ PET studies have shown that people with AD demonstrate a characteristic pattern of glucose hypometabolism temporo-parietally, with hypermetabolism occurring frontally as a compensatory mechanism [80, 82, 83]. → FDG PET has been able to distinguish the condition from healthy controls with 93 to 94 % sensitivity and specificity ranging between 93 to 99 % [84, 85].
CSF	p-tau and t-tau	Spinal Tap	→ The final major category for AD biomarkers is concerned with tau-related neurodegeneration [75, 76]. → The first major biomarker is concerned with increased levels of CSF phosphorylated tau (p-tau) and total tau (t-tau) [86].
Brain	Neurodegeneration	Magnetic Resonance Imaging (MRI)	→ The second major AD biomarker is concerned with measuring brain atrophy through structural MRI studies [87].

While these biomarkers have been validated for the inclusion in AD diagnostic criteria [88], there has been a shift in preference to move away from invasive, potentially risky procedures in diagnosing and managing chronic conditions [89]. This is especially the case in older adults, who are particularly vulnerable to minor stressor events such as spinal taps [90]. Furthermore, there have not yet been any cost-effectiveness analyses for the appropriate use of neuroimaging in the diagnosis of dementia published within the literature [91]. While PET scans have high diagnostic accuracy, health economics studies have indicated that adding it to the standard diagnostic protocol for AD would yield limited benefit in exchange for high costs [92]. The high cost of these diagnostic measures is reflected in Australia's response to dementia across 2018–2019, where $3 billion in spending was directly attributed to dementia. Specifically, spending on hospital services amounted to $383 million, while spending out-of-hospital medical services amounted to $99.2 million [4]. Accessibility has also been perceived as a significant barrier to medical care by older adults, specifically those that are homebound, regional, and remote [93, 94]. Travel-specific considerations include lack of public transportation, lack of wheelchair accessible vehicles, high cost of transportation services, and remoteness [93].

A potential avenue in this field is point-of-care testing (POCT). POCT is defined as medical diagnostic testing that is performed at the time and place of patient care being delivered. A key example of POCT is the application of personal glucometers to measure blood glucose [95]. It has been proposed that future analytes for POCT may include low-molecular weight metabolites, although a core limitation is bioanalytical performance [96]. The risk posed by invasive procedures, coupled with the health economics perspective and accessibility issues, stress the need for non-invasive or minimally invasive biomarkers for AD diagnosis, monitoring responsivity to treatment, and risk measurement; promising avenues include blood, urine, and saliva. Developing these biomarkers may improve patient outcomes and lessen the economic burden of medical diagnostics, hence alleviating the projected cost of over one trillion dollars incurred by the Australian government in the next four decades [1].

Several blood plasma biomarkers for AD have been identified, including Aβ, tau protein, and neurofilament light chain (Table 1.2) [76]. These findings support the investment into identifying a minimally invasive, cost-effective blood-based biomarker for AD [97-99]. It is essential to mention that plasma biomarkers also have cost-benefit advantages, as well as promising scalability, meaning that healthcare services may cover costs and thus the general public may have broader access [99].

Table 1.2. Current evidence for minimally and non-invasive biomarkers for AD.

Biofluid	Biomarker or Assay	Evidence for Use
Plasma	Aβ42:Aβ40	→ Recent clinical trial data suggest that the antibody-based removal of cerebral Aβ plaques may facilitate the clearance of tau tangles and slow cognitive decline [100]. → The difference in the plasma ratio of Aβ42 to Aβ40 in Aβ-positive and Aβ-negative individuals is modest, indicating that it may not be an efficient tool [99, 101-103].
Plasma	p-tau	→ The increase in phosphorylated tau-181 concentrations in the plasma is approximately threefold, showing high diagnostic accuracy for AD (ranging from 85 to 95 %) [100, 104, 105].
Urine	Genetic Analysis	→ A recent study has coupled experimental and computational methods to identify urine-based biomarkers for AD. → Tissue-based gene expression data within the brain were analysed to identify genes that may encode urine-excretory proteins that might act as candidate protein biomarkers for AD. → Three genes were differentially expressed in the urine of AD patients (secreted phosphoprotein-1; gelsolin; insulin-like growth factor-binding protein 7) [106]. → A recent transgenic mouse model (APP(swe)/PSEN1dE9) found thirteen proteins in the urine which may enable the detection of AD prior to Aβ deposition [107].
Saliva	p-tau, t-tau, Aβ, and Alpha-Synuclein	→ Saliva is also a rich source of potential biomarkers for AD detection and offers several practice advantages over CSF taps and venepuncture. → Total tau, phosphorylated tau, Aβ, and alpha-synuclein proteins have all been detected within the saliva during preliminary investigations [108].

Despite these promising biomarkers, developing accurate and specific blood biomarkers for AD is challenging, largely attributed to the fact that the peripheral circulation is not continuous with the brain extracellular fluid due to the blood-brain barrier and therefore does not experience the free exchange of molecules [76]. For instance, CSF levels of neuronal tau are measured at approximately 300 pg/mL, while plasma levels are measured at 5 pg/mL [109]. Further, the small concentrations of brain proteins which can enter the blood must be quantified in a medium containing high levels of common plasma protein, which introduces significant risk of interference during analytical detection. The minute concentrations of brain proteins present in the blood are also susceptible to being filtered by the kidneys, metabolised in the liver, or degraded by proteases. Together, these factors introduce variability that is difficult to account for in analytical or clinical studies [109, 110]. This is in comparison to metabolomics, which measures the unique biochemical profile within specific sample matrices and is not limited to describing the protein composition of the matrix. Minimally invasive routes, including biomarker identification in urine and saliva, are less studied (Table 1.2).

Although these core CSF and plasma biomarkers reflect the central pathophysiology of AD, the pathogenic heterogeneity of the disease, and often unspecific nature of the clinical symptomology, presents diagnostic difficulties [76]. There are also difficulties in correlating primary biomarker data with the clinical syndrome and disease progression [35]. For these reasons, there is an urgent need for new accurate and specific biomarkers to act as diagnostic tools across the AD continuum [76]. For instance, it is hypothesised that evidence of a subtle cognitive decline forms the final stage of pre-clinical AD, suggesting that the individual is approaching the border zone with MCI, which further increases AD risk [7, 111, 112] [113]. Therefore, identifying, understanding, and characterising these early disease phases before symptom onset is essential for early diagnosis and preventing future deterioration and decline.

1.5 Developing KP Biomarkers for AD

Developing and validating KP-specific biomarkers for AD poses many challenges with respect to the current literature published within the field, as there is extensive variability in terms of cohort characteristics and samples studied. It is essential to quantify these differences, as the KP metabolic complement is known to be affected by numerous factors. For example, sex disparities in KP metabolism have been observed due to the differential actions of gonadocorticoids [114]. This has been exemplified in a recent study exploring the serum concentrations of KP metabolites including TRP, KYN, 3-HK, 3-HAA, AA, QA, and picolinic

acid (PA) in older adults. All participants were recruited from the Kerr Anglican Retirement Village Initiative in Ageing Health (KARVIAH Cohort), had normal global cognition, and were categorised into groups based on their neocortical amyloid load (NAL). The low NAL group (NAL-; $n = 65$) and the high NAL group (NAL+; $n = 35$) were grouped using a standard uptake value ratio (SUVR) cut-off of 1.35. Findings showed that higher levels of kynurenine and neurotoxic AA were found in the female NAL+ subset [114]. This highlights both the influence of biological sex in AD pathogenesis, and the role of these KP metabolites as potential blood biomarkers for AD. Moreover, a 2021 study by Whiley and colleagues reported a reduction in the bioavailability of KP metabolites in the urine of individuals living with AD. Specifically, data indicate significantly lower concentrations of 5-hydroxyindoleacetic acid (5-HIAA), KA, TRP, xanthurenic acid (XA), and the TRP/KYN ratio. A decreasing trend in concentrations was observed in order of clinical diagnosis (healthy to MCI to dementia) [115], supporting the use of the KP as a measure of AD risk. This thesis will go on to further explore the KARVIAH Cohort dataset in Chapter 4.

Concentrations of KP metabolites within the saliva of AD patients are lesser known. Nonetheless, KP metabolites are detectable within the saliva and have been measured in clinical cohorts, including diabetic patients [116, 117]. It is also of interest to note that the KP profile varies within samples obtained from individual participants. Studies have found that the information contained in peripheral samples, such as urine and serum, are statistically independent [118]. It is strongly suggested that future research should seek to control for and clarify factors such as sex differences, lifestyle factors, and inter-sample variation when investigating the role of the KP in AD risk.

In recognition of the importance of the KP in improving health outcomes, the relationship between the KP and AD risk should be specifically determined. Mapping the sophisticated metabolic signature of the KP across sample types and cohorts may:

(i) Inform our understanding of underlying molecular mechanisms of AD, allowing the sub-classification of diseases according to the metabolic phenotype of the affected individual;

(ii) Provide precise prognostic and diagnostic biomarkers; and

(iii) Reveal appropriate biomarkers for a specific and targeted drug response [119].

1.6 Thesis Aims and Objectives

Compelling evidence has identified the KP as an avenue to predict, diagnose, and measure the progression of neurodegenerative diseases [120]. This provides promising directions for the development of novel and precise treatment options for individuals experiencing metabolic dysregulation and cognitive challenges. However, the complex interrelationship between the KP and AD risk has not been characterised, in part due to a lack of understanding of:

i) How normal KP function is characterised across the lifespan;

ii) A lack of evidence from 'at-risk' cohorts; and

iii) The heterogeneous and unvalidated methods used and the incomplete datasets available.

This thesis will address these research gaps by developing and applying validated targeted metabolomics approaches to assess the diagnostic accuracy of the KP as a non-invasive biomarker for AD risk across various sample types and cohorts. Outcomes from this research aims to show or predict the relationship between the KP and AD risk. There are three aims to this thesis that will be addressed across three studies:

Aim 1: Appraise and characterise the existing literature on how KP metabolites are measured and establish a set of normative data;

Aim 2: Identify and develop a validated analytical method to quantify KP metabolites in the liquid biopsies of healthy adults in a range of human biofluids going beyond blood samples; and

Aim 3: Analyse KP metabolites in the serum and urine of older adults at risk and not at risk of AD and assess their responsivity to treatment from clinical trial data.

Aim 1 will be carried out in the form of a systematic review and meta-analysis of KP function in healthy individuals from case-control and methodological validation studies (Chapter 2); Aim 2 will be investigated in the form of an empirical study conducted on the plasma, serum, saliva, and urine from healthy adults (Chapter 3); and Aim 3 will be investigated using archival data obtained from older adults participating in a clinical trial from the KARVIAH Cohort classified as at-risk and not-at-risk of AD via amyloid PET scan (Chapter 4).

The overarching aim of this thesis is to profile a variety of non-invasive and minimally invasive biofluids from healthy adults across the lifespan, as well as older adults at risk of AD, to characterise the relationship between the KP and AD risk. The purpose of this is to seed new directions for pragmatic biomarker discovery in the fields of AD risk prediction and the

monitoring of responsivity to treatment. As an additional note, this thesis will benefit from using analytical methods which have been objectively validated for diagnostic accuracy, and controlling for sex differences, lifestyle factors, and inter-sample variation.

Chapter 2: Validating the Instruments and Methodologies Used for the Quantification of Tryptophan and Kynurenine in Human Biofluids: A Systematic Review and Meta-Analysis

Foreword:

In this chapter, a normative dataset is generated from the published literature on the kynurenine pathway in healthy volunteer participants extracted from cross-sectional and methodological validation studies. Study characteristics were mapped, and studies were evaluated in terms of analytical rigour and methodological validation. Meta-analysis of variance between instruments, sample matrices, and metabolites was conducted. Regression analyses were applied to determine the relationship between metabolite, sample matrix, biological sex, participant age, and study age. The chapter is currently being prepared as a stand-alone journal article manuscript and first will be submitted to the preprint server *bioRvix*. A pilot study on the research conducted for this chapter was completed as part of a formative assessment for unit Experimental Design and Analysis B as part of the Master of Research degree.

Metri, N.J., Butt, A., Murali, A., Steiner-Lim, G.Z., Lim, C.K. (in preparation). Normative data on serum and plasma tryptophan and kynurenine concentrations from 8,089 individuals across 120 studies: A systematic review and meta-analysis. *bioRxiv.*

2.1 Introduction

Tryptophan (TRP), an essential amino acid, is largely derived from dietary proteins such as meat and dairy [121]. Peripheral concentrations of TRP are determined by the production of neurotransmitters serotonin and melatonin, and the balance between levels of dietary intake and the rate of removal from the plasma as part of protein biosynthesis [122]. Under normal physiological conditions, however, over 90 % of dietary tryptophan in the liver is metabolised along the kynurenine pathway (KP) into a variety of biologically active downstream metabolites (see Figure 2.1) [123, 124]. The KP has been implicated in an array of diseases, including depressive and psychotic disorders [125], neurodegenerative diseases [126], autoimmune diseases [127], cardiovascular diseases [128], renal diseases [129], metabolic disorders [130], gastrointestinal disease and distress [131], and cancers [132]. TRP metabolism and the KP have gained much interest in recent times given their role in modulating both innate and adaptive immune responses and inflammation [133, 134]. Given the link between the KP and multiple diseases, it has been suggested that the thorough analytical profiling of KP metabolites may improve patient care outcomes by revealing prognostic, diagnostic, and theragnostic biomarkers [135].

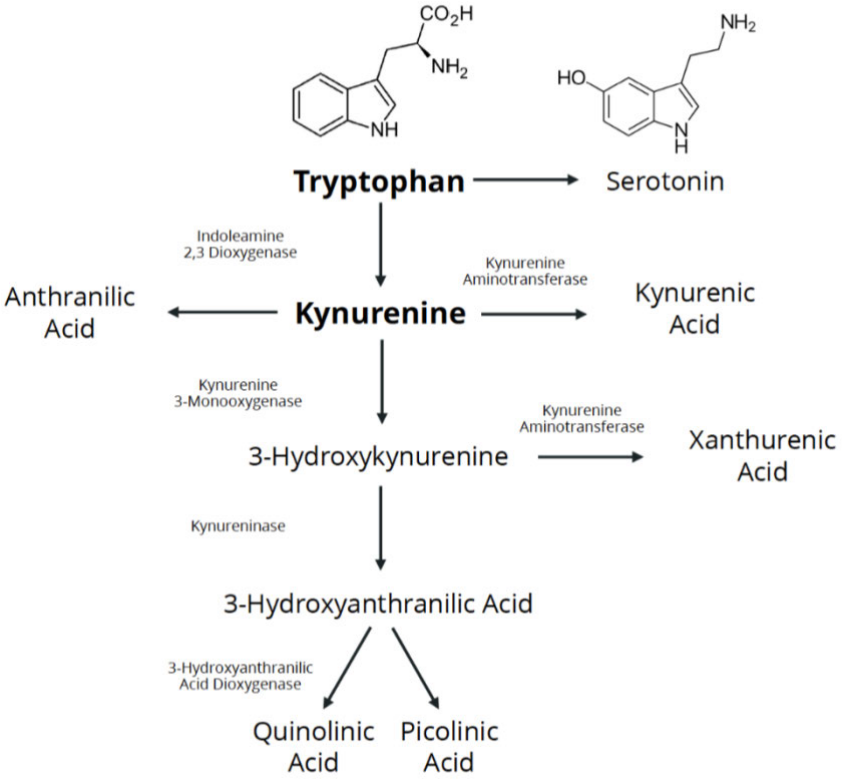

Figure 2.1. *Abbreviated schematic depicting the major metabolites and enzymes of the KP, adapted from Tan et al. (2012) [57].*

Meta-analytic evidence has shown extensive profiling of KP metabolites across various clinical cohorts within the plasma, serum, urine, and cerebrospinal fluid [125, 136-139]. This has been reported through various modes of detection (MODs), metabolites studied, sample matrices, and geographical locations. Despite the breadth of these data, there is a lack of information on healthy and younger cohorts (e.g., aged ≤ 50 years) with normal metabolic functioning. Inter-sample variation in KP profiling is also less characterised. For example, studies have found that the information contained in peripheral samples, such as serum and urine, are statistically independent [140]. Changes in the KP profile across the lifespan are even less characterised. These literature gaps are emphasised by the heterogeneity in methodological and analytical quality of the reported literature. Together, these factors mean that there is an absence of normative KP data, across the lifespan and across geographical regions, that is of high methodological and analytical rigour that may be referred to as a benchmark in future studies, clinical or otherwise.

The purpose of this study was to generate a set of KP normative values for the purpose of informing future biomarker investigations. This meta-analysis and systematic review therefore aimed to: (i) characterise and map the characteristics of studies reported within the literature, including MOD, metabolites studied, sample matrices, and geographical location; (ii) calculate normative means for TRP and kynurenine (KYN) across MOD, sample matrices, and geographical location; (iii) critically appraise the methodologies and data reported within the literature, in terms of author-reported methodological validation and through an independent risk of bias assessment; (iv) ascertain variance in sensitivity between MOD; and (v) determine the relationship between metabolite, biofluid, age, sex, and study year.

2.2 Methods and Materials

2.2.1 Study Protocol

This systematic review and meta-analysis was prospectively registered with the PROSPERO International Database of Systematic Reviews on 26 December 2021 (#CRD42021293595), and followed the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement (PRISMA) [141]. It is anticipated that this review will provide insight into the evidence required to inform the outcomes of biomarker development research.

2.2.2 Search Strategy

Initially, a scoping search was conducted using a comprehensive strategy to establish the breadth of the review on 15 November 2021. Based on this, a comprehensive search of articles was conducted on four electronic databases comprising PubMed, Web of Science, EMBASE, and Scopus. Databases were searched for articles from peer-reviewed journals from the databases' date of inception through to 27 December 2021. An example of the search algorithm from PubMed is described in Table 2.1. The same Medical Subject Headings (MeSH) and search strategy were applied across all four databases. However, minor adjustments were applied to allow for database-specific variations in the requirements for the search queries. Database alerts were set up to notify the authors of any new publications up until 1 October 2022, and citation searches of any reviews were performed.

Table 2.1. Population, Intervention, Comparison, Outcomes, Study Design (PICOS) framework illustrating the systematic search strategy for PubMed.

Interest
The use of liquid chromatography, mass spectrometry, or ELISA assay techniques. MeSH terms or other synonyms included "Chromatography, Liquid" OR "Mass Spectrometry" OR "Enzyme-Linked Immunosorbent Assay" OR "ELISA"
Outcome
The identification and quantification of tryptophan or kynurenine in human biofluids. MeSH terms or other synonyms included "Tryptophan" OR "Kynurenine"
Study Design
Cross-sectional cohort study. Data relating to the biofluids of healthy control groups will be extracted from clinical studies, or from analytical studies focused on healthy groups only. MeSH terms or other synonyms included "Blood" OR "Serum" OR "Plasma" OR "Urine" OR "Saliva" OR "Sebum" OR "Tears" OR "Sweat" OR "Cerebrospinal Fluid" OR "Feces" OR "Faeces"
Note: Interest, outcome, and study design searches were combined with the Boolean operator "AND". Comparison terms were not specified due to the broad nature of the review.

2.2.3 Study Selection

In total, 11,241 records were obtained from the systematic database search, indicating the feasibility of the review. All included studies were exported to a reference management

software (EndNote™ X9; Thomson Reuters, CA, USA). Using EndNote, duplicate references were removed, and remaining titles and abstracts were screened against study inclusion and exclusion criteria by the primary reviewer. The titles and abstracts of the remaining records were screened and if there was any doubt regarding their eligibility, the full text was retrieved for clarification. 20 % of articles deemed eligible to the primary reviewer were then confirmed by two independent reviewers to ensure inclusion criteria were met.

2.2.4 Eligibility Criteria

Inclusion criteria for clinical and/or analytical studies included: (i) quantifying KP metabolites, including TRP and KYN, in human biofluids including whole blood, blood serum, blood plasma, urine, saliva, sebum, tears, sweat, cerebrospinal fluid, and faeces via targeted metabolomic approaches; (ii) clinical case-control studies which included a healthy control group or analytical studies including only healthy adults with no known neurological, psychological, or medical condition; (iii) reported in the English language; (iv) using liquid chromatography (LC) coupled to mass spectrometry (MS), electron capture dissociation (ECD), spectroscopy, or enzyme-linked immunosorbent assays (ELISA); and (v) cross-sectional, observational studies with no intervention administered or methodological validation studies.

Exclusion criteria for publications included: (i) conference proceedings and papers, articles in press, editorials, letters, notes, short surveys, pre-prints, book chapters, and book series; (ii) publications that explore TRP or KYN identification and/or quantification in nutritional supplements, animals, food products, or following an intervention such as TRP loading or depletion; (iii) studies which explore TRP or KYN quantification in clinical cohorts only and did not include a healthy control group; and (iv) studies which utilise untargeted metabolomics due to potential imprecision in the metabolites characterised.

2.2.5 Data Extraction

Data extraction was then completed by the authorship team and double-checked by a second independent reviewer. Any disagreements were resolved by reviewing and discussing the articles with the research team. Study characteristics were extracting including author, year of publication, the instrument of detection, and the number of samples analysed. Concentrations of TRP and/or KYN in the biofluids of healthy adults were also extracted. In clinical studies, only data related to healthy controls were extracted and reported. The chromatographic conditions were extracted from eligible studies into standardised tables. To ensure the analytical rigour of the data, validation parameters of interest included: (i)

determining the robustness of the method by testing the variation in the instrumental parameters; (ii) limits of quantification (LOQ); (iii) limits of detection (LOD); (iv) precision; (v) accuracy of the method via recoveries for determining true values; (vi) assessment of the analyte's stability; (vii) calibration curve to test the response for a variety of standards; and (viii) the specificity such as matching the retention times of the samples and the standards [142].

2.2.6 Risk of Bias Evaluation

As not one appropriate tool was available to assess the methodological quality of studies, this was evaluated using a combination of tools, including the QUADOMICS tool [143], the QUADAS tool [144], and the Joanna Briggs Institute Checklist for Analytical Cross Sectional Studies [145]. The adapted tool consisted of six items which account for the particular methodological challenges posed by metabolomic approaches (Appendix B). Items were either scores as Yes, No, or NA/Unknown. Studies were then given an overall appraisal as either high quality or low quality by the authorship team.

2.2.7 Statistical Analyses

Studies were grouped via the MOD reported, by metabolite (TRP or KYN), by sample matrix, and by geographical region (America, Asia, Australia, Europe, Middle East). Findings from pooled data were compared by utilising established estimation and approximation methods reported in the literature [146]. Regarding this data set, this was mostly concerned with the conversion of median and interquartile range to mean and standard deviation (SD). Weighted mean (± SD) concentrations of TRP and KYN were calculated per MOD, sample matrix, and by geographical region reported. Variance in sensitivity between instruments was ascertained by a two-way ANOVA followed by Tukey's multiple comparison test. Only sample matrices with over 20 studies reported were included into the meta-analysis to ensure that there was enough statistical power to detect an effect. To determine the relationship between metabolite, biofluid, age, sex, and study year, an adjusted weighted-variance Ordinary Least Squares (OLS) regression analysis was performed. All statistical analyses were performed using GraphPad Prism v 9.0.0 (GraphPad Software, Inc., CA, USA) and Stata™ v17 (StataCorp, Texas, USA).

2.3 Results

The search strategy identified 11,241 publications. Following the application of the criteria, 531 full-text articles were assessed, of which 22.60 % (N = 120) met the inclusion criteria (Figure 2.2). Serum and plasma were the only sample matrices with over 20 studies reported for subsequent inclusion in the meta-analysis. Due to small sample sizes (and consequently a high level of variability), urine, CSF, saliva, tears, faeces, and whole blood studies were not synthesised and will not be discussed further.

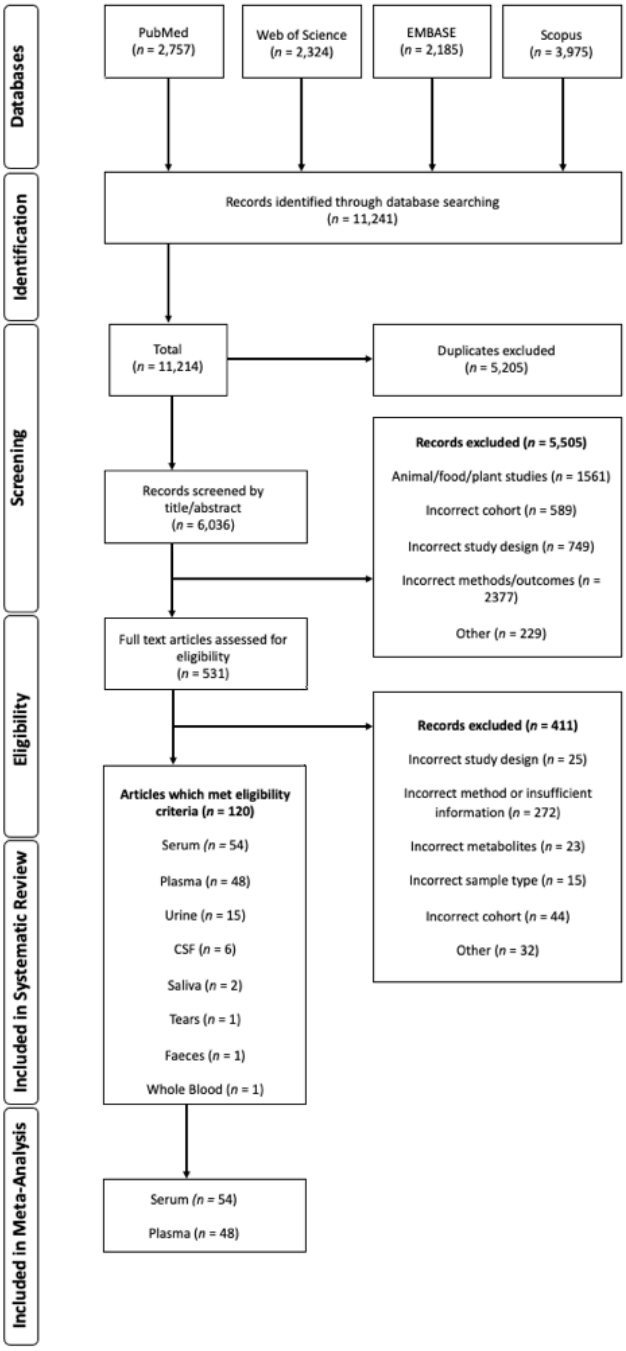

Figure 2.2. *Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram detailing the search, identification, screening, and eligibility processes.*

2.3.1 Study Characteristics

This meta-analysis reported on a grand total of 120 studies including 8,089 participants (*N* = 120; *n* = 8,089; Table 2.2). All citations are listed in Table 2.2. Studies were reported from across five regions, including America, Asia, Australia, Europe, and Middle East, and 31 countries in total. Studies were most reported from China (23.33 %; *n* = 28). The mean age across all studies was 47.35 ± 15.65 and 77.67 % of participants were female.

Regarding the quantification of TRP, a total of eight sample matrices were reported within the literature. This included serum (*n* = 54), plasma (*n* = 48), urine (*n* = 15), cerebrospinal fluid (CSF; *n* = 6), saliva (*n* = 2), tears (*n* = 1), faeces (*n* = 1), and whole blood (*n* = 1). A total of six sample types were reported in the quantification of KYN: serum (*n* = 34), plasma (*n* = 33), urine (*n* = 7), CSF (*n* = 6), saliva (*n* = 2), and faeces (*n* = 1). The most common MOD across metabolites reported within the literature included mass spectrometry (MS; *n* = 80). Other modes of detection reported include fluorescence detection (FL), ultra-violet visible spectroscopy (UV-Vis), ELISA, photo diode array (PDA), ECD, and electrochemiluminescence (ECL). The characteristics of the TRP and KYN quantification methods used in the included studies are described in Table 2.3, including MOD, column phase and diameter, particle size, mobile phases, flow rate, injection volume, column temperature, program time, and detection wavelength (where applicable).

Table 2.2. Study characteristics of included studies including the study type, mode of detection (MOD), country, specimen type, sample size, sex, and age.

Citation	Study Type	MOD	Country	Biospecimen	Population Size	Population Sex	Mean Age (SD)
Adrych et al., 2010 [147]	Clinical	MS	Poland	Serum	21	21M, 0F	34.00 (13.00)
Al Saedi et al., 2022 [148]	Clinical	UV-Vis + FL	Australia	Serum	28	4M, 24F	NR
Arnhard et al., 2018 [149]	Analytical	MS	Austria	Plasma	100	NR	NR
Bai et al., 2021 [150]	Clinical	ELISA	China	Urine	41	23M, 18F	66.83 (7.85)
Barry et al., 2009 [151]	Clinical	FL + PDA	Ireland	Plasma	36	26M, 10F	33.70 (6.60)
Bassi et al., 2017 [152]	Clinical	MS	USA	Urine	10	NR	NR
Bizzarri et al., 1990 [153]	Clinical	UV-Vis	Italy	Urine	9	NR	NR
Buczko et al., 2007 [154]	Clinical	UV-Vis	Poland	Saliva, Plasma	19	9M, 10F	61.00 (15.00)
Calvani et al., 2020 [155]	Clinical	MS	Spain, France	Serum	30	14M, 16F	74.60 (4.30)
Capuron et al., 2011 [156]	Analytical	UV-Vis + FL	France	Serum	284	87M, 197F	79.90 (4.50)
Chang et al., 2018 [157]	Clinical	MS	Taiwan	Plasma	82	40M, 32F	62.83 (12.66)
Chatterjee et al., 2019 [158]	Clinical	FL	Australia	Plasma	65	19M, 46F	77.61 (5.55)
Chen et al., 2019 [159]	Analytical	MS	China	Plasma	100	NR	NR

Chen et al., 2010 [160]	Clinical	UV-Vis + FL	Australia	CSF, Serum	35	NR	35.80 (3.00)
Chen et al., 2020 [161]	Clinical	MS	China	Plasma	75	32M, 43F	37.19 (9.66)
Cheng et al., 2015 [162]	Clinical	MS	China	Saliva	28	0M, 28F	NR
Clarke et al., 2009 [163]	Clinical	UV-VIS + FL	Ireland	Plasma	26	26M, 0F	32.20
Colle et al., 2020 [164]	Clinical	MS	France	Plasma	214	90M, 124F	45.50 (NR)
Crotti et al., 2019 [165]	Clinical	UV-VIS + FL	Italy	Plasma	5	NR	NR
Cseh et al., 2019 [166]	Analytical	MS	Hungary	Plasma	8	NR	NR
Curto et al., 2016 [167]	Clinical	MS	Italy	Serum	35	27M, 8F	44.80 (7.64)
Curto et al., 2016 [168]	Clinical	MS	Italy	Serum	84	15M, 69F	40.40 (9.43)
Domingues et al., 2015 [169]	Clinical	MS	Brazil	Plasma	38	NR	NR
Doolin et al., 2018 [170]	Clinical	MS	Ireland	Plasma	37	18M, 19F	80.86 (10.78)
Eniu et al., 2019 [171]	Clinical	MS	Romania	Serum	26	0M, 26F	NR
Fekkes et al., 1998 [172]	Clinical	FL	Netherlands	Plasma	17	17M, 0F	70.10 (1.30)
Fitzgerald et al., 2008 [173]	Clinical	FL	Ireland	Plasma	33	0M, 33F	41.30 (12.80)
Frick et al., 2004 [174]	Analytical	UV-Vis + FL	Austria	Serum	43	22M, 21F	66.3

Fukushima et al., 2014 [175]	Clinical	MS	Japan	Serum	27	12M, 15F	26.50 (5.60)
Furtado et al., 2017 [176]	Clinical	MS	Brazil	Plasma	18	7M, 11F	NR
Galla et al., 2021 [177]	Analytical	MS	Hungary	Urine	10	NR	NR
Geisler et al., 2015 [178]	Clinical	UV-Vis + FL	Austria	Serum	100	58M, 42F	49.00 (11.40)
Gevorkian et al., 2015 [179]	Clinical	ECD	USA	Plasma	140	72M, 68F	50.80 (8.80)
Girgin et al., 2020 [180]	Clinical	UV-Vis + FL	Turkey	Serum	30	20M, 10F	37.0 (1.30)
Gomez-Gomez et al., 2017 [181]	Clinical	MS	Spain	Urine	25	2M, 23F	NR
Gulaj et al., 2010 [182]	Clinical	UV-Vis + FL	Poland	Plasma	18	5M, 13F	76.17 (7.30)
Hajsl et al., 2020 [183]	Clinical	MS	Czech Republic	Plasma	25	14M, 11F	NR
Han et al., 2018 [184]	Clinical	MS	China	Serum	30	21M, 9F	NR
Henykova et al., 2016 [185]	Analytical	MS	Czech Republic	Serum	18	4M, 14F	NR
Huang et al., 2022 [186]	Clinical	MS	China	Serum	62	36M, 26F	43.40 (1.30)
Huang et al., 2021 [187]	Clinical	MS	China	Urine	40	NR	NR
Islam et al., 2020 [188]	Clinical	UV-Vis	Bangladesh	Serum	248	102M, 146F	NR
Jang et al., 2022 [189]	Clinical	MS	South Korea	Serum	35	32M, 3F	24.66 (2.99)

Kim et al., 2015 [190]	Clinical	MS	South Korea	Plasma	70	44M, 26F	63.20 (8.90)
Kim et al., 2009 [191]	Clinical	UV-Vis + FL	South Korea	Plasma	174	78M, 96F	32.49 (10.69)
Klatt et al., 2021 [192]	Clinical	MS	Australia	Serum	93	49M, 44F	NR
Koch et al., 1979 [193]	Analytical	ECD	USA	Urine, Plasma, Serum	11	NR	NR
Krasnova et al., 2000 [194]	Clinical	ECD	Russia	Serum	10	NR	NR
Kurgan et al., 2022 [195]	Clinical	MS	Turkey	Saliva	20	8M, 12F	39.60 (6.70)
Leichtle et al., 2012 [196]	Clinical	MS	Germany	Serum	58	26M, 32F	NR
Li et al., 2011 [197]	Clinical	FL	China	Serum	100	52M, 48F	NR
Lim et al., 2017 [198]	Clinical	MS	Australia	Serum	49	14M, 35F	45.29 (11.70)
Lionetto et al., 2021 [199]	Clinical	MS	Italy	Serum	239	87M, 152F	NR
Liu et al., 2018 [200]	Analytical	ECD	China	Plasma	NR	NR	NR
Lorite et al., 2007 [201]	Clinical	UV-Vis + FL	Spain	Serum	5	NR	NR
Lu et al., 2019 [202]	Clinical	MS	Singapore	Serum	76	NR	NR
Ma et al., 2009 [203]	Clinical	UV	China	Plasma	10	NR	NR
Malhotra et al., 2017 [204]	Clinical	MS	USA	Plasma	10	4M, 6F	44.00 (12.00)

Meng et al., 2022 [205]	Clinical	MS	China	Serum	96	96M, 0F	78.01 (6.99)
Michel et al., 2020 [206]	Clinical	MS	Germany	Serum	20	13M, 7F	NR
Mierzchala et al., 2020 [207]	Clinical	MS	Poland	Serum	30	NR	NR
Mu et al., 2012 [208]	Clinical	FL	China	Serum	110	58M, 52F	NR
Myint et al., 2007 [209]	Clinical	UV-Vis + FL	Korea	Plasma	80	40M, 40F	39.06 (8.75)
Myint et al., 2007 [210]	Clinical	UV-Vis + FL	Korea	Plasma	189	76M, 113F	32.49 (10.69)
Nakatsukasa et al., 2011 [211]	Clinical	MS	Japan	Tears, Plasma	34	17M, 17F	NR
Naz et al., 2019 [212]	Clinical	MS	Sweden	Serum	39	19M, 20F	NR
Ogawa et al., 2018 [213]	Clinical	FL	Japan	Plasma	217	100M, 117F	41.20 (13.90)
Oh et al., 2017 [214]	Clinical	MS	South Korea	Urine	163	NR	50.92 (15.39)
Ohashi et al., 2013 [215]	Analytical	MS	Japan	Serum	19	8M, 11F	23.60 (3.50)
Onesti et al., 2019 [216]	Clinical	MS	Belgium	Plasma	146	0M, 146F	NR
Palabiyik et al., 2016 [217]	Clinical	UV-Vis + FL	Turkey	Serum	30	13M, 17F	36.00 (2.00)
Panitz et al., 2021 [218]	Clinical	MS	Germany	Serum	43	19F, 24M	NR
Pertovaara et al., 2005 [219]	Clinical	UV-Vis + FL	Finland	Serum	309	170M, 139F	45.00 (11.00)

Primiano et al., 2020 [220]	Clinical	MS	Italy	Urine	12	4M, 8F	NR
Ren et al., 2011 [221]	Clinical	FL	China	Serum	120	62M, 58F	NR
Roca et al., 1999 [222]	Clinical	UV-Vis	Spain	Plasma	29	13M, 16F	NR
Rodrigues et al., 2021 [223]	Clinical	MS	UK	CSF, Plasma	20	NR	NR
Ruoppolo et al, 2014 [224]	Clinical	MS	Italy	Serum	76	35M, 41F	NR
Saito et al., 1979 [225]	Clinical	UV-Vis + FL	Japan	Serum	8	NR	NR
Saito et al., 2022 [226]	Clinical	MS	Japan	Serum	59	29M, 30F	52.50 (7.93)
Sakaguchi et al., 2011 [227]	Analytical	FL	Japan	Urine	7	7M, 0F	NR
Schwieler et al., 2020 [228]	Clinical	MS	Sweden	CSF	13	5M, 8F	40.40 (14.60)
Shi et al., 2019 [229]	Clinical	MS	China	Plasma	11	8M, 3F	51.09 (10.77)
Smolenska et al., 2020 [230]	Clinical	MS	Poland	Plasma	27	NR	NR
Sorgdrager et al., 2017 [231]	Clinical	MS	Netherlands	Serum	406	163M, 243F	42.90 (14.70)
Sorgdrager et al., 2019 [232]	Clinical	MS	Belgium	CSF, Serum	39	18M, 21F	71.30 (10.70)
Souissi et al., 2022 [233]	Clinical	UV-Vis	Tunisia	Plasma	50	NR	NR
Sousa et al., 2021 [234]	Clinical	UV-Vis + FL	Portugal	Urine	6	6M, 0F	NR

Sultana et al., 2012 [235]	Analytical	UV-Vis	Pakistan	Plasma	10	10M, 0F	NR
Sun et al., 2021 [236]	Clinical	MS	China	Plasma	401	163M, 238F	53.15 (6.64)
Sun et al., 2020 [237]	Clinical	MS	China	Faeces	38	24M, 14F	56.85 (10.99)
Suzuki et al., 2012 [238]	Clinical	MS	Japan	Serum	85	48M, 37F	NR
Suzuki et al., 2010 [239]	Clinical	MS	Japan	Serum	45	34M, 11F	63.40 (9.40)
Suzuki et al., 2011 [240]	Clinical	MS	Japan	Serum	64	39M, 25F	NR
Taherizadeh et al., 2020 [241]	Clinical	MS	Iran	Plasma	37	20M, 17F	64.24 (13.08)
Tcherkas et al., 2001 [242]	Clinical	ECD	Russia	Serum	16	NR	NR
Tezcan et al., 2022 [243]	Clinical	MS	Turkey	Serum	80	38M, 42F	35.09 (7.09)
Tong et al., 2018 [244]	Clinical	MS	China	Plasma	18	NR	NR
Trepci et al., 2021 [245]	Clinical	MS	Sweden	CSF	80	39M, 41F	NR
Tuka et al., 2021 [246]	Clinical	MS	Hungary	Plasma	34	0M, 34F	30.50 (12.77)
Uchikura et al., 2003 [247]	Analytical	ECL	Japan	Plasma	10	NR	NR
Valko et al., 2019 [248]	Clinical	FL	Slovakia	Urine	51	35M, 16F	36.60 (10.90)
Van Faassen et al., 2019 [249]	Clinical	MS	Netherlands	Plasma	68	35M, 33F	NR

Walser et al, 1993 [250]	Clinical	MS	USA	Serum	22	12M, 10F	NR
Wang et al., 2019 [251]	Analytical	MS	China	Plasma	475	189M, 286F	58.67 (6.30)
Wang et al., 2018 [252]	Clinical	MS	China	Serum	298	NR	62.00 (17.00)
Widner et al., 2000 [253]	Clinical	MS	Austria	Serum	20	10M, 10F	NR
Wu et al., 2022 [254]	Clinical	MS	China	Serum	10	NR	NR
Wu et al., 2020 [255]	Clinical	MS	China	Serum	36	6M, 30F	65.83 (7.30)
Wu et al., 2018 [256]	Clinical	MS	China	Serum	135	42M, 93F	66.99 (6.77)
Xu et al., 2012 [257]	Clinical	MS	China	Plasma	25	9M, 16F	32.12 (8.15)
Yan et al., 2017 [258]	Analytical	MS	Australia	Urine	10	6M, 4F	NR
Yao et al., 2010 [259]	Clinical	ECD	USA	Plasma	30	18M, 12F	NR
Yilmaz et al., 2020 [260]	Clinical	MS	USA	Urine	29	13M, 16F	79.12 (6.28)
Yoshitake et al., 2007 [261]	Analytical	FL	Japan	Urine	7	NR	NR
Zhang et al., 2020 [262]	Clinical	MS	China	Serum	79	42M, 37F	28.85 (9.43)
Zhao et al., 2011 [263]	Analytical	UV-Vis + FL	China	Urine	NR	NR	NR
Zhen et al., 2011 [264]	Clinical	UV-Vis	China	Plasma	20	10M, 10F	NR

Zhou et al., 2022 [265]	Clinical	MS	China	Plasma	60	30M, 30F	30.10 (11.50)
Zhou et al., 2019 [266]	Clinical	MS	China	Serum	72	41M, 31F	36.30 (11.90)

Note. ECD: Electron Capture Detector; ECL: Electrochemiluminescence; FL: Fluorescence Detection; MOD: Mode of Detection; MS: Mass Spectrometry; SD: Standard Deviation; UV-Vis: Ultraviolet-Visible Spectroscopy.

Table 2.3. Chromatographic conditions of the included studies, including MOD, column phase and diameter, particle size, mobile phases, flow rate, injection volume, column temperature, program time, and detection wavelength (where applicable).

Citation	MOD	Column Phase	Column Diameter (mm)	Particle Size (µM)	Mobile Phase A	Mobile Phase B	Flow Rate (mL/min)	Injection Volume (µL)	Column Temp. (°C)	Detection Wavelength (nm)	Program Time (min)
Adrych et al., 2010	MS	C_{18}	150 × 2.0	3	0 % to 60 % ACN	NR	0.2	NR	275	NA	12
Al Saedi et al., 2022	UV-Vis + FL	C_{18}	150 × 2.1	1.8	0.2mM SA	NR	0.75	20	38	KYN: 365 TRP: Ex/Em 280/438	12
Arnhard et al., 2018	MS	NH_2	150 × 1.0	3	50 % to 5 % ACN in AA	NR	0.035	5	50	NA	28.5
Bai et al., 2021	ELISA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Barry et al., 2009	FL + PDA	C_{18}	150 × 2.0	2	50mM Acetic Acid, 100mM ZA, 3 % (v/v ACN)	NR	0.3	NR	NR	FL: Ex/Em 254/404 PDA: 210 – 400	NR
Bassi et al., 2017	MS	NR	NR	NR	5mM AA, Methanol	NR	NR	20	NR	NA	NR
Bizzarri et al., 1990	UV-Vis	C_{18}	250 × 4.6	5	ACN, SA buffer (pH 4.76; 4:96, v/v)	NR	0.8 – 2.0	20	NR	280	24
Buczko et al., 2007	UV-Vis	C_{18}	150 × 4.6	NR	ACN, 0.1M AA, pH 4.65	NR	1.5	NR	NR	365	NR
Calvani et al., 2020	MS	C_{18}	150 × 2.1	1.6	0.1 % FA in water	0.1 % ACN in FA	0.5	NR	NR	NA	9
Capuron et al., 2011	UV-Vis + FL	C_{18}	244	5	0.015mol/L Degassed PP (pH 6.4)	27mL/L ACN	0.8	NR	25	KYN: 360 TRP: Ex/Em 285/365	NR
Chang et al., 2018	MS	C_{18}	100 × 2.1	1.8	0.5 % FA in water	0.5 % FA in ACN	0.4	NR	30	NA	NR
Chatterjee et al., 2019	FL	C_{18}	100 × 2.1	1.8	50nM SA, 25mM ZA, 2.25 % ACN	10 % ACN	0.75	NR	38	Ex/Em 344/388	10

Chen et al., 2019	MS	C_{18}	100 × 3.0	1.8	0.1 % FA in water, v/v	100 % ACN	0.3	2	30	NA	5
Chen et al., 2010	UV-Vis + FL	C_{18}	NR	NR	0.1M AA (pH 4.65)	NR	1	30	22	KYN: 360 TRP: Ex/Em 285/365	NR
Chen et al., 2020	MS	C_{18}	100 × 2.1	1.7	A: 0.1% FA in water	FA, ACN, water	0.2 – 0.4	1	40	NA	NR
Cheng et al., 2015	MS	C_{18}	100 × 2.1	1.7	10mM AF	ACN in water (95:5), 2mM AF	0.2	10	45	NA	NR
Clarke et al., 2009	UV-VIS + FL	C_{18}	150 × 2.0	2	50mM Acetic Acid, 100mM ZA, 3 % (v/v) ACN	NR	0.3	20	30	KYN: 330 TRP: Ex/EM 254/404	30
Colle et al., 2020	MS	C_{18}	100 × 2.1	1.7	0.1 % FA in water	0.1 % FA in ACN	0.5	5	40	NA	NR
Crotti et al., 2019	UV-VIS + FL	C_{18}	250 × 4.6	5	ACN, 0.005 Phosphate Buffer (15:85 v/v)	NR	1	NR	NR	KYN: 360 TRP: Ex/Em 285/345	NR
Cseh et al., 2019	MS	C_{18}	150 × 4.6	5	200mM ZA (pH 6.2)	NR	1.2	20	NR	KYN: 200 – 800 TRP: Ex/Em 300/495	NR
Curto et al., 2016	MS	PFP	100 × 2.1	2.6	0.1 % FA in water	100 % MeOH	0.3	80	25	NA	16
Curto et al., 2016	MS	PFP	100 × 2.1	2.6	0.1 % FA in water	100 % MeOH	0.3	80	25	NA	16
Domingues et al., 2015	MS	HILIC	100 × 4.6	2.7	10mmol/L AA	ACN, 10 % 10mmol/L AA (60:40, v/v)	0.5	5	NR	NA	NR
Doolin et al., 2018	MS	NR	NR	NR	NR	NR	NR	NR	NR	NA	NR
Eniu et al., 2019	MS	AAA-MS	250 × 3.0	NR	AA in water	10mM AF in MeOH	0.25	5	35	NA	24

Fekkes et al., 1998	FL	C_{18}	150 × 3.0	5	NR	NR	0.6	NR	28	NR	NR
Fitzgerald et al., 2008	FL	C_{18}	150 × 2.0	NR	50mmol/L Acetic Acid, 100mL/L ZA, 3 % (v/v) ACN	NR	NR	NR	NR	Ex/Em 254/404	NR
Frick et al., 2004	UV-Vis + FL	C_{18}	NR	NR	NR	NR	0.9	NR	NR	KYN: 360 TRP: Ex/Em 285/365	NR
Fukushima et al., 2014	MS	C_{18}	250 × 2.0	5	Water/ACN (80:20), 0.1 % Acetic Acid	ACN/water (80:20), 0.1% Acetic Acid	0.16	50	40	NA	NR
Furtado et al., 2017	MS	C_{18}	75 × 2.1	1.7	0.2 % FA in water	0.2 % FA in ACN	0.9	5	50	NA	5
Galla et al., 2021	MS	C_{18}	150 × 2.1	5	0.2 % FA in water	02 % FA in ACN	0.6	15	15	NA	NR
Geisler et al., 2015	UV-Vis + FL	C_{18}	NR	5	15mmol/L Acetic Acid-SA (pH 4.0)	NR	NR	NR	NR	KYN: 360 TRP: Ex/Em 286/366	NR
Gevorkian et al., 2015	ECD	C_{18}	250 × 4.6	5	NR	NR	0.7 – 1.2	NR	35	NA	NR
Girgin et al., 2020	UV-Vis + FL	NR	NR	NR	15mM pH 6.5 Monopotassium Phosphate, 0.7 % ACN	NR	0.8	NR	NR	KYN: 360 TRP: Ex/Em 286/Em	NR
Gomez-Gomez et al., 2017	MS	C_{18}	100 × 2.1	1.7	NR	NR	0.3	NR	NR	NA	NR
Gulaj et al., 2010	UV-Vis + FL	C_{18}	150 × 2.1	NR	KYN: 0.1M Acetic Acid, 0.1M AA, 2 % ACN TRP: 50mM Acetic Acid,	NR	0.2	NR	NR	KYN: 365 TRP: Ex/Em 254/404	NR

					0.25M ZA, 1.2 % ACN						
Hajsl et al., 2020	MS	C_{18}	150 × 2.1	5	0.1 % FA	0.1 % FA/ACN (v/v)	0.25	20	40	NA	20
Han et al., 2018	MS	CR-I(+)	150 × 3.0	5	Ethanol, Water, TFA (50:50:0.4, v/v/v/)	ACN, TFA (100:0.4, v/v)	0.4	5	15	NA	13
Henykova et al., 2016	MS	C_{18}	100 × 2.1	1.8	0.1 % FA	MeOH	0.3	10	30	NA	NR
Huang et al., 2022	MS	NR	NR	NR	NR	NR	NR	NR	NR	NA	NR
Huang et al., 2021	MS	C_{18}	150 × 4.6	3.5	Water	ACN	0.42	5	31	NA	24
Islam et al., 2020	UV-Vis	C_{18}	NR	NR	0.1 % FA in water	0.1 % FA in ACN	0.8	5	NR	NR	NR
Jang et al., 2022	MS	C_{18}	100 × 2.1	1.8	0.1 % FA in water	ACN	0.5	5	40	NA	NR
Kim et al., 2015	MS	C_{18}	100 × 2.1	3	0.2 % Acetic Acid in water	0.2 % Acetic Acid in ACN	0.2	3	40	NA	NR
Kim et al., 2009	UV-Vis + FL	C_{18}	100 × 4.7	NR	TRP: 57.2g Na2HPO412 H2O, 60mL ACN in water KYN: 250mM ZA in water	TRP: 420 water/280 CAN/320 MeOh KYN: 9 % ACN	NR	NR	NR	KYN: 365 TRP: Ex/Em 340/440	NR
Klatt et al., 2021	MS	C_{18}	50 × 2.1	1.8	0.1 % FA in water	0.1 % FA in ACN	NR	NR	NR	NA	NR
Koch et al., 1979	ECD	C_{18}	150 × 4.0	10	0.5M AA, 15 % MeOh	McIlvaine buffer, 20 % MeOh	1	20	NR	NA	NR
Krasnova et al., 2000	ECD	C_{18}	250 × 4.0	5	MeOh, Sodium Hydrophosp	NR	0.7	20	NR	NA	NR

					hate, Sodium Dihydrophosphate, 0.002M EDTA						
Kurgan et al., 2022	MS	C_{18}	10 × 2.1	3	Su (0.1 % FA)	MeOh Su (0.1 % FA)	0.3	10	15	NA	NR
Leichtle et al., 2012	MS	NR	NR	NR	1/1 Visopropanol/water	NR	0.08	25	NR	NA	NR
Li et al., 2011	FL	C_{18}	250 × 4.6	5	10 % ACN in water (v/v)	NR	1	20	NR	Ex/Em 285/353	NR
Lim et al., 2017	MS	C_{18}	100 × 2.1	1.8	50mM SA, 25mM ZA, 2.25 % ACN	10 % ACN	0.75	20	38	NA	NR
Lionetto et al., 2021	MS	F5	100 × 2.1	2.6	0.2 % FA	100 % ACN	0.3	10	NR	NA	NR
Liu et al., 2018	ECD	C_{18}	250 × 4.6	4	Acetate Buffer, MeOh (4:1, v/v)	NR	1	20	NR	NA	NR
Lorite et al., 2007	UV-Vis + FL	C_{18}	244 × 4.0	5	Phosphate	NR	0.8	100	NR	KYN: 360 TRP: Ex/Em 285/365	NR
Lu et al., 2019	MS	C_{18}	100 × 2.1	1.7	30 % ACN, 0.1 % FA, 10mmol/L AF	95 % ACN, 0.1 % FA, 10mmol/L AF	0.5	5	40	NA	NR
Ma et al., 2009	UV	C_{18}	125 × 4.0	5	50mmol/L SA, 6 % ACN (v/v)	NR	0.8	20	25	KYN: 360 TRP: 302	NR
Malhotra et al., 2017	MS	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Meng et al., 2022	MS	HILIC	150 × 2.1	2.6	75 % ACN, 7.5 % AF, 0.5 % FA	NR	0.25	2	20	NA	NR

Michel et al., 2020	MS	C_{18}	NR	NR	Water	0.1 % FA in ACN	NR	NR	NR	NA	8
Mierzchala et al., 2020	MS	C_{18}	50 × 1.0	1.8	0.1 % FA in water	0.1 % FA in ACN	0.07	3	50	NA	NR
Mu et al., 2012	FL	C_{18}	150 × 4.6	5	0.1mol/L Monopotassium Phosphate, MeOh (85:15, v/v)	NR	1	20	25	Ex/Em 200 – 450/250 - 500	12
Myint et al., 2007	UV-Vis + FL	C_{18}	100 × 4.7	5	50mM ZA, Acetic Acid, 1.0 % ACN	NR	1	100	NR	KYN: 365 TRP: Ex/Em 340/440	NR
Myint et al., 2007	UV-Vis + FL	C_{18}	100 × 4.7	NR	TRP: 57.2g NA2HPO41 2H2O, 160mL ACN in water KYN: 250mM ZA in water	TRP: 420 water/280 ACN/320 MeOh	NR	NR	NR	KYN: 365 TRP: Ex/Em 340/440	NR
Nakatsukasa et al., 2011	MS	C_8	50 × 1.2	3	25mM FA	60 % ACN in water	0.25	5, 2	40	NA	NR
Naz et al., 2019	MS	C_{18}	100 × 2.1	1.8	2.1 % FA in water	0.1 % FA in 95 % ACN	0.3	7.5	NR	NA	NR
Ogawa et al., 2018	FL	C_{18}	100 × 2.1	NR	NR	NR	NR	NR	NR	NR	NR
Oh et al., 2017	MS	T3	100 × 2.1	3	0.1 % FA	0.1 % FA in ACN	0.2	NR	30	NA	NR
Ohashi et al., 2013	MS	C_{18}	250 × 2.0	5	Water, ACN (80:20), 0.1 % Acetic Acid	ACN, Water (80:20), 0.1 % Acetic Acid	0.16	50	40	NA	NR
Onesti et al., 2019	MS	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Palabiyik et al., 2016	UV-Vis + FL	C_{18}	250 × 4.6	5	15mM Phosphate,	NR	0.8	100	NR	KYN: 360 TRP: Ex/Em 286/366	NR

					27mL/L ACN						
Panitz et al., 2021	MS	C_{18}	150 × 3.6	3	0.1 % FA in water	0.1 % FA in ACN	0.3	2	NR	NR	NR
Pertovaara et al., 2005	UV-Vis + FL	C_{18}	50 × 2.1	5	15mmol/L SA in Acetic Acid, 27mL/L ACN	NR	0.9	10	NR	KYN: 360 TRP: Ex/Em 266/366	NR
Primiano et al., 2020	MS	NR	NR	NR	NR	NR	NR	NR	NR	NA	NR
Ren et al., 2011	FL	C_{18}	250 × 4.6	5	10 % ACN (v/v)	NR	1.2	20	25	Ex/Em 285/353	NR
Roca et al., 1999	UV-Vis	C_{18}	150 × 3.9	4	0.14M SA, 0.5mL/L TEA	60 % ACN in water	NR	NR	NR	254	NR
Rodrigues et al., 2021	MS	NR	TRP: 100 × 3.0 KYN: 150 × 2.1	TRP: 2.5 KYN: 3	0.1 % FA in water	0.1 % FA in ACN	TRP: 0.3 KYN: 0.2	NR	TRP: 5 KYN: 25	NA	NR
Ruoppolo et al, 2014	MS	C_{18}	150 × 4.6	5	NR	NR	NR	NR	NR	NA	NR
Saito et al., 1979	UV-Vis + FL	CK-10-S	300 × 4.6	NR	NR	NR	0.73	NR	60	KYN: 280 TRP: Ex/Em 280/340	60
Saito et al., 2022	MS	PFP	100 × 2.1	1.9	0.3 % FA in water	0.3 % FA in MeOh	0.3	5	45	NA	10
Sakaguchi et al., 2011	FL	C_{18}	150 × 4.6	5	MeOh, water, TFA (2.5:97.5:0.05, v/v)	MeOh, water, TFA (60:40:0.05, v/v)	1	20	30	Ex/Em 280/320	NR
Schwieler et al., 2020	MS	C_{18}	150 × 2.1	1.8	0.6 % FA in water	0.6 % FA in MeOh	0.3	3	50	NA	13
Shi et al., 2019	MS	C_{18}	NR	NR	Methanol, 0.1 % FA in water (95:5, v/v)	NR	0.2	10	25	NA	4

Smolenska et al., 2020	MS	Hydro-RP	50 × 2.0	2.5	0 % to 60 % ACN	NR	0.2	NR	275	NA	12
Sorgdrager et al., 2017	MS	C_{18}	100 × 2.1	3	0.2 % FA in water	ACN	0.3	NR	25	NA	NR
Sorgdrager et al., 2019	MS	C_{18}	100 × 2.1	2.5	NR	NR	NR	0.15	NR	NA	NR
Souissi et al., 2022	UV-Vis	C_{18}	150 × 4.6	5	15mM Phosphate Buffer, 10.6 % ACN	NR	1.2	NR	30	KYN: 360 TRP: 280	KYN: 3 TRP: 5
Sousa et al., 2021	UV-Vis + FL	C_{18}	NR	3	20mM AF in 0.01 % FA in water, ACN, Ethanol (95/2/3, v/v/v)	NR	0.7	10	25	KYN: 365 TRP: Ex/Em 280/348	27
Sultana et al., 2012	UV-Vis	C_{18}	250 × 4.6	NR	Water, ACN (90:10, v/v)	NR	1.5	20	25	273	NR
Sun et al., 2021	MS	C_{18}	50 × 4.6	2.7	80 % MeOh/20 % water, 2.5 mM AF in MeOh	NR	1.6	15	20	NA	NR
Sun et al., 2020	MS	C_{18}	100 × 2.1	1.7	10mmol/L Ammonium Acetate, 0.1 % FA in water	0.1 % FA in ACN	0.3	10	40	NA	10
Suzuki et al., 2012	MS	T3	150 × 2.1	5	5mM AF with 0.01 % TFA, MeOh (80:20, v/v)	0.2	NR	NR	NR	NA	NR
Suzuki et al., 2010	MS	C_{18}	150 × 0.5	3	2.1 % FA	2.1 % FA, 40 % ACN	0.012	NR	NR	NA	NR
Suzuki et al., 2011	MS	C_{18}	150 × 0.5	3	2.1 % FA	2.1 % FA, 40 % ACN	0.012	NR	NR	NA	NR

Taherizadeh et al., 2020	MS	NR	NR	NR	NR	NR	1	NR	NR	NA	60
Tcherkas et al., 2001	ECD	C_{18}	250 × 4.0	5	0.01 – 0.02M Sodium Dihydrogenphosphate, 0.01 – 0.02 Disodium Hydrogenphosphate, 10 % - 19 % MeOh (v/v), 2mM Na2EDTA	NR	0.7	20	26	NA	60
Tezcan et al., 2022	MS	C_{18}	50 × 4.6	5	0.1 % FA in water (v/v)	0.1 % FA in ACN (v/v)	NR	30	350	NA	5
Tong et al., 2018	MS	C_{18}	50 × 2.1	5	0.1 % FA	0.1 % FA in ACN	0.5	5	40	NA	3.5
Trepci et al., 2021	MS	C_{18}	150 × 2.1	1.8	0.6 % FA in water	0.6 % FA in MeOh	0.3	NR	50	NA	13
Tuka et al., 2021	MS	PFP	NR	NR	FA in water, MeOh	NR	NR	NR	NR	NA	NR
Uchikura et al., 2003	ECL	C_{18}	150 × 4.6	NR	ACN, 10mM KH2PO4	NR	1	10	50	NA	NR
Valko et al., 2019	FL	C_{18}	250 × 4.0	5	15 % ACN	NR	0.8	40	30	Ex/Em 280 – 315/350 – 425	30
Van Faassen et al., 2019	MS	C_{18}	150 × 2.0	3	10mmol/L AA in 0.1 % FA	0.1 % FA in 95 % ACN	0.3	50	NR	NA	8.5
Walser et al, 1993	MS	C_{18}	250 × 4.6	NR	0.02M Sodium Phosphate	MeOh	NR	NR	NR	NA	NR
Wang et al., 2019	MS	C_{18}	150 × 4.6	5	0.1 % FA in water, 0.1 % HFBA	0.1 % FA in ACN, 0.1 % HFBA	0.8	1	50	NA	NR

Wang et al., 2018	MS	PFP	150 × 2.1	2.5	0.1 % FA in water	MeOh	0.230	10	15	NA	NR
Widner et al., 2000	MS	NR	NR	NR	NR	NR	NR	NR	NR	NA	NR
Wu et al., 2022	MS	C_{18}	150 × 2.1	5	NR	NR	NR	10	NR	NA	NR
Wu et al., 2020	MS	NR	NR	NR	NR	NR	NR	NR	NR	NA	NR
Wu et al., 2018	MS	NR	NR	NR	NR	NR	NR	NR	NR	NA	NR
Xu et al., 2012	MS	C_{18}	150 × 4.6	5	0.2 % FA, 0.005 % HFBA	0.1 % FA in ACN, 0.005 % HFBA	0.8	NR	50	NA	NR
Yan et al., 2017	MS	C_{18}	100 × 2.1	2.7	0.2 % FA in water (v/v)	0.1 % FA in ACN (v/v)	0.18	NR	NR	NA	8.5
Yao et al., 2010	ECD	C_{18}	250 × 4.6	5	10.3gL-1 Sodium Pentane Sulfonate, 5mL-1 Acetic Acid	MeOh/ACN/ Isopropanol (8/1/1), 8gL-1 Lithium Acetate, 20mL-1 Acetic Acid	NR	NR	35	NA	NR
Yilmaz et al., 2020	MS	NR	NR	NR	NR	NR	NR	NR	NR	NA	NR
Yoshitake et al., 2007	FL	C_{18}	250 × 4.6	5	0.04 % TFA/MeOh (99:1, v/v)	0.04 % TFA/MeOh (90:10, v/v)	1	20	21 - 25	NR	75
Zhang et al., 2020	MS	C_{18}	150 × 4.6	5	5mM AF in MeOh/water (45:55, v/v)	NR	0.5	NR	35	NA	NR
Zhao et al., 2011	UV-Vis + FL	C_{18}	250 × 4.6	5	20mmol/L NaAc, 30mmol/L HAc, 3 % MeOh	20mmol/L NaAc/HAc, 10 % MeOh, 19 % ACN	1	50	25	KYN: 365 TRP: Ex/Em 292/340	30
Zhen et al., 2011	UV-Vis	C_8	150 × 4.6	5	10mmol/L Acetate	NR	0.6	25	25	KYN: 360 TRP: 302	8

					Buffer, ACN (94:6, v/v)						
Zhou et al., 2022	MS	C_{18}	150 × 4.6	5	MeOh/water (45:55, in 0.005mol/L AF)	MeOh/water (35:65, in 0.005mol/L AF)	0.5	NR	35	NA	NR
Zhou et al., 2019	MS	C_{18}	150 × 4.6	5	MeOh/water (45:55, in 5mM AF)	MeOh/water (35:65, in 5mM AF)	0.5	NR	35	NA	NR

Note: AA: Ammonium Acetate; ACN: Acetonitrile; AF: Ammonium Formate; Ex/Em: Excitation/Emission; FA: Formic Acid; HFBA: Heptafluorobutyric Acid; MeOh: Methanol; PFP: Pentafluorophenyl; PP: Potassium Phosphate; SA: Sodium Acetate; TFA: Trifluoroacetic Acid; ZA: Zinc Acetate.

2.3.2 Risk of Bias Evaluation and Method Validation

Risk of bias varied across the reported studies and the breakdown of the appraisal is detailed for each study and item in Table 2.4. Over half of the included studies clearly defined their criteria for inclusion (59.17 %; *n* = 71), while only 28.33 % of studies (*n* = 34) described the study subjects and setting in detail. Majority of studies sufficiently described the type of sample (65.83 %; *n* = 79) and the handling and pre-analytical processing of the samples (72.5 %; *n* = 87). Only 34.17 % (*n* = 41) of included studies sufficiently described their methodological conditions. Indeed, 84.17 % (*n* = 101) of included studies reported their statistical analysis. Overall, 51.67 % (*n* = 62) of studies were subjectively appraised to be of high quality.

Reporting of independent method validation was highly varied (Table 2.5). Overall, 80.83 % (*n* = 73) of studies reported at least one parameter of their independent methodological validation. Most commonly, LOD was reported in 44.17 % (*n* = 53) studies. This was closely followed by the presentation of calibration curves (39.17 %; *n* = 47). Robustness of the methodology was least commonly validated in only 10.83 % (*n* = 13) of studies.

Table 2.4. Risk of bias across evaluation of included studies across the six bias domains and their overall rating.

Citation	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6	Overall
Adrych et al., 2010	N	Y	Y	N	N	N	Low
Al Saedi et al., 2022	Y	Y	Y	N	N	Y	High
Arnhard et al., 2018	N	N	N	N	Y	N	Low
Bai et al., 2021	Y	Y	Y	Y	Y	Y	High
Barry et al., 2009	Y	N	Y	Y	Y	Y	High
Bassi et al., 2017	Y	N	N	Y	N	Y	Low
Bizzarri et al., 1990	N	N	Y	Y	Y	N	Low
Buczko et al., 2007	N	N	Y	Y	Y	N	Low
Calvani et al., 2020	Y	Y	Y	Y	N	Y	High
Capuron et al., 2011	Y	Y	Y	Y	N	Y	High
Chang et al., 2018	Y	N	Y	Y	N	Y	High
Chatterjee et al., 2019	Y	Y	Y	Y	N	Y	High
Chen et al., 2019	N	N	N	Y	Y	Y	Low
Chen et al., 2010	Y	Y	N	N	N	Y	Low
Chen et al., 2020	Y	N	N	N	Y	Y	Low
Cheng et al., 2015	Y	N	Y	Y	Y	Y	High
Clarke et al., 2009	Y	N	Y	Y	Y	Y	High
Colle et al., 2020	N	N	Y	Y	N	Y	Low
Crotti et al., 2019	N	N	Y	Y	N	Y	Low
Cseh et al., 2019	N	N	Y	Y	Y	N	Low
Curto et al., 2016	Y	N	N	Y	N	Y	Low
Curto et al., 2016	Y	N	Y	Y	N	Y	High
Domingues et al., 2015	N	N	Y	Y	Y	N	Low
Doolin et al., 2018	Y	N	Y	Y	N	Y	High
Eniu et al., 2019	Y	N	Y	Y	N	Y	High
Fekkes et al., 1998	Y	N	Y	Y	N	Y	High

Fitzgerald et al., 2008	Y	N	Y	N	Y	Y	High
Frick et al., 2004	N	N	Y	N	N	Y	Low
Fukushima et al., 2014	N	N	Y	Y	N	Y	Low
Furtado et al., 2017	N	N	Y	Y	N	Y	Low
Galla et al., 2021	N	N	N	Y	Y	N	Low
Geisler et al., 2015	Y	N	N	N	Y	Y	Low
Gevorkian et al., 2015	N	N	N	Y	U	Y	Low
Girgin et al., 2020	Y	N	Y	Y	N	Y	High
Gomez-Gomez et al., 2017	Y	N	Y	Y	Y	Y	High
Gulaj et al., 2010	Y	N	Y	N	Y	Y	High
Hajsl et al., 2020	N	N	Y	Y	N	Y	Low
Han et al., 2018	N	N	N	Y	Y	Y	Low
Henykova et al., 2016	N	N	Y	Y	Y	N	Low
Huang et al., 2022	Y	Y	Y	N	N	Y	High
Huang et al., 2021	N	N	Y	Y	Y	Y	High
Islam et al., 2020	Y	N	Y	Y	U	Y	High
Jang et al., 2022	Y	N	N	Y	Y	Y	High
Kim et al., 2015	Y	N	Y	Y	N	Y	High
Kim et al., 2009	Y	Y	N	N	U	Y	Low
Klatt et al., 2021	N	N	N	N	N	Y	Low
Koch et al., 1979	N	N	N	Y	Y	N	Low
Krasnova et al., 2000	N	N	N	N	Y	N	Low
Kurgan et al., 2022	Y	Y	Y	Y	Y	Y	High
Leichtle et al., 2012	Y	N	N	Y	N	Y	Low
Li et al., 2011	Y	N	Y	Y	Y	Y	High
Lim et al., 2017	Y	Y	Y	Y	Y	Y	High
Lionetto et al., 2021	Y	Y	Y	Y	N	Y	High
Liu et al., 2018	Y	Y	Y	Y	N	Y	High
Lorite et al., 2007	Y	N	N	N	Y	Y	Low

Lu et al., 2019	N	N	Y	N	N	N	Low
Ma et al., 2009	N	Y	Y	Y	Y	Y	High
Malhotra et al., 2017	N	Y	Y	Y	N	Y	High
Meng et al., 2022	Y	Y	Y	Y	N	Y	High
Michel et al., 2020	Y	N	Y	Y	N	Y	High
Mierzchala et al., 2020	N	N	Y	Y	N	Y	High
Mu et al., 2012	Y	N	Y	Y	Y	Y	Low
Myint et al., 2007	Y	Y	Y	N	N	Y	High
Myint et al., 2007	Y	Y	Y	N	N	Y	High
Nakatsukas et al., 2011	Y	N	Y	Y	N	Y	High
Naz et al., 2019	Y	Y	N	N	N	N	Low
Ogawa et al., 2018	N	Y	Y	Y	N	Y	High
Oh et al., 2017	N	N	Y	Y	N	Y	Low
Ohashi et al., 2013	N	N	Y	Y	Y	Y	High
Onesti et al., 2019	Y	Y	N	Y	N	Y	High
Palabiyik et al., 2016	Y	N	Y	Y	N	Y	High
Panitz et al., 2021	N	Y	N	Y	N	Y	Low
Pertovaara et al., 2005	N	N	N	N	N	Y	Low
Primiano et al., 2020	Y	N	Y	N	N	Y	Low
Ren et al., 2011	Y	N	Y	Y	N	Y	High
Roca et al., 1999	Y	N	Y	U	N	Y	Low
Rodrigues et al., 2021	Y	N	Y	Y	N	Y	High
Ruoppolo et al., 2014	Y	N	Y	U	U	Y	Low
Saito et al., 1979	N	N	N	N	N	N	Low
Saito et al., 2022	N	N	N	Y	N	Y	Low
Sakaguchi et al., 2011	N	N	N	Y	Y	N	Low
Schwieler et al., 2020	Y	Y	Y	Y	Y	Y	High
Shi et al., 2019	Y	Y	N	Y	N	Y	High
Smolenska et al., 2020	Y	N	N	N	N	Y	Low

Sorgdrager et al., 2017	Y	N	Y	N	N	Y	Low
Sorgdrager et al., 2019	Y	Y	N	N	N	Y	Low
Souissi et al., 2022	Y	N	N	N	N	Y	Low
Sousa et al., 2021	Y	N	N	Y	Y	Y	High
Sultana et al., 2012	N	N	N	N	Y	Y	Low
Sun et al., 2021	Y	N	N	N	N	Y	Low
Sun et al., 2020	Y	Y	Y	Y	N	Y	High
Suzuki et al., 2012	N	Y	N	Y	N	Y	Low
Suzuki et al., 2010	N	Y	Y	Y	N	Y	High
Suzuki et al., 2011	N	Y	N	Y	N	Y	Low
Taherizadeh et al., 2020	Y	N	Y	Y	N	Y	High
Tcherkas et al., 2001	N	N	Y	Y	N	N	Low
Tezcan et al., 2022	Y	N	N	Y	N	Y	Low
Tong et al., 2018	Y	N	N	Y	Y	Y	High
Trepci et al., 2021	Y	N	Y	Y	N	Y	High
Tuka et al., 2021	N	N	Y	Y	Y	Y	High
Uchikura et al., 2003	N	N	N	Y	N	N	High
Valko-Rokytovska et al., 2019	Y	N	Y	Y	Y	Y	High
Van Faassen et al., 2019	N	N	Y	Y	Y	Y	High
Walser et al., 1993	N	N	Y	Y	N	N	Low
Wang et al., 2019	N	Y	Y	Y	N	Y	High
Wang et al., 2018	Y	Y	Y	N	U	Y	High
Widner et al., 2000	N	N	N	N	N	Y	Low
Wu et al., 2022	N	Y	N	Y	N	Y	Low
Wu et al., 2020	Y	N	Y	Y	N	Y	High
Wu et al., 2018	Y	N	Y	Y	N	Y	High
Xu et al., 2012	Y	N	Y	Y	N	Y	High
Yan et al., 2017	N	N	N	Y	Y	N	Low
Yao et al., 2010	Y	N	Y	Y	N	Y	High

Yilmaz et al., 2020	N	N	Y	Y	N	Y	Low
Yoshitake et al., 2007	N	N	N	Y	Y	N	Low
Zhang et al., 2020	Y	N	Y	N	N	Y	Low
Zhao et al., 2011	N	N	N	Y	Y	Y	Low
Zhen et al., 2011	Y	N	Y	Y	Y	Y	High
Zhou et al., 2022	Y	Y	Y	N	N	Y	High
Zhou et al., 2019	Y	Y	Y	Y	N	Y	High
Total (%)	71 (59.17)	34 (28.33)	79 (65.83)	87 (72.5)	41 (34.17)	101 (84.17)	62 (51.67)

Table 2.5. Reported method validation parameters of included studies, including specificity, calibration curve, stability, accuracy, precision, LOD, LOQ, and robustness.

Citation	Specificity	Calibration Curve	Stability	Accuracy	Precision	LOD	LOQ	Robustness
Adrych et al., 2010	NR	NR	NR	NR	NR	NR	NR	NR
Al Saedi et al., 2022	NR	NR	NR	NR	NR	NR	NR	NR
Arnhard et al., 2018	✓	✓	✓	✓	✓	✓	✓	NR
Bai et al., 2021	✓	NR	NR	NR	NR	NR	NR	NR
Barry et al., 2009	NR	NR	NR	NR	NR	NR	NR	NR
Bassi et al., 2017	NR	NR	NR	NR	NR	NR	NR	NR
Bizzarri et al., 1990	NR	✓	NR	✓	NR	✓	NR	NR
Buczko et al., 2007	NR	NR	NR	NR	NR	NR	NR	NR
Calvani et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Capuron et al., 2011	NR	NR	NR	✓	NR	NR	NR	NR
Chang et al., 2018	NR	NR	NR	NR	NR	NR	NR	NR
Chatterjee et al., 2019	✓	✓	NR	NR	NR	NR	NR	NR
Chen et al., 2019	✓	✓	✓	✓	✓	NR	✓	NR
Chen et al., 2010	NR	NR	NR	NR	NR	NR	NR	NR
Chen et al., 2020	NR	✓	✓	✓	✓	NR	✓	✓
Cheng et al., 2015	NR	✓	✓	✓	✓	✓	✓	NR
Clarke et al., 2009	NR	NR	NR	NR	NR	NR	NR	NR
Colle et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Crotti et al., 2019	NR	NR	NR	NR	NR	NR	NR	NR
Cseh et al., 2019	NR	✓	NR	✓	✓	✓	✓	NR
Curto et al., 2016	NR	NR	NR	✓	✓	✓	✓	NR
Curto et al., 2016	NR	NR	NR	✓	✓	✓	✓	NR
Domingues et al., 2015	NR	✓	NR	✓	✓	NR	✓	NR
Doolin et al., 2018	NR	NR	NR	NR	NR	NR	✓	NR

Eniu et al., 2019	✓	✓	NR	NR	NR	✓	✓	NR
Fekkes et al., 1998	NR	NR	NR	NR	NR	NR	NR	NR
Fitzgerald et al., 2008	NR	NR	NR	NR	NR	NR	NR	NR
Frick et al., 2004	NR	NR	NR	NR	NR	NR	NR	NR
Fukushima et al., 2014	✓	NR	NR	NR	NR	NR	NR	NR
Furtado et al., 2017	NR	✓	NR	✓	✓	✓	✓	NR
Galla et al., 2021	✓	✓	✓	✓	✓	✓	✓	NR
Geisler et al., 2015	NR	NR	NR	NR	NR	NR	NR	NR
Gevorkian et al., 2015	✓	NR	NR	NR	NR	NR	NR	NR
Girgin et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Gomez-Gomez et al., 2017	NR	NR	NR	NR	NR	NR	NR	NR
Gulaj et al., 2010	NR	NR	NR	NR	NR	✓	NR	NR
Hajsl et al., 2020	✓	✓	NR	✓	✓	NR	NR	NR
Han et al., 2018	✓	✓	✓	✓	✓	✓	NR	NR
Henykova et al., 2016	✓	NR	✓	✓	✓	✓	✓	NR
Huang et al., 2022	NR	NR	NR	NR	NR	NR	NR	NR
Huang et al., 2021	NR	✓	NR	✓	✓	✓	✓	NR
Islam et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Jang et al., 2022	NR	✓	NR	NR	NR	✓	✓	NR
Kim et al., 2015	NR	NR	NR	NR	NR	NR	NR	NR
Kim et al., 2009	NR	✓	NR	NR	NR	NR	NR	NR
Klatt et al., 2021	NR	NR	NR	✓	NR	NR	NR	NR
Koch et al., 1979	NR	✓	NR	NR	✓	NR	NR	NR
Krasnova et al., 2000	NR	NR	✓	NR	NR	✓	NR	NR
Kurgan et al., 2022	NR	NR	NR	NR	NR	✓	✓	NR
Leichtle et al., 2012	NR	NR	NR	NR	NR	NR	NR	NR
Li et al., 2011	✓	✓	NR	NR	✓	✓	NR	NR

Lim et al., 2017	NR	NR	NR	NR	NR	NR	NR	NR
Lionetto et al., 2021	✓	NR	NR	NR	NR	NR	NR	NR
Liu et al., 2018	✓	✓	NR	✓	✓	✓	NR	NR
Lorite et al., 2007	NR	✓	✓	NR	NR	✓	✓	✓
Lu et al., 2019	NR	✓	NR	✓	✓	✓	NR	NR
Ma et al., 2009	NR	✓	NR	✓	✓	✓	NR	NR
Malhotra et al., 2017	NR	NR	NR	NR	NR	NR	NR	NR
Meng et al., 2022	NR	✓	✓	NR	NR	✓	✓	✓
Michel et al., 2020	NR	NR	NR	✓	✓	✓	✓	NR
Mierzchala et al., 2020	NR	✓	✓	NR	NR	✓	✓	NR
Mu et al., 2012	✓	✓	NR	✓	✓	✓	✓	NR
Myint et al., 2007	NR	✓	✓	NR	NR	✓	✓	✓
Myint et al., 2007	✓	NR	NR	NR	NR	NR	NR	NR
Nakatsukasa et al., 2011	NR	✓	✓	NR	NR	✓	✓	✓
Naz et al., 2019	✓	✓	NR	NR	NR	✓	✓	✓
Ogawa et al., 2018	✓	✓	NR	NR	NR	✓	✓	✓
Oh et al., 2017	✓	✓	✓	✓	✓	✓	✓	NR
Ohashi et al., 2013	NR	✓	NR	NR	NR	✓	✓	NR
Onesti et al., 2019	NR	NR	NR	NR	NR	✓	NR	NR
Palabiyik et al., 2016	✓	✓	NR	NR	NR	✓	✓	✓
Panitz et al., 2021	✓	✓	NR	NR	NR	✓	✓	✓
Pertovaara et al., 2005	✓	✓	NR	NR	NR	✓	✓	✓
Primiano et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Ren et al., 2011	✓	NR	NR	✓	✓	✓	NR	NR
Roca et al., 1999	✓	NR	✓	✓	NR	✓	✓	✓
Rodrigues et al., 2021	NR	NR	✓	✓	NR	NR	✓	NR
Ruoppolo et al, 2014	NR	NR	NR	NR	NR	NR	NR	NR
Saito et al., 1979	NR	NR	NR	NR	NR	NR	NR	NR

Saito et al., 2022	NR	✓	NR	✓	✓	NR	NR	NR
Sakaguchi et al., 2011	✓	✓	NR	✓	✓	✓	NR	NR
Schwieler et al., 2020	✓	✓	✓	✓	✓	✓	✓	NR
Shi et al., 2019	✓	NR	NR	NR	NR	NR	NR	NR
Smolenska et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Sorgdrager et al., 2017	NR	NR	✓	✓	NR	✓	NR	NR
Sorgdrager et al., 2019	NR	NR	NR	NR	NR	NR	NR	NR
Souissi et al., 2022	NR	NR	NR	NR	NR	NR	NR	NR
Sousa et al., 2021	NR	✓	✓	✓	✓	✓	✓	NR
Sultana et al., 2012	✓	✓	✓	✓	✓	✓	✓	✓
Sun et al., 2021	NR	NR	NR	✓	✓	✓	✓	NR
Sun et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Suzuki et al., 2012	✓	NR	NR	NR	NR	NR	NR	NR
Suzuki et al., 2010	NR	NR	NR	NR	✓	✓	✓	NR
Suzuki et al., 2011	NR	NR	NR	NR	✓	✓	✓	NR
Taherizadeh et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Tcherkas et al., 2001	NR	NR	NR	NR	NR	NR	NR	NR
Tezcan et al., 2022	✓	NR	NR	NR	NR	NR	NR	NR
Tong et al., 2018	NR	✓	NR	✓	✓	NR	✓	NR
Trepci et al., 2021	NR	NR	NR	NR	NR	✓	✓	NR
Tuka et al., 2021	NR	✓	NR	✓	✓	✓	✓	✓
Uchikura et al., 2003	NR	NR	NR	✓	NR	✓	NR	NR
Valko et al., 2019	NR	✓	NR	✓	✓	✓	NR	NR
Van Faassen et al., 2019	✓	✓	✓	✓	✓	NR	✓	NR
Walser et al, 1993	NR	NR	NR	NR	NR	NR	NR	NR
Wang et al., 2019	NR	NR	NR	NR	NR	NR	NR	NR
Wang et al., 2018	NR	NR	NR	NR	NR	NR	NR	NR
Widner et al., 2000	NR	NR	NR	NR	NR	NR	NR	NR

Wu et al., 2022	NR	NR	NR	NR	NR	NR	NR	NR
Wu et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Wu et al., 2018	NR	NR	NR	NR	NR	NR	NR	NR
Xu et al., 2012	NR	NR	NR	NR	NR	NR	NR	NR
Yan et al., 2017	NR	✓	✓	✓	✓	✓	✓	NR
Yao et al., 2010	NR	NR	NR	NR	NR	NR	NR	NR
Yilmaz et al., 2020	✓	NR	NR	✓	NR	NR	NR	NR
Yoshitake et al., 2007	✓	✓	NR	✓	✓	✓	NR	NR
Zhang et al., 2020	NR	NR	NR	NR	NR	NR	NR	NR
Zhao et al., 2011	✓	✓	✓	✓	✓	✓	NR	NR
Zhen et al., 2011	✓	✓	NR	✓	✓	✓	✓	NR
Zhou et al., 2022	NR	NR	NR	NR	NR	NR	NR	NR
Zhou et al., 2019	NR	NR	NR	NR	NR	NR	NR	NR
Total (%)	35 (29.17)	47 (39.17)	23 (19.17)	43 (35.83)	39 (32.50)	53 (44.17)	44 (36.67)	13 (10.83)

2.3.3 Analysis of Variance

All grand means, MOD means, and region means for TRP and KYN are summarised in Table 2.6. The weighted grand mean concentration of TRP in serum across all MODs was calculated to be 60.52 ± 15.38 μM (see Figure 2.3). Mean reported TRP serum content in America, Asia, Australia, Europe, and the Middle East were 49.66 ± 2.75 μM, 60.30 ± 8.69 μM, 67.26 ± 11.19 μM, 60.73 ± 10.66 μM, and 54.01 ± 18.44 μM, respectively. Mean reported TRP content detected in the serum by MS, FL, and ECD were 60.39 ± 19.24 μM, 63.63 ± 15.36 μM, and 63.04 ± 20.23 μM, respectively. In comparison, the weighted grand mean concentration of TRP in plasma was calculated to be 51.45 ± 10.47 μm (Figure 2.4). Mean reported TRP plasma content in America, Asia, Australia, Europe, and the Middle East were 34.68 ± 9.90 μM, 53.81 ± 9.95 μM, 42.87 ± 8.51 μM, 52.92 ± 12.06 μM, and 34.82 ± 10.52 μM, respectively. Mean reported TRP content detected in the plasma by MS, FL, and ECD were 50.07 ± 16.65 μM, 59.26 ± 15.02 μM, and 26.49 ± 14.17 μM, respectively. MOD accounts for 2.96 % of the total variance seen within the data, $F(2, 6542) = 114.16$, $p < .001$. Sample matrix accounted for 3.23 % of the total variance seen within the data, $F(1, 6542) = 248.93$, $p < .001$. The interaction between MOD and sample matrix accounted for 1.53 % of the total variance seen in the data $F(2, 6542) = 58.94$, $p < .001$.

The weighted grand mean concentration of KYN in serum across all MODs was calculated to be 1.96 ± 0.51 μM (Figure 2.5). Mean reported KYN serum content in Asia, Australia, Europe, and the Middle East were 1.68 ± 0.43 μM, 2.43 ± 0.59 μM, 2.07 ± 0.52 μM, and 1.56 ± 0.62 μM, respectively. Mean reported KYN content detected in the serum by MS and UV-Vis were 1.90 ± 0.73 μM and 2.22 ± 0.69 μM, respectively. In comparison, the weighted grand mean concentration of KYN in plasma across all MODs was calculated to be 1.82 ± 0.54 μM (Figure 2.6). Mean reported TRP plasma content in America, Asia, Australia, Europe, and the Middle East were 0.94 ± 0.24 μM, 1.61 ± 0.34 μM, 2.12 ± 0.52 μM, 2.29 ± 0.73 μM, and 1.53 ± 0.72 μM, respectively. Mean reported KYN content detected in the plasma by MS and UV-Vis were 1.91 ± 0.87 μM and 1.95 ± 0.54 μM, respectively. MOD accounts for 1.30 % of the total variance seen within the data, $F(1, 3868) = 51.37$, $p < .001$. Sample matrix accounted for 0.71 % of the total variance seen within the data, $F(1, 3868) = 27.96$, $p < .001$. The interaction between MOD and sample matrix accounted for 0.79 % of the total variance seen in the data, $F(1, 3868) = 31.25$, $p < .001$.

Table 2.6. Normative data generated including grand mean concentrations (across all MODs and regions), region mean concentrations for TRP and KYN across serum and plasma, and MOD mean concentrations. Values presented are mean (μM) ± SD.

Mean	TRP Serum	TRP Plasma	KYN Serum	KYN Plasma
Grand Mean	60.52 (15.38)	51.45 (10.47)	1.96 (0.51)	1.82 (0.54)
By Region				
America	49.66 (2.75)	34.68 (9.90)	NR	0.94 (0.24)
Asia	60.30 (8.69)	53.81 (9.95)	1.68 (0.43)	1.61 (0.34)
Australia	67.26 (11.19)	42.87 (8.51)	2.43 (0.59)	2.12 0.52)
Europe	60.73 (10.66)	52.92 (12.06)	2.07 (0.52)	2.29 (0.73)
Middle East	54.01 (18.44)	34.82 (10.52)	1.56 (0.62)	1.53 (0.72)
By MOD				
MS	60.39 (19.24)	50.07 (16.65)	1.90 (0.73)	1.91 (0.87)
FL	63.63 (15.36)	59.26 (15.02)	NR	NR
UV-Vis	NR	NR	2.22 (0.69)	1.95 (0.54)
ECD	63.04 (20.23)	26.49 (14.17)	NR	NR

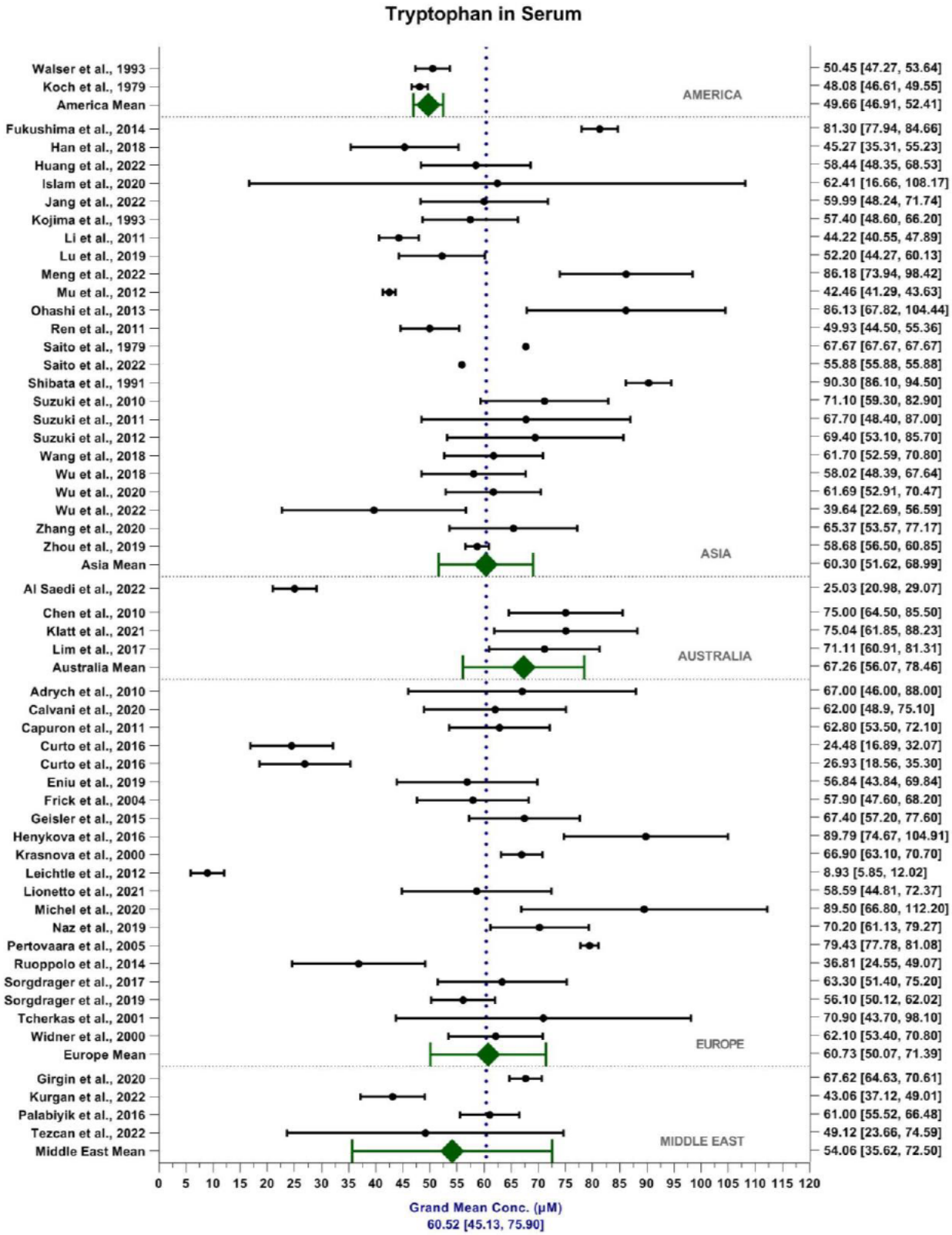

Figure 2.3. *Forest plot depicting weighted grand mean concentrations [95 % CI] of TRP in serum (µM) for each study together with region mean concentrations.*

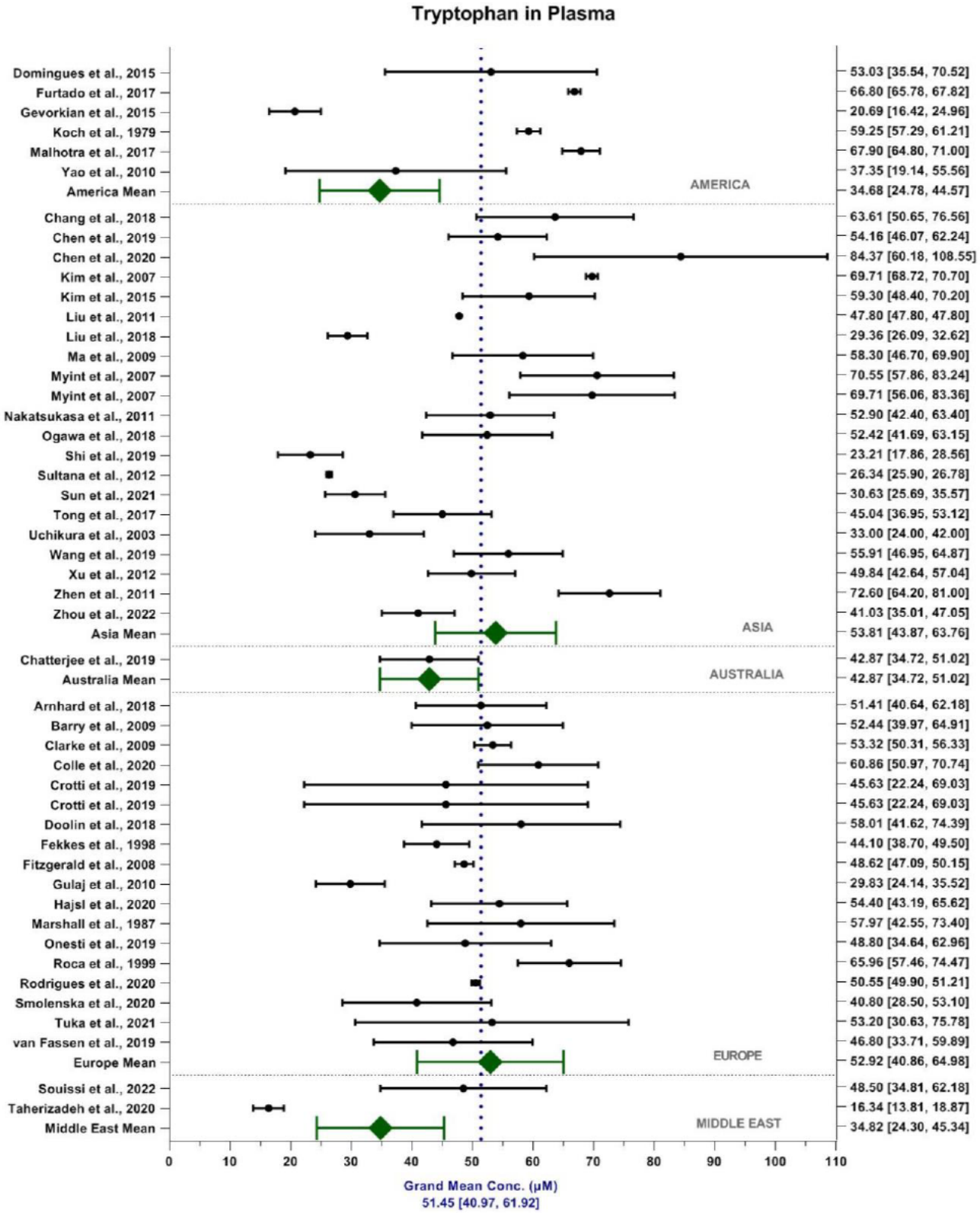

Figure 2.4. *Forest plot depicting weighted grand mean concentrations [95 % CI] of TRP in plasma (µM) for each study together with region mean concentrations.*

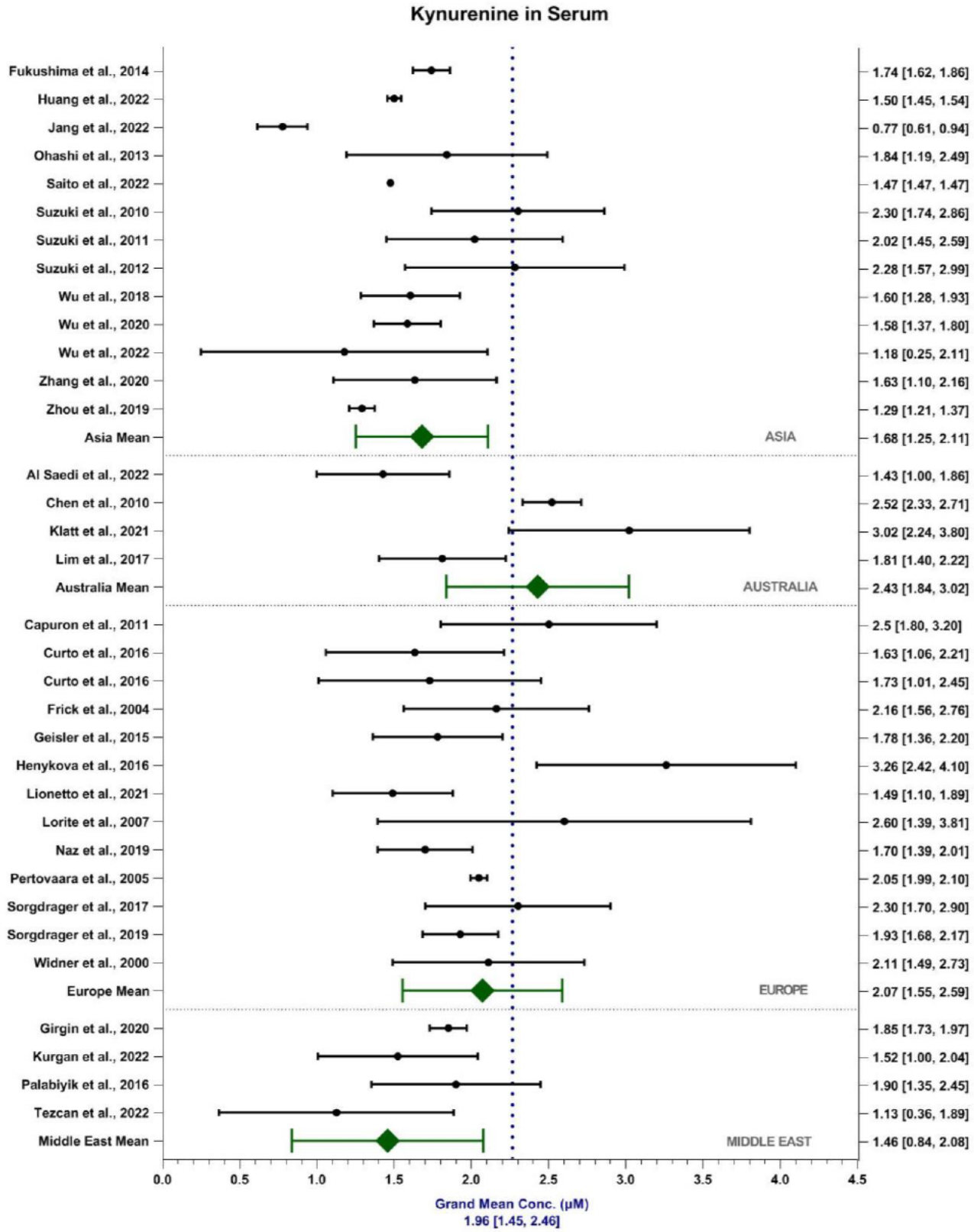

Figure 2.5. *Forest plot depicting weighted grand mean concentrations [95 % CI] of KYN in serum (μM) for each study together with region mean concentrations.*

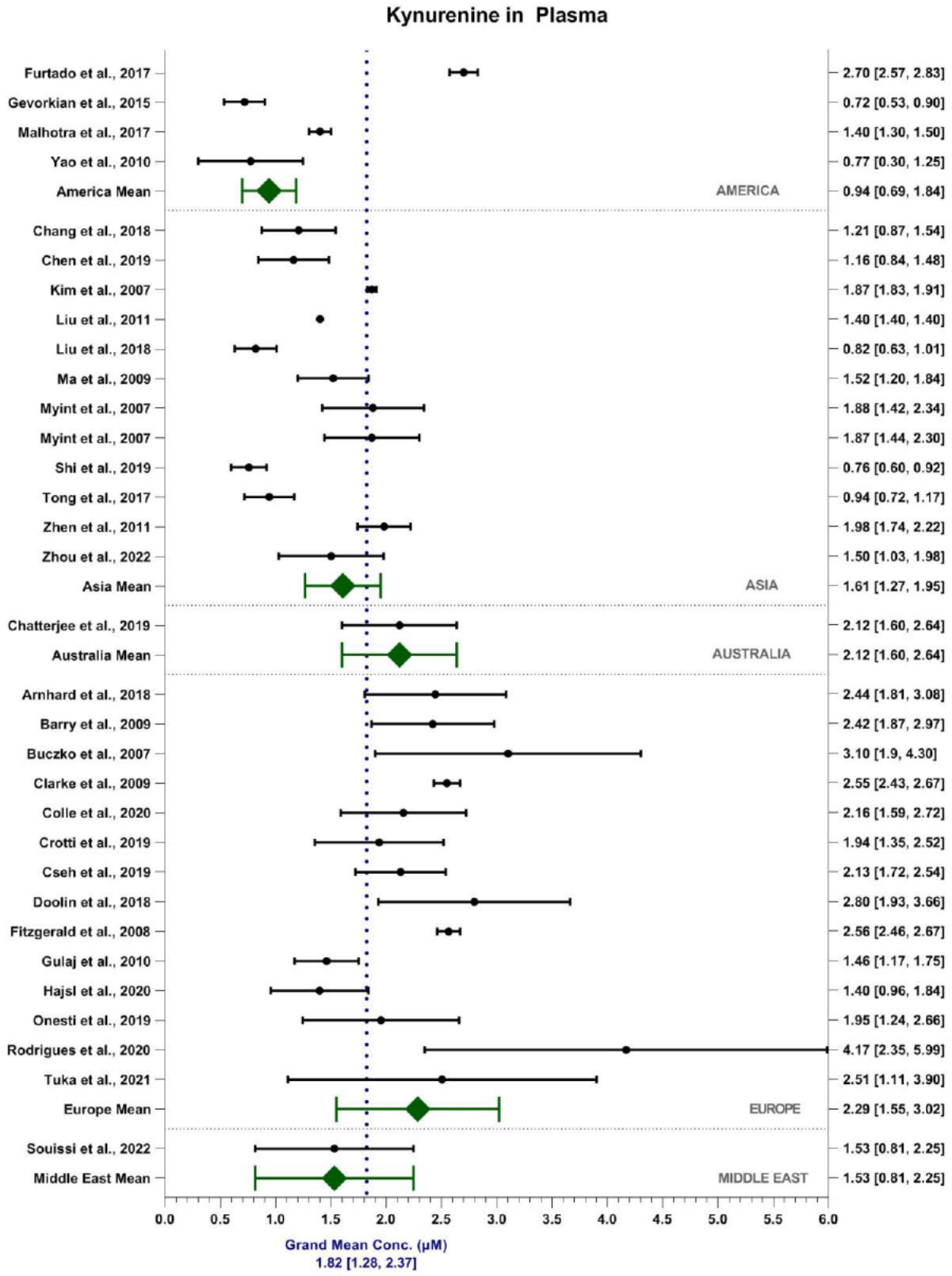

Figure 2.6. *Forest plot depicting weighted grand mean concentrations [95 % CI] of KYN in plasma (µM) for each study together with region mean concentrations.*

2.3.4 Regression Analyses

Outcomes from the adjusted weighted-variance OLS regressions including non-standardised β-coefficients and 95 % CIs are listed in Table 2.7, for TRP and KYN concentrations in serum, plasma, and blood, assessing predictors of biological sex, age, and study year, with *p*-values for statistically significant outcomes bolded. In summary, female sex, age, and study year were negatively correlated with TRP concentrations measured within the serum. Age and study year were also negatively correlated with TRP concentrations measured within the plasma, although no significant interaction was observed between biological sex and the concentrations measured. Across both serum and plasma, biological sex, age, and study year were correlated with TRP concentrations measured within the serum. These trends were repeated when considering KYN, although age was positively correlated with KYN concentrations. Interestingly, biological sex was not correlated with both TRP and KYN concentrations within the plasma.

Table 2.7. Adjusted weighted-variance OLS regression analyses for TRP and KYN. TRP blood and KYN blood are the pooled sample sizes of serum and plasma studies.

	β-Coeff [95 % CI]	***p***
	TRP Serum	
Female Sex	-0.22 [-0.39, -0.05]	**.012**
Age	-0.20 [-0.38, -0.01]	**.036**
Study Year	-1.37 [-1.67, -1.08]	**<.001**
	TRP Plasma	
Female Sex	-0.03 [-0.08, 0.02]	.266
Age	-0.74 [-0.86, -0.61]	**<.001**
Study Year	-1.58 [-1.87, -1.30]	**<.001**
	TRP Blood	
Female Sex	-0.14 [-0.19, -0.09]	**<.001**
Age	-0.55 [-0.65, -0.45]	**<.001**
Study Year	-1.35 [-1.55, -1.16]	**<.001**
	KYN Serum	
Female Sex	-0.05 [-0.05, -0.04]	**<.001**
Age	0.01 [0.00, 0.01]	**.002**
Study Year	-0.08 [-0.08, -0.07]	**<.001**
	KYN Plasma	
Female Sex	-0.00 [-0.00, 0.00]	.431
Age	0.02 [0.01, 0.02]	**<.001**

Study Year	-0.08 [-0.10, -0.07]	**<.001**
	KYN Blood	
Female Sex	-0.02 [-0.02, -0.02]	**<.001**
Age	0.01 [0.01, 0.01]	**<.001**
Study Year	-0.11 [-0.12, -0.11]	**<.001**

2.4 Discussion

Compelling evidence has identified certain KP metabolites as ideal targets for precision medicine initiatives [120], although these initiatives are currently limited by the availability and quality of the data summarising the KP profile [267, 268]. The current study addressed this gap by systematically mapping study characteristics according to MOD, metabolites studied, sample matrices, and geographical location, and calculating normative means across these variables. The methodologies and risk of bias reported within the literature were also critically appraised, the variance between MODs was ascertained, and the relationship between metabolite, biofluid, age, sex, and study year were determined.

The TRP and KYN metabolomic studies reported within this meta-analysis indicate that the most common MOD is MS (n = 80). This is in comparison to the popular but lesser common FL and UV-Vis. Qualitative appraisal of the data suggested that there was no link between study age and MOD. High resolution MS is commonly used in lipidomics [269], proteomics [270], and precision pharmacology [271]. It is thought that MS offers significant advantages in terms of sensitive analytical profiling [272], and that FL and UV-Vis MODs are limited in their sensitivity by the light-emitting capacity of the metabolite [273]. Nonetheless, the photophysical properties of TRP and KYN are well-characterised, and it is generally accepted that TRP and KYN are moderate emitters of intrinsic fluorescence and thus suitable for light-based detection [274]. FL and UV-Vis are suitable MODs for the analytical profiling of KP metabolites despite their less frequent use in the literature. Certain MOD averages reported within this meta-analysis, such as ECD, indicate extreme outliers. This may be due to the small number of studies reported within the literature, all of which vary in sample size.

It is essential to flag that MOD accounted for a small, but statistically significant proportion of the total variance within the data (2.96 %; p < .001). The MS means generally aligned best with the weighted grand mean calculations, which may be due to its greater sensitivity and specificity (i.e., accuracy and less variability across studies), given that the weighted mean accounted for the larger number of studies utilising MS. For example, the weighted grand mean concentration of TRP in serum across all MODs was calculated to be

60.52 ± 15.38 μM, while that in MS-specific studies was 60.39 μM. In plasma, the weighted grand mean concentration of TRP was 51.45 ± 10.47 μM, while the MS-specific mean was 50.07 μM. This trend was also observed within KYN in serum (grand mean concentration of 1.96 ± 0.51 μM versus an MS-specific mean of 1.90 μM) and KYN in plasma (grand mean concentration of 1.82 ± 0.54 μM versus an MS-specific mean of 1.91 μM). This suggests that MS consistently produces the most accurate results in measuring TRP and KYN. Sample matrix (serum vs. plasma) accounted for 0.71 % of the total variance ($p < .001$); a small proportion (albeit statistically significant) indicating their comparability. The interaction between MOD and sample matrix accounted for a small 0.79 % of the total variance ($p <.001$) suggesting that choice of MOD should not affect the comparability of results between serum and plasma. Considering that the intra-coefficient and inter-coefficient of variation for a single assay will typically vary within an acceptable range of 3–7 %, the < 1% variation between sample types, and the interaction between sample type and MOD observed here at the population level is negligible. These are novel findings, and the correlation between serum and plasma is further explored in Chapter 3 of this thesis.

Despite the significant variance observed, the findings reported in this meta-analysis are generally comparable to those reported within the literature. For instance, Geisler and colleagues [178] reported a mean TRP concentration of 67.41 ± 2.04 μM and KYN concentration of 1.78 ± 0.08 μM in the serum of 100 (42 % female) healthy adults. The variation in region means across the America, Asia, Australia, Europe, and Middle East regions may be attributed to dietary quality. For instance, across serum and plasma TRP and KYN concentrations, American and Middle Eastern data were consistently lower than the grand population mean. It is generally well-understood that high protein and fat intake may increase the availability of TRP in the plasma and hence upregulate the KP, and vice versa [37].

Furthermore, this meta-analysis reported on a total of eight sample types for the quantification of TRP, including serum ($n = 54$), plasma ($n = 48$), urine ($n = 15$), cerebrospinal fluid (CSF; $n = 6$), saliva ($n = 2$), tears ($n = 1$), faeces ($n = 1$), and whole blood ($n = 1$). By comparison, six sample types were reported in the quantification of KYN, including serum ($n = 34$), plasma ($n = 33$), urine ($n = 7$), CSF ($n = 6$), saliva ($n = 2$), and faeces ($n = 1$). Only serum and plasma TRP and KYN concentrations were meta-analysed to quantify the associations between the two matrices that are often used interchangeably [275]. Despite the heavy focus on metabolomic profiling within the blood, these results suggest that there is a growing interest in minimally invasive sample types, including saliva and urine. For instance,

the majority (66.67 %) of the TRP urine papers have been published since 2015 [152, 177, 181, 187, 214, 220, 234, 248, 258, 260], while all included TRP saliva studies were published within this timeframe [162, 195]. Saliva metabolomics is an emerging field of metabolomic research due to the diversity of metabolites present and the relative ease in sampling including decentralised approaches [276]. Interest in saliva metabolomic profiling has also piqued due reductions in the LOD of MS technologies and thus the improving analytical sensitivity [272]. Urine is also of interest in the field of biomarker discovery research as it is non-invasive, readily obtained in large quantities, and generally has high patient compliance [277]. Further, urine may provide a relatively richer matrix for analysis, given that concentrations of metabolites are often higher in the urine (due to their excretion) when compared to serum [278]. In states of homeostasis, this indicates that urine may be more susceptible to metabolic changes when compared to the serum [278]. Therefore, saliva and urine present rich matrices for simple disease diagnostics in a minimally invasive and generally inexpensive manner [276]. Future studies should thus seek to profile the KP metabolome within the saliva and urine across a broader range of metabolites than those presented within this meta-analysis and should seek to identify any correlations with traditional biofluids of interest such as serum and plasma (the primary aim of Chapter 3).

Moreover, while efforts are being made to improve the accuracy and sensitivity of KP metabolomics approaches, there are significant limitations, as detected by the author-reported validation parameters and the independent risk of bias assessment. Overall, 80.83 % (N = 73) of studies reported at least one parameter of their independent methodological validation, chosen in compliance with the guidelines for pharmaceutical and biotechnology industries [142]. Certain validation parameters were reported infrequently, such as robustness, defined as the ability of an analytical assay to obtain replicable values despite minor variations in the chromatographic conditions. Despite the dedicated experimental designs for robustness testing of LC-MS instruments reported within the literature [279], this parameter was only reported in 10.83 % of the included studies. Risk of bias appraisal also questioned the reliability of the results reported here. Overall, only 51.67 % of studies included in this meta-analysis were subjectively appraised to be of high quality. This was largely linked to poor reporting of methodological conditions (only reported in 34.17 % of included studies). It is generally acknowledged that MS data presents various complexities linked to the various independently developed acquisition methods, diverse workflows, and non-standardised manual curation [280]. Interestingly, the studies which were appraised to be of low quality often reported

significant deviations from the calculated grand population mean [167, 184, 193, 208, 254]. While this suggests that the results presented in this chapter should be interpreted with caution, this meta-analysis featured a very large sample size and TRP and KYN means are generally considered statistically reliable.

Regression analyses indicated various findings that may have implications for biomarker discovery research. Interestingly, age of the study (study year) was negatively correlated with concentrations of TRP and KYN within the serum and plasma. While this finding has not been directly replicated within the literature, it may be hypothesised that this is attributed to dietary changes. Changes in socio-economic status, urbanisation, and population growth have been linked to dietary and nutritional change in various regions including Asia and South America [281-283]. This may also be attributed to the increased analytical sensitivity, chromatographic separation efficiency, and speed which were developed and reported across 2000 to 2010 [284]. For example, most of the included studies published beyond 2020 have reported the use of use of columns with sub-2μm particles, ranging from 1.6–1.9 μm [148, 155, 161, 164, 189, 192, 207, 212, 226, 228, 237, 245]. Sub-2 μm particles have demonstrated significant gains in terms of faster chromatographic analysis, reduced peak dispersion and increased sensitivity, increased efficiency, and improved resolution [285, 286]. The optimisation of these conditions may be leading to the more selective measurement of metabolites.

Across the serum and plasma, multiple negative correlations were observed: increased percentage female biological sex (relative to male) was correlated with lower levels of TRP and KYN concentrations compared to male sex, while TRP concentrations decreased as age increased. The significant negative correlation between biological sex and TRP and KYN concentrations is generally accepted within the literature, where the concentrations in healthy females are often lower than that of healthy males [178]. In comparison, a positive correlation was observed between age and KYN concentrations, meaning that KYN concentrations in the serum and plasma increased with advancing age. The negative correlation between age and TRP concentrations is also supported by literature that shows the increased degradation of TRP in older age [287]. Furthermore, a recent study conducted in older adults identified a negative association between TRP and age, alongside a positive association between KYN and age [288, 289], suggesting that KP metabolites are strong predictors of age. These findings, together with those in the literature, help with the accurate profiling of the KP metabolome in diseases which show sex and age specific differences, such as AD. Age and sex specific data are

required for the development of accurate prognostic, diagnostic, and theragnostic biomarkers, as summarised in Table 2.2.

Strengths of the present study include a broad eligibility criteria and thorough search strategy that detected ample records; the inclusion of only normative data; the inclusion of only targeted metabolomics data to ensure that precise metabolites have been profiled; and rigorous appraisal of the risk of bias and the validation parameters applied for methodological appraisal. Furthermore, this study had a large sample size (n = 8,089) of relatively younger ($\leq$ 50 years) adults (47.35 ± 15.65), suggesting that it is statistically powered to detect accurate results. However, there was an unequal distribution of sexes, with 77.67 % of participants being of female biological sex. Moreover, meta-analysis was limited to serum and plasma data due to insufficient sample size in other minimally invasive sample types such as saliva or urine; a potential avenue for future empirical studies (see Chapters 3 and 4). This meta-analysis was also limited to only TRP and KYN concentrations, and future studies should seek to assess other biologically active KP metabolites, including KA and QA (as covered in Chapters 3 and 4). Majority of the normative data presented in the literature is investigated within cohorts that are deemed healthy as screened against a singular health concern. For example, many clinical studies focused on neurodegenerative disease will comment on metabolite concentrations in reportedly healthy older adults, screened only against neurodegenerative criteria, and without regard for other important considerations such as cardiovascular health or autoimmune diseases. This suggests that there may be some ambiguities in classifying normative data within the literature from the meta-analytic approach employed here. Nonetheless, this review was intended to inform future empirical research and method development studies and successfully synthesised pilot data.

Findings from this systematic review and meta-analysis have highlighted multiple future avenues for research. Importantly, future studies should seek to investigate and control for the relationship between geographical location and metabolite concentration, as well as clarify the relationship between study age and findings. Normative data should also be grouped into age and biological sex-specific brackets to ensure accuracy and specificity when being used for biomarker purposes. Specifically, studies should seek to report the metabolic profile of males and females separately, although the differences may not appear statistically significant within small sample sizes. Moreover, future method development studies should seek to standardise acquisition methods, workflows, and manual curation methods to improve the comparability of methodologies before moving into clinical and at-risk cohorts. Finally, future studies should consider comprehensively profiling the full KP metabolome (all major

metabolites) within an array of biological sample matrices and should seek to identify any correlations between samples to determine the diagnostic accuracy of the KP across a range of inflammatory health conditions.

Chapter 3: Tryptophan Metabolic Profiling in the Minimally Invasive Biofluids of Healthy Adults: Approach for Biomarker Discovery in Clinical Research

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Foreword:

In this chapter, all major biologically active metabolites within the kynurenine pathway were profiled in the serum, plasma, saliva, and urine of healthy adults in an effort to generate normative data. Data are reported descriptively and a correlation matrix for all data was calculated. Correlations were used to calculate the strength of association between all ten individual metabolite concentrations across the four sample types. This chapter is framed in the context of the thesis with reduced repetition of background information and a tailored discussion to improve readability. The chapter is currently being prepared as a stand-alone journal article manuscript that will initially be submitted to the preprint server *bioRvix*.

Metri, N.J., Christofides, K., Bustamante, S., Low, M., Steiner-Lim, G.Z., Lim, C.K (in preparation). Tryptophan metabolic profiling in the minimally invasive biofluids of healthy adults: Approach for biomarker discovery in clinical research. *bioRvix.*

3.1 Introduction

Tryptophan (TRP) metabolism and the kynurenine pathway (KP) have gained much interest in recent times given their role in health and disease via modulation of both innate and adaptive immune responses and inflammation [133, 134]. Given the link between the KP alterations and multiple diseases (see Chapters 1 and 2 for more detail), it has been suggested that the thorough characterisation of KP analytes may improve patient disease outcomes by revealing appropriate prognostic, diagnostic, and theragnostic biomarkers [290].

Meta-analyses have profiled KP metabolites across various clinical cohorts within the plasma, serum, urine, and cerebrospinal fluid [125, 136-139], and across the serum and plasma in healthy individuals (Chapter 2). Select studies have also profiled KP metabolites within the saliva of clinical cohorts [116]. Compared to the breadth of these clinical data, few studies have reported on the KP profile in the urine and saliva of healthy and younger cohorts (e.g., aged $\leq$ 50 years), as identified in Chapter 2. There is also a need to profile the KP within a cohort that has been comprehensively screened for all major illnesses, as this is a gap in the current literature (discussed in Chapter 2). Further, as also seen in Chapter 2, it is very rare for these studies to comprehensively map sample types together to see how they may compare. Specifically, no studies compared TRP and KYN concentrations within the saliva and urine.

The majority of KP metabolomic studies report on levels of TRP and kynurenine (KYN). However, biologically active downstream metabolites including kynurenic acid (KA), anthranilic acid (AA), 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), xanthurenic acid (XA), quinolinic acid (QA), and picolinic acid (PA) are not routinely assessed [125, 137-139]. Inter-sample variation in KP profiling is also less characterised, which is significant as studies have reported that the metabolite concentrations detected in separate peripheral samples, such as serum and urine, are statistically independent [140].

The present study aimed to identify and develop an analytical method to quantify KP metabolites, beyond TRP and KYN, in the liquid biopsies of healthy adults in a range of human biofluids going beyond blood samples. Objectives included examining KP metabolites in the liquid biopsies (blood plasma and serum, saliva, and urine) of healthy adults via an observational cross-sectional study design at one timepoint. The primary outcome from this research included the characterisation of normal KP function across minimally invasive sample types.

3.2 Methods and Materials

3.2.1 Study Setting & Participants

Recruitment and data collection were conducted from August to November 2022 in the HEADBOX Lab at the NICM Health Research Institute, Western Sydney University, Westmead campus. Human research ethics approval for the procedure followed by this study was granted by the Western Sydney University Human Research Ethics Committee (H14815). Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Biosafety approval was granted by the Western Sydney University Biosafety and Radiation Safety Committee (B14819).

Participants recruited were twenty adults with self-reported good general health and wellbeing assessed against the following inclusion criteria: (i) aged between 18 and 50 years; (ii) of good general health and wellbeing as determined by the research team based on participant self-report and the Short Form 36 Health Survey Questionnaire (SF-36); and (iii) English fluency; the SF-36 was modified for conciseness and therefore no cut-off score was applied. If required, participant responses were discussed with the wider research team for concordance in determining eligibility. Given this was a proof-of-concept study, a formal statistical power calculation was not performed [291].

For sample collection, all participants were instructed to: (i) to avoid moisturising their skin the day of sample collection; (ii) avoid eating, smoking, or brushing their teeth in the hour before sample collection; and (iii) rinse their mouth with water ten minutes prior to saliva collection. Participants then provided blood, saliva, and urine samples that were processed within 30 minutes of collection, and all samples were stored at at -80 ^{o}C until further analysis [292, 293].

3.2.2 Materials

A tourniquet was applied to the participant's arm (over the bicep muscle, proximal to the elbow). The prominent veins were located and palpated on the ante-cubital area. Warm packs were used if locating a prominent vein proved difficult. A 23 G × 19 mm butterfly needle with lure, one 10 mL SST™ II vacutainer collection tube, one 10 mL EDTA vacutainer collection tube, and vacutainer standard holder (Becton, Dickinson, and Company, USA) were used to collect two 10 mL tubes of whole blood from each participant. Blood samples were weighed and centrifuged at 4 ^{o}C for 10 minutes at 2500 rpm to separate the serum and plasma. Serum and plasma (500 μL) were aliquoted into 1500 μL Eppendorf tubes using 1000 μL pipette tips.

1mL of saliva was collected into 15mL Eppendorf tubes through passive drool. Participants were advised that they should spit into the jar until the amount of liquid saliva (not bubbles) reached the 1 mL fill line. Protein concentration within the saliva was quantified using a Pierce BCA Protein Assay Kit (ThermoScientific, Massachusetts, USA).

Mid-stream urine was collected into a sterile 50 mL Sarstedt collection container. Samples were then centrifuged for 10 minutes at 2500 rpm and 4 °C. Following centrifuging, 500 µL of the contents of the supernatant layer was aliquoted into 1500 µL Eppendorf tubes using 1000 µL pipette tips.

3.2.3 Reagents

All reagents used, including formic acid (FA), methanol (MeOH), and water, were of analytical mass spectrometry-grade and purchased from ChemSupply, NSW, Australia. Upon preparation, all solvents were sonicated (PowerSonic410, Thermoline Scientific, NSW, Australia) for 20 minutes prior to use.

3.2.4 Extraction of Polar Metabolites

10 µL of mixed internal deuterated standards consisting of 10 µM dTRP , dKYN, dAA and d5HIAA (CDN Isotopes, Canada) was dispensed into a 1500 µL Eppendorf tube. Then, 50 µL of plasma/serum/urine or 500 µL saliva was added to the Eppendorf tube containing the internal standards and vortexed thoroughly. 40 µL of MS-grade water containing 0.1 % formic acid (FA) was added to the tube, followed by 400 µL of freezer cold MS-grade methanol. The mixture was incubated in a -20 °C freezer for one hour to allow protein precipitation. Following complete protein precipitation, the mixture was centrifuged at 12,000 g for 10 minutes at 4 °C. The supernatant was collected into a 1500 µL Eppendorf tube and dried in a Speedvac concentrator (Savant DNA120 Speedvac Concentrator, ThermoScientific, Massachusetts, USA) on a high setting for 45 minutes until pelleted. Following concentration, the plasma, and serum residue was reconstituted in 100 µL of water containing 0.1 % FA. Saliva residue was reconstituted in 50 µL of water containing 0.1 % FA for a 4× concentration. Urine residue was reconstituted in 500 µL of water containing 0.1 % FA. 100 µL of the reconstituted urine residue was pipetted into a separate tube containing 900 µL of water containing 0.1 % FA to make a final 1/1000 dilution. The mixture was vortexed thoroughly to ensure that the residue was completely dissolved within the mobile phase, then transferred in whole to glass LC vials fitted with glass vial inserts (Shimadzu, Kyoto, Japan).

3.2.5 Metabolomic Analysis

Biospecimen analysis was conducted on a liquid chromatograph mass spectrometer (LCMS-8040; Shimadzu, Kyoto, Japan). Samples were chromatographed isocratically using a Luna 3 µm PFP(2) 100Å 100 × 2 mm column (Cat. #00D-4447-B0 Phenomenex, Australia), with a mobile phase consisting of 0.1 % FA in water at 0.3mL/min and solvent phase consisting of MS-grade 100% MeOH. Acquisitions were performed in ESI positive ionisation mode using an optimized protocol for establishing settings for each of the analytes individually, then collectively. Multiple-reaction-monitoring (MRM) parameters for each metabolite were established using commercial standards (Thermo Fisher Scientific, Massachusetts, USA) that were prepared at a concentration of 100 µM and run individually. Detected collision energies and mass/charge ratio (m/z) values were used to optimise ion fragmentation needed for the detection of low-level metabolites using the onboard method optimisation tool. Once individual MRM parameters were established for each metabolite, a combined MRM file for all the KP metabolites was piloted with the gradient elution of the solvent phase optimised to the following: 10 % at 0–7 minutes, 60 % at 7–8 minutes, 100 % at 8–8.5minutes, 10 % at 8.5–10 minutes (stop at 10 minutes). 10 µM of TRP was used as a positive control and was run at the beginning and end of each batch on each day. This positive control running standard was used to monitor within-day and between-day coefficients of variation. Determination of the limit of detection for each metabolite was based on a visual evaluation of the signal-to-noise ratio and the peaks detected. This analytical methodology is currently undergoing further validation testing and is being prepared for publication as a separate methodological validation study.

3.2.6 Statistical Methods and Variables

A data-driven approach was applied to the de-identified data in Stata™ v17 (StataCorp, Texas, USA). Potential confounders and effect modifiers, such as biological sex and age, were present although not controlled for due to the small sample size and proof-of-concept nature of this study. Data are reported descriptively and a correlation matrix for all data (sample types and KP metabolites) was calculated. Non-parametric Spearman correlations were used to calculate the strength of association between individual metabolite concentrations across sample types.

3.3 Results

3.3.1 Participant Demographics and Metabolite Concentrations

A total of 20 healthy participants were recruited for biological sample collection, ranging from 21 to 46 years of age. Blood was not able to be drawn from four participants (total $N = 16$), while one participant was unable to provide saliva and another urine (total $N = 19$). Participant demographics are presented in Table 3.1. Mean blood, saliva, and urine metabolite concentrations are presented in Tables 3.2–3.4.

Table 3.1. Participant demographics including age, sex, smoking status, and BMI.

	Participants (*N* = 20)
Mean Age (SD)	28.3 (7.8)
Sex (F/M)	10/10
Smoking Status (Y/N)	2/18
Mean BMI (SD)	27.7 (6.4)

Table 3.2. Mean blood metabolite concentrations in serum and plasma for CRE, TRP, KYN, 3-HK, KA, 3-HAA, AA, XA, PA, and QA.

Metabolite	Mean (SD) (*N* = 16)	
	Serum	**Plasma**
CRE (μM)	78.60 (24.18)	69.11 (20.47)
Male	98.93 (16.01)	78.83 (19.45)
Female	58.27 (7.22)	59.40 (17.43)
TRP (μM)	68.00 (14.99)	57.68 (15.89)
Male	76.24 (9.67)	63.93 (11.94)
Female	59.76 (15.26)	51.44 (17.59)
KYN (μM)	3.70 (0.84)	2.62 (0.54)
Male	3.81 (0.58)	2.69 (0.24)
Female	3.58 (1.08)	2.55 (0.75)
3-HK (nM)	50.47 (6.27)	42.19 (5.30)
Male	51.32 (3.79)	44.09 (5.09)
Female	49.61 (8.26)	40.28 (5.11)
KA (nM)	35.50 (30.91)	21.39 (7.49)
Male	33.75 (16.02)	24.73 (7.94)
Female	37.25 (42.23)	18.04 (5.61)
3-HAA (nM)	21.07 (16.03)	19.73 (6.76)
Male	26.72 (17.69)	21.77 (6.54)
Female	15.43 (12.85)	17.68 (6.77)
AA (nM)	172.60 (261.48)	76.18 (30.46)
Male	110.00 (67.98)	78.98 (19.47)
Female	235.21 (364.60)	73.39 (39.89)
XA (nM)	16.46 (21.49)	10.07 (7.65)
Male	18.34 (20.75)	11.44 (7.57)
Female	14.58 (23.48)	8.70 (7.99)
PA (nM)	144.18 (203.41)	225.21 (125.86)
Male	140.57 (193.01)	165.56 (83.52)
Female	147.80 (226.66)	284.85 (137.26)
QA (nM)	485.14 (91.39)	431.03 (82.33)
Male	517.40 (48.84)	462.99 (74.67)
Female	452.88 (114.60)	399.08 (81.32)

Note. 3-HAA: 3-Hydroxyanthranilic Acid; 3-HK: 3-Hydroxykynurenine; AA: Anthranilic Acid; CRE: Creatinine; KA: Kynurenic Acid; KYN: Kynurenine; PA: Picolinic Acid; QA: Quinolinic Acid; TRP: Tryptophan; XA: Xanthurenic Acid.

Table 3.3. Mean saliva metabolite concentrations for CRE, TRP, KYN, 3-HK, KA, 3-HAA, AA, XA, PA, and QA.

Metabolite	Mean (SD) (*N* = 19)
Protein (mg/L)	1345.39 (302.07)
Male	1380.27 (290.02)
Female	1306.63 (327.81)
CRE (μM)	2.98 (2.02)
Male	3.11 (2.39)
Female	2.83 (1.65)
TRP (μM/mg Protein)	6.66 (6.26)
Male	6.02 (6.73)
Female	7.37 (6.01)
KYN (nM/mg Protein)	81.53 (138.95)
Male	59.20 (124.57)
Female	101.06 (156.13)
3-HK (nM/mg Protein)	0.78 (1.04)
Male	0.56 (0.85)
Female	1.02 (1.22)
KA (nM/mg Protein)	326.80 (251.03)
Male	274.79 (321.54)
Female	384.59 (135.39)
3-HAA (nM/mg Protein)	50.01 (59.97)
Male	42.78 (60.31)
Female	58.04 (62.15)
AA (nM/mg Protein)	951.49 (1063.70)
Male	787.37 (1087.62)
Female	1133.83 (1069.54)
XA (nM/mg Protein)	77.28 (67.78)
Male	64.47 (73.65)
Female	91.51 (61.65)
PA (nM/mg Protein)	448.04 (374.34)
Male	368.62 (369.39)
Female	536.28 (380.97)
QA (nM/mg Protein)	94.01 (61.88)
Male	89.89 (67.85)
Female	98.60 (58.23)

Note. 3-HAA: 3-Hydroxyanthranilic Acid; 3-HK: 3-Hydroxykynurenine; AA: Anthranilic Acid; CRE: Creatinine; KA: Kynurenic Acid; KYN: Kynurenine; PA: Picolinic Acid; QA: Quinolinic Acid; TRP: Tryptophan; XA: Xanthurenic Acid.

Table 3.4. Mean urine metabolite concentrations for CRE, TRP, KYN, 3-HK, KA, 3-HAA, AA, XA, PA, and QA.

Metabolite	Mean (SD) (*N* = 19)
CRE (mM)	14.61 (10.08)
Male	16.95 (10.26)
Female	12.50 (9.95)
TRP (μM/mM CRE)	4.56 (2.44)
Male	4.27 (1.85)
Female	4.82 (2.95)
KYN (μM/mM CRE)	0.42 (0.25)
Male	0.31 (0.14)
Female	0.51 (0.29)
3-HK (nM/mM CRE)	59.62 (54.27)
Male	42.18 (20.05)
Female	75.31 (70.39)
KA (μM/mM CRE)	1.40 (0.75)
Male	1.06 (0.41)
Female	1.70 (0.88)
3-HAA (nM/mM CRE)	106.01 (96.62)
Male	66.59 (74.82)
Female	141.50 (103.63)
AA (nM/mM CRE)	1.89 (2.54)
Male	1.07 (1.44)
Female	2.62 (3.13)
XA (nM/mM CRE)	0.20 (0.11)
Male	0.17 (0.03)
Female	0.23 (0.14)
PA (nM/mM CRE)	28.65 (41.18)
Male	20.45 (34.32)
Female	36.03 (47.09)
QA (nM/mM CRE)	583.22 (370.81)
Male	492.79 (284.43)
Female	664.60 (433.07)

Note. 3-HAA: 3-Hydroxyanthranilic Acid; 3-HK: 3-Hydroxykynurenine; AA: Anthranilic Acid; CRE: Creatinine; KA: Kynurenic Acid; KYN: Kynurenine; PA: Picolinic Acid; QA: Quinolinic Acid; TRP: Tryptophan; XA: Xanthurenic Acid.

3.3.2 Correlation Analyses

The correlation matrix for all ten metabolites across all four sample matrices is presented in Figure 3.1. Blue cells refer to a negative correlation and red cells a positive correlation, with 0 indicating no relationship. The strength of the relationship is indicated by the intensity of the colour as noted in the scale. A total of eight significant correlations were found across sample matrices (Table 3.5). These included positive correlations between CRE serum and plasma, TRP serum and plasma, KYN serum and plasma, KA serum and plasma, KA saliva and urine, 3-HAA saliva and plasma, and PA plasma and saliva; and a negative correlation between AA serum and urine.

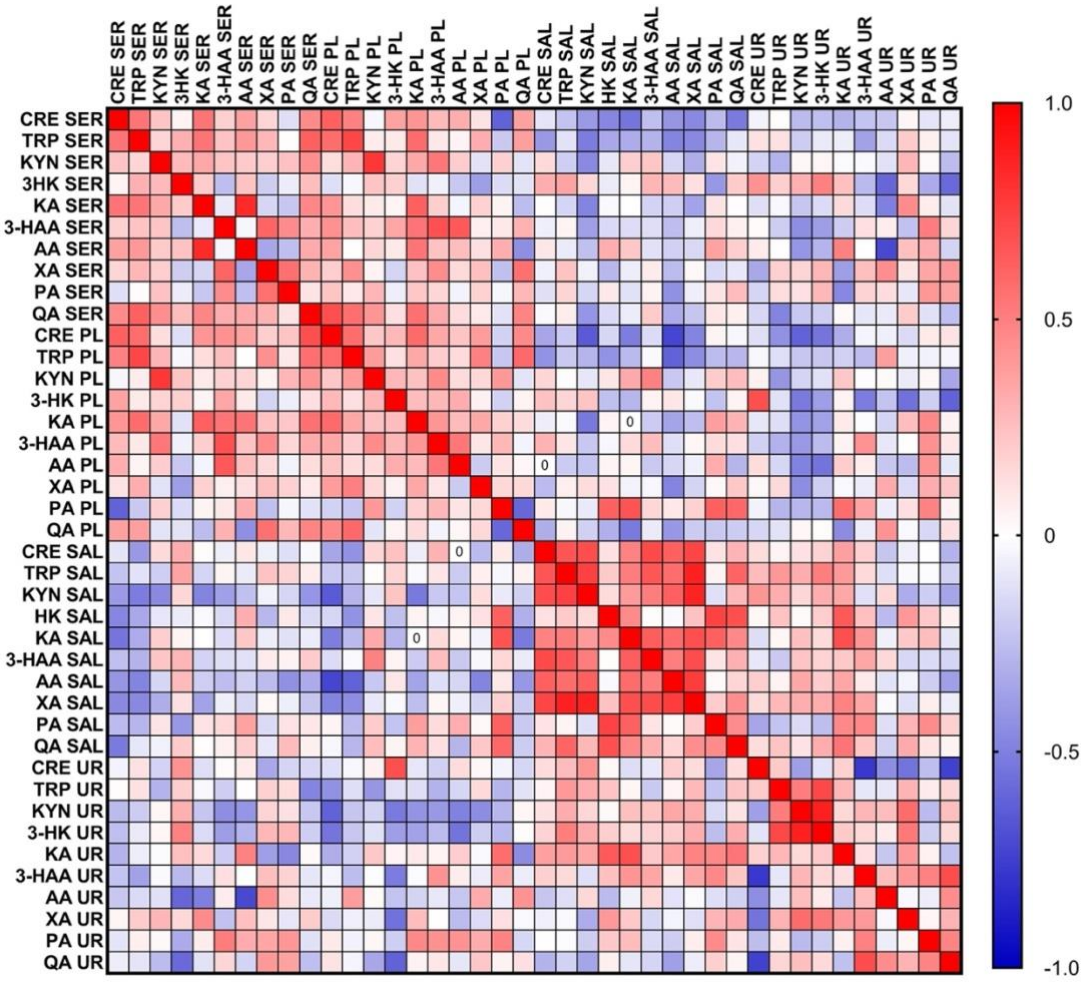

Figure 3.1. *Non-parametric Spearman correlation matrix metabolites across all four sample matrices for the 10 metabolites quantified; red = positive correlation; blue = negative correlation. Note. 3-HAA: 3-Hydroxyanthranilic Acid; 3-HK: 3-Hydroxykynurenine; AA: Anthranilic Acid; CRE: Creatinine; KA: Kynurenic Acid; KYN: Kynurenine; PA: Picolinic Acid; QA: Quinolinic Acid; TRP: Tryptophan; XA: Xanthurenic Acid; SER: Serum; PL: Plasma; SAL: Saliva; UR: Urine.*

Table 3.5. Statistically significant Spearman's correlation coefficients (ρ), 95 % CI and *p*-values across sample matrices.

Correlation	$\rho(df)$	95 % CI	*p*
CRE Serum vs. Plasma	0.6235(30)	0.1694, 0.8592	.0115
TRP Serum vs. Plasma	0.7206(30)	0.3357, 0.8993	.0023
KYN Serum vs. Plasma	0.7824(30)	0.4556, 0.9233	.0006
KA Serum vs. Plasma	0.6206(30)	0.1648, 0.8580	.0120
KA Saliva vs. Urine	0.6863(36)	0.3094, 0.8768	.0017
3-HAA Serum vs. Plasma	0.6799(30)	0.2630, 0.8829	.0048
AA Serum vs. Urine	-0.7157(33)	-0.8974, -0.3267	.0027
PA Plasma vs. Saliva	0.6235(33)	0.1694, 0.8592	.0115

3.4 Discussion

This proof-of-concept study was the first research to compare nine KP metabolites and creatinine within the serum, plasma, saliva, and urine of healthy adults (aged 18 to 50 years), and report on sex-specific data. Sex-specific data were not able to be calculated in the meta-analysis reported in Chapter 2 due to reporting style of the data, and therefore this was a novel contribution by the present study.

Serum and plasma concentrations of TRP observed here were elevated when compared to those reported within Chapter 2 (Table 2.7). Evidence from the meta-analysis reported in Chapter 2 has shown that the mean concentration of TRP in the serum and plasma of healthy adults is 60.52 μM and 51.45 μm, respectively. This is in comparison to the serum and plasma concentrations reported within this study (68.00 μm, and 57.68 μm, respectively). However, when compared to the Australian data presented in Chapter 2 (Table 2.7; 67.26 μm), the TRP serum values reported in this study (68.00 μm) align well. Saliva concentrations observed here were also significantly higher when compared to the normative data reported within other cross-sectional studies (6.66 μm/mg protein vs. 0.24–1.50 μm [162, 195]). As discussed in Chapter 2, urine TRP concentrations are extremely varied with high levels of between-study variability, meaning that data could not be meaningfully synthesised. Therefore, future studies should seek to clarify this by assessing this in larger cohorts and controlling for major influencing factors such as diet.

The KYN concentrations within the serum and plasma reported in this study are also elevated when compared to the normative data reported in Chapter 2 (Table 2.6). Evidence in

the meta-analysis reported in Chapter 2 has shown concentrations of 1.96 μM within the serum and 1.82 μM within the plasma. In comparison, this study has reported relatively higher concentrations (3.70 μM and 2.62 μM within the serum and plasma, respectively). However, when compared to the Australian data presented in Chapter 2 (Table 2.7; 2.43 μM in the serum and 2.12 μM in the plasma), these values are in closer agreement. Saliva concentrations were also elevated when compared to those reported within the literature [154, 195]. Urine KYN concentrations within the literature are varied, as reported within the meta-analysis, but the results presented within this empirical study are comparable [177, 263].

The values reported within this empirical study align closely with the Australian specific data presented in the meta-analysis (Chapter 2; Table 2.6). This supports the hypothesis that dietary factors (i.e., linked to geographical location) may play an important role in the up-regulation or down-regulation of the KP [281-283]. Further, this current study showed elevated concentrations of TRP and KYN within the serum (relative to the plasma), which are in accordance with the meta-analysis reported in Chapter 2 and the wider literature. Studies have reported that the metabolome profiles of serum and plasma are distinct, with many metabolites showing significantly higher concentrations within the serum relative to the plasma [275, 294].

There is little consensus within the reported literature on the normative concentrations of 3-HK, KA, 3-HAA, AA, XA, PA, and QA within the serum, plasma, saliva, and urine of healthy adults. While these concentrations are often profiled in disease cohorts, including psychiatric, neurological, and neurodegenerative diseases (NDDs) such as Alzheimer's disease (AD) [295, 296], there is a scarcity of information characterising normal KP function across the lifespan. Novel data generated from the current study is essential for providing a benchmark for future investigations assessing the full gamut of KP metabolites in clinical and at-risk cohorts across a range of sample types. A priority for future research is to profile these metabolites in a larger sample with a wider age range (i.e., full lifespan including children and older adults) to characterise the transition from healthy to at-risk to disease states.

A major outcome from this study was to determine whether a correlation existed between traditional sample matrices of interest, including serum and plasma, and novel, minimally invasive matrices, such as saliva and urine. In terms of the relationship between KP metabolites in serum and plasma, a total of five significant positive correlations were found for CRE, TRP, KYN, KA, and 3-HAA. The strong relationship between serum and plasma for TRP and KYN replicates what we observed in the meta-analysis reported in Chapter 2. Despite

the differences in absolute concentration of KP metabolites in the serum compared to the plasma (serum is often higher), a high overall correlation has been observed in human serum and plasma metabolite profiles [275].

Interestingly, a total of three significant and novel relationships were found involving saliva and urine. This included a positive correlation between KA in saliva and urine and PA in saliva and plasma, and a negative correlation between AA in serum and urine. KA belongs to the neuroprotective branch of the KP and is often decreased centrally (within the cerebral cortex) and peripherally (within the serum) of people living with an NDD, potentially reflecting the state of neuroinflammation [297, 298]. KA concentrations in the saliva and urine were more closely related than KA in the serum and plasma (r = .6863 vs. .6206), and interestingly, KA levels in saliva and urine were not associated with KA in the serum and plasma. KA, as a metabolite is largely excreted from the body in the urine, and studies exploring inflammatory conditions have found increased KA excretion within the urine [299]. While no specific causal relationship has been observed, this may suggest that the decreased concentrations of KA observed in people with NDDs could be linked to decreased rates of excretion within the urine. Speculatively, this may suggest that urinary KA, and by extension, salivary KA, are both promising options for monitoring the state of inflammation within the body.

PA is another endogenous metabolite that has been shown to possess an array of immunological and neuroprotective effects in the central nervous system [300]. Recent research has shown that plasma levels of PA in people living with AD are inversely correlated with neurotoxic tau in the cerebrospinal fluid (CSF) [120]. Considering the relationship between the levels of PA in the saliva and plasma, further investigation examining the use of salivary PA in AD contexts is warranted. There are several advantages of this, for example, it could be speculated that levels of PA in the saliva (which correlate to the plasma) are inversely related to levels of neurotoxic tau in the CSF [120]. In this instance, lower levels of PA in the saliva may be indicative of greater AD pathophysiology within the central nervous system, although this would require extensive validation from future research. Nevertheless, indexing tau neurotoxicity through measuring PA in the saliva would eliminate the need for a spinal tap to obtain CSF. This is especially important in older adults who are particularly vulnerable to minor stressor events such as spinal taps [90]. The role of AA in health and disease is debated, with some studies reporting attenuation of inflammation and oxidative stress [301], with others reporting that elevated circulating levels of AA in the plasma increases dementia risk by up to 40 % [302]. Our observation shows that the levels of AA in the serum are inversely associated with AA levels in the urine in a healthy state; this will be explored further in Chapter 4.

The correlations observed between saliva and urine, saliva and plasma, and serum and urine can be contextualised by the complex biochemical matrix in the body that extends beyond tissue or fluid borders [303]. It has been proposed that comprehensive approaches that combine the analysis of multiple sample matrices, and the correlations between them, are required to generate an integrative understanding of the body's metabolic processes [303]. While several studies have collected KP metabolomic data across several sample types (see Chapter 2), between-fluid correlation networks are less common in the KP-context. For example, plasma concentrations of several KP metabolites have been shown to significantly correlate with their respective levels within the CSF [120]. Although the relationship between central and peripheral KP concentrations is well-characterised, little is known about the between-fluid KP correlations in the saliva and urine. The current study adds new knowledge on these relationships by being the first study reported within the literature to observe a positive correlation between urine and saliva. Future studies should seek to clarify any sex-specific differences in the identified correlations.

Strengths of the present study include being the first to map the profile of all nine major KP metabolites, alongside CRE, in the serum, plasma, saliva, and urine of healthy adults. This study was also the first to consider a between-fluid correlation matrix in healthy adults. While promising, this was a small, proof-of-concept study that should be replicated in a larger cohort and include other specimen types (such as sebum), to clarify the relationship between central and peripheral KP markers. Additionally, the analytical profiling methods used in this study are yet to be independently validated according to the criteria discussed in Chapter 2 (currently being prepared for publication as a separate method validation study). Future studies should seek to further clarify the nature and mechanism of this relationship in a KP context. Comprehensively profiling the salivary and urinary metabolome in healthy adults, as well as people who are at-risk of diseases such as AD, is an essential pre-requisite for accurate biomarker discovery research.

Chapter 4: Measuring Response to Treatment Using Kynurenine Pathway Biomarkers in People With and Without AD Risk

Foreword: In this chapter, all major biologically active metabolites within the kynurenine pathway were profiled in the serum and urine of cognitively healthy older adults who had their risk of Alzheimer's disease characterised. Data presented are from at two timepoints (before and after a 12-month curcumin intervention). Group differences in the mean change of cognitive scores, Alzheimer's disease risk pathology, and serum kynurenine pathway metabolites were assessed. Between-group differences in urine metabolites were assessed at endpoint only. Correlation analyses were conducted to determine the relationship between metabolites, biospecimen types, and clinical markers. The chapter is currently being prepared as a stand-alone journal article manuscript that will be first submitted to the preprint server *bioRvix*.

Metri, N-J., Chatterjee, P., Goozee, K., James, I., Shen, K., Sohrabi, H.R., Shah, T., Asih, P.R., Dave, P., ManYan, C., Taddei, K., Martins, R.N., Steiner-Lim, G.Z., Lim, C.K (in preparation). Measuring response to treatment using KP biomarkers: A sub-study of a 12-month randomised controlled trial of curcumin for cognitive function in people with and without AD risk. *bioRvix.*

4.1 Introduction

4.1.1 KP Profiling in AD Risk

The kynurenine pathway (KP) has been implicated in an array of diseases, including neurodegenerative diseases such as Alzheimer's disease (AD) and its clinically relevant prodromal phase mild cognitive impairment (MCI) [126]. In neuroinflammatory conditions such as AD, the KP is significantly upregulated. This upregulation has been linked to the increased production of neuroactive metabolites that may be immunomodulatory, neuroprotective, or neuroactive in effect [304]. It is essential to flag that the up regulation of the KP manifests both centrally within the central nervous system and peripherally within circulation [305-307].

It is generally thought that the preclinical-AD phase is characterised by relatively higher levels of neocortical-amyloid load (NAL), and that this accumulation may commence up to three decades prior to any clinically-recognisable cognitive decline [304, 308, 309]. Higher ratios of core KP metabolites, tryptophan (TRP) and kynurenine (KYN), have been observed within the serum and plasma of both AD and MCI cohorts [310, 311]. This is clinically relevant as this ratio is inversely correlated with cognitive performance [310]. A recent study conducted in the KARVIAH Cohort (outlined in Chapter 1) explored the potential for KP upregulation in cognitively normal older adults with higher NAL as detected by positron emission tomography (PET) [304]. That study was conducted on the baseline data from participants enrolled in a 12-month double-blind placebo-controlled clinical trial investigating the therapeutic efficacy of ingesting oral curcumin on cognitive function and NAL. In that study, the authors adjusted for age and AD risk genes and found higher levels of KYN and anthranilic acid (AA) in at-risk females, but not males [304], providing insight into the role of the KP on disease pathophysiology and sex differences. Curcumin therapy is relevant to the KP as it has been shown to have a direct relationship with indoleamine 2,3-dioxygenase (IDO), a regulator of the KP. For instance, multiple curcumin studies have highlighted the inhibition of IDO [312, 313], suggesting that the KP may have potential in measuring the therapeutic response of interventions, however, it has not been investigated in this theragnostic context to date.

The KP profile is not well-characterised across sample types, such as urine. Numerous studies have shown that the targeted metabolic profiling of urine may provide a potential biomarker panel for cognitive dysfunction [115, 314, 315]. For example, a recent study exploring the spectrum of clinical diagnosis found lower levels of TRP in the urine of participants with AD compared to healthy controls [115]. Interestingly, a decreasing trend in

concentrations was observed in accordance with the spectrum of clinical diagnosis (e.g., healthy vs. MCI vs. AD) [115]. While this trend was also observed within the serum [115], other studies have found that the information contained in peripheral samples, such as urine and serum, are statistically independent [118]. Furthermore, defining biomarkers that may predict treatment response is an essential aspect of developing personalised and point-of-care-testing (POCT) [316]. For instance, TRP has been previously identified as a sensitive biomarker for treatment response in personalised cancer treatments [316]. To date, no such studies have been reported in the context of the AD-risk trajectory.

4.1.2 Aims and Objectives

The current study utilised archival data collected from the KARVIAH Cohort in New South Wales, Australia (2015) [304, 317]. The overarching goal of this study was to assess response-to-treatment in a clinical trial to determine whether the KP may be considered a marker for treatment responsivity in an AD-risk context. We conducted a sub-study on the clinical trial data from the curcumin intervention study and aimed to evaluate the predictive role of KP metabolites by exploring: (a) whether there were any differences in the serum and urine KP profiles post-curcumin intervention; and (b) the clinical and pathological relevance of any KP perturbations by assessing their relationship with cognition, as measured by the Mini-Mental State Examination (MMSE), and AD risk determined by PET NAL standardised uptake value ratio (SUVR) values.

4.2 Methods and Materials

4.2.1 Participants

Volunteers (N = 206) underwent screening for inclusion in the KARVIAH Cohort, a healthy older cohort that was screened for AD risk factors. Inclusion criteria for participants included no objective cognitive impairment as confirmed by a score of ≥26 on the Montreal Cognitive Assessment (MoCA) [318]; aged between 65 to 90 years; of good general health and wellbeing with no known significant cerebral vascular disease and corrected vision and hearing; and fluent in English [304, 317]. Exclusion criteria included uncontrolled hypertension (systolic > 170 mmHg or diastolic > 100 mmHg); severe depressive illness as measured by the Depression, Anxiety, and Stress Scale (DASS); an acute functional psychiatric illness; lifetime history of a psychotic disorder such as schizophrenia; lifetime history of stroke; or the diagnosis of dementia according to the revised criteria determined by the National Institute on Aging (Alzheimer's Association) [304, 317, 319].

Volunteers who met the criteria for the KARVIAH cohort (n = 134) underwent blood collection and neuroimaging, as well as further neuropsychometric testing using the MMSE score to confirm normal global cognition (≥26) [304, 320]. Cognitively normal volunteers were included in the current study (n = 97). Their participation was ethically reviewed by the Macquarie University Human Research Ethics Committee, NSW, Australia (No. 5201701078) and the Bellberry Human Research Ethics Committee, NSW, Australia (No. 2012-09-1086). Use of the archival data was approved by the Western Sydney University Human Research Ethics Committee, NSW, Australia (No. H14815). All participants provided written informed consent prior to participating.

4.2.2 Intervention

Participants were enrolled in a 12-month intervention double-blind placebo-controlled clinical trial (full details reported in the ANZCTR: ACTRN12613000681752). During the study, participants ingested oral curcumin (BCM-95™, 1.5mg daily) or a biologically inactive placebo. Following the completion of the trial period, participants were re-assessed using the same testing batter and neuroimaging.

4.2.3 Sample Collection and Genotyping

Study participants fasted overnight for ten hours prior to blood collection (80 mL) using standard venepuncture approaches and processing techniques for blood serum [317]. Bloods were collected at baseline and endpoint. Midstream urine was collected at the time of blood collection only at endpoint as the decision was made to include urine analytes as tertiary outcome measures partway through the trial. Urine was collected into a sterile collection container and centrifuged for 10 minutes at 2500 rpm and 4 °C. All samples were collected in 2015 and stored at -80 ^{o}C until they were thawed and analysed for KP metabolites in 2017.

Apolipoprotein E (*APOE*) was genotyped using purified gDNA extracted from 500 μL whole blood. Participant samples were genotyped and profiled for the ε2, ε3, and ε4 *APOE* variants as per the TaqMan SNP genotyping assays for rs7412 (C 904973) and rs429358 (C 3084793; AB Applied Biosystems by Life Technologies, Victoria, Australia) [304].

4.2.4 Neocortical Amyloid-β Load and Hippocampal Volumes via PET and MRI

Study participants underwent neuroimaging within three months of sample collection at the Macquarie Medical Imaging facility in Sydney, Australia. Neuroimaging measures included PET using ^{18}F-Florbetaben (FBB) and magnetic resonance imaging (MRI) [304]. FBB was administered via intravenous bolus over 30 seconds while seated in a rested position (n = 100). Images were acquired over a twenty-minute scan via five-minute acquisitions,

beginning fifty minutes post-injection. NAL was calculated as the average SUVR (cut-off 1.35) of the ligand within the anterior and posterior cingulate, lateral occipital, lateral temporal, superior parietal, and frontal regions normalised with that in the cerebellum using CapAIBL [304, 321, 322]. Participants were grouped into either the 'at risk' group (NAL+) or the 'not at risk' group (NAL-). Participants who met all standard MRI inclusion and exclusion criteria (n = 96) underwent MRI using a General Electric 3Tesla Scanner (Model 750W), as previously described but not reported further here [304, 323].

4.2.5 Reagents and Metabolomic Analysis

All KP metabolite standards and reagents used were analytical mass-spectrometry reagent grade and purchased from Sigma-Aldrich (St Louis, Missouri, USA). Deuterated internal standards were purchased from Medical Isotopes, Inc (Pelham, New Hampshire, USA). Serum samples were prepared for KP metabolite analyses using the additional volume of 10 % (w/v) TCA.

KP metabolites TRP, KYN, AA, 3-hydroxykynurenine (3-HK), and 3-hydroxyanthranilic acid (3-HAA) were analysed simultaneously using ultra-high performance liquid chromatography (20 μL injection volume), as reported elsewhere [324]. Kynurenic acid (KA) separation was carried out following injection (10 μL) into a Poroshell RRHT C_{18} column (1.8 μM, 2.1 × 100 mm; Agilent Technologies, Inc, Santa Clara, California, USA) using fluorescence detection with an Ex/Em of 344/388 nm and 1.5-minute retention time, as published previously [304]. Concentrations were analysed using Agilent OpenLAB CDS ChemStation (v C.01.04) [304]. Picolinic acid (PA) and quinolinic acid (QA) were analysed using a combination of electron-capture negative-ion gas chromatography coupled with mass spectrometry (GCMS), as described elsewhere [325]. Minor modifications were made using an Agilent 7890 A GC system coupled with an Agilent 5975 C mass spectrometry detector and an Agilent 7693 A autosampler (Agilent Technologies, Inc, Santa Clara, California, USA) [304]. Agilent GC/MSD ChemStation (v. 02.02.1431) was used to determine concentrations.

4.2.6 Statistical Analysis

A data-driven approach was applied to the de-identified data in Stata™ v17 (StataCorp, Texas, USA). Normality checks were performed for all variables via visual inspection of histograms prior to statistical analyses to determine whether parametric of non-parametric approaches should be used. To compare participant baseline characteristics, two-tailed independent groups *t*-tests with equal variances assumed were used for all continuous variables

(age, education, MMSE), and chi-squared tests were conducted for categorical variables (sex, *APOE-ε4* status, amyloid PET AD risk).

The concentrations of various enzymes and ratios were also calculated. IDO was calculated by dividing KYN concentrations by TRP concentrations, while kynurenine aminotransferase (KAT) was calculated by dividing KA concentrations by KYN concentrations. 3-HK concentrations were divided by KYN concentrations to quantify kynurenine 3-monooxygenase (KMO), while kynureninase (KYNU) was quantified by dividing 3-HAA concentrations by 3-HK. 3-hydroxyanthranilic acid dioxygenase (3-HAO) was quantified by dividing QA by 3-HAA, and the QA-KA ratio (QKR) was calculated by dividing QA by KA.

Two-tailed independent groups *t*-tests with equal variances assumed were then used to assess the group differences (curcumin vs. placebo) in the mean difference of MMSE scores and amyloid PET SUVR values, as well as KP serum metabolite concentrations; appropriate non-parametric analysis was performed for any variables violating normality assumptions and reported accordingly below. Between-group differences in urine KP metabolites were assessed at endpoint only. Exploratory post-hoc tests were conducted for significant outcomes and stratified for AD risk (positive vs. negative amyloid PET) and sex. Participant characteristics including differences in KP concentrations at baseline were checked as effect modifiers and confounders against all variables and controlled for where necessary. Mean differences, SDs, 95 % confidence intervals, and effect sizes are reported; medians and ranges are reported for non-parametric assessments.

To determine the relationship between KP biospecimen types (serum and urine) and any clinical relevance of findings, correlation analyses were also conducted for continuous variables including MMSE, PET, and KP metabolites across and within groups. Non-parametric Spearman's correlations were used for all correlational analyses. Statistically significant results were stratified by sex and AD risk. This study was exploratory in nature, thus no correction for multiple comparisons was applied and all tests were conducted two-tailed ($\alpha = 0.05$).

4.3 Results

4.3.1 Participant Demographics and Clinical Characteristics

Participant demographics, clinical characteristics, and their statistical comparisons are detailed in Table 4.1. There were no significant differences between groups indicating

successful randomisation and good compatibility between individuals randomised to curcumin and placebo groups at baseline.

Table 4.1. For placebo and curcumin groups, baseline participant demographics, clinical characteristics, and their statistical comparisons.

Participant Characteristics	**Curcumin (*n* = 48)**	**Placebo (*n* = 49)**	***p***
Age, years mean ± SD	79.04 ± 6.01	79.27 ± 5.23	0.845
Education, years mean ± SD	14.33 ± 2.86	14.76 ± 3.65	0.529
Baseline MMSE, mean ± SD	28.48 ± 1.15	28.69 ± 1.16	0.362
Sex, F:M (% F)	33:15 (68.75)	33:16 (67.35)	0.882
APOE Status, Pos:Neg (% Pos)	8:40 (16.67)	11:38 (22.45)	0.473
PET, Normal:Amyloid (% Amyloid)	33:15 (31.25)	34:15 (30.61)	0.946

Note. The low NAL group (NAL-; $n = 65$) and the high NAL group (NAL+; $n = 35$) were grouped using a standard uptake value ratio (SUVR) cut-off of 1.35.

4.3.2 Mean Changes in Clinical Characteristics and Serum KP Metabolite Concentrations

Change in PET score was found to be positively skewed with one significant outlier present ($z = 6.679$), thus non-parametric analysis was used (Mann-Whitney U-test) and the outlier was removed; removal of the outlier did not affect the final statistical result. All other data were found to be normally distributed, thus parametric analysis was utilised. Other than baseline levels of AA, there was no evidence of effect modification or confounding from baseline participant characteristics, so these were subsequently not controlled in further analyses (apart from AA).

Table 4.2 details the outcomes and estimations of all MMSE and PET scores, as well as KP concentrations within the serum. Data are presented as mean change (endpoint minus baseline) with standard deviation and 95 % confidence interval for placebo and curcumin groups. The between groups difference (curcumin minus placebo) is shown together with degrees of freedom (*df*), *p*-value (bolded for statistical significance at $p < 0.05$, two-tailed), and Cohen's *d.*

As shown in Table 4.2, the mean change in TRP ($p = 0.033$) and KYN ($p = 0.030$) differed significantly between groups from baseline to endpoint. The significant changes in TRP and KYN were driven by a greater increase in the treatment group after 12-months curcumin treatment, relative to placebo. The larger increase in the curcumin group (*cf.* placebo) in KYN was in females only ($p = .045$).

There was a mean reduction in AA concentration from baseline to endpoint that was greater in the placebo compared to curcumin group. Given that the placebo group had significantly higher AA levels at baseline, baseline AA scores were subsequently controlled for using ANCOVA, which indicated that the group differences in AA at endpoint were explained by the higher baseline levels in the placebo group, $F(3, 61) = 15.06$, $p < 001$; $p_{group} = .902$; $p_{baseline\ AA} < .001$; $p_{group*baseline} = .884$). Changes in AA were not associated with sex differences. Post-hoc analyses stratified by AD risk, revealed no significant differences between curcumin and placebo group in the change in TRP, KYN, and AA concentrations (all $p > .077$)

Table 4.2. Outcomes and estimations of all MMSE scores, amyloid PET SUVR value, and KP concentrations within the serum. Data are presented as mean change (endpoint minus baseline), standard deviation, and 95 % confidence interval for placebo and curcumin groups. TRP and KYN are presented in μM, while KA, 3-HK, 3-HAA, AA, PA, and QA are reported in nM.

	Mean Change (Endpoint – Baseline)		**Between Group Difference**			
	Curcumin	**Placebo**	**Curcumin – Placebo**	***p***	***df***	**Cohen's *d***
	M_{Diff} ± SD	**M_{Diff} ± SD**	**M_{Diff}**			
	[95 % CI]	**[95 % CI]**	**[95 % CI]**			
MMSE	0.31 ± 1.29	0.18 ± 1.52	0.13	0.655	95	0.67
	[-0.06, 0.69]	[-0.25, 0.62]	[-0.36, 0.53]			
PET*	-1.12	-1.10		0.738	95	0.03
	[-1.33, -0.36]	[-1.35, -0.40]				
3-HK	2.57 ± 120.15	-15.00 ± 31.61	17.56	0.325	95	0.20
	[-32.32, 37.45]	[-24.08, -5.92]	[-17.69, 52.82]			
3-HAA	-11.55 ± 9.07	-12.71 ± 8.15	1.16	0.511	95	0.13
	[-14.19, -8.92]	[-15.05, -10.39]	[-2.32, 4.63]			
AA	-8.66 ± 12.02	-15.12 ± 18.69	6.46	**0.046**	95	0.41
	[-12.15, -5.17]	[-20.49, -9.75]	[0.12, 12.81]			
KYN	0.46 ± 0.43	0.28 ± 0.38	0.18	**0.030**	95	0.45
	[0.33, 0.58]	[0.17, 0.39]	[0.02, 0.34]			
TRP	4.41 ± 7.25	1.56 ± 5.61	2.85	**0.033**	95	0.44
	[2.31, 6.52]	[-0.05, 3.18]	[0.24, 5.46]			
PA	-7.90 ± 54.10	-20.87 ± 74.99	12.97	0.341	92	0.20
	[-23.97, 8.17]	[-42.64, 0.91]	[-13.92, 39.85]			
QA	41.86 ± 128.85	7.33 ± 104.43	34.52	0.156	92	0.30

	[3.59, 80.12]	[-22.99, 37.66]	[-13.43, 82.47]			
KA	-14.43 ± 26.99	-15.89 ± 18.14	1.47	0.754	95	0.06
	[-22.26, -6.59]	[-21.10, -10.68]	[-7.79, 10.72]			
IDO	5.42 ± 12.44	4.43 ± 8.16	0.99	0.644	95	0.09
	[1.81, 9.03]	[2.09, 6.77]	[-3.24, 5.22]			
KAT	-9.21 ± 9.99	-8.47 ± 7.24	-0.74	0.675	95	-0.09
	[-12.11, -6.31]	[-10.54, -6.39]	[-4.25, 2.77]			
KMO	-9.59 ± 43.07	-11.16 ± 13.29	1.57	0.808	95	0.05
	[-22.09, 2.92]	[-14.98, -7.34]	[-11.22, 14.37]			
KYNU	-0.08 ± 0.07	-0.10 ± -0.07	0.01	0.302	95	0.21
	[-0.10, -0.06]	[-0.12, -0.08]	[-0.01, 0.04]			
3-HAO	3.32 ± 1.29	3.34 ± 1.31	-0.02	0.934	84	-0.02
	[2.92, 3.72]	[2.94, 3.75]	[-0.58, 0.53]			
QKR	2.76 ± 4.08	2.20 ± 4.57	0.56	0.536	92	0.13
	[1.54, 3.97]	[0.87, 3.53]	[-1.22, 2.33]			

* Non-parametric analysis with reporting of median, range, *N*, and effect size calculated as $z/\sqrt{N}$.

Note. MMSE: Mini-Mental State Examination; PET: Positron Emission Tomography; 3-HK: 3-Hydrokynurenine; 3-HAA: 3-Hydroxyanthranilic Acid; AA: Anthranilic Acid; KYN: Kynurenine; TRP: Tryptophan; PA: Picolinic Acid; QA: Quinolinic Acid; KA: Kynurenic Acid; IDO: Indoleamine Dioxygenase; KAT: Kynurenine Aminotransferase; KMO: Kynurenine 3-Monooxygenase; KYNU: Kynureninase; 3-HAO: 3-Hydroxyanthranilic Acid Dioxygenase; QKR: QA/KA Ratio

4.3.3 Group Differences in Urine KP Metabolite Concentrations

All urine KP metabolites did not follow a normal distribution, thus non-parametric tests (Mann-Whitney U-tests) were utilised for all metabolites. Table 4.3 details the outcomes and estimations of all KP concentrations within the urine from endpoint (12 months) only. Data are presented as median and range for placebo and curcumin groups together with the *N*, *p*-value (bolded for statistical significance at $p < 0.05$, two-tailed), and effect size. There was a significant difference for AA only ($p = .016$), with greater urine concentrations in the curcumin group compared to the placebo group at 12-months; this was apparent in men ($p = .010$), but not women, and did not differ with AD risk.

Table 4.3. Outcomes and estimations of all KP concentrations within the urine at endpoint (12 months). Data are presented as median and range for placebo and curcumin groups, together with relevant *p*-value, *N*, and effect size. TRP and KYN are reported in μM/mM CRE, while KA, 3-HK, 3-HAA, AA, PA, and QA are reported in nM/mM CRE.

	Curcumin Median [Range Min, Max]	**Placebo Median [Range Min, Max]**	***p***	***N***	**Effect size: $z/\sqrt{N}$**
TRP	5.31 [0.92, 13.82]	5.00 [1.97, 16.57]	0.596	35	.10
KYN	0.36 [0.13, 1.23]	0.45 [0.12, 1.07]	0.855	35	-.03
KA	1.36 [0.82, 2.65]	1.25 [0.55, 2.04]	0.094	35	-.28
HK	58.67 [0.00, 518.83]	116.02 [13.23, 346.29]	0.337	35	.16
3-HAA	713.21 [191.99, 2872.46]	673.85 [218.95, 1921.62]	0.843	35	.03
AA	40.65 [23.72, 182.86]	32.93 [13.65, 64.49]	**0.016**	35	-.41
PA	250.52 [139.13, 447.49]	261.47 [114.98, 461.77]	0.466	35	-.12
QA	1910.04 [1160.37, 8360.61]	1909.01 [743.72, 5721.23]	0.791	35	-.04
IDO	81.74 [19.59, 216.57]	66.21 [29.06, 170.87]	0.974	35	.01
KAT	2.94	3.20	0.466	35	-.12

	[0.85, 10.46]	[1.07, 12.42]			
KMO	148.51	259.56	0.136	35	.25
	[0.00, 592.72]	[77.82, 473.22]			
KYNU	0.36	0.45	0.855	35	-.03
	[0.13, 1.23]	[0.12, 1.07]			
3-HAO	2.71	2.97	0.691	35	-.07
	[0.83, 9.73]	[1.43, 6.77]			
QKR	1398.39	1567.97	0.233	35	.20
	[711.27, 5230.67]	[782.66, 4974.98]			

Note. 3-HK: 3-Hydrokynurenine; 3-HAA: 3-Hydroxyanthranilic Acid; AA: Anthranilic Acid; KYN: Kynurenine; TRP: Tryptophan; PA: Picolinic Acid; QA: Quinolinic Acid; KA: Kynurenic Acid; IDO: Indoleamine Dioxygenase; KAT: Kynurenine Aminotransferase; KMO: Kynurenine 3-Monooxygenase; KYNU: Kynureninase; 3-HAO: 3-Hydroxyanthranilic Acid Dioxygenase; QKR: QA/KA Ratio

4.3.4 Correlation Analyses

Non-parametric Spearman correlations were used to calculate the strength of association between all significant findings from Table 4.2 (TRP, KYN, AA) and Table 4.3 (AA). KP metabolites in the serum and urine were correlated with MMSE scores and PET SUVR values across groups (curcumin and placebo), and separately within groups.

Significant correlations from the mean difference data (endpoint – baseline) across and within groups are depicted in Table 4.4 for the serum KP markers. Across groups, KYN was positively correlated with amyloid PET SUVR values in the mean difference data (endpoint – baseline; p =.027); this relationship was not present at the individual group level (both p > .089). Serum KYN concentrations were positively correlated with PET SUVR values in female participants only (p = .004). A significant positive correlation was found between change in MMSE scores and change in serum AA concentrations in the placebo group only (p = .041); this did not differ with sex or AD risk. There were no significant associations between the change in PET and MMSE values with the change in serum TRP concentrations either across groups or within groups.

Table 4.4. Significant correlation outcomes from the mean difference data (endpoint – baseline) across and within groups, including MMSE, PET, and KP metabolites in serum.

Correlation	Group	*rho*	*p*
PET vs. KYN in Serum	Across Groups	.2267	.027
MMSE vs. AA in Serum	Placebo	.2928	.041

Note: AA: Anthranilic Acid; KYN: Kynurenine; MMSE: Mini-Mental State Examination; PET: Positron Emission Tomography.

Statistically significant correlation outcomes from the endpoint data across and within groups are depicted in Table 4.5 including both serum and urine KP markers. Across groups, MMSE scores at endpoint were positively correlated with TRP concentrations in serum (p = .001) and AA concentrations in urine (p = .001). These relationships were found in the curcumin group only (Table 4.5) and did not differ with sex or AD risk. There were no further significant correlations across or within groups for endpoint serum TRP, KYN, AA, or urine AA with MMSE or PET values.

Table 4.5. Significant correlation outcomes from the endpoint data across and within groups, including MMSE, PET, and KP metabolites in serum and urine.

Correlation	Group	ρ	p
MMSE vs. TRP in Serum	Across Groups	.3224	.001
MMSE vs. AA in Urine	Across Groups	.5581	.001
MMSE vs. TRP in Serum	Curcumin	.4020	.005
MMSE vs. AA in Urine	Curcumin	.7242	.001

Note: MMSE: Mini-Mental State Examination, TRP: Tryptophan, AA: Anthranilic Acid

4.4 Discussion

This was the first study to assess response-to-treatment in a clinical trial to determine whether the KP may be considered a viable biomarker for treatment responsivity in people with and without risk of AD. While the present research is a sub-study of an archival dataset, this is the first to characterise the KP profile in urine in the KARVIAH cohort [326].

Overall, no positive results were observed in this clinical trial in terms of improvements in cognitive outcomes (as measured by MMSE) or AD risk (as measured by amyloid PET as a continuous variable) linked to a 12-month curcumin intervention. Between-group analyses indicated that an increase in TRP and KYN concentrations was greater in the curcumin group relative to placebo, irrespective of AD risk. KYN increases were apparent in women only. However, the reduction in concentrations of AA in the serum were larger in the placebo than the curcumin group, which were linked to higher levels at baseline, regardless of sex or AD risk. There were greater AA concentrations in the curcumin compared to placebo group in the urine at endpoint, this was apparent in men and not women, and was irrespective of AD risk.

Curcumin therapy is relevant to the KP as it has been shown to have a direct relationship with IDO, the first major rate-limiting enzyme in the KP. The upregulation of the KP reported in the present study is contrary to the current understanding reported in the literature, however, these findings are largely generated from preclinical research, and have highlighted the inhibition of IDO [312, 313]. In general, it is understood that curcumin supplementation inhibits the KP [327], and this known pharmacological mechanism cannot be reconciled with our observation of elevated concentrations of TRP and KYN in the treatment group following 12-months oral curcumin. Nevertheless, our findings were in an otherwise healthy cohort with normal global cognition (MMSE ≥26) [304, 320], justifying raised levels of TRP and KYN. Further research is required in human participants to replicate this finding and elucidate the underling mechanism to bridge the gap with this preclinical work.

A significant positive correlation was observed between change in KYN concentrations and NAL across (but not within) groups, indicating that KYN levels within the serum increase as NAL increases in the brain of cognitively normal older adults, irrespective of curcumin treatment. Importantly, this relationship was observed only in female participants. Our finding is in accordance with the literature that has identified elevated KYN in cognitively healthy females at risk of AD and KYN as a significant and independent predictor of NAL-associated AD risk [326]. The link between changes in amyloid in the neocortex and serum KYN has been attributed to the increased degradation of TRP (as a precursor to KYN) and hence the upregulation of the KP in females who are at risk of AD [326]. This finding is difficult to reconcile with our observation that both serum TRP and KYN increased with curcumin treatment. Nevertheless, the isolated effect observed in females reported in this study is supported by the wider literature that has reported both the general influence of biological sex on the KP profile in the serum [328]. Findings suggest that KYN warrants further exploration as a minimally invasive biomarker for AD-risk in older, cognitively normal females.

MMSE scores at endpoint were higher with increased concentrations of TRP in serum and AA in urine. This was reported across groups but appears to be driven by the increases in the curcumin group. No significant sex-specific correlations were reported for this relationship, as supported by the findings reported within the literature [326], and findings did not differ with AD risk. While the clinical trial had a negative outcome (indicated by no significant changes in MMSE scores or PET NAL attributed to curcumin treatment), findings suggest that serum TRP and urine AA may be proxies for cognitive function and potentially as markers of response-to-treatment associated with curcumin intervention in older people with and without a risk of AD. This finding is in accordance with the findings in Chapter 2 that show that TRP concentrations in the serum and plasma decrease with increasing age, and the general literature that shows that higher levels of TRP may be protective of cognition. For instance, research has shown that TRP loading (in the form of enriched foods) improves nocturnal sleep, mood, total antioxidant capacity levels, as well as melatonin and serotonin levels in older adults; all correlated with AD risk [329].

In the Framingham study, greater levels of plasma AA increase the risk of incident dementia in older people by up to 40 % after controlling for sex, education, age, *APOE-ε4* status, and total homocysteine levels [302]. Here, we observed that better cognitive function in older people was linked to more AA in the urine. Taken together with our observation in Chapter 3 that serum and urine levels of AA are inversely related, this suggests that excreting AA (rather than retaining) may offer some protective benefits on cognition, particularly for

men. Given that findings come from correlational analyses and that little is known about the physiological relevance of AA in health and disease, further research is required to implicate causality and identify the mechanism by which AA is linked to cognitive (dys)function and clarify sex-specific effects.

Strengths of the present study include being the first to assess treatment responsivity in people at risk of AD by comprehensively assessing interventional data and determining response-to-treatment by the KP. This was also the first study explore the clinical and pathological relevance of any KP profile alterations by assessing their relationship with cognition in the form of MMSE test scores and AD pathophysiology in the form of NAL measured by PET. It is important to reiterate that the clinical trial reported in this study did not have any positive results in terms of improved cognitive or pathophysiological outcomes. This has implications for the KP findings given the lack of variability in clinical and pathological outcomes (e.g., MMSE $\geq$ 26). Future research should seek to clarify response-to-treatment in studies which have had positive primary outcomes to improve the veracity of the data and link KP activity to functional changes. Moreover, the MMSE is not considered a sensitive tool to detect cognitive changes in cognitively healthy older adults, as well as older adults with MCI [330]. This suggests that the potentially subtle cognitive changes occurring in cognitively healthy older adults may not have been adequately measured and characterised, potentially explaining the negative effects on cognition reported in this trial. Future research should seek to utilise a comprehensive neuropsychological test battery with composite cognitive domain scores to delineate any domain-specific changes associated with curcumin intervention and link these to KP biomarkers [331]. As this was an exploratory study and there were no between-group differences at baseline, demographics (including age and APOE status) were not adjusted for. Future research should seek to consider a wide range of potential confounders and effect modifiers beyond those reported in this study and adjust for these potential confounders or effect modifiers using an appropriate regression model (as done in other studies assessing the KARVIAH cohort [326]).

Nonetheless, the aim of this study was to assess treatment responsivity in people with and without risk of AD, and promising KP metabolites for determining response-to-treatment were successfully identified (TRP, KYN, and AA), together with sex-specific effects. Future studies should focus on exploring other interventions with potentially larger effect sizes and extending the research across the AD pathology continuum (e.g., healthy vs. at-risk vs. MCI vs. AD).

5. General Discussion

Foreword:

In this chapter, a general discussion is provided that contextualises the findings from the overall thesis with each of the individual aims. First, the normative data generated from Chapters 2 and 3 are discussed, followed by the prognostic and theragnostic markers from Chapter 4. Overall, a variety of non-invasive and minimally invasive biofluids (including serum, plasma, saliva, and urine) were profiled in healthy adults across the lifespan, as well as in older adults with and without risk of AD undergoing treatment in a clinical trial. This chapter highlights that the thesis achieved its overarching aim of seeding new directions for pragmatic biomarker discovery in the fields of AD risk prediction and the monitoring of responsivity to treatment. Future directions for research are proposed.

This thesis aimed to characterise the relationship between the kynurenine pathway (KP) and Alzheimer's disease (AD) risk through profiling a variety of minimally invasive biofluids from healthy adults across the lifespan, as well as older adults at risk of AD. This aim was interrogated through three studies that systematically mapped the state of the targeted metabolomics literature and meta-analysed normative data for tryptophan (TRP) and kynurenine (KYN) within the serum and plasma (Chapter 2); applied a validated analytical method to quantify the major biologically active KP metabolites (beyond TRP and KYN) of healthy adults in a range of human biofluids (Chapter 3); and analysed the full suite of KP metabolites in the serum and urine of older adults with and without risk of AD to assess their responsivity to treatment from interventional data (Chapter 4). These aims were successfully met through their respective chapters, and their separate and combined findings are discussed below.

A major outcome from this thesis was to generate normative data on the KP. A summary of these norms and region means for TRP and KYN in the serum and plasma generated within Chapter 2 is presented in Table 5.1. A list of the mean KP metabolite concentrations in the serum, plasma, saliva, and urine of healthy adults is presented in Table 5.2. Generating normative ranges, such as those presented in Tables 5.1 and 5.2, is an essential component of developing routine diagnostic tests. Normative data are needed not only to benchmark and evaluate clinical tests as we move towards incorporating KP into point-of-care testing (POCT) [332], but also add great value for researchers for future clinical, diagnostic, prognostic, and theragnostic studies who may look to these data as a guideline in characterising their KP results.

Table 5.1. Normative data generated including grand mean concentrations and region mean concentrations for TRP and KYN across serum and plasma (Chapter 2). Values presented are mean and SD (μM).

	TRP Serum	**TRP Plasma**	**KYN Serum**	**KYN Plasma**
Grand Mean	60.52 (15.38)	51.45 (10.47)	1.96 (0.51)	1.82 (0.54)
America Mean	49.66 (2.75)	34.68 (9.90)	NR	0.94 (0.24)
Asia Mean	60.30 (8.69)	53.81 (9.95)	1.68 (0.43)	1.61 (0.34)
Australia Mean	67.26 (11.19)	42.87 (8.51)	2.43 (0.59)	2.12 0.52)
Europe Mean	60.73 (10.66)	52.92 (12.06)	2.07 (0.52)	2.29 (0.73)
Middle East Mean	54.01 (18.44)	34.82 (10.52)	1.56 (0.62)	1.53 (0.72)

Table 5.2. Normative metabolite concentrations in the serum, plasma, saliva, and urine of healthy Australian adults for CRE, TRP, KYN, 3-HK, KA, 3-HAA, AA, XA, PA, and QA (Chapter 3).

	Serum[a]	Plasma[a]	Saliva[b]	Urine[c]
CRE	78.60 (24.18)	69.11 (20.47)	2.98 (2.02)	14.61 (10.08)
TRP	68.00 (14.99)	57.68 (15.89)	6.66 (6.26)	4.56 (2.44)
KYN	3.70 (0.84)	2.62 (0.54)	81.53 (138.95)	0.42 (0.25)
3-HK	50.47 (6.27)	42.19 (5.30)	0.78 (1.04)	59.62 (54.27)
KA	35.50 (30.91)	21.39 (7.49)	326.80 (251.03)	1.40 (0.75)
3-HAA	21.07 (16.03)	19.73 (6.76)	50.01 (59.97)	106.01 (96.62)
AA	172.60 (261.48)	76.18 (30.46)	951.49 (1063.70)	1.89 (2.54)
XA	16.46 (21.49)	10.07 (7.65)	77.28 (67.78)	0.20 (0.11)
PA	144.18 (203.41)	225.21 (125.86)	448.04 (374.34)	28.65 (41.18)
QA	485.14 (91.39)	431.03 (82.33)	94.01 (61.88)	583.22 (370.81)

Note. 3-HAA: 3-Hydroxyanthranilic Acid; 3-HK: 3-Hydroxykynurenine; AA: Anthranilic Acid; CRE: Creatinine; KA: Kynurenic Acid; KYN: Kynurenine; PA: Picolinic Acid; QA: Quinolinic Acid; TRP: Tryptophan; XA: Xanthurenic Acid.

[a]Serum and plasma values are presented as means and SD. CRE, TRP, and KYN means are presented in μM. 3-HK, KA, 3-HAA, AA, XA, PA, and QA means are presented in nM.

[b]Saliva values are presented as means and SD. CRE means are presented in μM. TRP is presented in μM/mg protein, while KYN, 3-HK, KA, 3-HAA, AA, XA, PA, and QA are presented in nM/mg protein.

[c]Urine values are presented as means and SD. CRE means are presented in mM. TRP, KYN, and AA are presented in μM/mM CRE. 3-HK, 3-HAA, AA, XA, PA, and QA are presented in nM/mM CRE.

Alongside the normative data presented in Table 5.1, the systematic review and meta-analysis reported in Chapter 2 successfully appraised and characterised the existing literature on how and where KP metabolites are measured. Regarding mode of detection (MOD), mass-spectrometry (MS) was identified as the most common modality reported within the literature and was also best aligned with the weighted grand mean calculations. However, there was limited variability between MODs (< 3 %), suggesting that the choice of MOD is not essential

to the successful and standardised quantification of TRP and KYN in the serum and plasma, which may increase their uptake and utilisation as potential POCT.

Despite not being meta-analysed, Chapter 2 highlighted the growing interest in profiling the metabolome of minimally invasive sample types beyond blood. For instance, the majority (66.67 %) of the TRP urine papers have been published since 2015 [152, 177, 181, 187, 214, 220, 234, 248, 258, 260], while all included TRP saliva studies were published within this timeframe [162, 195]. Across all MODs and sample matrices reported in Chapter 2, significant limitations in the methodological rigour were identified, alongside potential bias in approximately half of the included studies. While the risk of bias suggests that the data presented in Chapter 2 should be interpreted with caution, regression analyses suggested various associations that may have strong implications for future biomarker research. For instance, TRP and KYN concentrations were found to decrease in studies that were published more recently. It is speculated that this may be linked to changes in socio-economic status, urbanisation, dietary quality, and/or the increased analytical sensitivity reported recently [281-284]. Moreover, the correlations between metabolite, biological sex, and age reported in Chapter 2 are in accordance with pre-existing literature [287]. The findings reported in this chapter improve the availability and quality of the data summarising the normative KP profile and support the initiative to identify a suitable KP target for precision medicine approaches.

The findings in the systematic review and meta-analysis reported in Chapter 2 highlighted that future studies should aim to profile KP metabolites beyond TRP and KYN in minimally invasive sample types; should seek to investigate and control for the relationship between biological sex, age, and geographic location and metabolite concentrations; and should also seek to identify any associations with traditional sample matrices of interest (i.e., serum and plasma). These directions for research became the objectives for the empirical research conducted and reported in Chapter 3, which successfully contributed to developing an analytical method to quantify KP metabolites in the liquid biopsies of healthy adults in human biofluids (going beyond blood samples). Alongside the normative data presented in Table 5.2, a total of three significant and novel relationships were found involving saliva and urine. This included a positive correlation between kynurenic acid (KA) in saliva and urine and picolinic acid (PA) in saliva and plasma; and a negative correlation between anthranilic acid (AA) in serum and urine. While little is known about the between-fluid saliva and urine associations in a metabolomics setting, Chapter 3 adds new knowledge to these relationships by being the first to observe a positive correlation between KA in urine and saliva.

The aim of Chapter 4 was to expand on the findings of the previous empirical study reported in Chapter 3 by examining the theragnostic potential of KP markers in relation to AD risk, which was successfully achieved. This next sub-study utilised archival clinical trial data from a 12-month curcumin intervention to determine whether the KP may be considered a marker for treatment responsivity in an AD-risk context. While no positive results were observed in terms of improvements in cognitive outcomes or AD risk, serum TRP and KYN increased with curcumin treatment over 12-months, compared to controls. KYN levels within the serum were found to increase as neocortical amyloid load (NAL) increased in the brain in females, irrespective of treatment group. This is in accordance with the literature that has identified elevated KYN in cognitively healthy females at risk of AD and KYN as an independent predictor of amyloid deposition in the neocortex [326], hence supporting the role of KYN as a minimally invasive biomarker for NAL in older cognitively healthy females. Further, MMSE scores were found to increase (i.e., improve, indicating better cognitive outcomes) as concentrations of TRP in serum and AA in urine increase. These AA findings are in line with those reported in Chapter 3 which showed that AA levels in the serum decrease as urine levels increase, suggesting that excreting AA via the urine may be protective in terms of cognitive outcomes. Together, the findings presented in Chapters 3 and 4 support those reported from the Framingham study, that higher levels of AA in the blood is associated with greater dementia risk [302]. Within-group analyses showed that these correlations occurred within the curcumin treatment group only, and not the placebo group (despite the overall negative outcome from the trial), regardless of AD risk. Taken together, this suggests that serum TRP and urine AA may be useful proxy markers of cognitive function, and may have utility in monitoring response to treatment in either curcumin interventional studies or in studies exploring older people with and without risk of AD.

In conclusion, the findings reported in this thesis provide important directions for the development of POCT for diagnostic, prognostic, and theragnostic purposes in an AD setting. Together, the outcomes reported seed new directions for pragmatic biomarker discovery in the fields of AD prevention, risk reduction, and the monitoring of responsivity to treatment. Future studies should seek to comprehensively profile the salivary and urinary metabolomes in multiple cohorts along the AD pathophysiology continuum and clarify response-to-treatment in studies which have had positive primary outcomes to further explore the potential of KP metabolites as theragnostic markers.

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Appendices

Appendix A. Statement of Authorship

Study 1 (Chapter 2): NJM and CKL conceptualised this study. NJM performed the database searches and record screening. NJM, ASB, and AM performed the data extraction and risk of bias appraisals. NJM and CKL performed the data analyses and interpretation of outcomes. The chapter was drafted and finalised by NJM following critical revisions by GSL and CKL.

Study 2 (Chapter 3): NJM, CKL, and GSL conceptualised this study. Collection protocol for the biospecimens was developed and optimised by NJM, GSL, and CKL. Biospecimens were collected by NJM and GSL. Samples were extracted and prepared for analysis by NJM, and analysed by NJM and KC. The analytical methods (including sample extraction) were developed and optimised by KC and SB. Technical oversight was provided by ML. NJM and CKL performed the data analyses and interpretation of outcomes. The chapter was drafted and finalised by NJM following critical revisions by GSL and CKL.

Study 3 (Chapter 4): This study utilised archival data from a study conceptualised and analysed by PC, KG, IJ, KS, HRS, TS, PRA, PD, CMY, KT, RNM, and CKL. This sub-study was conceptualised by NJM, CKL, and GSL. NJM, CKL, and GSL performed the data analysis and interpretation of outcomes. The chapter was drafted and finalised by NJM following critical revisions by GSL and CKL.

Appendix B. Risk of Bias Evaluation

The following items are adapted from the Joanna Briggs Institute Critical Appraisal Checklist for Analytical Cross-Sectional Studies, the Quality Assessment of Diagnostic Accuracy Assessment (QUADAS), and the QUADOMICS Tool. All items should be scored as either Y, N, or NA/UNKNOWN.

1. **Were the criteria for inclusion in the sample clearly defined? (JBI)**

The authors should provide clear inclusion and exclusion criteria that they developed prior to the recruitment of the study participants. The inclusion and exclusion criteria should be specified with sufficient detail and all the necessary information critical to the study. In the instance of healthy cohorts, the general definition of 'healthy' and the screening tools used to determine this should be described e.g., cognitively healthy older adults, metabolically healthy younger adults.

2. **Were the study subjects and the setting described in detail? (JBI)**

The study sample should be described in sufficient detail so that other researchers can determine if it is comparable to the population of interest to them. The authors should provide a clear description of the population from which the study participants were selected or recruited, including demographics, location, and period. In the instance of healthy cohorts, the authors should describe the age, biological sex, and geographical location of the cohort.

3. **Was the type of sample used fully described? (QUADOMICS)**

To score positively in this item, the report should present a details description of the type of sample e.g., serum, plasma, urine, saliva, etc. Moreover, the authors should specifically list the type of plasma specimen (e.g., EDTA, heparin, citrate) or urine specimen (e.g., midstream urine). Clinical and physiological factors should also be described e.g., fasting status.

4. **Were the handling of specimens and pre-analytical procedures reported in sufficient detail and similar for the whole sample? (QUADOMICS)**

To score positively on this item, the study should describe any process related to the pre-analytical handling of the samples that could affect the results (e.g., number of freezing cycles, timing and storage of specimens, time from blood draw until centrifugation and storage, details on centrifugation conditions).

5. **Was the execution of the index test described in sufficient detail to permit replication of the test? (QUADAS & QUADOMICS)**

The assay used to determine the concentrations of the metabolites in the samples should be described thoroughly. A citation to a technical article is not considered sufficient detail. In terms of mass spectrometry, description of the use of particular technologies (e.g., column chromatography, capillary electrophoresis) should be outlined. Analytical variability of the test should be described and controlled. The authors should explicitly describe the degree of instrument or observer variation and the methods used to control this variation e.g., validation parameters such as sensitivity, specificity, and calibration curves. A chromatogram should be provided.

6. **Was the statistical analysis reported? (JBI)**

The methods section should be details enough for reviewers to identify which analytical techniques were used and how specific confounders were measured. For example, the authors should state whether regression or stratification analysis was used.

OVERALL APPRAISAL: High Quality / Low Quality