School of Public Health

An investigation of a vitamin D, ionised calcium and parathyroid hormone regulatory axis of cerebral capillary function: Implications for cognitive performance in ageing

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Declaration

To the best of my knowledge and belief, this thesis titled 'An investigation of a vitamin D, ionised calcium and parathyroid hormone regulatory axis of cerebral capillary function: Implications for cognitive performance in ageing', contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

The animal research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013) and the United Kingdom Animals (Scientific Procedures) Act 1986, as modified by the European Directive (ASPA) (revised 2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number #N34-10 and AEC_2011_30A, and the Charles River United Kingdom Home Office Ethics Committee, Project License #70/7221.

The clinical research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007). The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number #HR97/2011.

Virginie Lam
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Abstract

Dementia, a term used to describe a clinical syndrome caused by neurodegeneration, is characterised by the progressive deterioration of an individual's cognitive function and the development of behavioural abnormalities that affect memory, intellect and/or capacity for independent living. The worldwide prevalence of dementia is increasing due to population ageing and the number of individuals living with dementia has been expected to exceed 115 million by the year 2050. Therefore, identifying risk factors, primary and secondary prevention strategies are global health priorities.

Late-onset Alzheimer's disease (AD) and vascular dementia (VaD) are considered the two most common forms of age-related dementia. Over the past few decades, the majority of AD-biomedical research has primarily focused on considering the putative causal association of brain parenchymal proteinaceous deposits with disease onset and progression. However, accumulating evidence has implicated a vascular origin for AD. Cerebral capillary dysfunction and neurovascular inflammation may be one of the earliest indicators of AD onset, preceding amyloidosis and neurodegeneration. Pathological dysfunction of cerebral capillary integrity has been demonstrated to coincide with the progressive decline in cognitive function and indeed, extensive capillary damage has been reported in brain regions specifically involved in cognition. Consistent with a causal role, experimental and clinical studies have shown therapeutic benefit in AD progression if cerebrovascular disturbances are corrected or attenuated.

Epidemiological, clinical and animal model studies show that nutritional status is relevant to risk for AD/VaD and cognitive decline. A vascular axis is commonly purported to be central to nutritional mediators of central nervous system integrity. Vitamin D (VD) has generated considerable interest in the dementia field based principally on its supposed beneficial extra-skeletal roles in the brain and on cognitive performance. A number of experimental studies have demonstrated beneficial effects of VD, typically in the context of correcting for VD deficiency. However, paradoxical observations have been reported. Emerging evidence suggests

that higher VD concentrations may be associated with cerebrovascular and global cognitive abnormalities and interestingly, hypervitaminosis D has been causally related with a premature ageing phenotype in genetically modified mice. The anti-oxidant and immunomodulatory effects of VD have been considered in the context of CNS function and cognition, however the effects of VD on cerebral capillary endothelium have not been previously considered.

The complexity of the VD homeostatic system involves the regulatory effects of active calcium metabolites (ionised calcium, iCa) and parathyroid hormone (PTH). Indeed, aberrations in iCa and PTH homeostasis are both indicated in risk for several neurodegenerative disorders including AD/VaD. The interactive effects of the VD/iCa/PTH have not been considered in the context of cerebrovascular integrity and cognitive function.

This thesis explores the novel hypothesis '*The Vitamin D - Calcium - Parathyroid Hormone endocrine axis regulates cerebral capillary integrity and is associated with neurocognitive performance*'. The hypothesis is further supported by an in-depth review of the literature presented in Chapter 1. The main objectives of this thesis were to investigate the interactive and/or independent effects of hypervitaminosis D, iCa and PTH on the modulation of cerebral capillary integrity and function in genetically un-manipulated rodent models, presented in Publication 1. The second primary objective of this thesis was to explore the potential association between VD, iCa and PTH homeostasis with neurocognitive function, as shown in Publication 2.

To explore the putative regulatory and integrative effects of exogenous VD, iCa and PTH, on the function of cerebral capillary endothelium and neurovascular inflammation, clinically relevant states of dietary-induced hypervitaminosis D, endocrine mediated hypo - and - hyper - parathyroidism were modeled in two genetically un-manipulated rodent models for a duration of either 6, 12 or 24 weeks (Publication 1). Parenchymal extravasation of plasma-derived immunoglobulin G (IgG) was used as a surrogate marker of blood-to-brain cerebral capillary permeability and neuro-inflammation was determined by quantification of glial

fibrillary acidic protein (GFAP), representative of astroglial activation. Substantial cerebral capillary disturbances were demonstrated in the cerebral cortex and hippocampal formation in two rodent species in association with the dosage of VD supplement provided. Greater doses of VD were also associated with increased serum iCa and reduction in serum PTH. A dose response was suggested and parenchymal effects persisted for up to 24 weeks of the dietary intervention. Conversely, the provision of exogenous PTH did not increase cerebral capillary permeability despite a substantial increase in serum iCa concentration. Greater capillary permeability was observed in hypoparathyroid animals subjected to parathyroid gland ablation, concomitant with a reduction in serum iCa. Both VD supplemented and parathyroidectomised intervention groups showed modest increases in astroglial activation, whereas the provision of exogenous PTH reduced GFAP-expression. The overall findings from this study demonstrate that the provision of exogenous VD at levels that markedly suppress serum PTH and increase serum iCa can be detrimental and significantly compromise the barrier properties of cerebral capillary vessels but do not promote neurovascular inflammation per se. Moreover, the data from this study suggests PTH may have vasculo-protective effects and reiterates the importance of considering the synergistic and/or independent effects of metabolites and hormones related to VD homeostasis in the investigation of vascular-neurodegenerative conditions.

Serum VD homeostasis was considered in context of neurocognitive performance in healthy, middle-aged and older aged adults; presented in Publication 2. Based on the principle findings of Publication 1, iCa and PTH, critical regulators of VD homeostasis, were taken into consideration when exploring the putative effects of VD on cognitive performance. A cross-sectional sample cohort of 181 individuals (116 female; 65 male) between the ages of 43 to 84 years of age was included in the sample analysis. All participants provided a fasted blood sample and completed cognitive measures of verbal episodic learning and memory (considered as the most sensitive and specific marker of age-related cognitive decline). The principle findings of this study suggest an association between higher VD status and poorer performance on verbal episodic memory in middle-aged and older individuals with normal VD - iCa - PTH homeostasis and indicate the provision of VD

supplements in individuals with adequate VD status may cause adverse cognitive outcomes.

Taken together, the primary findings presented in Publication 1 and Publication 2 of this thesis support the broad hypothesis that the 'Vitamin D – Calcium - Parathyroid Hormone' endocrinal axis has vascular modulating effects on cerebral capillary permeability. The experimental data from the animal studies provide novel insight into the detrimental effects of supplementary VD on cerebral capillary integrity and function, which may subsequently modulate neurocognitive function in a clinical context. The findings emphasize the importance of establishing an optimal concentration for serum VD that is ideal for cerebral capillary functioning and cognitive health.

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List of primary publications

This PhD-by publication includes 5 first author peer-reviewed publications to address my candidacy objectives. The following articles have been published in each of the journals listed below. Author contributions and copyright authorisation for each publication is detailed in **Appendix I.**

1. **Lam V**., Takechi R., Pallebage-Gamarallage M., Giles C., & Mamo J. C. (2015). The vitamin D, ionised calcium and parathyroid hormone axis of cerebral capillary function: therapeutic considerations for vascular-based neurodegenerative disorders. *Plos One, 10*(4), e0125504.

   [Impact Factor: 3.534]

2. **Lam V**., Albrecht M. A., Takechi R., Prasopsang P., Lee Y. P., Foster J. K., & Mamo J. C. L. (2016). Serum 25-hydroxyvitamin D is associated with reduced cognitive performance in healthy, middle-aged and older adults. *European Journal of Nutrition*, *55*(4), 1503-1513.

   [Impact Factor: 3.84]

3. **Lam V**., Dhaliwal S. S., & Mamo J. C. (2013). Adjustment of ionized calcium concentration for serum pH is not a valid marker of calcium homeostasis: implication for identifying individuals at risk of calcium metabolic disorders. *Annals of Clinical Biochemistry*, *50*(Pt 3), 224-229.

   [Impact Factor: 2.080]

4. **Lam V**., Albrecht M. A., Takechi R., Giles C., James A. P., Foster J. K., & Mamo J. C. (2013). The serum concentration of the calcium-binding S100B is positively associated with cognitive performance in older adults. *Frontiers in Aging Neuroscience*, *5*, 61.

   [Impact Factor: 2.843]

5. **Lam V**., Albrecht M. A., Takechi R., Heidari-Nejad S., Foster J. K., & Mamo J. C. (2014). Neuropsychological performance is positively associated with plasma albumin in healthy adults. *Neuropsychobiology*. *69*(1), 31-38.

[Impact Factor: 2.303]

List of secondary publications

The following publications are not directly linked to my candidacy objectives however are complimentary and demonstrate my research productivity and engagement with other relevant research activities during the course of my project.

1. Elahy M., Jackaman C., Mamo J. C., **Lam V.**, Dhaliwal S. S., Giles C., Nelson D., & Takechi R. (2015). Blood-brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. *Immunity and Ageing, 12,* 2.

   [Impact Factor: 2.32]

2. Elahy M., **Lam V**., Pallebage-Gamarallage M. M., Giles C., Mamo J. C., & Takechi R. (2015). Nicotine attenuates disruption of blood-brain barrier induced by saturated-fat feeding in wild-type mice. *Nicotine and Tobacco Research, Pii*, ntv044.

   [Impact Factor: 2.805]

3. Takechi R., Pallebage-Gamarallage M. M., **Lam V**., Giles C., & Mamo J. C. (2014). Long-term probucol therapy continues to suppress markers of neurovascular inflammation in a dietary induced model of cerebral capillary dysfunction. *Lipids in Health and Disease, 13,* 91.

   [Impact Factor: 2.31]

4. Takechi R., Pallebage-Gamarallage M. M., **Lam V**., Giles C., & Mamo J. C. (2013). Nutraceutical agents with anti-inflammatory properties prevent dietary saturated-fat induced disturbances in blood-brain barrier function in wild-type mice. *Journal of Neuroinflammation, 10*, 73.

   [Impact Factor: 4.902]

5. Takechi R., Galloway S., Pallebage-Gamarallage M. M., **Lam V.**, Dhaliwal S. S., & Mamo J. C. (2013). Probucol prevents blood-brain barrier dysfunction in wild-type mice induced by saturated fat or cholesterol feeding. *Clinical and Experimental Pharmacology and Physiology, 40*(1), 45-52.

[Impact Factor: 2.405]

6. Takechi R., Pallebage-Gamarallage M. M., **Lam V.**, Giles C., & Mamo J. C. (2013). Aging-related changes in blood-brain barrier integrity and the effect of dietary fat. *Neurodegenerative Diseases, 12*(3), 125-135.

[Impact Factor: 3.454]

7. Pallebage-Gamarallage M., **Lam V**., Takechi R., Galloway S., Clark K., & Mamo J. (2012). Restoration of dietary-fat induced blood-brain barrier dysfunction by anti-inflammatory lipid-modulating agents. *Lipids in Health and Disease, 11,* 117.

[Impact Factor: 2.31]

8. Pallebage-Gamarallage M., **Lam V.**, Takechi R., Galloway S., & Mamo J. C. (2012). A diet enriched in docosahexanoic acid exacerbates brain parenchymal extravasation of apo B lipoproteins induced by chronic ingestion of saturated fats. *International Journal of Vascular Medicine, 2012*, 647689.

9. **Lam V**., Takechi R., Pallebage-Gamarallage M. M., Galloway S., & Mamo J. C. (2011). Colocalisation of plasma derived apo B lipoproteins with cerebral proteoglycans in a transgenic-amyloid model of Alzheimer's disease. *Neuroscience Letters, 492*(3), 160-164.

[Impact Factor: 2.055]

10. Pallebage-Gamarallage M. M., Takechi R., **Lam V.**, Galloway S., Dhaliwal S. S., & Mamo J. C. (2010). Post-prandial lipid metabolism, lipid-modulating agents and cerebrovascular integrity: implications for dementia risk. *Atherosclerosis Supplements, 11*(1), 49-54.

[Impact Factor: 9.667]

11. Takechi R., Galloway S., Pallebage-Gamarallage M. M., **Lam V**., & Mamo J. C. (2010). Dietary fats, cerebrovascular integrity and Alzheimer's disease risk. *Progress in Lipid Research, 49*(2), 159 -170.

[Impact Factor: 12.963]

Abbreviations

$1, 25(OH)_2D_3$	1α, 25-dihydroxyvitamin D_3/calcitriol
25(OH)D	25-hydroxyvitamin D_3/calcidiol
2-D	Two-dimensional
3-D	Three-dimensional
APP	Acute-phase proteins
AD	Alzheimer's disease
APOE	Apolipoprotein E
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BNT	60-item Boston Naming Test
Ca	Calcium
CDR	Cognitive drug research test battery
CNS	Central nervous system
CRP	C-reactive protein
CSR	Calcium sensing receptor
CSF	Cerebrospinal fluid
CTX	Cerebral cortex
DASS	Depression, Anxiety and Stress Scales
D-KEFS	Delis-Kaplan Executive Function System verbal fluency subsets
ELISA	Enzymatic-linked immunosorbent assay
FGF-23	Fibroblast growth factor 23
GFAP	Glial fibrillary acidic protein
H-PTH	Primary hyperparathyroidism
HDI	Highest density intervals
HIV	Human immunodeficiency virus
HPF	Hippocampal formation
iCa	Ionised calcium
iCa_{raw}	Unadjusted ionised calcium
iCa_{pub}	Ionised calcium using published normative equations
iCa_{regr}	Ionised calcium values predicted using regression model

IEP	Ion-electrode potentiometry
IgG	Immunoglobulin G
IU	International units
LDFR	Long delay free delayed recall
LOESS	Local polynomial regression fitting
MCI	Mild cognitive impairment
MCMC	Markov Chain Monte Carlo
MMSE	Mini Mental State Examination
NART	National Adult Reading Test
PTH	Parathyroid hormone
PTX	Parathyroidectomy
RAVLT	Rey Auditory Verbal Learning Test
ROS	Reactive oxygen species
SD	Standard deviation
SDFR	Short delay/immediate free recall
T1	Learning trial 1 of RAVLT
T2	Learning trial 2 of RAVLT
T3	Learning trial 3 of RAVLT
T4	Learning trial 4 of RAVLT
T5	Learning trial 5 of RAVLT
T6	Short delay free recall
T7	Long delay free recall
VaD	Vascular dementia
VD	Vitamin D
VDD	Vitamin D deficiency
VDP	Vitamin D binding protein
VDR	Vitamin D binding receptor
WAIS-III	Wechsler Adult Intelligence Scale – Third edition
WBC	White blood cell count

Table of Contents

Chapter 1

Chapter 1: Review of the Literature

1.1 Introduction

Population ageing is a growing global phenomenon in which the prevalence of chronic diseases has considerably increased (Cardoso, Cominetti, & Cozzolino, 2013; Hasnain & Vieweg, 2014). Dementia is the progressive decline in cognitive function and represents one of the greatest causes of institutionalisation, morbidity and mortality among the elderly. Indeed, the occurrence of dementia is rising; according to the World Health Organization, there are currently over 44 million people worldwide affected by dementia (Alzheimer's Disease International, 2009). Without further advances to current preventative measures, this number is expected to exceed 115 million by the year 2050, accounting for over 20% of the world's population (Alzheimer's Disease International, 2009; Prince et al., 2013).

Presently, dementia is regarded as the greatest cause of disability and dependence in older Australians aged over 65 years and the second leading cause of death in Australia (The Australian Institute of Health and Welfare, 2015). In 2014, approximately 332, 000 Australians were living with dementia (The Australian Institute of Health and Welfare, 2014). Reflecting a growing socio-economic burden on the health/residential aged care system, approximately $5 billion was reportedly spent on dementia-related health care between 2009-10 (The Australian Institute of Health and Welfare, 2012, 2014). Presently, there is no cure for dementia. Despite significant research and development of several pharmacological strategies, effective disease-modifying therapies have been largely unsuccessful in terms of slowing disease progression. Clearly, identifying preventative strategies to reduce dementia risk and delaying disease onset and progression are global public health priorities to address the 'most significant health crisis of the 21st Century' (Alzheimer's Disease International, 2010).

1.1.1 Dementia

Dementia is the term used to describe a clinical syndrome caused by neurodegeneration, more commonly affecting the older population. Dementia is clinically characterised by the progressive deterioration of an individual's cognitive capacity and behavioural abnormalities that affect memory, intellect and ability to rationalise, physical functioning and capacity for independent living (Grand, Caspar, & Macdonald, 2011). Late-onset Alzheimer's disease (AD) and vascular dementia (VaD) are widely recognised as the most common causes of age-related dementia, accounting for over two-thirds of diagnosed cases (Fotuhi, Hachinski, & Whitehouse, 2009; Kelley & Petersen, 2007). The pathological hallmarks of AD and VaD frequently coexist and only a small percentage of individuals diagnosed with dementia exhibit AD without vascular abnormalities (de la Torre, 2004; Duron & Hanon, 2008; Fotuhi et al., 2009; Iadecola, 2010).

1.1.1.2 Pathophysiology of late-onset Alzheimer's disease

The underlying pathophysiology of late-onset AD-type remains complex and is still not entirely understood. Potential causative mechanisms for late-onset AD have been extensively considered throughout the literature and include neuro-inflammation, oxidative neuronal damage, neurotoxicity, and genetic vulnerability (Grammas, 2011; Zlokovic, 2008).

Late-onset AD is pathologically defined by hallmark features including brain tissue atrophy, cerebral extracellular deposition of amyloid-β peptide, intra-neuronal neurofibrillary tangles consisting of hyper-phosphorylated microtubule-associated protein *tau*, neuronal degeneration, disrupted cholinergic neurotransmission, and extensive cerebrovascular abnormalities including neurovascular damage and inflammation (Ellis et al., 1996; Fukuoka, Nakayama, & Doi, 2004; Heneka et al., 2015; Mattson, 2004; Miyakawa, 2010; Mufson, Counts, Perez, & Ginsberg, 2008; Perl, 2010; Takata & Kitamura, 2012). Neuronal cell death has been associated with the development of senile plaques and tangles, particularly in the hippocampal and cortex regions of the brain, mainly associated with learning and memory (Mattson, 2004). Whilst AD research over the past few decades has primarily focused on the

latter clinical stages of the disease related to aberrant deposition of protein constituents, mounting evidence has implicated a vascular origin for the disease as cerebrovascular changes may precede the clinical diagnosis of AD by years (Blennow et al., 1990; Dickstein et al., 2010; Grammas, 2000; Kalaria, 1992, 1999; Zlokovic, 2008).

1.2 Barriers of the central nervous system

In order to maintain central nervous system (CNS) homeostasis, it is crucial the composition of brain extracellular fluid is kept within precise physiological range, independent of fluctuations in the systemic circulation. There are three barriers of the CNS, namely, the arachnoid barrier, the blood-cerebrospinal fluid barrier (BCSFB) and the blood-brain barrier (BBB). These barriers work in conjunction to maintain a steady-state fluid ionic microenvironment for optimal cerebral function, to protect the CNS from any chemical insults by preventing the entry of pro-inflammatory mediators, macromolecules and neurotoxins into the brain, and to ensure the brain receives adequate nutrition from the peripheral circulation (Abbott & Friedman, 2012; Abbott, Patabendige, Dolman, Yusof, & Begley, 2010).

1.2.1 Arachnoid epithelium and blood-cerebrospinal fluid barrier

The arachnoid membrane essentially 'envelops' the brain beneath the dura mater layer and separates extracellular fluid of the CNS from the peripheral circulation (Abbott & Friedman, 2012). The avascular nature of the multi-layered epithelial layer and its small surface area relative to the blood-brain barrier does not represent a significant interface for blood-brain exchange (Cipolla, 2009).

Cerebrospinal fluid (CSF) is separated from systemic circulation and cerebral blood flow by the BCSFB, located at the choroid plexuses of the brain. The BCSFB is comprised of tight junctions formed between the epithelial cells of the CSF-facing surface of the epithelium that exclude the diffusion of large or hydrophilic molecules into the CSF whilst allowing transport of hydrophobic molecules and metabolic products via specific protein transporters (Abbott et al., 2010).

1.2.2 Blood-brain barrier

The BBB represents the major interface between the brain parenchyme and blood (Hawkins & Davis, 2005). The total length of cerebral capillaries in an adult human brain is approximately 600 km and the combined surface area of the BBB constitutes the largest surface area for blood-tissue exchange, averaging between 150 and 200 cm^2g^{-1}, a total area of 15 m^2 for exchange per average adult (Nag & Begley, 2005). Unlike capillaries in other parts of the body, cerebral capillary vessels are comprised of tightly apposed endothelial cells that effectively 'bind' the adjacent cells and regulatory transport systems that restrict ion flux, paracellular diffusion and transcytosis, in order to regulate the neuronal microenvironment as demonstrated in **Figure 1** (Abbott, Ronnback, & Hansson, 2006; Bradbury, 1993; Egleton & Davis, 1997). The combination of specific carrier-mediated systems and short distance between adjacent capillary vessels (~ 40 μm) facilitates the rapid diffusion-mediated exchange of essential nutrients such as glucose, amino acids and vitamins into brain interstitial fluid, rapid diffusion of neurotransmitters and the active transport of metabolic waste products from neurons back to the systemic circulation (Abbott & Friedman, 2012; Abbott et al., 2010).

The permeability of the BBB is modulated by the expression of tight junction and adherens junction proteins in order to protect the brain from systemically derived harmful neurotoxic and pro-inflammatory agents. Molecules greater than 400 kda in size are unable to permeate across the cerebral capillary layer (Hawkins & Davis, 2005; Pardridge, 2012). The cerebral capillary endothelium is lined by the basement membrane separating astrocytes and pericytes of the outer lining of the BBB. Both astrocytes and pericytes are critically important in maintaining cell integrity, synaptic signal transmission, neuronal plasticity, and providing immune functionality to capillaries; often referred as the neurovascular unit (Abbott et al., 2006; Zlokovic, 2005).

A highly selective barrier system resulting from the combination of structural, chemical and functional characteristics of the arachnoid epithelium, the BCSFB and the BBB allow strict regulation between components of blood, brain and CSF. Physiological and pathological states can alter the strict microenvironment in

the CNS by modulating barrier permeability (Abbott et al., 2006; Weiss, Miller, Cazaubon, & Couraud, 2009; Zlokovic, 2008).

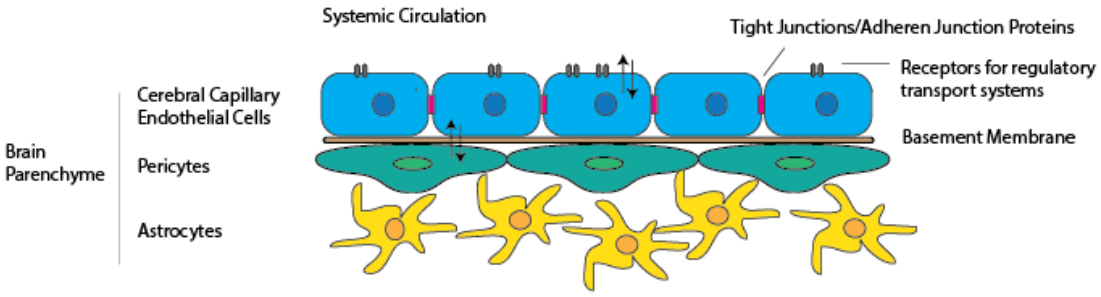

Figure 1. Diagrammatic representation of the Blood-Brain Barrier.

1.2.2.1 Cerebrovascular dysfunction & neurovascular inflammation in Alzheimer's disease

Structural damage or dysfunction of the cerebral capillary endothelium perturbs cerebral cellular homeostasis, transporter and enzymatic systems and subsequently limits the ability of the barrier to restrict blood-borne neurotoxic substances from entering the brain parenchyme (Herve, Ghinea, & Scherrmann, 2008; Huber, Egleton, & Davis, 2001). A compromised BBB can range from mild, transient tight junction opening to chronic barrier dysfunction, where the latter has been implicated in the pathophysiology of a number of neurodegenerative and neuro-inflammatory diseases (Forster, 2008; Persidsky, Ramirez, Haorah, & Kanmogne, 2006; Petty & Lo, 2002).

In primary neurodegenerative conditions such as AD, VaD, multiple sclerosis and epilepsy; and in secondary neurodegenerative disorders such as stroke, cerebrovascular integrity is impaired and consequently causes inappropriate blood-to-brain parenchyme protein trafficking, a decrease in tight junction protein expression, neurovascular inflammation and if persistently exaggerated, neuronal apoptosis (Kalaria, 2010; Zlokovic, 2011). Pathological alterations associated with

altered cerebral capillary permeability include vascular endothelial and smooth muscle cell proliferation and has been consistently indicated in individuals with dementia of AD-type ((Ellis et al., 1996). Substantial endothelial cell necrosis reportedly coincided with BBB disruption in AD subjects (Claudio, 1996). Moreover, blood-specific plasma proteins have been reported in the brain parenchyme of AD subjects, observations consistent with impaired cerebral capillary endothelium (Kalaria, 1992; Wisniewski, Vorbrodt, & Wegiel, 1997; Zipser et al., 2007).

Based on the highly vascularised nature of the BBB, evolving research and supporting evidence suggests compromised cerebral capillary vessel integrity may be one of the primary causative factors of AD-dementia and may precede pathophysiological changes such as amyloidosis and tau-tangles (Bell & Zlokovic, 2009; Brown & Thore, 2011; Gorelick et al., 2011; Zlokovic, 2008). These findings are further supported by recent novel findings from our laboratory of an age-associated increase in cerebral capillary permeability and heightened neurovascular inflammation in otherwise healthy wild-type mice, preceded neuropathological sequeale (Takechi, Pallebage-Gamarallage, Lam, Giles, & Mamo, 2012). Studies by Chen et al. (2009) reported the extent of BBB breakdown coincides with the severity and duration of vascular disruption (Chen et al., 2009). Consistent with a causative role, recent experimental and clinical studies have shown therapeutic benefit in AD progression if cerebrovascular disturbances are corrected or attenuated (Gorelick et al., 2011; Takechi, Pallebage-Gamarallage, Lam, Giles, & Mamo, 2014). Nonetheless, modulation of cerebral capillary function with age-related diseases remains a poorly understood phenomenon.

A chronic, heightened state of inflammation involving the up-regulation of complement molecules, pro-inflammatory cytokines, acute phase reactants and other inflammatory mediators have been extensively reported in the AD literature (Meraz-Rios, Toral-Rios, Franco-Bocanegra, Villeda-Hernandez, & Campos-Pena, 2013; Zlokovic, 2011). Post-mortem AD tissue and experimental findings derived from small vessel disease murine models are consistent with impaired BBB integrity coinciding with the entry of neurotoxic blood-derived products into the brain

parenchyme (Erickson & Banks, 2013; Sengillo et al., 2013). The entry of neurotoxic molecules into the brain parenchyme causes the paracrine activation of surrounding astrocytes and pericytes, ultimately causing neuronal damage/death (Bell et al., 2010; Zipser et al., 2007). In cell culture studies, the exposure of inflammatory factors to bovine brain capillary endothelial cells, as an *in vitro* BBB model, directly increased the permeability of the cell layer and thereby modulated barrier function (Deli et al., 1995; Grammas, 2011; Mark & Miller, 1999). Additionally, enhanced tight junction protein breakdown caused by extensive leucocyte recruitment upon exposure to inflammatory molecules was reported in an *in vivo* rat model (Bolton, Anthony, & Perry, 1998). Cerebral capillary dysfunction induced as a consequence of a chronically heightened state of systemic inflammation is positively associated with AD (Drake et al., 2011; Stolp et al., 2011; Takechi et al., 2010a). Moreover, the expression of inflammatory markers in the early stages of AD is associated with greater disease progression (Grammas, 2011). A number of population and experimental studies have reported the efficacy of anti-inflammatory/anti-oxidative pharmacological agents and certain environmental and nutritional factors in positively regulating BBB integrity via modulation of systemic inflammatory pathways (Ifergan et al., 2006; Kalayci et al., 2005; Pallebage-Gamarallage et al., 2012; Pallebage-Gamarallage et al., 2010; Takechi, Galloway, Pallebage-Gamarallage, Lam, & Mamo, 2010b; Takechi, Pallebage-Gamarallage, Lam, Giles, & Mamo, 2013b; Takechi et al., 2014).

1.2.2.2 Blood-brain barrier and cognition

Cognitive functioning is entirely dependent on the ability of neurons to form, maintain and break synaptic connectivity to other neurons (Lee & Silva, 2009). These processes are crucial for motor learning in the cerebellum and memory formation in the hippocampus and frontal cortex. Vascular risk factors have been associated with increased risk of cognitive impairment in both cross-sectional and longitudinal studies (Iadecola, 2013; Lorius et al., 2015). Many researchers have suggested that a 'breached' BBB increases the risk of neuronal damage and cognitive decline, however until recently, the initial area of damage was unknown. Montagne and colleagues (2015) reported cerebral capillary endothelium is firstly compromised in the hippocampal formation, the hippocampal sub-region CA1 and the dentate

gyrus, in older aged adults and thought to contribute to the early stages of age-related cognitive impairment (Montagne et al., 2015). Moreover, pericyte injury was shown to correlate with the extent of BBB damage (Montagne et al., 2015). Previous studies by Wang and colleagues (2006) also reported increased hippocampal endothelial permeability in subjects with mild cognitive impairment (defined as the clinical stage prior to progression to AD) when compared to age-matched controls (Wang, Golob, & Su, 2006). On this basis, it is important to address vascular risk factors involved in BBB dysfunction in aim to reduce the prevalence of cognitive impairment and AD progression.

1.3 Vitamin D

1.3.1 Importance of nutrition in brain function

Over the past few decades, there has been extensive research in the field of nutrition and the ageing population (Cardoso et al., 2013; Scott et al., 2006; Swaminathan & Jicha, 2014; Tucker et al., 1990). Whilst ageing is considered a primary risk factor of AD, there is strong epidemiological evidence and intervention trial data linking nutritional deficiencies with the exacerbation of neurodegenerative disease progression and cognitive deterioration (Cherubini et al., 2005; da Silva et al., 2014; Solfrizzi et al., 2011; Tucker, Qiao, Scott, Rosenberg, & Spiro, 2005). Elderly individuals with insufficient dietary intake of certain micronutrients and vitamins generally perform poorer in cognitive assessments when compared to controls (Requejo et al., 2003; Rosenberg & Miller, 1992; Tucker et al., 2005). AD-individuals commonly present with inadequate levels of specific micronutrients and vitamins and may be associated with increased risk of adverse brain function and thereby disease progression. However, cognitive dysfunction is commonly associated with progressive changes in eating behaviour and dietary deficiencies may therefore be consequential. Currently, widespread use of vitamin and mineral supplements has been promoted among the community to promote brain/cognitive function (Buell et al., 2010). Vitamin D (VD) has generated substantial interest in the dementia field based principally on its non-classical roles in the brain and neurocognitive health.

1.3.2 Sources and metabolism of vitamin D

Vitamin D is a lipid soluble vitamin first discovered by McCollum and colleagues in 1922 (McCollum, Simmonds, Becker, & Shipley, 1922). This steroid hormone is involved in the regulation of various essential physiological functions related to the maintenance of mineral ion homeostasis and skeletogenesis (Bikle, 2014). In recent years, VD has been implicated with a number of functions unrelated to its classical role in systemic calcium homeostasis including neuroprotection and modulating inflammation (Holick, 2003).

1.3.2.1 Sources of vitamin D

Vitamin D is obtained from two distinct sources; either from dietary sources or via skin photosynthesis. The main forms of VD present in foods are cholecalciferol (vitamin D_3), derived from animal sources, and ergocalciferol (vitamin D_2), of plant-based origin. Vitamin D_3 can also be synthesized endogenously through exposure to sunlight. The latter is considered the main source of VD for the human body as the human diet is not rich in either forms of VD, hence the widespread promotion of food fortification and supplement usage in some urbanised settings. Upon exposure of the subcutaneous VD prehormone 7-dehydrocholesterol to ultraviolet B radiation (wavelength 290 – 320 nm), endogenous vitamin D_3 is rapidly formed (DeLuca, 2004). Feedback mechanisms ensure the degradation of VD to inactive photoproducts upon excessive exposure to sunlight minimises the risk of VD intoxication (Holick, 2007).

1.3.2.2 Metabolism of vitamin D

Vitamin D_3 is considered a pro-hormone rather than a true vitamin and requires two sequential hydroxylations for conversion to its active metabolite, calcitriol (1α, 25-dihydroxyvitamin D_3). VD is transported through the circulation bound to its carrier protein known as the VD binding protein and undergoes the first hydroxylation in the liver or locally in other organs, converting VD to calcidiol (25-hydroxyvitamin D (25(OH)D); considered the stable marker of VD status) by 25-hydroxylase followed by renal hydroxylation by the 1 α-hydroxylase enzyme, CYP27B1, which generates calcitriol responsible for regulating gene expression; see

Figure 2 (Boucher, 2012; DeLuca, 2004; Lips, 2006; Norman, 2008) A number of VD metabolites are generated as a result of activation the VD precursors and whilst it has been proposed all hydroxylated forms of VD can bind to the VD receptor (VDR), serum concentrations of these metabolites are generally too low to exert a significant biological response (Tuohimaa, Keisala, Minasyan, Cachat, & Kalueff, 2009).

The conversion of VD to calcidiol is uncontrolled and the rate of conversion is dependent on the exogenous supply of VD. Whilst this metabolite is considered as the storage form of the pre-hormone prior activation, renal calcitriol synthesis is strictly controlled by endocrinal feedback mechanisms. The synthesis of VD and its metabolism is closely coupled to calcium homeostasis and is modulated by parathyroid hormone (PTH), calcium and phosphorus concentrations. Calcium and PTH positively regulate CYP27B1 activity, whilst negative regulators of this enzyme include phosphate, fibroblast-growth-factor 23, klotho and calcitriol itself, inducing VD catabolism (Dusso, Brown, & Slatopolsky, 2005; Holick & Chen, 2008; Kovesdy & Quarles, 2013). Calcitriol acts as a nuclear hormone and is the only high affinity ligand for the VDR (Haussler et al., 1998; Pike & Meyer, 2010). The effects of vitamin D are mediated via the binding of calcitriol to the VDR. For example, a decrease in circulating calcium elicits PTH release by the parathyroid glands thereby stimulating renal CYP27B1 activity to induce synthesis of calcitriol. To reinstate calcium levels, calcitriol reduces renal calcium excretion, increases intestinal calcium absorption and stimulates osteoclastic activity for calcium release in bones. Interestingly, VDR is expressed in a number of human tissues and cell types, including those irrelevant to bone formation and calcium homeostasis.

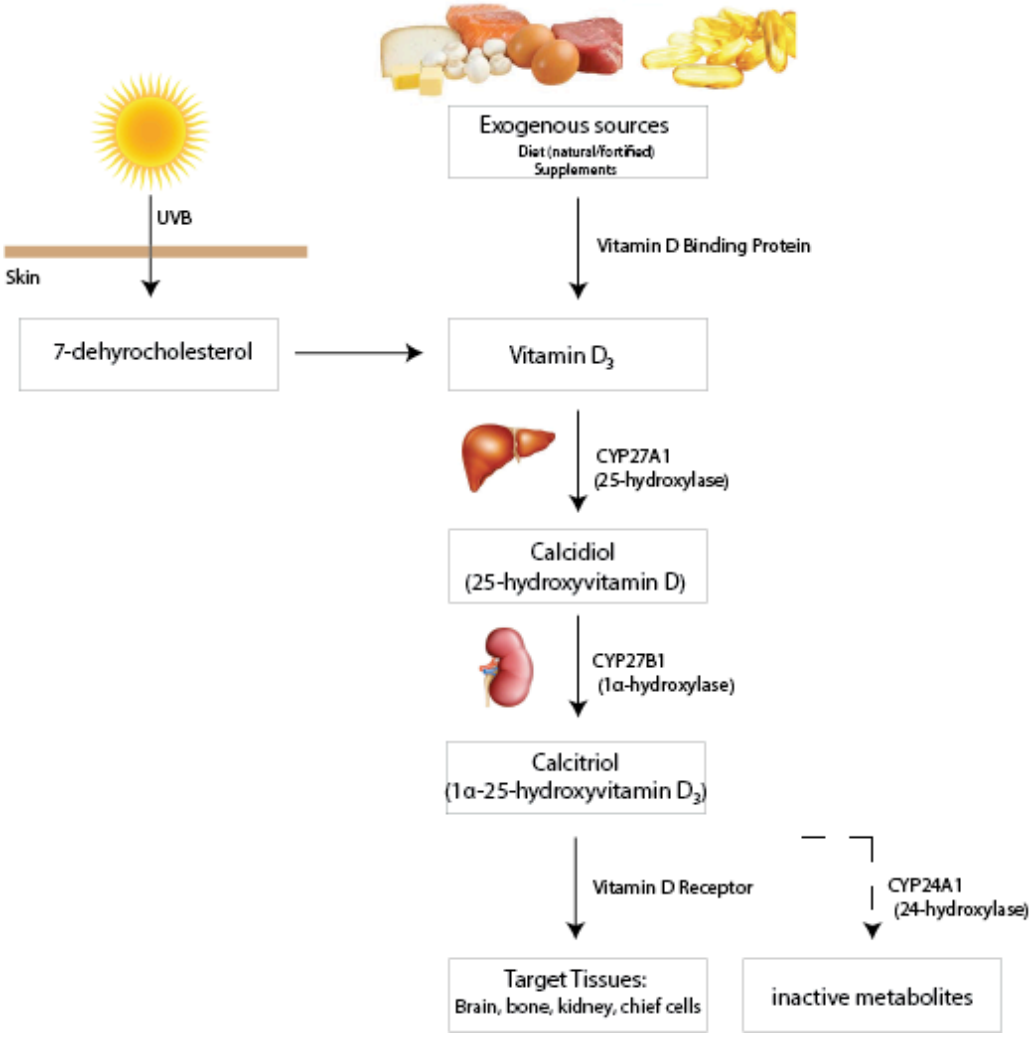

Figure 2. Activation pathway of vitamin D metabolism

1.3.3 Vitamin D in the central nervous system

The human brain is capable of locally synthesizing calcitriol and indeed, the expression of the VDR is widely expressed in both neuronal and glial cells within particular brain structures particularly vulnerable to age-related degeneration, namely, the cortex, hippocampus and the cerebellum (Eyles, Smith, Kinobe, Hewison, & McGrath, 2005; Wang, Zhu, & DeLuca, 2012; Zehnder et al., 2001). Furthermore, the VDR and its enzyme are colocated in the brain. The widespread distribution of the VDR in both humans and rodents are similar and indeed, strong

VDR expression in the neuroepithelium of neonatal rats during early neurogenesis has also been reported (Eyles et al., 2005; Veenstra et al., 1998). In addition to the discovery of VDR and its enzyme in brain tissue, VD has been shown to cross the BBB and thus suggesting a physiological functional effect of VD on the CNS (Holmoy et al., 2009). According to Holmoy and colleagues (2009), the concentration of VD in the CSF positively correlates with that in serum under physiological conditions (Holmoy et al., 2009). Indeed, epidemiological and experimental studies have postulated VD generally has a protective role in neurocognitive function and cerebrovascular disease although the mechanisms for these purported benefits remain unclear (Annweiler et al., 2009; Farid et al., 2012; Tuohimaa et al., 2009).

1.3.3.1 Neuroprotective properties of vitamin D

Recent prospective studies have shown a beneficial association of VD and numerous age-related disorders and suggest that VD may be neuroprotective via vascular mechanisms (Buell & Tucker, 2011). Data from animal studies have implied that VD may modulate the expression of neurotrophins and calcium binding proteins to avoid excitotoxicity for normal brain function (Brewer et al., 2001; Eyles, Brown, Mackay-Sim, McGrath, & Feron, 2003; Ibi et al., 2001). There is also evidence that VD exhibits anti-oxidant and immuno-regulatory properties to suppress oxidative damage and the expression of pro-inflammatory cytokines (Garcion et al., 1998; Mathieu et al., 2004; Wobke, Sorg, & Steinhilber, 2014). Briones et al. (2012) and others reported that VD may modulate an exaggerated pro-inflammatory state associated with ageing, which also coincided with attenuation of amyloid accumulation (Briones & Darwish, 2012; Durk et al., 2014; Yu et al., 2011). Moore et al. (2005) successfully demonstrated that VD acts as an anti-inflammatory agent by attenuating the age-related up-regulation of inflammatory cytokines and down-regulation of anti-inflammatory cytokines (Moore, Piazza, Nolan, & Lynch, 2007; Moore, Piazza, McCartney, & Lynch, 2005). In addition, VD was found prevent ischemic-induced BBB dysfunction in cultured cerebral endothelial cells by protecting them from reactive oxygen species (ROS) and pro-inflammatory cytokines (Won et al., 2015). Experimental findings demonstrated significant up-regulation of CYP27B1 and VDR expression upon exposure of cultured brain

pericytes to inflammatory mediators suggesting VD-related autocrine/paracrine feedback mechanisms in the BBB (El-Atifi, Dreyfusm, Berger, & Wion, 2015; Nissou et al., 2014). Conversely, recent findings by Barker and colleagues (2015) showed the treatment of VDD with VD supplementation increase the pro-inflammatory phenotype (Barker et al., 2015). Additional neuroprotective attributes of VD may include regulation of neurophysiological mechanisms via enhanced neurotransmission, synthesis of neurotrophic factors, up-regulated dendritic growth and neuronal preservation (Buell & Dawson-Hughes, 2008; McCann & Ames, 2008).

1.3.4 Global prevalence of vitamin D deficiency

It has been estimated over 1 billion individuals worldwide are either vitamin D deficient (VDD) or insufficient and this phenomenon is particularly prevalent in the ageing population (Boucher, 2012; Holick et al., 2012; Holick & Chen, 2008; van Schoor & Lips, 2011). Circulating concentrations less than 50 nmol/L and 75 nmol/L of VD generically define clinical VDD and VD insufficiency, respectively. Lips and colleagues (2006) report that major causes of VDD include inadequate sun exposure, dietary deficiencies, impaired absorption, increased secretion, obesity, and advanced age (Lips, 2006).

The interest in the potential importance of VD in human health continues to expand, particularly in the prevention of neurodegenerative diseases. Increasing evidence has implicated VDD as a substantial risk factor of inflammatory-related disorders/diseases including, cancer, diabetes, obesity, hypertension, cardiovascular disease, and all-cause mortality (Clemente-Postigo et al., 2015; Schottker et al., 2013; Weng et al., 2013; Yin & Agrawal, 2014). Moreover, numerous cross-sectional and longitudinal studies have shown an inverse correlation between VD status and disorders of the CNS such as multiple sclerosis, stroke, schizophrenia, Parkinson's disease, and AD (Annweiler, Llewellyn, & Beauchet, 2013a; Ascherio et al., 2014; Balion et al., 2012; DeLuca, Kimball, Kolasinski, Ramagopalan, & Ebers, 2013; Dickens, Lang, Langa, Kos, & Llewellyn, 2011; Eyles, Burne, & McGrath, 2013; Itzhaky et al., 2012; Suzuki et al., 2013). The widespread insufficiency of VD suggests a range of physiological functions related to this vitamin. Collectively, these

findings are suggestive that greater VD status may have beneficial effects for brain heath, however larger clinical and randomised controlled trials are required to elucidate whether these effects are causally associated to disease pathogenesis or a marker of health.

1.3.4.1 Cell culture and animal models of vitamin D deficiency

A number of experimental studies have demonstrated the neuroprotective features of VD. Findings by Wang et al. (2001) reported the pre-treatment of VD attenuated the effects of various stressors, including 6-hydroxydopamine-induced neurotoxicity (Wang et al., 2001). In addition, adult VDD was strongly associated with substantial changes in behaviour and brain neurochemistry in the mouse (Groves et al., 2013). Studies by Latimer et al. (2014) postulated a causal relationship between VD status and cognitive function via VD-mediated of hippocampal gene expression in aged rats models with altered VD homeostasis (Latimer et al., 2014). VDD in adult rats have been shown to exacerbate stroke-related cerebrovascular injuries, accompanied by severe post-stroke behavioural abnormalities (Balden, Selvamani, & Sohrabj, 2012). Keeney and colleagues (2013) reported chronic VDD causes elevated tyrosine nitration stress, alterations in glucose metabolism and mitochondrial changes in the brain of middle to older aged rats (Keeney et al., 2013a). Calcitriol reportedly reduced cerebral amyloid accumulation and improved cognitive functioning in mouse models of AD (Durk et al., 2014). VDR and 1α-hydroxylase knockout mice also exhibit a premature ageing phenotype coinciding with VDD accompanied by severe hypocalcemia, hypophosphatemia and secondary hyperparathyroidism (Keisala et al., 2009; Lanske & Razzaque, 2007). Conversely, a recent study based on 16 to 20 week-old VDD rats subjected to chronic consumption of VDD diets showed no major impairment in behaviour, although subtle effects were observed in tasks related to attention processing and neurotransmitter signalling in the striatum (Byrne et al., 2013). Moreover, Brouwer-Browlsma et al. (2014) found no differential effects between 22-month old mice fed either control or a VDD diet for 12 months (Brouwer-Brolsma et al., 2014). An interesting study by Solomon et al. (2011) demonstrated VD dietary restriction improved early disease severity and delayed disease onset in a mouse model of amyotrophic lateral sclerosis when compared to a diet with adequate VD, however

performance functional outcomes were reduced upon disease onset (Solomon, Gianforcaro, & Hamadeh, 2011).

Collectively, experimental studies investigating the mechanisms of VD neuroprotection remain inconclusive. It is clear however, no studies to date have explored if there is a direct effect of VD on the permeability of the cerebral capillary endothelium, which is purportedly associated with cognitive performance and progression of some neurodegenerative disorders. There is emerging evidence that higher concentrations of VD may also be associated with an increased risk of chronic diseases.

1.3.4.2 Hypervitaminosis D

Based on the tight regulation of VD activation, hypervitaminosis is rare in humans based on dietary sources. The prevalence of VD supplementation during the early periods of synthetic fortification, early ageing, hypercalemia, cardiovascular complications (vascular-related) and early death was reported in children, supporting the association between hypervitaminosis D and accelerated ageing (Markestad et al., 1987; Oliveri, Cassinelli, Mautalen, & Ayala, 1996). Furthermore, mutant mice with accelerated ageing phenotypes occur concomitant with hypervitaminosis D (Tuohimaa et al., 2009).

Fibroblast growth factor 23 (FGF-23) and Klotho are secretory proteins involved in the regulation of mineral homeostasis and have recently emerged as key mediators in early ageing (Medici et al., 2008; Razzaque & Lanske, 2006; Tsujikawa, Kurotaki, Fujimori, Fukuda, & Nabeshima, 2003). Genetic ablation of FGF-23 or Klotho genes in rodent models result in hypervitaminosis D, hypercalcemia and hyperphosphatemia; corresponding with a phenotype consistent with premature ageing (Medici et al., 2008; Razzaque & Lanske, 2006; Torres et al., 2007). Tsujikawa et al. (2003) and others reported dietary restriction of VD reverses the premature-ageing phenotypes and prolongs survival (Madathil, Coe, Casu, & Sitara, 2014; Tsujikawa et al., 2003). These findings provide strong evidence that hypervitaminosis D may be causally associated with the ageing process. Furthermore, Huebbe et al. (2011) presented experimental evidence that the APOE

ε4 allele, a positive risk factor of AD, is associated with higher serum VD levels (Huebbe et al., 2011). Increased levels of VD can trigger apoptosis and result in tissue atrophy upon periods of prolonged exposure (Johnson, Muindi, Hershberger, & Trump, 2006; Medici et al., 2008; Narvaez & Welsh). Excessive activation of the VDR causes gene transcription associated with mitochondrial export of cytochrome C and subsequent cleavage of caspase-9, which consequently promotes DNA fragmentation and thereby apoptosis (Demay, 2006). On the basis of these findings, it appears an optimal concentration of VD must be determined for optimal brain function due to the complications involved with hypo-and-hypervitaminosis.

1.3.4.3 Vitamin D and cognition

Higher vitamin D status, represented by greater serum concentrations of 25(OH)D has been associated with better cognitive performance in some, though not all studies (Bartali, Devore, Grodstein, & Kang, 2014; Buell et al., 2010; Granic et al., 2015b; Littlejohns et al., 2014; Oudshoorn, Mattace-Raso, van der Velde, Colin, & van der Cammen, 2008). An international task force considering VD and cognition in the older population recently concluded that hypovitaminosis increases the risk of cognitive decline and dementia in older adults (Annweiler et al., 2015). Indeed, hypovitaminosis D is associated with purportedly 2.4 times higher risk of cognitive impairment, specifically with AD, compared to individuals with normal levels of the vitamin (Annweiler et al., 2009; Annweiler et al., 2013a; Annweiler et al., 2013b; Etgen, Sander, Bickel, Sander, & Forstl, 2012; Littlejohns et al., 2014).

Hooshmand and colleagues (2014) reported positive associations between VD status with cognitive function, CSF amyloid-β and brain tissue volumes (Hooshmand et al., 2014). Regional cerebral blood flow and brain function was found positively correlated with VD concentrations in AD patients (Farid et al., 2012). A lower concentration of serum VD correlates well with certain deficits in executive functioning (a heterogeneous set of higher level processes that control and regulate other abilities and behaviour), however, episodic memory was found to be unaffected (Annweiler et al., 2013b; Brouwer-Brolsma et al., 2013; Granic et al., 2015b). Conversely, results also indicated cognitive impairment was more prevalent in individuals with higher levels of VD, especially those taking VD supplements

(Granic et al., 2015b; McGrath et al., 2007). A number of prospective cohort studies have failed to show a correlation between VD and cognitive performance, ranging from early childhood to advanced aged individuals (Dean et al., 2011; Maddock, Geoffroy, Power, & Hypponen, 2014; Schneider et al., 2014; Tolppanen, Williams, & Lawlor, 2011).

Given that sun exposure and subsequent skin synthesis is a major source of VD, studies (especially cross-sectional and short-follow ups) exploring the association of VD homeostasis with cognition in late-aged individuals may be confounded by 'reverse causation' due to lifestyle changes and immobility. Moreover, the ability to synthesize endogenous VD declines with age and may be due to morphological changes due to biological ageing (Holick, Matsuoka, & Wortsman, 1989; MacLaughlin & Holick, 1985). As a lipid soluble vitamin, VD is typically stored in adipose tissue. As ageing is generally associated with increased overall body fat composition, it is possible that individuals diagnosed as VDD are in fact VD adequate and incorrectly diagnosed (Konradsen, Ag , Lindberg, Hexeberg, & Jorde, 2008).

As the majority of the available literature is primarily focused on VDD populations, evidence suggests individuals with low VD levels are likely to benefit from supplementation. A biphasic U-shaped relationship has been used to describe the association between VD status and the risk incidence of chronic diseases such as cardiovascular disease, prostate cancer, and all-cause mortality (Durup et al., 2015; Tuohimaa et al., 2004). A biphasic relationship has also been suggested between VD status and global cognition in the elderly, whereby moderate VD concentrations are deemed beneficial but in cases of deficits or excess the putative neuroprotective properties of VD are lost (Granic et al., 2015b). **Figure 3** represents the purported U-shaped association between serum VD concentration and the risk of chronic diseases and cognitive dysfunction. Indeed, results from the Women's Health Initiative found that VD and calcium supplementation for over a period of 8 years had no effects on cognition (Rossom et al., 2012). Moreover, studies by Stein et al. (2011) show that supraphysiological doses of VD may not be essential to exert a beneficial cognitive effect (Stein, Scherer, Ladd, & Harrison, 2011). Further increasing VD beyond

sufficient levels evidently leads to adverse cognitive outcomes. The health risks and benefits from VD and calcium supplementation remain controversial where numerous clinical studies have implicated increased risk of vascular events and white matter lesions.

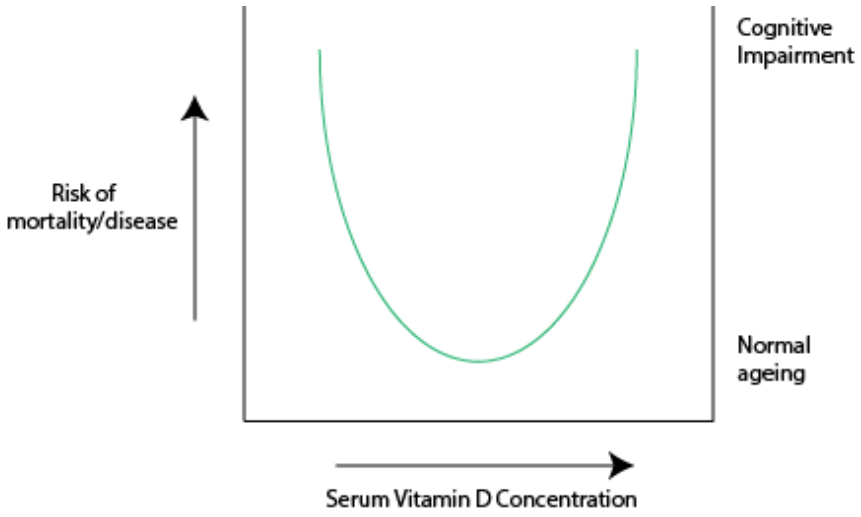

Figure 3. Purported biphasic U-shaped relationship between Vitamin D status and the risk of chronic diseases and cognitive decline

1.4 Calcium

An equivocal function of VD is to regulate calcium metabolism. Calcium is the fifth most abundant element in the body and arguably the most widespread and ubiquitous signalling molecules in mammalian cells and has been implicated in a number of neurodegenerative diseases in the past few decades, particularly dementia (Berridge, Bootman, & Lipp, 1998; Berridge, Lipp, & Bootman, 2000; Peacock, 2010).

1.4.1 Calcium physiology

A complex, integrated system involving the actions of VD, PTH and calcitonin, is required for the strict regulation of calcium homeostasis. PTH and VD

are the main regulators of calcium homeostasis and have major regulatory effects on each other (Chattopadhyay, 2000; Silver, Yalcindag, Sela-Brown, Kilav, & Naveh-Many, 1999).

The majority of calcium of the body is stored as skeletal hydroxyapatite and a small portion is rapidly exchangeable calcium is found between extracellular fluid in bone found between osteoblasts, osteocytes and bone matrix. Only a small percentage of calcium comprises the extracellular and intracellular calcium concentration of the body, indicative of its potency and importance in cellular and vascular functions.

1.4.1.1 Extracellular calcium

Extracellular calcium is ultimately the source of all intracellular calcium ions and is crucial in blood coagulation, maintenance of skeletal integrity, intercellular adhesion and plasma membrane integrity (Brown, 1999). Extracellular serum calcium exists in three main fractions; approximately one half of total serum calcium is bound primarily to plasma proteins such as albumin, a small percentage is complexed to anions such as phosphate, bicarbonate, sulphate, and lactate, and the remaining one half circulates as the free divalent cation, ionised calcium. Ionised calcium (iCa) is the physiologically active fraction whereas the protein-bound and complexed forms of calcium are considered biologically inert and used as storage or buffering systems (Shane & Irani, 2006).

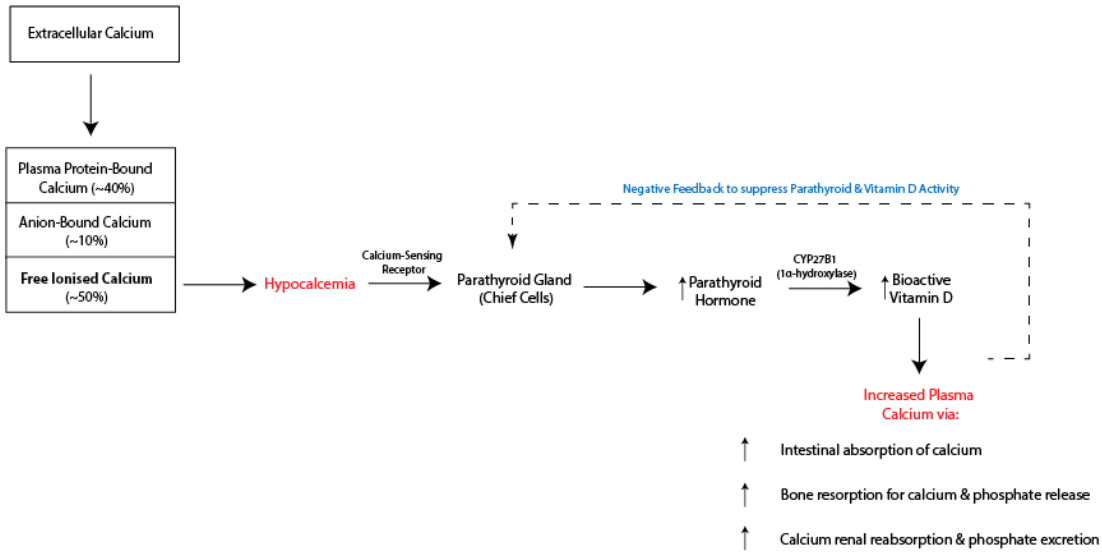

Figure 4. Extracellular calcium homeostasis feedback regulation

Under normal homeostatic mechanisms, total serum calcium is kept between 2.1 to 2.5 mM whilst iCa is strictly controlled by its hormonal counterparts between 1.1 to 1.3 mM. The calcium-sensing receptor (CSR) is abundantly distributed in all tissues related to calcium control and small changes in circulating calcium levels trigger intracellular signalling pathways to exert feedback mechanisms (see **Figure 4**). In response to low serum levels of iCa, chief cells of the parathyroid gland promote the synthesis and secretion of PTH, which rapidly stimulates the activation of VD. VD and PTH thereafter synergistically increase serum iCa via enhancing intestinal absorption of dietary calcium, increasing osteoclast activity to liberate calcium and reducing renal calcium excretion. Once calcium levels have been restored, negative endocrinal feedback mechanisms take place and suppress PTH synthesis and secretion.

1.4.1.2 Intracellular calcium signalling

Intracellular calcium acts as an important secondary messenger and involved in a number of crucial cellular functions including, gene transcription, cell proliferation, cell migration, neurotransmission and of particular interest, the modulation of the permeability of the brain capillary endothelium (Abbott, 1998;

Berridge, Bootman, & Roderick, 2003). The concentration of calcium and the signalling over cellular compartments is strictly controlled via diffusional and active transport systems that allow rapid removal of intracellular calcium from cytosolic space. The flux of calcium across plasma membrane and between intracellular compartments generates synaptic signalling and plays a vital role in neuronal functioning. Cytosolic calcium levels are maintained at extremely low concentrations of ~100 nm, approximately 10,000 to 20 000-fold less than the extracellular milieu during resting state (Zündorf & Reiser, 2011). This gradient is maintained by calcium-binding buffering proteins and via intrinsic membrane transport systems responsible for transport into organelles for intracellular storage or removal of calcium from cytosol back into extracellular space (Abbott, 1998).

It is crucial local and global levels of calcium are strictly controlled at temporal and spatial levels as increases in calcium can lead to the activation of numerous cellular signalling pathways including those for learning and memory, as well as apoptotic pathways (Green, 2009).

1.4.2 Central theory of calcium and ageing

The potential critical role of cellular calcium in neuronal ageing was first postulated almost three decades ago and commonly referred to as the 'Calcium Hypothesis of neuronal ageing' (Khachaturian, 1987; Landfield, 1987). Based on the role of calcium as an upstream messenger in multiple signalling cascades, dysregulation of calcium homeostasis modulates cellular physiology, molecular functions and ultimately cell structure (Toescu, Verkhratsky, & Landfield, 2004). Whilst brief elevations of intracellular calcium are required for controlling membrane excitability and modulating essential processes such as learning, memory and gene transcription, chronic elevations in intracellular calcium triggers neurotoxic signalling cascades that lead to neuronal death (Arundine & Tymianski, 2003; Delorenzo, Sun, & Deshpande, 2005). The 'Calcium Hypothesis' states that impaired calcium signalling and synaptic plasticity caused by sustained elevations of calcium transients associated with ageing consequently compromises neuronal communication resulting in impaired cognition, memory formation and

neurodegeneration (Mattson, 2007; Mattson & Chan, 2003). Indeed, experimental and clinical studies have demonstrated brain neuronal calcium dyshomeostasis in both models of ageing and AD (Bruno et al., 2012; Hopp et al., 2015; Murchison et al., 2009; Thibault, Gant, & Landfield, 2007). Electrophysiological studies of hippocampal neurons have consistently shown significant altered calcium influx and transients (Disterhoft, Moyer, Thompson, & Kowalska, 1993; Raza et al., 2007; Thibault, Hadley, & Landfield, 2001).

1.4.3 Calcium and the blood-brain barrier

Strict regulation between extracellular and intracellular calcium concentrations is crucial in maintaining the paracellular permeability of the BBB by moderating junctional and cytoskeletal protein components (De Bock et al., 2013; Somjen, 2004). Normal BBB function is perturbed when extracellular calcium concentration is either decreased and/or intracellular free calcium concentration is increased, though the exact mechanisms involved require clarification (De Bock et al., 2013). Both hyper - and - hypocalcemic conditions have been shown to disrupt cell-cell contact of the junction protein formation and thereby increasing permeability and reducing trans-epithelial resistance of the cell layer; restoration of calcium levels restored normal barrier function (Brown & Davis, 2002).

The importance of maintaining calcium homeostasis is further reinforced by the pioneering studies by Murphy and colleagues who demonstrated the role of the BBB and BCSFB in regulating the CNS during chronic hypo-or-hypercalcemic conditions (Murphy & Rapoport, 1988; Murphy, Smith, & Rapoport, 1986; Murphy, Smith, & Rapoport, 1988; Murphy, Smith, & Rapoport, 1989) It is still unclear whether serum calcium is directly related to the extracellular levels of calcium in the brain. It was reported that calcium levels in the brain and CSF do not change in proportion to acute or chronic changes of plasma iCa (despite chronic changes of up to 50% in plasma calcium), thus suggesting that calcium transport is primarily regulated by saturable transport mechanisms at the BBB (Murphy, Smith, & Rapoport, 1991). On the contrary, Tai et al. (1986) reported that calcium ions could

enter the brain via passive diffusion through the BBB, where serum calcium directly correlated with brain and CSF concentrations (Tai, Smith, & Rapoport, 1986).

1.4.4 Calcium and cognitive function

Consistent with the 'Calcium Hypothesis' several studies have implicated an association between serum iCa and global cognition. Schram et al. (2007) and others reported greater baseline serum calcium concentration associated with impaired cognitive performance in older aged adults (Schram et al., 2007; Tilvis et al., 2004). Moreover, the APOE ε4 allele (related to a 3-fold increase of developing AD) has also been associated with higher serum calcium and cognitive decline (van Vliet, Oleksik, Mooijaart, de Craen, & Westendorp, 2009). Neurotoxic effects of APOE ε4 were linked with disrupted neuronal calcium homeostasis by increasing the intracellular calcium levels by influx of extracellular calcium. Experimental studies have shown amyloid-β aggregation may be involved in the disruption of intra-neuronal calcium levels, by forming calcium permeable channels into neuronal membranes (Green, 2009). As per biphasic responses of nutrients and vitamins, high concentrations of calcium are considered harmful. Excess calcium causes aberrant aggregation of proteins and nucleic acids, modulates the integrity of lipid membranes and promotes phosphate precipitation (Verkhratsky, Rodriguez, & Parpura, 2012).

A recent meta-analysis observed calcium supplementation (both with and without co-administration of VD) is associated with an increased risk of vascular-related events (Bolland et al., 2010; Bolland, Grey, Avenell, Gamble, & Reid, 2011). Payne and colleagues (2008, 2013, 2014) have published several cross-sectional studies relating exaggerated iCa serum concentrations with cerebral white matter lesions (often associated with cognitive decline), larger brain lesion volumes and the induction of the apoptotic cascade (de Groot et al., 2000; Payne, Anderson, & Steffens, 2008; Payne, McQuoid, Steffens, & Anderson, 2014; Payne, Pierce, McQuoid, Steffens, & Anderson, 2013). Elevated serum calcium levels have been linked with higher risk of myocardial infarctions, other cardiovascular events, stroke and mortality (Jorde, Sundsfjord, Fitzgerald, & Bonaa, 1999; Leifsson & Ahren, 1996; Wang et al., 2014). Consistent with the literature, a pro-inflammatory and pro-

oxidant phenotype was found to be associated with hypercalcemia (Orrennius, Burkitt, Kass, Dypbukt, & Nicotera, 1992; Rezic-Muzinic et al., 2013). However, data reported by Talmor-Barkan et al. (2009) demonstrated hypocalcemia was associated with an inflammatory state (Talmor-Barkan et al., 2009a).

If iCa is central to the neurovascular effects of VD, then by extension of clinical observations, VD supplementation would notionally be beneficial for subjects with hypocalcemia, but possibly harmful in subjects with elevated or adequate levels of serum calcium. Similar to VD, the effects of iCa on capillary permeability and inflammation have not been directly investigated and based on the interplay of both metabolites, their interactive effects need to be considered in conjunction with one another.

1.4.5 Calcium paradox

The calcium paradox is a well-known pathophysiological phenomenon based on the observations made on a calcium-depleted isolated rat heart preparation where severe myocardial injury and necrosis occurred in response after calcium repletion (Zimmerman et al., 1967). Thus if considered in context of AD, the calcium paradox is the parathyroid hormone-induced neuronal intracellular calcium overload triggered by calcium deficiency, which ultimately relates back to the 'Calcium Hypothesis'.

1.5 Parathyroid hormone

As an important regulator of calcium homeostasis and potential role in the calcium paradox, PTH has been positively implicated with cognitive decline and dementia (Braverman et al., 2009; Dotzenrath et al., 2006). The role of PTH function has not been investigated independently in context of neurodegeneration as the manifestations of hyperparathyroidism are commonly thought to be secondary to hypercalcemia or VDD.

1.5.1 Parathyroid hormone physiology

The main physiological role of PTH is to function as a 'calciostat' to maintain extracellular levels of circulating calcium (Tabatabai & Jan De Beur, 2005).

The hormone is synthesized as a pre-propeptide subsequently cleaved to a biologically active 84 amino acid peptide. Full biological activity resides in the first 34 amino-terminal amino acids of the PTH molecule. PTH is secreted by chief cells of the parathyroid glands and is tightly regulated on a transcriptional and post-transcriptional level, dependent on calcium concentrations. Moreover, PTH regulates phosphorus levels and reciprocally, increased levels of phosphorus stimulate the synthesis and secretion of PTH due to reduced circulating calcium. PTH does not have a binding hormone and binds to PTH receptors on osteoblasts and epithelial cells of the nephron, where the conversion of VD is metabolised to calcitriol. To ensure steady-sate, PTH and VD mediate the mobilisation of calcium from osteoblasts and to increase renal reabsorption of calcium. PTH activity is down regulated by increased calcium, increased VD and possibly increased FGF-23. The secretion of PTH is never fully suppressed and has an extremely short half-life of ~ 4 minutes.

1.5.2 Parathyroid hormone and neurocognition

Emerging evidence has suggested PTH may directly affect neurovascular integrity independent of VD homeostasis. PTH has been shown to cross the BBB and an exaggerated level of PTH within the CSF appears to cause neuronal degeneration due to cellular iCa overloading (Hirasawa et al., 2000; Khudaverdian & Chursina, 1996). Indeed, hyperparathyroidism is the most common cause of hypercalemia. In a number of clinical studies, hyperparathyroidism has been reported as an independent risk factor of age-related cognitive decline (Bjorkman, Sorva, & Tilvis, 2008; Braverman et al., 2009). Furthermore, case-controlled findings show improved cognitive performance in some subjects with hyperparathyroidism following parathyroid surgery, though not all (Bollerslev, Rolighed, & Mosekilde, 2011; Coker et al., 2005; Walker et al., 2009). PTH has been shown to stimulate the endothelial expression of vascular growth factor and may play a role in endothelial dysfunction pathophysiology (Rashid, Bernheim, Green, & Benchetrit, 2008). Conversely, PTH has a purported role as a modulator of the functional activity of neurons (Khudaverdyan & Ter-Markosyan, 2000). Thus, it is possible that studies reporting detrimental effects of VDD may be a surrogate marker of PTH-induced sequelae.

1.6 Conclusion

Based on experimental and clinical evidence, it is clear cerebral capillary integrity is pivotal to the risk and/or progression of a range a neurodegenerative disorders including Alzheimer's disease. Taken together, it seems obvious to investigate the putative association of the highly integrated Vitamin D – Calcium - Parathyroid Hormone endocrinal axis and related metabolites with cerebral capillary vessel permeability. The study of this integrated system in terms of neuropathology will be exceedingly informative with regards to possible translational clinical interventions and may provide insight on potentially modifiable risk factors in the restoration/modulation of neurovascular integrity and function prior to disease diagnosis or progression.

Thesis hypothesis and specific objectives

The pre-clinical studies presented in this thesis were aimed to principally explore the potential interactive and/or independent effects of the Vitamin D - Calcium - Parathyroid Hormone endocrine axis on cerebral capillary function. A secondary objective was to consider in a clinical context, cognitive performance relative to serum Vitamin D - Calcium - Parathyroid Hormone homeostasis.

HYPOTHESIS: The Vitamin D - Calcium - Parathyroid Hormone endocrine axis regulates cerebral capillary integrity and is associated with neurocognitive performance.

The above hypothesis was investigated by the following objectives:

Objective 1

To explore the effects of hypervitaminosis D, calcium and the calcium counter-regulatory hormone, parathyroid hormone, on the modulation of cerebral capillary integrity and function. Wild-type mice and rats were randomised to dietary or endocrinal/surgical interventions to mimic either hypervitaminosis D, hyper -or - hypo- parathyroid states, concomitant with altered calcium homeostasis. Groups of mice and rats were assigned to one of the following treatments: dietary supplementation of vitamin D_3 ranging from 1,000 to 120,000 IU/kg of diet, exogenous infusion of parathyroid hormone or parathyroid tissue ablation. After 6, 12 or 24 weeks of dietary or surgical intervention, the functional integrity of the BBB and neurovascular inflammation was assessed principally by three-dimensional immunofluorescent microscopy. Vitamin D - Calcium - Parathyroid Hormone status was determined by measuring serum concentrations of bioactive ionised calcium and parathyroid hormone.

Objective 2

To investigate the putative association between Vitamin D - Calcium - Parathyroid Hormone homeostasis and neurocognitive performance. Serum levels of vitamin D, ionised calcium and parathyroid hormone, and a panel of cognitive measures (assessing primarily verbal episodic learning and memory) were analysed in a cross-sectional sample of healthy middle-aged and older adults.

Chapter 2

Chapter 2: Vitamin D - Calcium - Parathyroid hormone endocrine axis and the modulation of cerebral capillary integrity in rodents

The content of this chapter is covered by Publication 1:

Lam V., Takechi R., Pallebage-Gamarallage M., Giles C., & Mamo J. C. (2015). The vitamin D, ionised calcium and parathyroid hormone axis of cerebral capillary function: therapeutic considerations for vascular-based neurodegenerative disorders. *Plos One, 10*(4), e0125504.

Synopsis:

Background

Cerebrovascular dysfunction characterised by the abnormal transport of plasma proteins into the brain parenchyme, is a common pathological hallmark of vascular-based neurodegenerative disorders whereby increasing clinical and experimental evidence suggests cerebral capillary barrier dysfunction may occur in the developmental/pre-clinical stages of dementia (Lorius et al., 2015; Zlokovic, 2011). As discussed in Chapter 1, VD has received much attention in the dementia field due to its association with a number of neuro-beneficial properties including modulation of immuno-regulatory factors, neurotrophin synthesis and neuronal protection (Buell & Tucker, 2011; Garcion et al., 1998; McCann & Ames, 2008).

Experimental evidence of whether greater VD levels and its corresponding metabolites are beneficial to neurovascular integrity and function is lacking. Moreover, the major counter-regulatory metabolites and hormones involved in VD homeostasis namely, calcium and PTH, have been implicated in the pathogenesis of neurodegenerative diseases (Bjorkman et al., 2008; Hirasawa et al., 2000; Payne et al., 2013; Schram et al., 2007; Tilvis et al., 2004), so synergistic and/or interactive effects need to be considered. Prior to the studies presented in the following

publication, no existing evidence had considered the endocrinal axis of Vitamin D - Calcium - Parathyroid Hormone in a hypervitaminosis D context in relation to vascular-related neurodegenerative disorders.

To investigate the major components of the integrated endocrinal system and its association with neurovascular integrity and function, vitamin D and parathyroid hormone intervention studies were explored in rodent models.

Methods in brief

2 rodent species (wild-type C57BL/6J mice and Sprague-Dawley rats) were randomised to one of the following interventions: dietary VD supplementation across a normal to high dosage range, parathyroid gland ablation or infusion of exogenous PTH, for a duration of either 6, 12 or 24 weeks. Three-dimensional (3-D) immunofluorescent quantification was used to assess cerebral capillary integrity by measuring the parenchymal abundance of plasma protein IgG, a marker of BBB leakage, whereas neuro-inflammation was determined by quantification of glial fibrillary acidic protein (GFAP), representative of astroglial activation. Blood biochemistry was used to determine the calcemic status of each animal.

Results in brief

Significant cerebrovascular disturbances were seen in both experimental species in association with the dosage of VD supplement provided. Greater doses of VD were also associated with increased serum iCa and suppression of serum PTH. Sustained treatment effects were evident up to 24 weeks of intervention. Ablation of parathyroid glands increased the parenchymal abundance of IgG, concomitant with a reduction in serum iCa. With the provision of exogenous PTH, iCa levels increased, however cerebral capillary permeability of the PTH-infused animals were comparable to their respective controls. Both VD supplemented and PTX groups showed modest increases in astroglial activation, whereas PTH-infused groups showed a reduction in the expression of GFAP.

Discussion and conclusion in brief

This pre-clinical study explored whether dietary induced hypervitaminosis D or endocrine mediated hypo - and - hyper - parathyroidism can modulate the permeability of the cerebral capillary layer and possibly neurovascular inflammation. The principal findings of these experiments demonstrate VD can significantly compromise cerebral capillary integrity and function, via a mechanism independent of calcium homeostasis. In addition, the data suggests PTH may have vasculo-protective effects. Increased capillary permeability was not associated with neurovascular inflammation contrary to previous studies in rodent models where compromised BBB and heightened inflammation were reported (Freeman & Granholm, 2012; Takechi et al., 2012).

Collectively, the results from this study support the role of VD in modulating the cerebral capillary function and permeability. The provision of supplemental VD at doses that increase serum iCa and suppress serum PTH concentration compromise cerebrovascular integrity but do not promote neurovascular inflammation per se. The information gained from this highly relevant publication provides future directions to explore the potential mechanisms related to the modulation of the BBB in relation to the VD and its hormonal counterparts, particularly in the investigation of vascular-based neurodegenerative disorders.

PLOS | ONE

RESEARCH ARTICLE

The Vitamin D, Ionised Calcium and Parathyroid Hormone Axis of Cerebral Capillary Function: Therapeutic Considerations for Vascular-Based Neurodegenerative Disorders

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Abstract

Blood-brain barrier dysfunction characterised by brain parenchymal extravasation of plasma proteins may contribute to risk of neurodegenerative disorders, however the mechanisms for increased capillary permeability are not understood. Increasing evidence suggests vitamin D confers central nervous system benefits and there is increasing demand for vitamin D supplementation. Vitamin D may influence the CNS via modulation of capillary function, however such effects may be indirect as it has a central role in maintaining calcium homeostasis, in concert with calcium regulatory hormones. This study utilised an integrated approach and investigated the effects of vitamin D supplementation, parathyroid tissue ablation (PTX), or exogenous infusion of parathyroid hormone (PTH) on cerebral capillary integrity. Parenchymal extravasation of immunoglobulin G (IgG) was used as a marker of cerebral capillary permeability. In C57BL/6J mice and Sprague Dawley rats, dietary vitamin D was associated with exaggerated abundance of IgG within cerebral cortex (CTX) and hippocampal formation (HPF). Vitamin D was also associated with increased plasma ionised calcium (iCa) and decreased PTH. A response to dose was suggested and parenchymal effects persisted for up to 24 weeks. Ablation of parathyroid glands increased CTX- and HPF-IgG abundance concomitant with a reduction in plasma iCa. With the provision of PTH, iCa levels increased, however the PTH treated animals did not show increased cerebral permeability. Vitamin D supplemented groups and rats with PTH-tissue ablation showed modestly increased parenchymal abundance of glial-fibrillary acidic protein (GFAP), a marker of astroglial activation. PTH infusion attenuated GFAP abundance. The findings suggest that vitamin D can compromise capillary integrity via a mechanism that is independent of calcium homeostasis. The effects of exogenous vitamin D supplementation on capillary function and in the context of prevention of vascular

neurodegenerative conditions should be considered in the context of synergistic effects with calcium modulating hormones.

Introduction

In primary neurodegenerative conditions such as vascular-dementia, Alzheimer's disease, multiple sclerosis and epilepsy; and in secondary neurodegenerative disorders such as stroke, cerebral capillary function is impaired, resulting in inappropriate blood-to-brain parenchyme protein trafficking, neurovascular inflammation and if persistently exaggerated, cellular apoptosis [1, 2]. Consistent with a causal association of capillary dysfunction in some neurodegenerative disorders, there is an accumulating body of literature from clinical studies and in animal models showing therapeutic benefit in disease progression if blood-brain barrier (BBB) disturbances are corrected or attenuated [3, 4].

Epidemiological and experimental studies suggest that vitamin D generally has a protective role in neurodegenerative disorders although the mechanism(s) for these purported benefits are not clear [5–7]. Analyses of transcription factors and signaling pathways support the contention that a key effect of vitamin D is via positive regulation of genes broadly involved in neurovascular inflammation [8]. However, other mechanisms relevant to neurodegenerative conditions may include modulation of p-glycoprotein expression; up-regulation of serotonin synthesizing genes; protein oligomerization (such as beta-amyloid) and apoptosis [9–11]. Some reported positive downstream effects of vitamin D include restoration of capillary function in models of multiple sclerosis and cessation of disease progression; enhanced cognitive performance in subjects with mild cognitive impairment; decreased risk for Alzheimer's disease; and in some psychiatric disorders, an improvement in behavior [12–17].

A consequence of the perceived positive effects of vitamin D in reducing risk of neurodegenerative disorders has generated momentum in developed nations with aging populations, to consider adopting policies that promote vitamin D supplementation. However, this is a contentious issue, as robust physiological studies to investigate potential vitamin D toxicology are not yet realized and the 'optimal' vitamin D level remains controversial [5–7]. Moreover, the effects of vitamin D may be via regulation of calcium homeostasis, or the effect of calcium regulatory hormones, rather than via direct effects of the vitamin per se. Presently, there is little evidence to support the hypothesis that vitamin D at greater than ordinary physiological concentrations is likely to be beneficial.

An equivocal function of vitamin D is to regulate calcium metabolism. In response to low serum levels of ionised calcium (iCa), sensor cells within parathyroid tissue promote secretion of parathyroid hormone (PTH), which rapidly stimulates the conversion of vitamin D to its bioactive form 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Bioactive vitamin D and PTH will synergistically increase plasma iCa via enhancing intestinal absorption of dietary calcium, increasing osteoclast activity to liberate calcium and reducing renal calcium excretion. The provision of supplemental vitamin D progressively increases iCa and concomitantly suppress PTH secretion. Clearly then, cerebral capillary effects of exogenous vitamin D supplementation cannot be considered in isolation.

Calcium is pivotal to neuronal excitability and indeed the changes that underlie learning and memory [18–20]. In addition, calcium ions are critical in maintaining the integrity of the BBB [21, 22]. Several studies have implicated an association between elevated serum iCa and global cognitive decline [23–25]. Moreover, at greater concentrations and when chronically

exaggerated, serum iCa is positively correlated with cerebral white matter lesions, greater lesion volume and induction of the apoptotic cascade [19, 26–29]. If iCa is central to the neurovascular effects of vitamin D, then by extension of the clinical observations, supplementation with vitamin D would notionally be beneficial in subjects with hypocalcemia, but possibly harmful in subjects with raised, or adequate levels of serum calcium. Tai et al reported that the influx of iCa into the CNS is not regulated by a saturable mechanism that is sensitive to acute changes in plasma concentration, but more likely to occur through passive diffusion [30]. The CNS would therefore notionally be protected from rapid acute changes in iCa [31, 32]. However, chronic heightened effects may be realized. The effects of iCa on capillary permeability and on neurovascular inflammation per se, have not been directly investigated.

Several lines of evidence also suggest that PTH may directly affect neurovascular integrity independent of vitamin D homeostasis. PTH has been shown to cross the BBB and an exaggerated level of PTH within CSF appears to cause neuronal degeneration due to cellular iCa overloading [33, 34]. In clinical studies, increased levels of PTH have been reported as an independent risk factor of age-related cognitive decline [35, 36]. Furthermore, case-controlled findings show improved cognitive performance in subjects with hyperparathyroidism following parathyroid surgery [37, 38]. Therefore, it is possible that studies reporting detrimental effects of vitamin D deficiency may be a surrogate marker of PTH induced sequelae.

Whilst capillary integrity is considered pivotal to risk or progression of a range of neurodegenerative disorders, regulation of capillary function via the vitamin D-iCa-PTH axis is neither established nor elucidated. However, delineation of the putative synergistic effects of vitamin D-iCa-PTH on capillary integrity may be of significant therapeutic importance. To this effect, this study adopted intervention models of dietary vitamin D supplementation; exogenous parathyroid hormone supplementation or parathyroid tissue ablation in two genetically unmanipulated rodent models, to investigate capillary permeability and neurovascular inflammation within CNS regions of interest that are relevant to learning and memory.

Materials and Methods

Animals and dietary/hormonal interventions

All animals were housed in accredited animal holding facilities in individual ventilated cages with 12-h light/dark cycle, controlled air temperature (21°C) and air pressure (Curtin Animal Holding Facility, Perth and Charles River Laboratories, Kent). All animals were given ad libitum access to specified diets and water. Dietary protocols and surgical procedures described in this study were approved by NHMRC accredited Curtin Animal Ethics Committee (approval no. N34-10 [mice] and AEC_2011_30A [rats]) and Charles River UK Ethics Committee (project Licence no. 70/7221), respectively. All surgical and endpoint protocols were performed under strict anaesthetic protocols to minimize pain and stress.

Dietary Vitamin D (VD) supplementation

7-week-old female wild-type C57BL/6J mice and Sprague-Dawley rats were purchased from Animal Resources Centre (Murdoch, W.A, Australia). Animals were randomly assigned in groups of 8 to one of the following semi-purified A1N93G diets: Control 0.5% Calcium, 0.35% phosphorus containing 1000 IU; 20,000IU; 40,000 IU; 80,000 IU or 120, 000 IU vitamin D_3/kg diet (Specialty Feeds, Glenn Forrest, W.A, Australia). Eight animals from each group were sacrificed at either 6, 12 or 24 weeks after commencement of dietary intervention. The approximate daily intake of VD per animal (international units) is listed in Table 1.

Table 1. Daily intakes of vitamin D.

Study	Vitamin D_3 (VD) per kg of diet (IU)	Control (1, 000)	20, 000	40, 000	80, 000	120, 000
Mice	**Daily intake of VD (IU/day)**	2	40	80	160	240
	Intake of VD/kg/day (IU)	100	2, 000	4, 000	10, 000	16, 000
Rats	**Daily intake of VD (IU/day)**	15	300	600	1, 200	1, 800
	Intake of VD/kg/day (IU)	75	1, 500	3, 000	6, 000	9, 000

The approximate international units (IU) of vitamin D consumed by Sprague-Dawley rats and C57BL6/J mice per respective treatment group are shown as units consumed per day or daily units consumed per kilogram of weight.

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Parathyroid Gland Ablation

Seven week-old female Sprague-Dawley rats (200–250g) were randomly assigned as control animals or subjected to selective parathyroidectomy (PTX) (Charles River Laboratories, Kent, UK). Briefly, rats were placed under general anaesthesia via isofluorane and with the aid of a dissecting microscope; parathyroid glands were identified, dissected and ablated from the surrounding tissue. The skin wounds were closed using wound clips. Thereafter, animals were provided with 1.5% calcium lactate solution 3 days post-operation. Both control and PTX groups were given ad libitum access to standard SDS-VRF-1 diet (Calcium 1% Phosphate 0.6%) manufactured by Special Diet Services, UK and sacrificed at either 12 or 24 weeks. Successful parathyroid tissue ablation was confirmed by determining circulating serum PTH. The PTX treatment group data is indicative of rats with a PTH concentration <65pg/ml. At 12 weeks and 24 weeks post surgery data is presented for 4-PTX and 7-PTX rats, respectively. Eight controls were studied alongside each PTX treatment group, at 12 and 24 weeks.

Exogenous Parathyroid Hormone Infusion

Seven week-old female Sprague-Dawley rats were randomly allocated to either control or PTH-infused groups. All animals were placed under general anaesthesia and Alzet mini osmotic pumps (2ML) were implanted subcutaneously between the scapulae of each animal (Charles River Laboratories, Kent, UK), containing either saline and 2% cysteine (control group) or rat 1–34 PTH fragment diluted in isosmotic saline with 2% cysteine at a concentration of 0.332ug/hour/rat (Bachem Labs, Switzerland). Skin wounds were closed using wound clips. All osmotic pumps were primed and filled under aseptic conditions. Animals were given ad libitum access to standard SDS-VRF-1 diet (Special Diet Services, UK) and sacrificed at either 6 (control n = 9; PTH- infused n = 8) or 12 weeks (control n = 8; PTH-infused n = 4). Treatment at 24 weeks could not be investigated as this would have required subsequent replacement of the mini osmotic pumps, a procedure not permitted by the ethics protocols. Rats with incomplete wound healing at one week post pump insertion were humanely euthanized under general anaesthesia. As exogenous PTH cannot be detected by PTH assays, we like other laboratories utilized serum measures of ionised calcium and total calcium as a surrogate marker of efficacy. In addition residual osmotic pump reservoir volume and weight confirmed delivery of the vehicle buffer/hormone.

Sample collection and preparation

At each intervention endpoint, rats were anaesthetized with 75mg/kg ketamine and 10mg/kg xylazine whilst mice were anaesthetized with pentobarbitone (45 mg/kg) followed by exsanguination via cardiac puncture. An initial 100ul of fresh whole blood was collected into plain syringes for immediate analysis of blood ionised calcium whist the remaining sample was left to

clot at room temperature for 30 minutes and centrifuged for serum extraction. Thereafter, samples were aliquoted and stored at -80°C until further analysis. Brain specimens were carefully extracted and fixed in 4% paraformaldehyde (w/v in PBS, pH 7.2) for 24h, followed by cryoprotection with 20% sucrose for 72 h at 4°C. The brain specimens were then frozen in dry ice/isopentane and stored at -80°C.

Cerebral Extravasation of Immunoglobulin G

The integrity of cerebral capillaries was assessed by measuring cerebral perivascular extravasation of the plasma protein immunoglobulin G (IgG), a marker of unspecific blood-to-brain leakage of plasma proteins and macromolecules by 3-dimensional (3-D) semi-quantitative immunomicroscopy, using methods as previously described, with minor modifications [39]. Briefly, 20μm brain cryosections were blocked with 10% goat serum in PBS for 30 min and thereafter incubated with polyclonal goat anti-mouse IgG^1 or goat anti-rat IgG^1 antibodies conjugated with Alexa 488 fluorochrome (1:100; Invitrogen, USA) at 4°C for 20 h. Nuclear counterstains were performed for detection of cerebrovascular endothelial cells and the sections were mounted with anti-fade mounting medium. Negative controls were included with all the experiments, which included the replacement of the primary antibody with either buffer or an irrelevant serum. No fluorescent staining was observed in any of the negative controls.

Representative 3-D immunofluorescent micrographs were captured with AxioVert 200M (Carl-Zeiss, Germany) coupled with an mRM digital camera and ApoTome optical sectioning system. Each 3-D image was captured at a magnification of x200 (Plan-Neofluar 20x objective lens) and consisted of at least 12 2-dimensional Z- stack images with a 1.225μm axial distance optimized by Nyquist overlap theory. For each region of interest in the brain, a minimum of 10–30 3-D images was randomly captured from the cortex or hippocampal formation per animal. Capillary vessel fluorescence was excluded based on conservative threshold exclusion settings and confirmed manually for each image processed as previously indicated [4, 39, 40], avoiding confounders associated with extensive and incomplete perfusion with wash-out buffers. All 3-D images were used for the subsequent quantitative analysis. The voxel intensity of the fluorescent dye was calculated with Volocity 6.1 3-D image analysis software (PerkinElmer, UK) and expressed per volume unit. The average of the total fluorescent intensities of all the images in cortex and hippocampal formation regions were calculated within each animal and thereafter compared between each of the treatment groups.

Immunofluorescent Analysis of Cerebral Inflammation

Similar to cerebral IgG immunomicroscopy, the cortex and hippocampal expression of glial fibrillary acidic protein (GFAP) was determined as a surrogate marker for astrocyte activation by utilizing immunodetection techniques as described previously [39, 41]. 20um brain cryosections were blocked with 10% goat serum and incubated with polyclonal rabbit anti-mouse GFAP (1:200; Abcam, UK) for 20 h at 4°C. Polyclonal goat anti-rabbit IgG conjugated with Alexa488 (1:200) was then applied to the sections for 2h at RT followed by DAPI nuclear counterstain. Slides were mounted with anti-fade medium. Negative controls were run with every experiment, which included the replacement of the primary antibody with either buffer or an irrelevant serum. No fluorescent staining was observed for control tissues with the indicated capture settings.

Biochemistry Analyses

Blood Ionised Calcium. Serum ionised calcium levels were measured to determine the calcemic status of each animal. Whole blood was collected via cardiac puncture into non-

coagulated syringes and immediately analysed with a hand-held VetScan iSTAT analyser and CG8+ blood gas cartridges (Abbott Point of Care, Australia) in accordance with the manufacturer's instructions. Appropriate aqueous controls were run at every endpoint.

Serum Total Calcium and Inorganic Phosphate. Serum total calcium and phosphate were analysed by quantitative colorimetric assays by QuantiChrom Calcium and Phosphate Assay kits, respectively, according to manufacturer instructions (BioAssay Systems, CA).

Serum Parathyroid Hormone. Serum PTH was measured with ELISA kits specific for the detection of intact rat PTH 1–84 molecule (Immutopics Rat Intact PTH ELISA, San Clemente, CA). The kit is supplied with prepared standards and two control sera and has a sensitivity of 1.6pg/ml and range to 3000pg/ml for serum or plasma. Intra-assay precision of <2.4% and inter-assay variability of <6.0%. Reported values of PTH in rats have been reported between 40–400pg/ml [42–44]. In this study, serum was collected under identical conditions and PTH determined on the same day for all treatment groups indicated.

Statistical Analysis

For quantitative immunomicroscopy, a minimum of 10 and up to 30 3-D images was captured from each mouse and rat per region of interest, respectively. In total over 800 (mice) and 4000 (rat) 2-D images were taken per group. All raw data was log transformed and the arithmetic mean was used as a measure of central tendency. Thereafter, all statistical analyses were done with non-parametric statistics by one-way analysis of variance (ANOVA) analyses followed by Tukey's post hoc tests. Pearson's correlation analysis was used to determine the association between parenchymal expression of IgG and specified plasma biomarkers. Data was considered to be statistical significant where p value <0.05. Unless otherwise stated, all data are expressed as mean +/ SEM. Statistical analyses were performed with IBM SPSS Version 22 (SPSS Inc., Chicago, USA).

Results

Parenchymal abundance of IgG was used to assess cerebral capillary permeability in wild-type mice and rats maintained on diets supplemented with vitamin D. Fig 1 depicts the abundance of IgG within cortex (CTX) and hippocampal formation (HPF) for the respective treatment groups. The findings show a strong positive association between parenchymal IgG within CTX and HPF and the dose of exogenous vitamin D provided. However, subtle differences in response to vitamin D supplementation were observed between Sprague-Dawley rats and C57BL/6J mice. Abundance of IgG within both CTX and HPF were markedly elevated within 6 weeks of treatment in rats consuming chow containing 80,000 IU VD/kg of diet, whereas the treatment effect was not seen in mice until approximately 12 weeks of feeding. Thereafter, both species showed stabilization in the abundance of parenchymal IgG, with persistently exaggerated amounts relative to control groups at 24 weeks of feeding, but comparable to vitamin D supplemented animals at 12 weeks of feeding.

Greater dosages of vitamin D in rats were required to increase serum iCa above controls compared to mice, however this may have reflected the markedly lower baseline values in control mice compared relative to control rats (Fig 2). Similarly, serum total Ca was progressively increased and phosphate decreased respectively in mice, whereas little change in rats was indicated at equivalent treatment dosages of vitamin D. A duration-of-treatment effect for the concentration of iCa, serum total calcium and phosphate is indicated in Fig 3. Vitamin D provided at 80,000IU VD/kg significantly increased bioactive iCa, compared to control group in mice within 6 weeks of treatment. However, effects were significant at 12 weeks for both species. Thereafter, stabilization was indicated for the duration of treatment (up to 24 weeks). In mice,

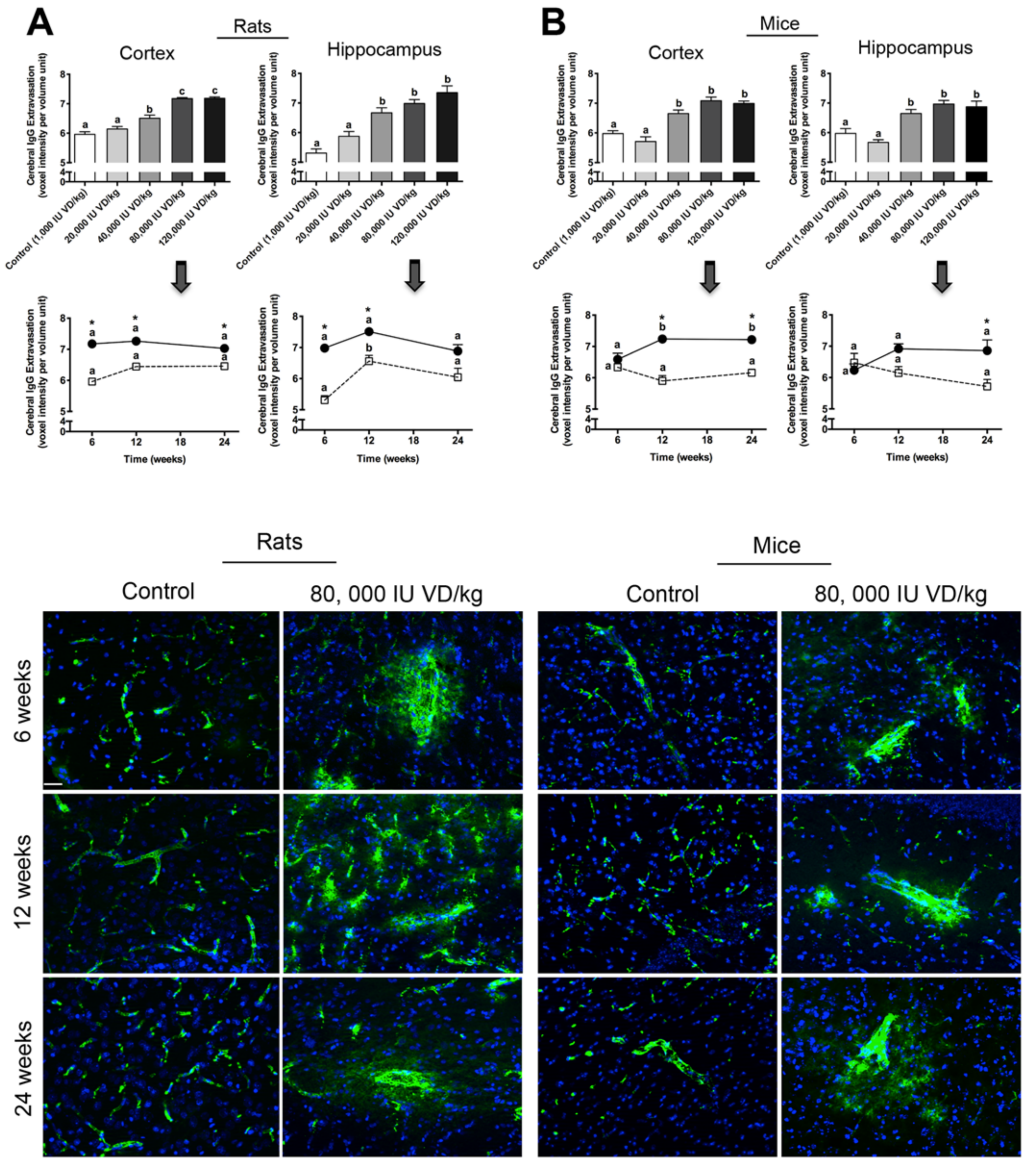

Fig 1. Cerebral Capillary Integrity. Cerebral capillary vessel integrity was assessed using 3-dimensional semi-quantitative fluorescent immunomicroscopy by measurement of parenchymal extravasation of plasma macromolecule IgG in the cerebral cortex (cortex) and hippocampal formation (hippocampus) of wild-type rats (A) and mice (B) supplemented with units of dietary vitamin D (VD) as indicated and measured in voxels per volume unit. The bar graphs illustrate a strong dosage effect of vitamin D concentration and abundance of cerebral IgG in the cortex and hippocampal formation in both species; statistical significance is denoted by different letters ($p<0.05$; $n = 8$; one way ANOVA followed by Tukey's post hoc analysis). The bottom graphs demonstrate duration-

of-treatment effects in both rodent species fed either control (open square/dotted lines) or diets enriched with 80, 000 IU VD/kg (black circles/solid lines) for 6, 12 and 24 weeks. Different letters show $p<0.05$ for duration effects within each group ($n = 8$) whilst an asterisk show statistically significant different dietary effects between control group and its' relevant treatment group at each time point ($p<0.05$; $n = 8$; one-way ANOVA followed by Tukey's post hoc analysis). Data is shown as mean ± SEM. Representative 2-dimensional extended-focus fluorescent immunomicrographs (magnification x 200) of cerebral IgG distribution in animals fed control and 80, 000 IU VD/kg diets are shown in the last frame; IgG is shown in green and DAPI nuclei counterstaining is shown in blue. Scale bar represents 100μm.

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total calcium was also exaggerated compared to controls, but there was no difference in total Ca in rats. Serum levels of phosphate essentially remained the same for the duration of treatment for both species studied.

The effect of dietary vitamin D supplementation on circulating PTH concentration in rats is provided in Fig 4. A marked dose response is indicated with serum PTH levels significantly attenuated as a consequence of vitamin D supplementation. Marked suppression of PTH was evident within six weeks of intervention and persistent for the treatment duration of 24 weeks in animals kept on exogenous vitamin D of 80, 000 IU VD/kg. The reported concentration of PTH in rats reported over several decades is variable, indicative of different methods of immunodetection [45, 46]. Nonetheless, vitamin D treatment effects on serum/plasma PTH concentration are as in this study consistently substantive.

The effects of chronically suppressed serum PTH on capillary permeability without the confounder of dietary supplementation of vitamin D, was explored in other experimental groups, that is in rats with surgical ablation of parathyroid tissue. Fig 5 shows that the parenchymal expression of IgG within CTX and HPF 12 weeks post parathyroidectomy was over 2-fold greater compared to control animals. These effects persisted but were not significantly amplified further when investigated at 24 weeks of intervention. Increased capillary permeability occurred in PTH ablated rats concomitant with a reduction in iCa and to a lesser extent, total Ca. The latter is a contraindication compared to the vitamin D intervention experiments, where increased capillary permeability occurred concomitant with elevated iCa. We note that the serum PTH concentration in control rats for PTH ablation (Fig 5) indicated a lower PTH concentration than in control rats for vitamin D intervention (Fig 4), despite identical serum preparation and measures of PTH being done for all groups simultaneously. With a coefficient of variation for this assay of less than 3%, our interpretation for differences in the concentration of PTH between control groups is the possibility of strain differences in rats hosted at two sites (Charles River, UK for PTH interventions and vitamin D treated groups hosted at Curtin University, Western Australia). Nonetheless, irrespective of this observed difference between the two control group PTH measures, comparison of treatment versus respective control is a valid comparison.

Exogenous provision of PTH via the placement of mini-osmotic pumps predictably increased serum iCa and total Ca. However, consistent with the findings in parathyroidecomized rats, there was no suggestion that capillary permeability had been compromised as a consequence of heightened serum iCa. Rather, parenchymal abundance of IgG within CTX and HPF were comparable between rats given PTH and sham operated control rats hosting osmotic pumps filled only with saline.

Table 2 depicts correlation analysis of cerebral capillary permeability with vitamin D, iCa, total Ca, PTH and phosphate for all vitamin D supplemented and parathyroid-intervention experimental groups. The vitamin D studies in rats and mice suggest that provision of vitamin D and the serum concentration of iCa are strongly associated with increased capillary permeability within CTX and HPF. In addition, the vitamin D supplementation studies also show a marked negative correlation between capillary permeability and the serum concentration of PTH. However, the PTH intervention experiments do not support the contention that the positive

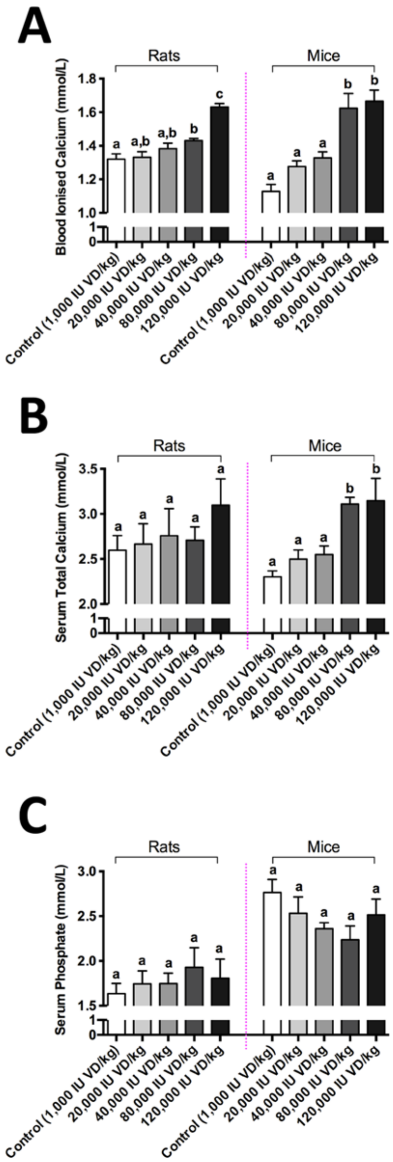

Fig 2. Blood biochemistry analyses. Blood biomarkers were measured in wild-type rats and mice supplemented with different doses of dietary vitamin D (VD) to assess calcemic status. Blood Ionised Calcium (B), serum Total Calcium (C) and Serum Phosphate were measured using either an iSTAT point-of-care analyser or commercially available colorimetric kits. Data is shown as mean ± SEM. Statistical significance is denoted by different letters ($p<0.05$; $n = 8$; one-way ANOVA followed by Tukey's post hoc test).

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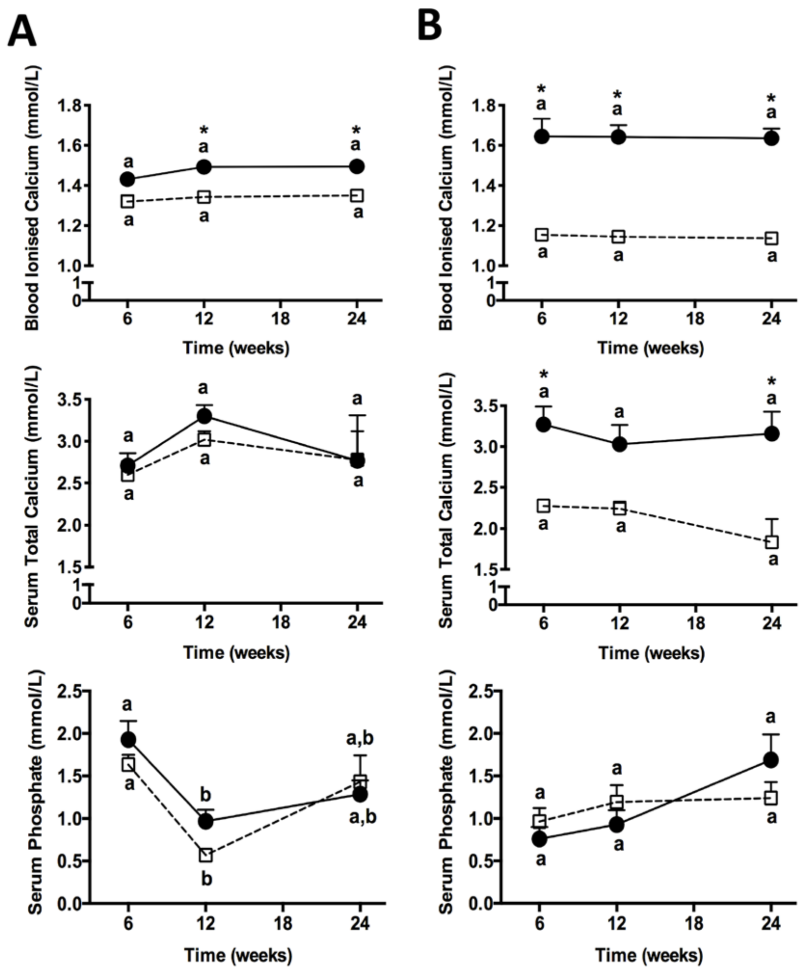

Fig 3. Duration-of-treatment effects of exogenous vitamin D on calcemic status. The duration-of-treatment effects of dietary vitamin D on blood ionised calcium, serum total calcium and serum phosphate in either control animals (open square/dotted lines) or animals fed 80, 000 IU vitamin D/kg (black circles/solid lines) at 6, 12 and 24 weeks of age are shown. Column (A) represents data derived from Sprague-Dawley rats and column (B) of C57BL/6J mice. $p<0.05$ for duration effects within each group is represented by different letters (n = 8; one-way ANOVA). * $p<0.05$ compared to relevant control at each corresponding time point (dietary effects; n = 8; one-way ANOVA). Data is shown as mean ± SEM.

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association between iCa and capillary integrity indicated in the vitamin D studies is causal. Provision of PTH had markedly increased serum iCa to a comparable degree as dietary vitamin D supplementation, however, PTH treated rats did not demonstrate compromised capillary permeability. Consistent with the findings in PTH treatment of rats, parathyroidectomy-induced disturbances in capillary function also did not support an association of serum iCa with

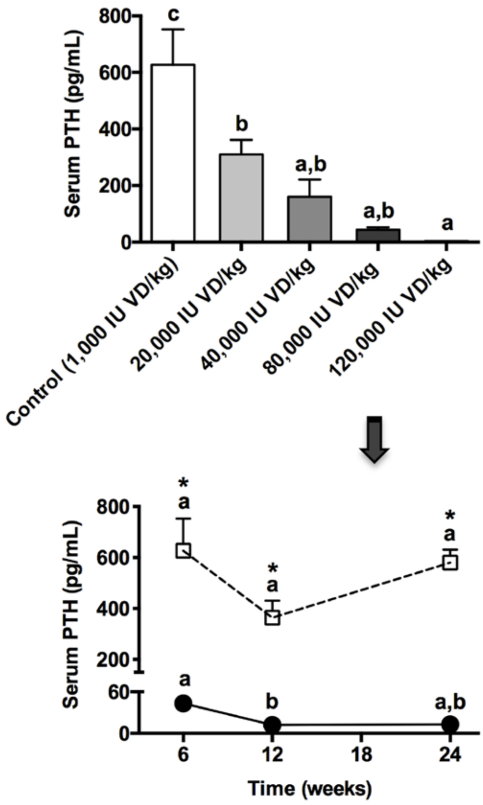

Fig 4. Measures of Parathyroid Hormone (PTH) in vitamin D-supplemented rats. After 12 weeks of intervention, circulating serum concentrations of intact parathyroid hormone (PTH) were determined via commercially available ELISA kits in each of the dietary vitamin D (VD) groups. Data shown as mean ± SEM is indicated in the top graph. Different letters denote statistical significance between groups ($p<0.05$; n = 8; one-way ANOVA followed by Tukey's post hoc analysis). Duration-of-treatment effects in each representative group are shown in the bottom frame for either 6, 12 or 24 weeks (control, shown as open squares/dotted line or 80,000IU VD/kg, shown as black circles/solid black line). Different letters are indicative of $p<0.05$ for duration effects within each group (n = 8; one-way ANOVA) whereas * show $p<0.05$ compared to respective controls (n = 8; one-way ANOVA). All data is shown as mean ± SEM.

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increased capillary permeability. Rather, iCa, total Ca, PTH and cerebral capillary integrity were negatively correlated (or showed no correlation) with parenchymal abundance of IgG. Across all experimental treatment groups, the consistent finding was a negative effect of vitamin D and a positive effect of PTH on capillary integrity.

Capillary dysfunction characterized by inappropriate extravasation of plasma proteins is postulated to contribute to neurovascular inflammation. Parenchymal abundance of GFAP within CTX and HPF was used as a marker of astroglial activation. Fig 6, demonstrates a modest level of heightened GFAP in rats and mice treated with a supplementary vitamin D regimen that had resulted in increased capillary permeability. In this study, the distribution of

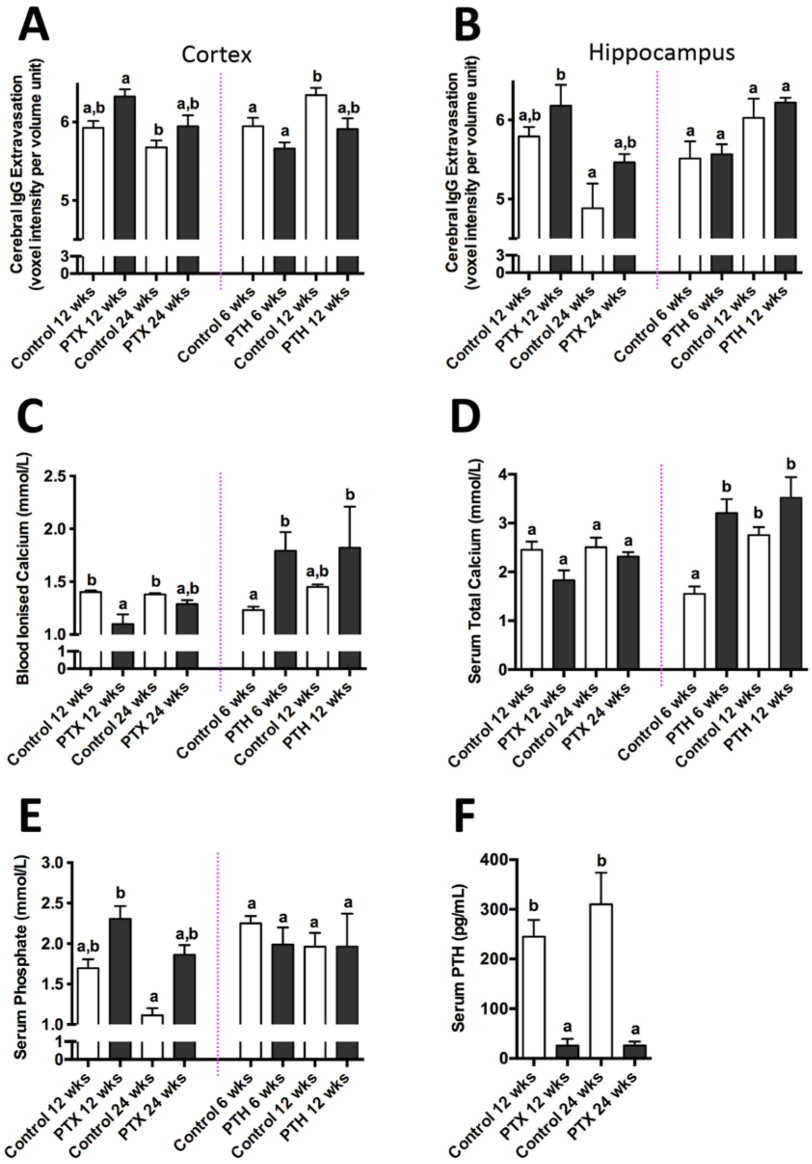

Fig 5. Parathyroidectomy and infusion of Parathyroid Hormone (PTH) studies. Cerebral parenchymal abundance of IgG in the cerebral cortex and hippocampal formation of Sprague-Dawley rats subjected to ablation of parathyroid tissue (PTX) or exogenous infusion of parathyroid hormone (PTH) and their respective controls at either 6, 12 or 24 weeks are shown in graphs A and B. Blood ionised calcium (C), serum total calcium (D), serum phosphate (E) and serum intact PTH (F) were also analysed in each intervention group. Different letters denote statistical significance between groups ($p<0.05$; n = 4–10; one-way ANOVA followed by Tukey's post hoc analysis). The data shown are mean ± SEM.

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Table 2. Correlation Table of Cerebral Capillary Permeability with markers of Vitamin D-Calcium-Parathyroid hormone homeostasis.

Animals	Intervention	Region of Interest	Vitamin D	Total Calcium	Ionised Calcium	Parathyroid Hormone	Phosphate
Rats	Vitamin D 6 weeks	CTX	0.887**	0.063	0.613**	-0.62**	0.163
		HPF	0.803**	0.253	0.679**	-0.68*	0.005
	Vitamin D 12 weeks	CTX	0.851**	0.619**	0.775**	-0.61**	-0.172
		HPF	0.666**	0.529**	0.567**	-0.543**	-0.213
	Vitamin D 24 weeks	CTX	0.728**	-0.337	0.448	-0.662**	-0.106
		HPF	0.534*	-0.174	0.363	-0.349	-0.173
Mice	Vitamin D 6 weeks	CTX	0.300	0.172	0.145	-	-0.164
		HPF	-0.191	-0.127	-0.116	-	0.002
	Vitamin D 12 weeks	CTX	0.768**	0.609**	0.730**	-	-0.291
		HPF	0.692**	0.513**	0.613**	-	-0.175
	Vitamin D 24 weeks	CTX	0.919**	0.565	0.884**	-	-0.106
		HPF	0.679*	0.715*	0.765**	-	0.006
Rats	PTX 12 weeks	CTX	-	-0.458	-0.605	-0.608*	0.475
		HPF	-	-0.722*	-0.502	-0.079	0.726*
	PTX 24 weeks	CTX	-	-0.366	-0.629*	-0.228	0.482
		HPF	-	-0.674*	-0.099	-0.48	0.325
Rats	PTH Infusion 6 weeks	CTX	-	-0.182	-0.573	-	0.001
		HPF	-	-0.051	0.401	-	-0.02
	PTH Infusion 12 weeks	CTX	-	-0.212	-0.127	-	-0.228
		HPF	-	0.208	0.014	-	0.026

Correlation coefficients of cerebral capillary permeability (IgG) in both cerebral cortex (CTX) and hippocampal formation (HPF) with vitamin D, blood ionised calcium, serum total calcium, parathyroid hormone and serum phosphate for all vitamin D supplemented experimental groups (rats and mice), parathyroid gland ablated (PTX) and parathyroid hormone-infused (PTH-infusion) groups are represented in this table. (**p<0.01; *p<0.05; n = 4–10; Pearson's analysis).

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parenchymal abundance of IgG was frequently but not consistently associated with GFAP abundance. There was no evidence of an association between parenchymal GFAP with serum levels of iCa, total Ca, serum PTH or with vitamin D treatment per se. Rather, the provision of exogenous PTH to otherwise normal rats was found associated with mild suppression of GFAP activity at 12 weeks in both CTX and HP and conversely, a slight increase in GFAP activity was reported in parathyroidectomized animals.

Discussion

Increasing evidence suggests that disturbances in blood-brain barrier integrity may increase risk for vascular-based neurodegenerative conditions such as vascular dementia and Alzheimer's disease. The main objective of this study was to investigate putative regulatory and integrative effects of exogenous vitamin D_3, calcium and parathyroid hormone on the function of cerebral capillary endothelium and neurovascular inflammation.

Parenchymal abundance of plasma derived IgG was utilized as a surrogate marker of blood-to-brain capillary permeability. Within both the hippocampal formation and cortex of wild-type rats and mice, the findings demonstrate that provision of exogenous dietary vitamin D can significantly compromise the barrier properties of cerebral capillary vessels. Rats appeared to have been more susceptible to the effects of exogenous vitamin D as significant differences compared to control animals were realised within 6 weeks of commencement of the diet, whereas the effect was not realized in mice until 12 weeks of intervention. Furthermore, whilst

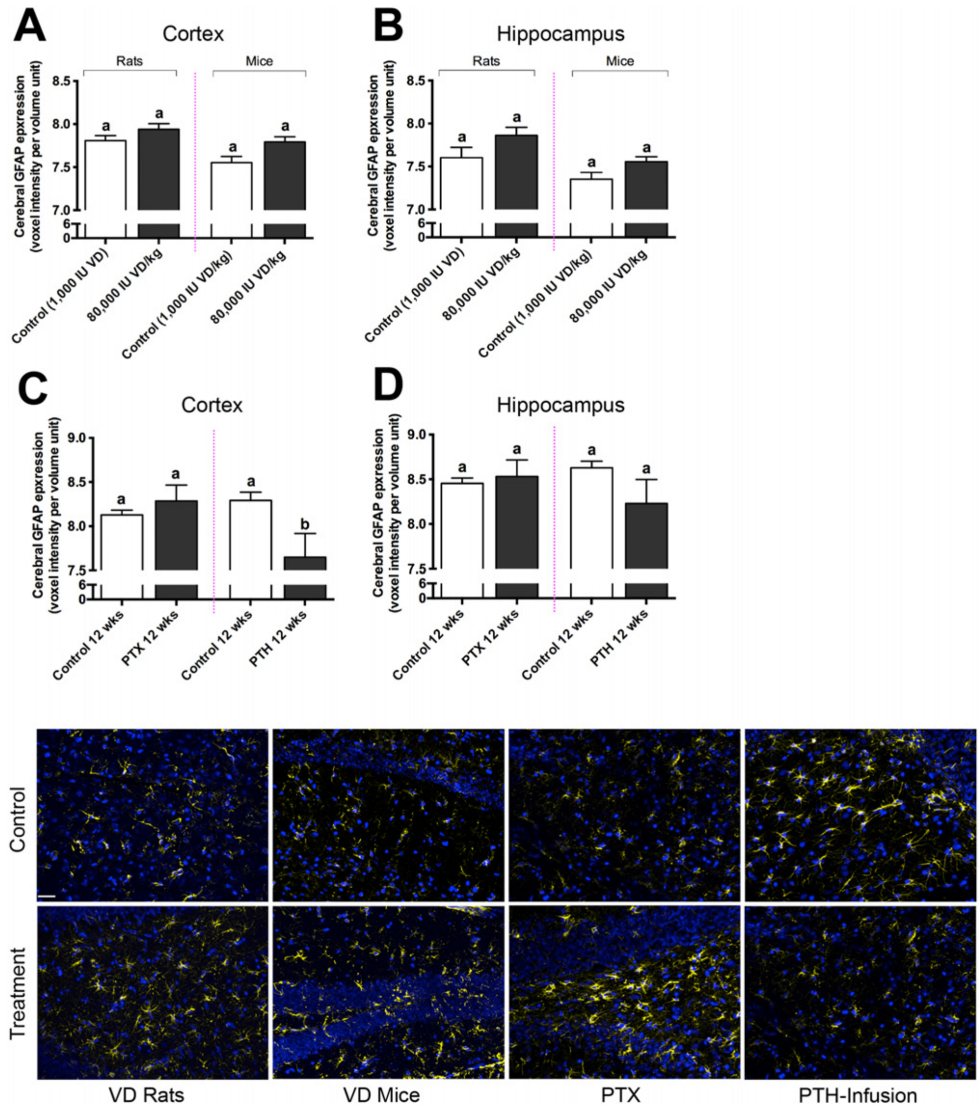

Fig 6. Measure of Neuroinflammation. Neuroinflammation was assessed by measuring the voxel intensity of cerebral glial fibrillary acidic protein (GFAP) expression in cerebral cortex (A) and hippocampal formation (B) in Sprague-Dawley rats and C57BL/6J mice fed 80,000 IU vitamin D (VD) per kilogram of diet and their respective controls, expressed as voxels per volume unit. Cerebral GFAP expression in cortex (C) and hippocampal formation (D) in parathyroid gland ablated (PTX) rats and parathyroid hormone (PTH) infused rats with their controls are also shown. Statistical difference represented by different letters ($p<0.05$; $n = 4–10$; one way ANOVA followed by Tukey's post hoc test). Data is shown as mean ± SEM. Representative 2-dimensional immunofluorescent micrographs of neuroinflammatory GFAP, in extended focus, are shown in the last frame. Each intervention (vitamin D enrichment; PTX; PTH-infusion) and their respective controls are indicated. GFAP is shown in yellow and nuclei in blue. Scale bar indicates 100μm.

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the concentration of vitamin D incorporated into the diets was equivalent for the two species studied, lower rates of feed consumption relative to body weight, resulted in rats receiving a comparatively lower dose of exogenous vitamin D for each respective treatment arm (Table 1). In both species, the effects of exogenous vitamin D supplementation on parenchymal abundance of IgG were fully realized within 12 weeks of feeding. Thereafter, steady-state levels of parenchymal IgG were indicated within 24 weeks of treatment, suggesting that efflux of parenchymal IgG via either CSF exchange or degradation within epithelial cells of the choroid plexus, may be compensating for blood-to-brain extravasation of some plasma proteins and macromolecules.

Regulation of capillary function or permeability by vitamin D or its bioactive metabolites has not been previously reported, however several lines of evidence suggest the possibility of direct effects on endothelial function. The vitamin D receptor is widely distributed including on vascular endothelial cells, smooth muscle cells, microglia and cerebral neurons [47–50]. It has been demonstrated that 1,25(OH)D_3 and its precursor 25(OH)D_3 both directly increase endothelial mediated conversion of vitamin D to the potent metabolite, 1,25(OH)$_2D_3$, by stimulating l-alpha-hydroxylase activity. In rat and human brain endothelial cell cultures, 1,25(OH)$_2D_3$ was reported to increase p-glycoprotein expression and activity [51]. Increased blood-to-brain kinetics of IgG could occur as a consequence of upregulated p-glycoprotein pathway, although this would seem unlikely as it's considered to be relatively specific. Other potential mechanisms of increased blood-to-brain trans-capillary transport that might be influenced by vitamin D metabolites include modulation of tight junction proteins or adherin expression, or non-specific transcytotic mechanisms. Gascon-Barre and Huet (1983) reported that vitamin D delivery to brain parenchyme is principally free circulating 1,25(OH)$_2$D via high affinity binding sites and not associated with plasma levels of 1,25(OH)D per se [52]. If this was the case, then sub-endothelial bioactive metabolites may also influence capillary permeability by modulating pericyte and astrocyte function. However, these alternate regulatory pathways of trans-endothelial transport are yet to be experimentally investigated.

The dose range of exogenous vitamin D_3 provided to rats and mice in this study was within, or considerably greater than what is ordinarily indicated for clinical therapeutic use due to greater tolerance of vitamin D reported in rodents (Table 1) [53, 54]. Studies suggest that because of differences in l-alpha-hydroxylase activity and in the concentration of chaperone binding proteins, comparably greater amounts of exogenous vitamin D are required to significantly increase serum levels of bioactive vitamin D metabolites [54, 55]. Indeed, clinical studies also demonstrate significant heterogeneity between individuals in serum active vitamin D metabolites with dietary supplementation doses ranging between 1,000 IU to 20, 000 IU VD to increase vitamin D and/or calcium status [56, 57].

Many studies suggest that physiological and toxicological effects of vitamin D are mediated as a consequence of altered cellular loading with iCa. Intracellular iCa is ordinarily orders of magnitude less than in serum, but is generally positively associated with the extracellular iCa concentration. In this study, baseline levels of serum iCa in rats and mice was markedly different, with significantly lower levels in mice maintained on control diets. Provision of dietary vitamin D indicated a dose effect on serum iCa and total Ca, particularly in mice. Similar effects were seen in rats, but the effect was only realized at the higher concentrations of exogenous vitamin D provided. Consistent with the iCa and total Ca data, serum phosphate was reduced in mice with increasing provision of vitamin D, but showed little effect in rats. The relatively weak effects of exogenous vitamin D on iCa is consistent with the species differences indicated and is comparable to the variability reported in humans.

Differences in iCa (rats and mice) and total Ca (mice) compared to control treatment groups were realised within six weeks of commencement of vitamin D intervention and

remained relatively constant for up to 24 weeks of feeding. Tai et al reported that acute (15 min changes) in serum calcium did not alter integrity of the BBB indicated by permeability of radiolabelled sucrose, however potential capillary effects with chronically raised serum iCa have not been reported [30]. Clinical studies have however shown a correlation between serum iCa and CSF/serum albumin ratio consistent with direct positive effects on capillary permeability [58]. However, for the latter study caution must be exercised as these subjects had primary hyperparathyroidism. In kidneys, elevated PTH stimulates conversion of calcidiol to the active metabolite ($1,25(OH)_2D_3$). In cell culture studies, direct but paradoxical effects of iCa on endothelial function were demonstrated by Rezic-Muzinic et al who showed the transformation of endothelial cells to a pro-inflammatory phenotype with both sub-optimal and exaggerated exposure to iCa [59]. Parenchymal iCa mediated effects of endothelium, endothelial regulator cells (pericytes/astrocytes) and inflammatory cells (glia) cannot be ruled out. However, in rodent studies maintained on supraphysiological doses of vitamin D, Murphy et al reported that rats had similar levels of CSF and brain extracellular iCa to control rats [31]. The study by Murphy et al suggested that diffusion is the primary modality of trans-endothelial transport of iCa [31]. However, significant regional differences in 45Ca uptake into the CNS have also been reported. The frontal cortex primarily reflects transport across cerebral capillary endothelium, whereas uptake into ventricular CSF reflects transport across the choroid plexuses [30].

In vitamin D supplemented rats and mice, CTX and HPF abundance of IgG was strongly and consistently associated with elevated serum iCa consistent with a causal association. However, this interpretation of the findings was not supported in rats infused with PTH 1–34, where substantial increases in iCa were realized without increased abundance of parenchymal IgG. Similarly, rats with surgical ablation of PTH tissue showed mild increased of IgG expression within CTX and HPF, concomitant with a reduction in iCa. Although it cannot be excluded from these findings, it is unlikely iCa is the primary causative factor in breakdown of capillary endothelium but rather may exacerbate cellular dysfunctioning through promotion of neurotoxic signalling cascades.

A strong negative correlation between capillary permeability and serum levels of PTH was reported in vitamin D supplemented animals and it has been indicated that a causal effect is unlikely to be via regulation of iCa. Whilst it is tempting to suggest the mechanisms may be direct given the substantial ingestion of dietary-derived vitamin D, lower levels of the free active metabolites of vitamin D as a consequence of profound PTH suppression also cannot be excluded.

Direct effects of PTH on capillary function are not reported. However, cell culture studies suggest that epithelial cells possess abundant PTH-receptor 1 and that PTH stimulates vascular endothelial growth factor (VEGF-165), a critical modulator of endothelial cell proliferation [60, 61]. PTH alters the ceramide/sphingosine-1-phosphate rheostat; potentially a key modulator of endothelial function and PTH promotes endothelial nitric-oxide production, a potent vasodilator [61, 62]. Whilst these studies suggest that PTH generally promotes vascular integrity, other reports suggest detrimental physiological effects of exaggerated PTH [34]. However, the latter is indicated only if overloading of cellular iCa is occurring. In this study, infusion of exogenous PTH 1–34 fragment to intact rats maintained on standard chow containing 1,000 IU of vitamin D per kg, increased the serum concentration of iCa to a level comparable to rats maintained on 120, 000 IU vitamin D/kg, but without detrimental effects on cerebral capillary permeability. Therefore, the findings do not support the notion that in this species, hyperparathyroidism directly compromises capillary integrity.

Collectively, capillary permeability appears to be increased in rats and mice maintained on a regimen of vitamin D that is likely to increase active metabolites of vitamin D and suppress

PTH synthesis and secretion. The effects appear to plateau, suggesting chronic homeostatic effects. Parenchymal abundance of IgG is used as a surrogate marker of non-specific blood-to-brain protein kinetics, hence presumably inappropriate delivery of other plasma proteins and possibly macromolecules may be occurring. Many studies support the contention that persistent and exaggerated parenchymal abundance of serum derived neurotoxic substances could induce neurovascular inflammation and promote progression of several neurodegenerative disorders. However, in this study, the abundance of GFAP within CTX or HPF was found not to markedly differ in rodent treatment groups of exogenous vitamin D, PTX or PTH treated animals compared to controls. Irrespective of mechanisms, in the rodent species studied the results do not suggest that exaggerated provision of diets enriched in vitamin D significantly promote neurovascular inflammation. However, equally the study design does not investigate if vitamin D per se will attenuate neurovascular inflammation. Previous studies report increased capillary permeability induced by diets enriched in pro-atherogenic lipids and substantial neurovascular inflammation [40, 63, 64]. Clearly, it is a reasonable proposition that excessive vitamin D could have synergistic effects with other nutrients that compromise cerebral capillary integrity. The latter proposition may intuitively seem unlikely given the substantial body of evidence that links low levels of vitamin D with heightened inflammation. However, provision of vitamin D above physiological levels should not be assumed to be beneficial. Indeed, in a cross-sectional study completed as part of a longitudinal clinical study of late-life depression, vitamin D consumption was found to be positively associated with brain lesions in elderly subjects even after controlling for potentially explanatory variables [65]

Conclusion

The findings from this study reiterate the substantial limitations when considering putative associations of between serum vitamin D, calcium and parathyroid hormone with physiological, pathological or cognitive sequelae. A recent meta-analysis that concluded vitamin D deficiency is associated with a substantially increased risk of all-cause dementia and Alzheimer disease, did not take into consideration an endocrine axis of effects [17]. This study suggests that provision of exogenous vitamin D at levels that suppress PTH secretion and increase iCa concentration compromise the permeability of cerebral capillary vessels but do not promote neurovascular inflammation per se. Potential synergistic effects of vitamin D in heightened inflammatory states should be investigated to further support the putative efficacy of vitamin D supplementation.

Author Contributions

Conceived and designed the experiments: VL RT JM. Performed the experiments: VL RT MPG CG. Analyzed the data: VL CG JM. Contributed reagents/materials/analysis tools: VL RT CG JM. Wrote the paper: VL RT JM.

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Supplementary methodology

The following section provides detailed information on the pilot studies undertaken to determine the dosage range and tolerability of VD supplementation for the dietary intervention to model human VD status in experimental rodents. In addition, further details regarding parathyroid gland surgery and provision of exogenous PTH fragment infusion methodology are provided.

Vitamin D supplementation pilot studies

Pilot studies were undertaken to evaluate the tolerability and effects of high-dose oral vitamin D_3 supplementation administered to young wild-type C57BL/6J mice to mimic a state of hypervitaminosis D that is of clinical relevance.

Strong evidence suggests rodents are extremely tolerant and resistant to high dietary VD intake across a range of concentrations (Fleet et al., 2008; Rowling, Gliniak, Welsh, & Fleet, 2007). The species variation is thought to be achieved by suppression of renal hydroxylase activity which subsequently limit the levels of its bioactive metabolites, regardless of the presence of high circulating 25(OH)D (Feldman, Pike, & Adams, 2011). Indeed, Vieth et al. (1990) reported in humans, free circulating 25(OH)D is 5-fold greater than in other species such as the rat (Vieth, Kessler, & Pritzker, 1990). Despite the low affinity of 25(OH)D for VDR, chronic heightened levels of the circulating pre-hormone may exceed the binding capacity of the VDR, override these mechanisms and cause direct gene transcription effects in target tissues when 25(OH)D enters the cell (Feldman, Pike, & Glorieux, 2005; Jones, 2008; Uchida, Ozono, & Pike, 1994). Thus to achieve comparable high VD status in rodents, the following dietary regimen was undertaken.

Each group of mice were randomly assigned to a semi-purified diet formulation consisting of the following vitamin D_3 concentrations (IU/kg of diet): 1,000 (control diet as per NHMRC animal care guidelines); 20,000; 40,000; 80,000 or 120,000 (Specialty Feeds, Glenn Forrest, W.A, Australia). Calcium and phosphorus content was consistent for all diet formulations at 0.5% and 0.35%, respectively.

Using 3-D immunofluorescent methodology as indicated in Publication 1, the figure below (**Figure 5**) indicates the dose-response observed in terms of capillary permeability and dietary VD concentration. The pilot studies indicated the VD supplemented diets were well tolerated by the animals.

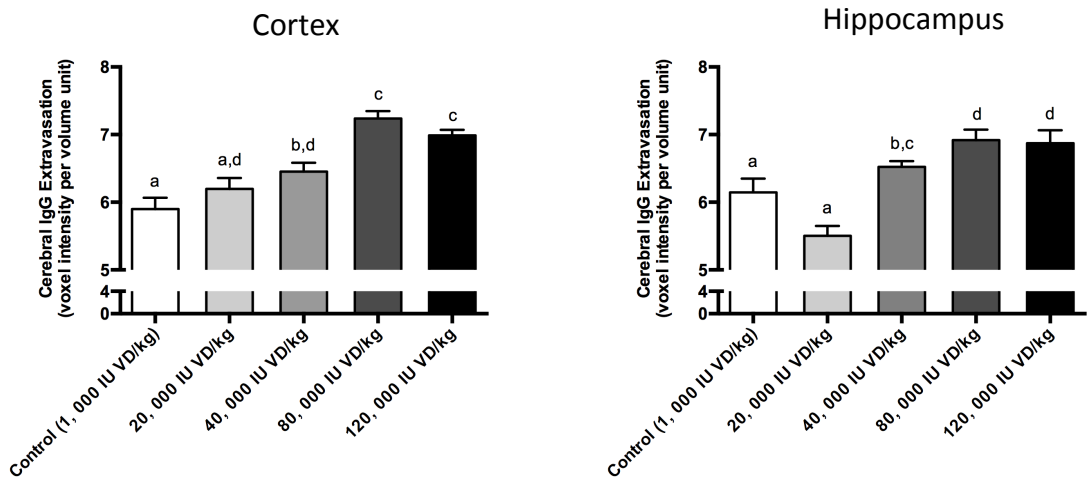

Figure 5. The graphs depict a strong dose-response of dietary vitamin D_3 supplementation and parenchymal extravasation of IgG in the cerebral cortex and hippocampal formation in C57BL/6J mice after a 12-week dietary intervention. Statistical significance is represented by different letters where $p<0.05$ (n = 8; one-way ANOVA followed by Tukey's post hoc analysis).

Parathyroid gland ablation

To study the effects of clinically comparable hypocalcemia, the PTX rat model, a widely used endocrine model to study the biological effects of PTH suppression *in vivo* via removal of PT glands was utilized (Lempert, Scharla, Minne, & Ziegler, 1991). The suppression of concentrations of serum PTH and iCa are routinely used as surrogate markers of the removal of the PT glands (data shown in Publication 1).

Exogenous parathyroid hormone infusion

To mimic a clinically relevant model of chronic primary hyperparathyroidism with typical clinical manifestations such as excessive secretion of PTH and subsequently hypercalemia and suppressed VD status, circulating levels of PTH levels were increased by the controlled continual infusion of supraphysiological doses of exogenous PTH fragment 1-34 (Bachem Labs, Switzerland) at a constant rate for either 6 to 12 weeks delivered by 2ML Alzet mini osmotic pumps (Alzet, Durect Corporation, CA).

The concentration of PTH fragment solution and more importantly the dose of PTH delivered per hour used to modulate circulating PTH levels were based on pioneer studies published by Berdud et al. (1998), summarised in **Table 1** (Berdud et al., 1998). The calculations of pump reservoir concentrations are based on a 200g rat. Briefly, the specified weight of PTH per dosage was dissolved in sterile isosmotic saline (0.9% sodium chloride) with 2% cysteine and adjusted to a pH of 1.4 by addition of hydrochloric acid. The control vehicles were filled to equivalent volumes with isosmotic saline and 2% cysteine. All pumps were pre-conditioned by incubation in the same buffer for 24 hours at 37°C prior implantation to equilibrate the flow rate of each pump. Thereafter, pumps were inserted within the subscapular pocket of each of the animals per experimental group after recovery inhalation anaesthesia.

Dose of PTH (µg/hour/200g rat)	0.33
Concentration of PTH solution (µg/µL)	0.13
PTH (µg) solubilised in solution	265.60

Table 1. Dosage of PTH fragment 1-34 to model primary hyperparathyroidism in Sprague-Dawley rats

Chapter 3

Chapter 3: Serum 25-hydroxyvitamin D and neurocognitive performance

This chapter is comprised of the following articles:

Lam V., Albrecht M. A., Takechi R., Prasopsang P., Lee Y. P., Foster J. K., & Mamo J. C. L. (2016). Serum 25-hydroxyvitamin D is associated with reduced cognitive performance in healthy, middle-aged and older adults. *European Journal of Nutrition, 55*(4), 1503-1513.

Lam V., Dhaliwal S. S., & Mamo J. C. (2013). Adjustment of ionized calcium concentration for serum pH is not a valid marker of calcium homeostasis: implication for identifying individuals at risk of calcium metabolic disorders. *Annals of Clinical Biochemistry, 50*(Pt 3), 224-229.

Publication 2:

Lam V., Albrecht M. A., Takechi R., Prasopsang P., Lee Y. P., Foster J. K., & Mamo J. C. L. (2016). Serum 25-hydroxyvitamin D is associated with reduced cognitive performance in healthy, middle-aged and older adults. *European Journal of Nutrition*, *55*(4), 1503-1513.

Synopsis:

Background

Over the past decade, there has been a considerable amount of interest in the field of VD and neurocognition, with particular interest in older-aged individuals (Annweiler et al., 2013a; Buell et al., 2010). The majority of the literature, experimental and epidemiological, has been focused upon the neuro-beneficial properties of VD in context of correcting low VD status. Vitamin D supplementation has been widely promoted in the community to 'improve' cognition and reduce the risk factors of a number of chronic age-related diseases and overall mortality (Annweiler et al., 2015; Schottker et al., 2013). Conversely, very few studies have reported the effects of higher VD status and cognitive performance. Recent data from two large cross-sectional studies indicated cognitive impairment is more prevalent in individuals with higher levels of 25(OH)D, particularly those taking VD supplements (Granic et al., 2015b; McGrath et al., 2007). Based on these findings, it seems obvious VD supplementation must be addressed appropriately as further increasing 25(OH)D levels beyond sufficient levels is associated with cognitive dysfunction.

The pathophysiology associated with AD-related cognitive impairment is complex and involves a number of mechanisms including disruption of cerebral capillary integrity and function, neuro-inflammation, oxidative stress, and neuronal damage (Baierle et al., 2015; Ewers, Mielke, & Hampel, 2010; Keller, 2006). Indeed, vascular damage that causes dysfunction of the BBB can influence cognitive performance (Pop et al., 2013). In context of the primary findings of Publication 1, where supraphysiological levels of VD were associated with compromised BBB, it

may be plausible to implicate higher serum VD concentration and cognitive impairment.

The main aim of this study was to explore whether there is an association between individuals with 'normal' VD homeostasis and cognitive function. Moreover, the principle regulators of VD homeostasis, iCa and PTH were taken into consideration when exploring the putative effects of VD on cognitive performance.

Methods in brief

181 individuals between the ages of 43 to 84 (116 Females, 65 Males) were recruited for this cross-sectional study. Vitamin D status, determined by serum 25(OH)D, iCa and PTH, was explored in association with cognitive assessments evaluating verbal episodic learning and memory via the Rey Auditory Verbal Learning Test (RAVLT), commonly used as the most sensitive and specific marker of age-related cognitive change. National Adult-Reading Test (NART), Depression, Anxiety and Stress Scales (DASS), age, gender, iCa and PTH were used a covariates. Hierarchical Bayesian modeling was used to analyse the relationship between serum VD homeostasis and cognitive performance.

Results in brief

Individuals with serum VD concentrations in the upper end of normal range were associated with poorer episodic memory performance, in particularly the trial 5 of RAVLT and long delay free recall. Serum iCa had modest positive associations with certain measures of episodic memory, whereas no correlation was evident between PTH and any cognitive variables.

Discussion and conclusion in brief

Firstly, the findings from this study require replication in other study populations of larger sample size to confirm the statistical relationships identified. The overall results suggest higher VD status may exert negative effects on cognitive performance in individuals with normal Vitamin D - Calcium - PTH homeostasis.

Whilst the study findings require further validation, the outcomes are particularly important from a cognitive context, where a large body of evidence associates VD deficiency with age-related cognitive impairment. Despite the uncertainty of the optimal concentration of VD for optimal brain function and the possibility of 'reverse causation', the use of VD supplements is widely promoted to 'optimise' brain/cognitive health. Overall, the administration of VD supplementation in individuals with adequate serum VD levels should be re-considered as the data from this study suggest higher VD status may not be beneficial from a cognitive perspective.

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ORIGINAL CONTRIBUTION

Serum 25-hydroxyvitamin D is associated with reduced verbal episodic memory in healthy, middle-aged and older adults

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Abstract
Background There is increasing evidence supporting an association of higher serum vitamin D concentration with better cognitive performance in older individuals. However, to date, consideration of the putative association between vitamin D and cognition has been based principally on studies investigating clinical participant samples manifesting vitamin D deficiency, particularly in older people. Moreover, relationships between vitamin D and cognition are typically not considered in the context of counter-regulatory calcium-modulating hormones or calcium homeostasis.
Objective Serum vitamin D/bioactive (ionised) calcium/parathyroid hormone homeostasis was considered in the context of cognitive performance in healthy, middle-aged and older individuals.
Design A cross-sectional sample of 179 participants between the ages of 47–84 years was recruited for this study (114 females, 65 males). Participants provided fasting blood samples for analysis of serum 25-hydroxyvitamin D levels, ionised calcium (iCa) and parathyroid hormone (PTH) and completed cognitive measures of verbal episodic learning and memory.
Results Serum 25-hydroxyvitamin D concentrations were negatively associated (with and without covariates of age, gender, depression and NART scores, iCa, and PTH) with measures of verbal episodic learning and memory, in particular with trial 5 of the Rey Auditory Verbal Learning Test (RAVLT) and long-delay free recall on the RAVLT.
Conclusion Overall, the findings from this study suggest an association between higher vitamin D status and poorer performance on verbal episodic memory in middle-aged and older individuals with normal vitamin D–calcium–PTH homeostasis. Despite requiring replication in other participant samples, this is a potentially important finding as it indicates that it may not be beneficial from a cognitive perspective to provide vitamin D supplements in individuals with already adequate vitamin D status.

Keywords Vitamin D · Serum 25-hydroxyvitamin D · Cognition · Ionised calcium · Parathyroid hormone · Verbal episodic memory

Abbreviations

iCa	Ionised calcium
PTH	Parathyroid hormone
25(OH)D	25-Hydroxyvitamin D
RAVLT	Rey Auditory Verbal Learning Test
DASS	Depression, Anxiety and Stress Scales

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NART National Adult Reading Test-Second Edition
CDR Cognitive drug research test battery

Introduction

There is accumulating evidence supporting an association of higher serum vitamin D concentration with better cognitive performance in older individuals [1–4]. Putative mechanisms for the purported effects include enhanced neurotransmission, increased synaptogenesis or neurogenesis and/or inhibition of degenerative processes including apoptosis [5, 6]. However, consideration of the relationship between vitamin D and cognitive status in the extant literature has been based principally on studies investigating individuals manifesting vitamin D deficiency, particularly in older people. For example, just under half of the sample cohort in [2] report vitamin D deficiency (<25 nmol/L; 14.5 %) and/or insufficiency (25–50 nmol/L; 44.3 %). Similarly, Lee and colleagues report that a positive association between vitamin D and cognition is only evident in the deficient/insufficient group (<50 nmol/L) [3]. The majority of cross-sectional and longitudinal studies that have been conducted in this field to date suggest a potentially beneficial association of vitamin D homeostasis and neuropsychological performance. Hypovitaminosis D is highly prevalent in older adults aged 65 years and above [7, 8] and indeed, an international task force considering vitamin D and cognition in older adults recently concluded that hypovitaminosis D increases the risk of cognitive decline and dementia in older adults [9].

Recent reports include a study undertaken by Taylor and Mulligan [10], who showed in a geriatric population that lower levels of vitamin D were associated with poorer cognitive performance. Deficient/insufficient serum 25-hydroxyvitamin D (25(OH)D) status, which was noted in 41.3 % of tested individuals (i.e. vitamin D <50 nmol/L), was also reported to be associated with poorer cognitive function in a population at risk for both functional mobility and cognitive decline [11]. Hooshmand et al. [12] suggested that vitamin D may be positively associated with cognitive status, cerebrospinal fluid amyloid β (1–42) levels and brain tissue volumes after comparing deficient/insufficient (<50 nmol/L) participants with participants manifesting vitamin D levels within the normal range. In a prospective study including 1185 women aged 60–70 years using data derived from the Nurses' Health Study, the quintile with the highest levels of plasma vitamin D (96 nmol/L) was noted to manifest better cognitive function approximately 10 years post-baseline compared to the lowest quintile vitamin D participants (35 nmol/L) [13].

Studies exploring the association of vitamin D homeostasis with cognition in advanced age individuals may, however, be confounded by 'reverse causation'. For example, individuals with poorer/declining cognition may perform fewer daily sunshine exposing activities, thereby reducing their vitamin D synthesis. Indeed, results from clinical trials in older participants administered vitamin D show no positive effect with respect to cognitive outcomes [14]. In a prospective cohort of late-middle-aged subjects, there were no significant associations between lower levels of 25(OH)D and cognitive test scores at baseline, change in cognitive scores over time, or dementia risk [15]. Similarly, Taylor and Mulligan [10] found that vitamin D supplementation did not improve cognition in patients diagnosed with vitamin D deficiency. Graf et al. [16] reported no association between vitamin D homeostasis and increased risk for mild cognitive impairment (MCI), nor did vitamin D homeostasis predict progression from the clinical status of cognitively healthy or MCI to dementia. In a prospective study of 775 aged participants, Granic et al. [17] reported that individuals within both the lowest (approximately <20 nmol/L) and highest (approximately >60 nmol/L) season-specific serum 25(OH)D quartiles had an increased risk of cognitive impairment compared with those in the middle quartiles, when the data were adjusted for socio-demographic, health and lifestyle confounders. Moreover, in this particular cohort an increased risk of poorer global cognition and attention amongst those in the highest 25(OH)D quartile was only observed in users of vitamin D supplements/medication. The latter observations suggest detrimental rather than beneficial effects associated specifically with the provision of exogenous vitamin D [18]. Taken together, serum 25(OH)D levels that are both too high and too low may therefore be harmful with respect to functional capacity. Interestingly, McGrath et al. [19] demonstrated that older individuals with the highest quintile of vitamin D plasma concentrations (>85.4 nmol/L) performed more poorly on learning and memory as measured by the logical memory test than individuals in the two quintiles with concentrations between 55.8 and 85.4 nmol/L. Moreover, the figures reported in [3] hint at a possible negative association between functional status and vitamin D levels in participants at the upper concentration range, with the upper limit at 200 nmol/L.

A key physiological consideration in the present context concerns the relationship between vitamin D, parathyroid hormone (PTH) and calcium. Low serum levels of ionised calcium (iCa) trigger cells within parathyroid tissue to promote secretion of parathyroid hormone (PTH), which rapidly stimulates the conversion of vitamin D to its bioactive form (1,25-dihydroxyvitamin D3). Thereafter, bioactive vitamin D and PTH effectively restore blood iCa by enhancing intestinal absorption of dietary calcium, increasing osteoclast activity to liberate calcium and reducing renal calcium excretion. Provision of exogenous dietary

vitamin D will progressively increase iCa and suppress PTH secretion. Clearly, the relationship between vitamin D homeostasis and neuropsychological performance should not be considered without taking into consideration potential interactions with iCa and the calcium regulatory hormone, PTH.

The ionised form of calcium globally regulates neuronal excitability and neurotransmitter release, and acute changes in iCa have been reported to influence learning and memory [20–22]. In addition, calcium ions are critical in maintaining the integrity of the neurovascular junction [23, 24]. Several studies have implicated an association between elevated serum iCa and global cognitive decline [25–27]. If iCa is central to the purported benefits of vitamin D on cognitive performance, then by extension supplementation with vitamin D would notionally be beneficial in subjects with hypocalcemia, but possibly harmful from a functional perspective in subjects with raised (or adequate) levels of serum iCa.

Similar to the potential confounding effects of iCa when considering the possible association between vitamin D and cognitive performance, clinical studies have shown that increased levels of PTH are an independent risk factor for age-related cognitive decline [28, 29]. Indeed, case-controlled findings in participants with hyperparathyroidism show improved cognitive performance following parathyroid surgery [30, 31]. Therefore, it is possible that studies reporting an association between hypovitaminosis D and cognition may represent a surrogate association that is mediated via hyper-PTH-induced effects.

Cognition is a multifactorial entity that reflects *inter alia* the domains of perceptual speed, primary memory, secondary memory, verbal abilities and executive functioning. With respect to age-related cognitive status, the hippocampus is one of the earliest brain regions to undergo ageing processes and verbal episodic learning and memory has been regularly cited as the most sensitive and specific marker of age-associated cognitive change that may foreshadow the emergence of neurodegenerative decline. Despite a bank of data supporting the correlation between vitamin D status and executive dysfunction/global memory, the association with the verbal learning and memory domain remains uncertain [17, 32, 33]. Furthermore, recent data suggest an association between measures of attention and vitamin D status [34]. In this study, we therefore evaluated verbal episodic learning and memory as well as a measure of attention and working memory, and explored whether there was an association between cognitive performance and serum vitamin D homeostasis whilst taking into account the possible influence of serum iCa and PTH concentration on this association. The hypothesis was that, in generally healthy middle-aged and older subjects, vitamin D would correlate positively with verbal episodic learning and memory as well as attention and working memory.

Methods

Participants

The study was approved by the Curtin University Human Research Ethics Committee (HR97/2011). A total of 250 participants (96 males, 154 females) over the age of 40 (range = 43–84 years) were recruited to the study from December 2011 to February 2012. All participants provided written informed consent and completed a medical history and medications questionnaire and were interviewed to confirm the information provided. Exclusion criteria for the study were: major surgery or another significant clinical event within the previous 6 months; current diagnosis with a psychiatric disorder and/or currently taking psychotropic medications; head injury within the past 5 years; diagnosis at any stage of life with haemophilia, cancer or HIV; renal impairment; liver dysfunction; endocrine disorders (type I or II diabetes, hyper/hypo-thyroidism or hyper/hypo-parathyroidism); Mini-Mental State Examination (MMSE) score <24. Of the 210 participants who satisfied inclusion criteria, 2 participants did not complete the NART (a key index of premorbid capacity) and 23 participants did not complete the RAVLT long-delay free recall (a key index of verbal episodic memory) and were therefore also excluded. This left 179 participants included in the final analysis. We tested for differences between participants who completed the RAVLT long delay and participants who did not on a range of demographic variables as well as the included biomarkers to determine whether there were systematic differences between these two groups. There was no significant difference between completers and non-completers on age, gender, vitamin D concentration, PTH concentration, iCa concentration, NART, MMSE, DASS Anxiety, DASS Depression, DASS Stress, RAVLT trial 1, RAVLT trial 5, RAVLT List B or RAVLT short-delay free recall. All participant data were collected between December 2011 and August 2012, involving two study visits. The first visit took place at the PathWest collection centre of Royal Perth Hospital, Western Australia, for blood sampling, whilst neuropsychological assessments were completed at Curtin University, Western Australia. On average, participants were tested 6.1 weeks between each visit (median = 4.6 weeks, SD = 6.6 weeks, range = 0–38 weeks). Eighty per cent of participants completed both cognitive assessments and blood sampling within 2 months of each other.

Serum vitamin D and parathyroid hormone

Peripheral venous samples were collected into serum separator Vacutainer™ tubes (Becton–Dickinson, Franklin Lakes, NJ, USA) following an overnight fast. After collection, samples were left to clot at room temperature for 30 min and thereafter serum was collected post-low-speed centrifugation. Serum samples were stored at −80 °C until analysis was undertaken. Serum 25-hydroxyvitamin D (25(OH)D), which is a routine biomarker of vitamin D status, was quantified by a widely used and commercially available enzyme-immunoassay kit (Immunodiagnostic Systems, United Kingdom) according to manufacturer's instructions. These kits have a specified detection limit of 5 nmol/L. Parathyroid hormone (PTH) was measured with commercially available ELISA kits from Immutopics Inc (San Clemente, CA). These kits measure human bioactive intact PTH fragment 1–84 with a sensitivity of 13 pg/mL.

Serum ionised calcium

Fasting blood samples were collected and analysed as previously described [35]. All samples were analysed on the ABL800 FLEX series auto-analyser (Radiometer Medical, Copenhagen, Denmark) as specified by the manufacturer and were excluded from analysis if sample pH < 7.2 or >7.6.

Neuropsychological measures

Trained staff under supervision of a registered clinical neuropsychologist administered the Rey Auditory Verbal Learning Test (RAVLT), digit span forwards and backwards, Depression, Anxiety and Stress Scales (DASS) and National Adult Reading Test-Second Edition (NART). The RAVLT was chosen as a widely used, reliable and valid measure of verbal episodic learning and memory, whilst digit span was included as a previous study had shown an association between vitamin D and a measure of attention [17]. Measures from the RAVLT included in the statistical analyses were as follows: items recalled from learning trial 1, items recalled from learning trial 5, total items recalled across the 5 learning trials, learning rate (this was modelled by fixing the intercept for each individual using their recall score after trial 1 and modelling the scores on the remaining trials as a function of the square root of the trial number, as this approach was able to best fit the learning rate across trials), items recalled from the interference list (list B) and items recalled on short-delay free recall of the target list, long-delay free recall of the target list and recognition 'hits' for the target list. In addition, for the target list a short-delay forgetting score [learning trial 5 score—short-delay score] and a long-delay forgetting score [learning trial 5 score—long-delay score] were computed. The DASS and NART were chosen as key covariates that may have a significant impact on cognitive performance; these measures evaluated current symptoms of depression, anxiety and stress and estimated premorbid cognitive ability, respectively.

Statistical analysis

The relationship between serum vitamin D and verbal episodic learning and memory performance was considered using a hierarchical Bayesian mixed-model [36]. Hierarchical models can increase power by pooling outcome estimates across individual cognitive tests within a cognitive domain towards each other, reducing Type S (sign) and Type M (magnitude) errors through shrinkage towards common estimates [37]. The model focused on the relationship between vitamin D and cognitive outcomes. Covariates included were age, gender, NART score, DASS scores (Anxiety, Depression and Stress scales entered separately), iCa and PTH. iCa and PTH were included as additional biological covariates because these biomarkers are critically involved in the regulation of vitamin D homeostasis, as previously discussed. Interaction terms between vitamin D and age, iCa and PTH were also fitted, but there was no evidence found for a credible interaction; therefore, these terms are not reported on further. The model was formed with one parameter estimating the overall effect of vitamin D (as well as for each covariate), two parameters estimating the domain level effect of vitamin D (and each covariate) on digit span and episodic learning and memory, and finally, nested within this domain level estimate were measurement level parameters describing the relationship between each individual outcome measure and vitamin D (as well as for each covariate). The model structure constrains the estimate of each cognitive outcome estimate (e.g. for long-delay free recall) by shrinking the estimate towards the domain level effect and the overarching effect of vitamin D (and each covariate).

All priors used in the Bayesian model could be described as being weakly informative for the scale of the data, i.e. any bias induced by the priors was towards 0 not towards any specific effect of vitamin D. The model included: an overall intercept for the model, an intercept for each participant, an overarching beta for vitamin D and each covariate, a domain level beta for vitamin D and each covariate and an outcome level beta for vitamin D and each covariate. The prior for the overall intercept was described by a normal distribution with mean = 0 and SD = 1. The prior for each participant's intercept was described by a normal distribution centred on the overall intercept and with a SD generated from a uniform distribution bounded by 0 and 20. The overarching prior for the effect of vitamin D and

each covariate was described by normal distributions with a mean of 0 and SD = 5. The parameter estimate from the overarching effect informed each of the domain and outcome level priors, which were described by normal distributions with a mean equal to the higher-order estimate and a SD generated by a half Cauchy distribution with scale = 2. Finally, outcome level errors were modelled as being derived from a *t*-distribution to render the analysis robust to outliers [38]. Where appropriate, covariates were logarithmically transformed to reduce skew; this included vitamin D. (However, demographics are here reported on the original scale.) Each variable was then scaled to a mean of 0 and a standard deviation of 1. If a smaller score on any neuropsychological measure indicated 'better' performance, the score was inverted. After 1250 adaptation steps per chain, a further 1250 samples were saved for each of 4 chains for the final parameter estimates, yielding a total of 5000 samples describing the posterior. The posterior was monitored for convergence by examining plots of the posteriors and by using the Gelman–Rubin diagnostic. All posterior distributions used for inference had a minimal effective sample size of at least 1000. The means ± 95 % highest density intervals (HDI) of the posterior distributions were used to describe the credibility interval for each of the parameter estimates. A sensitivity analysis of the hierarchical model was performed by evaluating two further models: the first increased the scale of all priors by a factor of 2, and the second reduced the scale of all priors by a factor of 2. This did not appreciably alter the effect estimates within the hierarchical model; mean difference in parameter estimates <0.006. All statistical analyses were conducted in R version 3.1.1 using the 'rstan' package (Stan development team 2014).

The fitted curves in Fig. 1 were generated using 1500 bootstrapped samples of the data, with fits derived from (1) local polynomial regression fitting (LOESS) with span equal to 2 and degree of polynomial equal to 2, and (2) normal linear regression.

Results

Participant demographics and vitamin D

Of the final 179 participants included in this analysis, 114 were female and 65 were male. The mean age of participants was 65.7 (SD = 7.3). Descriptive demographic data (Age, NART, Education, Race, Depression, Anxiety and Stress) and group performance for measures from the Rey Auditory Verbal Learning Test are presented in Table 1. The concentration of vitamin D ranged from 31 to 334.4 nmol/L, with a mean = 84.7 (SD = 43.1) and median = 74.2. There were only 10 participants in our sample who demonstrated deficient/insufficient vitamin D status, defined as <50 nmol/L. The concentration of PTH ranged from 6.7 to 783 pg/mL. The concentration of iCa ranged from 1.13 to 1.42 mmol/L. Of the 'biological' covariates, vitamin D was significantly associated with PTH ($\rho = -0.18$, $p = 0.018$), but not with iCa ($\rho = -0.053$, $p = 0.48$). None of the remaining covariates (age, gender, NART or DASS) were significantly associated with vitamin D.

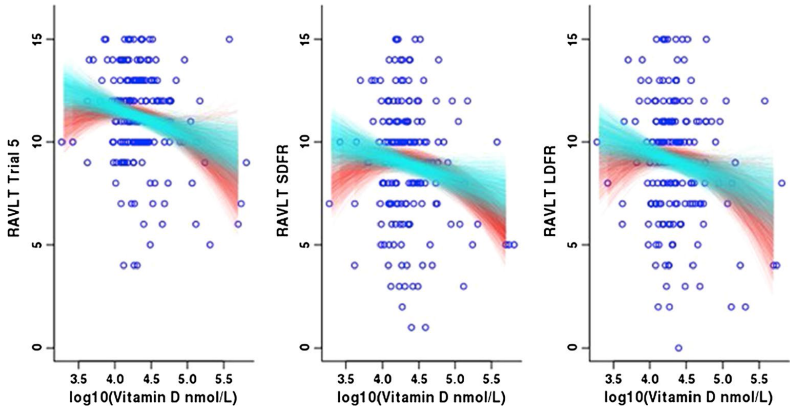

Fig. 1 Scatter plots showing the relationship between vitamin D and three episodic memory items with the largest effect size. *Red lines* illustrate the loess fitted curves generated from 1500 bootstrapped samples. *Blue lines* illustrate the fitted lines from a simple linear model also generated from 1500 bootstrapped samples

Table 1 Participant demographic characteristics, serum biomarkers of vitamin D-calcium-parathyroid hormone status and measures of episodic memory items from RAVLT [mean (SD); range; and % of total sample age band]

	Mean (SD)	Range	% <1.5 SD	Age band (years) 48–60	% of total sample	Age band (years) 60–70	% of total sample	Age band (years) 70–84	% of total sample
Demographics and biomarkers									
Gender	114 F|65 M			31 F|11 M	27.00	57F|30 M	49.00	26 F|24 M	28.00
Age (years)	65.7 (7.3)	48–84		56.0 (3.1)		65.06 (2.8)		74.5 (3.4)	
NART (errors)	13.6 (6.4)	3–39		13 (5.6)		14.1 (6.9)		13.3 (6.2)	
Education (years)	13.4 (2.7)	10–18		14.1 (2.5)		13.2 (2.7)		13.0 (2.7)	
DASS Depression	3.6 (5.0)	0–28		2.5 (3.7)		3.8 (5.0)		4.1 (5.8)	
DASS Anxiety	3.4 (3.3)	0–20		3.5 (3.1)		3.7 (3.7)		2.7 (2.7)	
DASS Stress	8.2 (6.4)	0–31		7.6 (6.9)		8.4 (6.8)		8.1 (5.5)	
iCa (mmol/L)	1.3 (0.05)	1.13–1.42		1.3 (0.04)		1.3 (0.04)		1.3 (0.06)	
PTH (pg/mL)	66.5 (104)	7–783		44.2 (22.3)		67.0 (97.4)		84.4 (146.6)	
Vitamin D (nmol/L)	84.7 (43.1)	31–334		79.5 (38.1)		83.3 (34.7)		91.5 (57.8)	
Race (Caucasian| other| NA)	165|3|11								
RAVLT (max score = 15)									
T1 correct	5.4 (1.6)	1–10	12.00	5.8 (1.4)	5.00	5.4 (1.7)	10.00	4.9 (1.7)	20.00
T5 correct	11.2 (2.4)	4–15	7.00	12.1 (2.1)	5.00	11.3 (2.2)	6.00	10.2 (2.7)	10.00
Sum of trials 1–5	44.3 (9.3)	16–63		48.9 (7.7)		45.0 (8.6)		39.4 (9.7)	
Learning rate [items/sqrt(trial)]	2.9 (1.1)	0–6		3.3 (0.95)		2.9 (1.1)		2.5 (1.2)	
List B correct	5.0 (1.8)	1–10	12.00	5.9 (1.7)	2.00	5.0 (1.9)	20.00	4.4 (1.6)	2.00
Short-delay free recall	9.0 (3.0)	1–15	7.00	10.0 (2.4)	2.00	9.2 (3.1)	8.00	7.7 (2.9)	6.00
Long-delay free recall	9.0 (3.1)	0–15	9.00	10.3 (2.7)	5.00	9.2 (3.0)	7.00	7.7 (3.1)	12.00
Recognition 'hits'	13.5 (2.1)	8–15	14.00	13.7 (1.3)	7.00	13.9 (2.4)	11.00	12.7 (1.9)	22.00
Forgetting T6–T5	−2.3 (2.0)	−11–3		−2.1 (1.8)		−2.2 (2.2)		−2.5 (1.9)	
Forgetting T7–T5	−2.1 (2.1)	−8–3		−1.8 (1.9)		−2.2 (2.1)		−2.5 (2.1)	
Forgetting T7–T6	0.1 (1.6)	−5–4		0.3 (1.6)		0.0 (1.6)		0.0 (1.5)	
Digit span (max score = 16)									
Digits forward	10.4 (2.1)	6–14		10.8 (2.2)		10.4 (2.3)		10.0 (1.6)	
Digits backward	6.9 (2.3)	3–3		7.4 (2.1)		6.6 (2.2)		6.8 (2.4)	

Relationship between vitamin D and episodic memory

Figure 1 presents scatter plots of the relationships between vitamin D and three RAVLT variables, with the largest effect size overlaid with LOESS fits (red) and linear fits (blue). The relationship between vitamin D and cognitive performance for these variables was approximately linear, as can be seen by the large overlap between the red and the blue fits. A formal test for a quadratic relationship was also applied and was not significant.

Figure 2 presents the mean estimate and 95 % HDI of the association between vitamin D and each RAVLT variable both with and without the covariates age, gender, NART, DASS, iCa and PTH. As can be seen from Fig. 2, the inclusion of the covariates did not substantially modify the relationship between vitamin D and episodic memory performance. Of greatest note, the findings revealed a negative relationship between vitamin D and episodic memory performance; more specifically, the 95 % HDI for number of items recalled correctly on trial 5 of the RAVLT (no covariates: standardised parameter estimate = −0.211, 95 % HDI = −0.340, −0.075; with all covariates: standardised parameter estimate = −0.135, 95 % HDI = −0.242, −0.021), short-delay free recall (no covariates: standardised parameter estimate = −0.142, 95 % HDI = −0.266, −0.025; with all covariates except PTH: standardised parameter estimate = −0.104, 95 % HDI = −0.215, −0.004) and long-delay free recall (no covariates: standardised parameter estimate = −0.139, 95 % HDI = −0.260, −0.021; with all covariates except PTH: standardised parameter estimate = −0.096, 95 % HDI = −0.206, −0.001) excluded 0. Cognitive performance as evaluated on the remaining learning and memory variables showed a consistently negative association with vitamin D, with similar effect sizes to those noted with respect to the association between learning trial 5 correct responses and long-delay free recall, as just specified.

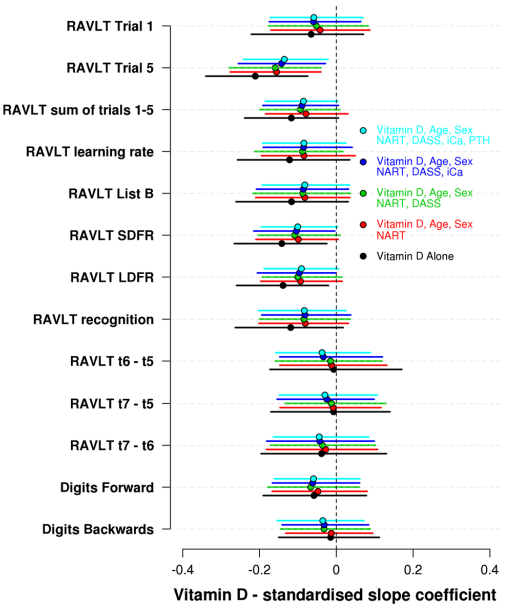

Fig. 2 Standardised effect coefficients (+95 % HDI) from the hierarchical Bayesian model showing the relationship between vitamin D and performance on each measure (and derived measure) from the RAVLT and digit span forward and backwards. The *black lines* and *points* illustrate the association without including covariates in the model, whilst the *coloured lines* and *points* illustrate the effect whilst sequentially adding covariates up to the full model which includes: age, gender, NART, DASS Depression, DASS Anxiety, DASS Stress, ionised calcium and parathyroid hormone

Relationship between vitamin D and digit span

The bottom of Fig. 2 presents the mean estimate and 95 % HDI of the effect of vitamin D on the two digit span measures both with and without the covariates age, gender, NART, DASS, iCa and PTH. As can be seen from Fig. 2, there was no credible influence of vitamin D on digit span.

iCa and PTH association with episodic memory and digit span

We further examined the relationship between the covariates (iCa and PTH) and episodic memory performance within the full model (i.e. vitamin D plus all covariates). It was found that iCa had modest positive associations with some measures of episodic memory (RAVLT trial 5 = 0.120, 95 % HDI = 0.004, 0.235; RAVLT sum of trials 1–5 = 0.119, 95 % HDI = 0.013, 0.227). By contrast, all PTH credible intervals substantially included 0 for all RAVLT measures, indicating no systematic relationship. All credible intervals estimating the association between iCa or PTH and digit span included 0, indicating no credible relationship between these biomarkers and attentional measures.

Discussion

In this study, we explored whether there was an association between serum vitamin D homeostasis verbal episodic memory and attention. Predicated on existing literature, we hypothesised that in generally healthy middle-aged and older subjects, vitamin D would correlate positively with cognitive function. The findings of this study are important as, conversely, they demonstrated that vitamin D was

negatively associated with verbal episodic memory performance, whilst simultaneously taking into account critical vitamin D regulatory factors (serum PTH and iCa) as covariates. Although somewhat surprising in the context of the experimental hypothesis, the current findings are consistent with two large, independent cross-sectional and prospective studies which reported that individuals with higher vitamin D plasma concentrations performed more poorly on measures of global cognition, verbal episodic memory and attention [17, 19].

The associations between serum vitamin D and verbal episodic memory noted in the current study were negative across all RAVLT test items, with the strongest relationships observed on total number of items recalled during the fifth learning trial and during the long-delay free recall. However, the association between vitamin D and cognitive performance on these two variables could not be clearly dissociated from the association with the remaining episodic memory measures, as there was significant overlap of the credible intervals estimating the association between vitamin D and performance on all episodic memory measures. Indeed, it is highly unlikely that the influence on vitamin D is specific to these two variables within the domain of episodic memory, given that the neurological substrates mediating all of the episodic memory variables and the theoretical constructs indexed by these variables are considered to be closely inter-related.

In this study, we did not find an association between vitamin D status and our measures of attention and working memory. Previously, Granic et al. [17] found a negative association between vitamin D and performance on the cognitive drug research test battery (CDR). It may be that our measure of attention was not as sensitive as that used by Granic et al. [17], or it is also likely that they test different aspects of attention. Another consideration is that the CDR battery uses computer administration, whereas we tested attention and memory using face-to-face testing in the current study.

These results need to be considered in the context of the increasing body of literature reporting that low-vitamin D status is associated with impaired cognitive function in older, frail populations [2, 39, 40]. In the current study, it is possible that individuals that we have reported with 'higher' vitamin D status may have previously been vitamin D deficient and that, prior to sampling, vitamin D concentrations in these individuals were optimised through supplements, dietary modification or sun exposure. However, this speculative possibility seems unlikely, as the vitamin D concentrations in our study cohort lay well within the reference range for the population at large. Interestingly, despite 25-hydroxyvitamin D being used as the universal indicator of long-term vitamin D status, it is known that the hormonally active form of vitamin D (1,25-dihydroxycholecalciferol) is the metabolite that mediates transcriptional events through the vitamin D receptor in its target tissues. Indeed, recent reports from the prospective ARIC-Brain MRI cohort failed to identify significant associations between lower vitamin D status and cognitive test scores at baseline or over time [15]. Additionally, findings obtained from several other studies undertaken in a wide span of age groups (from young to older aged adults) do not support the notion of positive effects of vitamin D supplementation on brain health or cognitive function [41–43]. Moreover, studies by Stein et al. [44] show that supraphysiological doses of vitamin D may not be essential to exert a beneficial cognitive effect. Indeed, a U-shaped relationship between vitamin D and global cognition has been suggested in the elderly, whereby moderate vitamin D concentrations are deemed beneficial, but in cases of deficits or excess the putative neuroprotective properties of vitamin D are lost [17, 18, 45].

One consideration alluded to previous concerns of the possibility of 'reverse causation' with respect to previous reports of an association between vitamin D levels and cognition in advanced age individuals. Namely, individuals with poorer/declining cognitive capacity may ingest a modified diet with lower vitamin D intake and/or engage in reduced outdoor activities resulting in lower exposure to sunlight, subsequently adversely affecting vitamin D levels. However, in the current study we in fact observed a negative relationship between cognitive performance and vitamin D levels in late-middle-aged healthy individuals. In interpreting these findings with respect to putative 'reverse causation', one possible line of argument is that higher functioning late-middle-aged healthy individuals may be engaged in more 'white collar' occupational activities which may entail long hours of work predominantly undertaken indoors, thereby limiting (in relative terms) the exposure of these individuals to daily sunshine exposing activities and reducing their vitamin D synthesis. This possibility cannot be completely discounted in the present study. However, it should be noted that the cohort recruited for the current study included people who were still working full-time as well as semi-retired and fully retired individuals, such that this line of argument would appear less plausible to account for the findings obtained across the current cohort considered as a whole.

Our analysis of relevant covariates in the current study showed that ionised calcium concentrations at the upper end of the normal range were correlated with better cognitive performance. Amongst the components of the RAVLT, the strongest associations with ionised calcium were evident for the RAVLT learning trials (i.e. trial 5 and sum of trials 1–5). These findings differ from two previously mentioned prospective studies that reported an association between higher levels of serum calcium and greater

cognitive decline in older adults [25, 26]. These apparent discrepancies may be due to different cut-off reference values being applied for ionised calcium in different studies. Calcium may exert a protective effect on verbal episodic memory capacity through its feedback regulation of vitamin D. Once adequate calcium levels are achieved, it is known that PTH secretion is downregulated, thereby decreasing vitamin D activity. In addition, vitamin D may play a role in neuronal calcium regulation through it actions on L-type voltage-sensitive calcium channels at relatively low concentrations [45].

In the current investigation, PTH levels were not correlated with better cognitive performance. The literature indicates that there is generally an inverse relationship between vitamin D and its counter-regulatory hormone, PTH [46]. It is possible that no associations were evident between elements of verbal episodic memory and PTH status in the current study due to the nature of our study cohort. This cohort comprised healthy middle-aged and older individuals with relatively normal vitamin D–calcium homeostasis. Therefore, the current findings do not necessarily rule out an association between PTH and cognition in other circumstances. Whilst some studies have reported elevated PTH as a major risk factor of dementia [28, 47], contradictory evidence has indicated that PTH may exert beneficial properties such as maintaining vascular integrity and acting as an anti-inflammatory protein [48, 49].

The findings of the present study suggest caution is warranted in attempting to actively elevate vitamin D concentrations above normal ranges, at least in healthy middle-aged and older individuals. The 'optimal' concentration of vitamin D for ideal brain/cognitive health remains unclear [9]. Indeed, whilst a number of animal studies support the neuroprotective effects of vitamin D [50, 51], other studies have reported very subtle or no beneficial effects of vitamin D on cognitive functioning [52, 53]. Moreover, there is increasing evidence that supraphysiological doses of vitamin D are not necessarily beneficial [44]. Consistent with this notion, recent rodent studies from our laboratory reported that vitamin D supplementation at high-end ranges is detrimental to cerebral capillary health; moreover, capillary changes are thought to precede age-related cognitive decline [54]. In addition, population studies have implicated higher vitamin D intake with increased brain lesion volume [55].

The findings of the current study are potentially important. However, some limitations of the current study should be acknowledged. As stated, this was a generally healthy middle-aged and older cohort in which the range in scores on the different measured variables was more limited than would be expected in clinical samples. This restricted range may have limited some of the observed associations that were reported. Nevertheless, significant associations were identified amongst the cognitive and biological variables that were analysed. In addition, only a single measurement of serum samples in each individual was used to assay vitamin D, iCa and PTH levels in the current study. A greater number of samples would have offered greater potential accuracy. However, single samples have also been used in many other previous studies [2, 19, 56]. Finally, the current findings require replication in other populations in order to delineate in more detail the nature of the statistical relationships that have been identified here.

Overall, the findings from this study suggest higher vitamin D status may exert negative effects on verbal episodic memory in individuals with normal vitamin D–calcium–PTH homeostasis. Although requiring replication in other participant samples, this is a potentially important finding as it indicates that it may not be beneficial from a cognitive perspective to provide vitamin D supplements in individuals with already adequate vitamin D status.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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Publication 3:

Lam V., Dhaliwal S. S., & Mamo J. C. (2013). Adjustment of ionized calcium concentration for serum pH is not a valid marker of calcium homeostasis: implications for identifying individuals at risk of calcium metabolic disorders. *Annals of Clinical Biochemistry, 50*(Pt 3), 224-229.

Synopsis:

Background

Ionised calcium and PTH are important regulatory counterparts of VD homeostasis. Aberrant homeostasis of both serum biomarkers have been individually implicated as potential risk factors of age-related cognitive impairment (Braverman et al., 2009; Schram et al., 2007). Based on the potential confounding effects of iCa and PTH on VD homeostasis and cognitive performance, both biomarkers were included as biological covariates for data consideration in Publication 2.

Ionised calcium is regarded as the biologically active fraction of serum calcium and routinely measured via ion-electrode potentiometry (IEP) in diagnostic laboratories. Presently, the analysis of iCa is based on published algorithms used to correct for *in vivo* or *ex vivo* sampling confounders in relation to sample pH. However the validity and clinical relevance of reporting iCa adjusted to a pH of 7.4 in conjunction with natural variability in serum pH remains unclear and may lead to inaccurate result interpretation. Based on the importance of considering serum iCa as a biological covariate of in the investigation of VD in relation to cognitive performance, the aim of this study was to investigate the validity of the algorithm pH-adjusted iCa value.

Methods in brief

A cross-sectional sample of 285 individuals was included in the study analysis. Fasted blood samples were collected, handled and analysed in strict accordance to the International Federation of Clinical Chemistry and Laboratory Medicine Guidelines (Boink et al., 1992). Serum iCa and pH were measured by an IEP-auto analyser hosted at an accredited diagnostic laboratory. The association between unadjusted-iCa values, pH-adjusted iCa values and predicted-iCa values using a regression equation factoring in unadjusted-iCa, and serum pH were analysed.

Results in brief

The unadjusted-iCa concentrations and predicted-iCa values were comparable however adjusted-iCa values were significantly lower than unadjusted-iCa. 15% of subject sample analyses were underestimated with hypercalemia and over 60% of the subjects' calcemic status was overestimated when using the published algorithm.

Discussion and conclusion in brief

The findings of this study demonstrated the discordance between pH adjusted-iCa and unadjusted-iCa values and do not support the utilisation of published equations in iCa analysis and interpretation. Expressing unadjusted-iCa is likely to be a more valid and physiologically relevant biomarker of calcium status when standardised sample collection and protocols are in practice.

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Original Article

Adjustment of ionized calcium concentration for serum pH is not a valid marker of calcium homeostasis: implications for identifying individuals at risk of calcium metabolic disorders

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Abstract

Background: Ionized calcium (iCa) is the biologically active form of this micronutrient. Serum determination of iCa is measured via ion-electrode potentiometry (IEP) and reporting iCa relative to pH 7.4 is normally utilized to avoid the potential confounding effects of *ex vivo* changes to serum pH. Adjustment of iCa for pH has not been adequately justified.

Methods: In this study, utilizing carefully standardized protocols for blood collection, the preparation of serum and controlling time of collection-to-analysis, we determined serum iCa and pH utilizing an IEP-analyser hosted at an accredited diagnostic laboratory.

Results: Regression analysis of unadjusted-iCa (iCa_{raw}) concentration versus pH was described by linear regression and accounted for 37% of serum iCa_{raw} variability. iCa_{raw} was then expressed at pH 7.4 by either adjusting iCa_{raw} based on the linear regression equation describing the association of iCa with serum pH (iCa_{regr}) or using IEP coded published normative equations (iCa_{pub}). iCa_{regr} was comparable to iCa_{raw}, indicating that blood collection and processing methodologies were sound. However, iCa_{pub} yielded values that were significantly lower than iCa_{raw}. iCa_{pub} did not identify 15% subjects who had greater than desirable serum concentration of iCa based on iCa_{raw}. Sixty percent of subjects with low levels of iCa_{raw} were also not detected by iCa_{pub}. Determination of the kappa value measure of agreement for iCa_{raw} versus iCa_{pub} showed relatively poor concordance ($\kappa = 0.42$).

Conclusions: With simple protocols that avoid sampling artefacts, expressing iCa_{raw} is likely to be a more valid and physiologically relevant marker of calcium homeostasis than is iCa_{pub}.

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Introduction

Calcium has multiple biological effects influencing cell function and systems physiology.[1,2] Plasma calcium concentration is tightly regulated and in healthy subjects, is kept within a range of between 2.2 and 2.6 mmol/L. Half of the total plasma calcium is bound to proteins or anions. Ionized or 'diffusable' calcium (iCa) is the biologically active fraction.[1–3] The plasma concentration of iCa in healthy subjects is ordinarily maintained between 1.12 and 1.30 mmol/L. However, disruptions to this homeostatic system lead to disorders of calcium metabolism that may precipitate, or exaggerate progression of several chronic disease conditions.[1,4,5]

Primary hyperparathyroidism (h-PTH) is the third most commonly reported endocrine disorder and is characterized by frank clinical features including markedly elevated serum iCa.[6] However, some h-PTH subjects are reported with normal or mildly elevated serum calcium.[7] Elevated PTH in the absence of hypercalcaemia may be a consequence of secondary mediators such as vitamin D deficiency, insufficient dietary intake of calcium or hypercalciuria.[7] However, when secondary causes of h-PTH have been excluded, serum total calcium may still paradoxically remain within normal limits.[7,8] While it is presently unclear whether there is an obligatory increase in total serum calcium in response to PTH hypersecretion, a greater proportion of serum calcium nonetheless tends to be ionized, emphasizing the importance of regulating the concentration of the biologically active form.

Chronically low levels of serum total calcium are also a risk factor for chronic disorders including osteoporosis, nephrolithiasis, cardiovascular and neurodegenerative conditions.[9] Hypocalcaemia is commonly a consequence of inadequate dietary intake, or insufficient vitamin D, which stimulates PTH secretion to achieve normocalcaemia

through calcium reabsorption via kidney, bone resorption and calcium absorption via the bowel.[3,6]

The physiological relevance of determining iCa was established decades ago.[10] However, until the advent of ion-selective electrode direct potentiometry (IEP), measures of serum iCa were not common because of technical difficulties in adjusting for confounders.[11] The advent of IEP analysers with contemporary measures of endocrine modulators of calcium metabolism and vitamin D homeostasis provide a platform for more detailed distinction of calcium metabolic disorders.[12]

The measure of iCa in serum can be significantly affected by pH due to the competition for protein binding sites between calcium and hydrogen ions.[11,13] Therefore, to avoid potential confounding effects due to pre-analytical collection and transport of blood specimens, iCa is normally referenced relative to a serum pH of 7.4. The iCa value is typically adjusted based on published algorithms which describe the correlation of iCa and serum pH.[14,15]

The validity and clinical relevance of reporting iCa adjusted to pH 7.4 has not been justified. In controlled animal models, Wolfe and Weir[16] showed significant inter-generational strain differences in mean serum pH, suggesting that variability in serum pH is a naturally occurring phenomenon. Several studies including that of Barth *et al.*[17] also reported significant non-linear and regression differences between laboratories when adjusting total serum calcium for albumin concentration according to published normative data. Moreover, others have reported that up to one-third of subjects measured via IEP-based iCa analysis may have venous-serum samples that fall outside the pH reference limits utilized in published algorithms to adjust iCa relative to a serum pH of 7.4.[18]

The ABL800 FLEX auto-analyser produced by Radiometer is a reputable product used by many accredited diagnostic laboratories. When calculating iCa, the ABL800 FLEX auto-analyser utilizes a linear regression correction for pH, claimed to be valid for pH values between 7.2 and 7.6.[14,19] In this study, we determined the putative validity of the calculated corrected value for iCa generated by the ABL800 FLEX auto-analyser (iCa_{pub}). The findings suggest that a linear adjustment of iCa relative to a pH of 7.4 may result in an under-estimation of subjects with hyper- or hypocalcaemia. Uncertainty in the validity of expressing iCa adjusted for pH may contribute to paradoxical observations of calcium homeostasis in subjects with parathyroid hormone disorders and our understanding of the role of iCa in physiological function and disease risk.

Methods

Subjects

A total of 285 otherwise healthy participants aged between 18 and 80 y of age were recruited for the study approved by the Curtin University Human Research Ethics Committee (approval number HR97/2011). Potential participants completed a medical history and medications questionnaire and were interviewed to confirm the information provided. Subjects who had major surgery or a clinical event 'in the last six months'; haemophilia; cancer/chemotherapy or with HIV were not included in the study.

Blood sampling

Participants arrived for provision of fasting blood samples between 8:00 and 10:00 in collection rooms adjacent to the accredited diagnostic centre following an overnight fast (minimum 8 hours). Participants were asked to avoid prolonged strenuous exercise and to limit alcohol intake to two standard drinks 24 h prior blood sampling.

Fasting blood samples were collected and handled under standardized preanalytical conditions to prevent interference of sampling artefacts that may affect pH and iCa values.[20,21] A venous sample was drawn from each subject after being seated for 10 min into serum separator Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA). All specimens were collected under consistent conditions where tourniquet use was avoided where possible, care was taken to avoid muscular contraction in the limb and blood tubes were filled to capacity without the 'ingress' of air into the tube at the end of sampling. In cases where a tourniquet was used, a discard tube was drawn before the sample after release for 30 s prior sampling. Samples were allowed to clot for 30 min and immediately centrifuged at 3800 rpm for eight minutes at room temperature and thereafter analysed on the ABL800 FLEX series auto-analyser (Radiometer Medical, Copenhagen, Denmark) as described by the manufacturer. The average total time of sampling-to-analysis was 60 min. Samples exceeding two hours since collection were excluded.

iCa determination

The analyser algorithm for pH adjustment to 7.4 programmed into the ABL800 FLEX auto-analyser is based on the algorithm provided by Fogh-Andersen *et al.*[15] and commonly used in many IEP devices. The equation is: Corrected iCa^{2+} (pH 7.4) = Measured iCa^{2+} [1 – 0.53 × (7.40 – measured pH)]. Note that this equation is only applicable to samples with pH values within 7.2–7.6, otherwise the conversion of the measured iCa concentration to pH 7.4 is considered invalid. Samples outside the indicated reference range were not included in the analyses presented.

Statistical analysis

Regression analysis was performed using unadjusted-iCa (iCa_{raw}) as the dependent variable and pH as the independent variable. In addition to the linear effect of pH, the deviations from the linear were assessed by adding in the square of pH into the regression model. The linear model was considered to be an adequate fit if the deviations or the quadratic effect was not significant ($P > 0.05$). The predicted values from the regression model were determined (iCa_{regr}). Adjusted R^2 was calculated to represent the percentage of variance in iCa_{raw} that was accounted by the independent variable pH.

Table 1 Mean and standard deviation for measures of ionized calcium (iCa)

	pH	iCa_{raw} (mmol/L)	iCa_{regr}(mmol/L)	iCa_{pub}(mmol/L)
Mean ± SD	7.367	1.278 ± 0.045	1.278 ± 0.036	1.255 ± 0.036*
Minimum	7.080	1.130	1.162	1.136
Median	7.360	1.280	1.275	1.253
Maximum	7.750	1.420	1.437	1.412

Mean and standard deviation, median and range for measures of ionized calcium (iCa_{raw}), adjusted to a serum pH value of 7.4 by published normative algorithms (iCa_{pub}), or utilizing the regression equation which described the association of iCa_{raw} with pH (shown in Figure 1) (iCa_{regr}). Asterix (*) indicates that the mean iCa_{pub} was significantly different from the mean of iCa_{raw} or iCa_{regr} at $P < 0.0001$

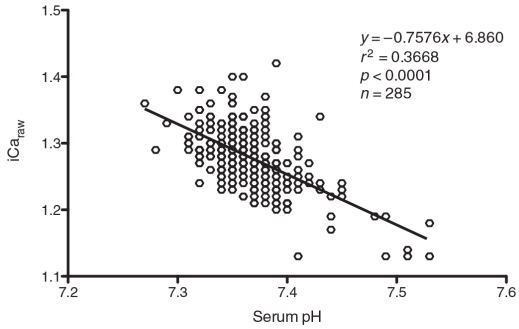


Figure 1 Correlation of unadjusted ionized calcium (iCa_{raw}) and serum pH in 285 otherwise healthy subjects. The regression equation describing the association of iCa_{raw} with serum pH in this cohort is indicated. The mean of iCa_{pub} was significantly different from iCa_{raw} at $P < 0.0001$

Comparisons between the iCa_{raw}, iCa_{regr} and iCa_{pub} were performed using paired *t*-tests, since each subject had all three measurements. Cohen's kappa measure of agreement between the two measures iCa_{raw} and iCa_{pub} was determined. The equation for κ is $\kappa = \frac{Pr(a)-Pr(e)}{1-Pr(e)}$: where Pr(*a*) is the relative observed agreement among measures, and Pr(*e*) is the hypothetical probability of chance agreement. If the measures are in complete agreement, then $\kappa = 1$. If there is no agreement among the measures other than what would be expected by chance (as defined by Pr(*e*)); $\kappa = 0$.

A frequency distribution of iCa_{raw} and iCa_{pub} were each expressed into three categories: below 1.20 mmol/L (Hypo-iCa); within the desirable reference range of 1.20 mmol/L ≤ iCa ≤ 1.29 mmol/L (Normal-iCa); or with serum iCa ≥ 1.30 mmol/L, were compared. The three reference ranges indicated were selected for consistency with other studies in the absence of consensus-based values.[6,22] *P*-values less than 5% were considered as statistically significant. All analyses were conducted using SPSS version 10.

Results

The cohort of 285 subjects investigated had a mean serum pH of 7.367, but despite strict blood collection protocols to avoid sample collection artefacts, the pH range was nonetheless significant (7.08–7.75; Figure 1). The association of unadjusted serum ionized calcium (iCa_{raw}) versus pH of serum measured by the ABL800 FLEX auto-analyser is depicted in Figure 1. The association of iCa_{raw} with pH was significant and best described by linear regression analysis. However, it was notable that serum pH only described 37% of subject iCa_{raw} variability.

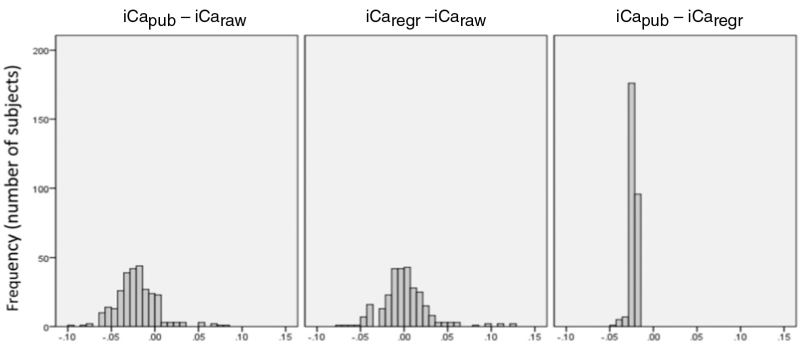
Table 1 lists the mean + SD, the median and range for iCa_{raw}; following adjustment based on the linear regression analysis depicted in Figure 1 (iCa_{regr}); or based on the published normative corrections utilized in the adjustment settings of the ABL800 FLEX auto-analyser (iCa_{pub}). The mean and median of iCa_{raw} versus iCa_{regr} were not significantly different for this cohort. However, in contrast the mean and median of iCa adjusted to pH 7.4 according to published normative equations was significantly lower than the unadjusted value (iCa_{raw}), or iCa_{regr}. Figure 2 depicts the subject frequency distribution of the delta difference between iCa_{pub} versus iCa_{raw}. Paired analysis found that iCa_{pub} was significantly different from iCa_{raw} by approximately −0.22 mmol/L. In contrast, the frequency distribution of the delta difference between iCa_{regr} versus iCa_{raw} shown in Figure 2 second frame, was not significantly different (0.00018 mmol/L).

Consideration of iCa adjusted to pH 7.4 is illustrated in Figure 3 as a scatter plot relative to individuals iCa_{raw} value. The data demonstrated that irrespective of an individual's serum pH, iCa_{pub} consistently underestimates the serum concentration of iCa.

Table 2 shows the distribution of subjects with iCa_{raw} versus iCa_{pub} stratified according to a serum concentration of iCa (<1.20 mmol/L); a serum concentration of iCa (≥1.20 and ≤1.29 mmol/L); or above the upper reference limit of ≥1.30 mmol/L. Of the 285 subjects studied, 70 (24.6%) were identified as having serum iCa ≥1.30 mmol/L based on iCa_{raw}. In contrast, iCa_{pub} identified 26 individuals (9.1% of subjects) with iCa ≥ 1.30 mmol/L. Conversely, six of 10 subjects with iCa below the lower desirable concentration of 1.20 mmol/L based on iCa_{raw} were classified as normocalcaemic when reported according to published normative corrections (iCa_{pub}). Determination of Cohen's measure of agreement between iCa_{raw} and iCa_{pub} showed relatively poor concordance between two measures iCa_{raw} and iCa_{pub} (κ measure of agreement = 0.42).

Discussion

This study determined the best-fit association of uncorrected iCa versus serum pH, generated by a commonly used IEP-analyser, the ABL800 FLEX auto-analyser by Radiometer. The association of iCa versus the independent



Bonferroni's multiple Comparison test	Mean diff. (mmol/L)	Significant?
iCa_{pub} versus iCa_{raw}	0.0226	0.0001
iCa_{regr} versus iCa_{raw}	0.0001	No
iCa_{pub} versus iCa_{regr}	0.0224	0.0001

Figure 2 Subject frequency plot of the delta between the alternate measures of ionized calcium (iCa). The top frame depicts a subject frequency plot of the delta between the alternate measures of iCa. iCa_{raw} adjusted to pH 7.4 based on published normative corrections (iCa_{pub}), or the equation describing the association of iCa_{raw} versus serum pH (shown in Figure 1) (iCa_{regr}) are indicated. The table depicts the mean delta difference and significance versus iCa_{raw}

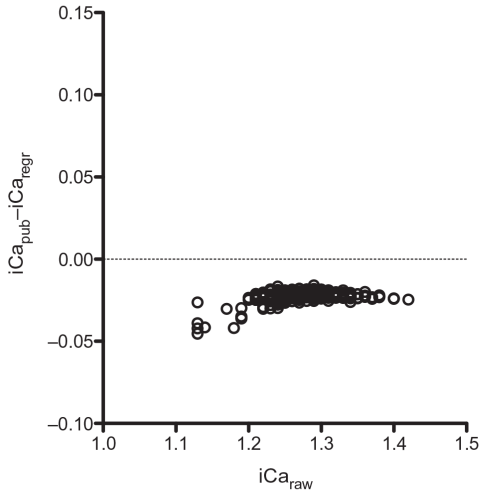


Figure 3 Scatter plot depicting the difference in iCa_{pub} and iCa_{regr} relative to iCa_{raw}. Scatter plot for subjects depicting the difference in ionized calcium adjusted to a serum pH value of 7.4 by published normative algorithms (iCa_{pub}), or utilizing the regression equation which described the association of iCa_{raw} with pH (shown in Figure 1) (iCa_{regr}), relative to the unadjusted value (iCa_{raw})

variable (pH) was significant but only accounted for approximately 37% of the measures recorded in a cohort of 285 subjects. The findings reiterate that serum iCa is significantly regulated by factors other than serum pH.

In this study, the cohort had an unsurprising mean serum pH of 7.367, but despite controlling for potential sample collection confounders, variability around the mean serum pH nonetheless was as high as 5.2% (pH 7.08–7.75). Serum samples with $7.1 > pH > 7.6$ were not included in the analyses; however, if variability in serum pH does indeed represent natural biological variation between individuals, then in absolute terms this would reflect in this cohort potential differences of iCa_{raw} of 0.51 mmol/L (estimated on the linear regression equation generated). The findings suggest that if there is confidence in the integrity of blood sample collection and analysis, then adjustment of measured iCa_{raw} relative to an *ex vivo* pH cannot be justified. Indeed, in a physiological context it is the absolute tissue exposure to iCa_{raw} that needs to be considered.

Stratification of subjects based on iCa_{raw} identified that approximately 25% of subjects (70 of 285 individuals) had a serum iCa above the upper reference limit of 1.29 mmol/L, whereas the adjusted value based on published algorithms (iCa_{pub}) identified just 9.1%. All of the latter individuals were confirmed as being in the hyper-iCa_{raw} category. Although fewer in number than the hyper-iCa_{raw} group, six out of 10 individuals identified by iCa_{raw} as having lower than desirable levels of iCa, were within the normal range of 1.20–1.29 mmol/L according to the iCa_{pub} adjustment. Clearly, utilizing iCa_{raw} rather than iCa_{pub} may alter clinical management of individuals. Moreover, utilizing a robust measure of iCa_{raw} may alter our understanding of how iCa influences physiological systems and disease risk.

Sustained hyper- or hypocalcaemic disorders have significant physiological effects that can contribute to onset and

Table 2 Frequency of subjects identified as having serum iCa_{raw} and iCa_{pub} below (Hypo), within (Normal); or above (Hyper) the normal reference range

	iCa_{pub}			
	Hypo-iCa	Normal-iCa	Hyper-iCa	Total
iCa_{raw}				
Hypo-iCa	4 (1.4%)	6 (2.1%)	0 (0%)	10 (3.5%)
Normal-iCa	9 (3.2%)	196 (68.8%)	0 (0%)	205 (71.9%)
Hyper-iCa	0 (0%)	**44 (15.4%)**	26 (9.1%)	70 (24.6%)
Total	13 (4.6%)	246 (86.3%)	26 (9.1%)	285 (100%)

Table 2 depicts the frequency of subjects identified as having serum iCa_{raw} or iCa_{pub} below 1.20 mmol/L (Hypo-iCa); within the reference range of 1.20 mmol/L $\leq$ iCa $\leq$ 1.29 mmol/L (Normal-iCa); or with serum iCa $\geq$ 1.30 mmol/L. Shaded frequencies show where there is agreement between the two measures. The highlighted frequency shows significant divergence between iCa_{raw} and iCa_{pub} and identifies subjects in this cohort with notionally elevated levels of iCa

progression for a range of chronic disorders.[6,9] Confidence in the validity of iCa measures are therefore pivotal to optimize clinical management. Our data suggest that adoption of published generalized linear models to adjust the raw measured value of iCa is not appropriate. Rather, standardizing blood sampling and management protocols will provide confidence that IEP measures of iCa_{raw} are biologically relevant. If an adjusted value of iCa relative to serum pH 7.4 is to be considered, then the data suggest that adjustment should be at the very least reiterated per IEP-unit to ensure comparability of research findings. In this study, the linear regression equation that described iCa_{raw} versus pH (iCa_{regr}) was significantly different from the commonly utilized algorithm (iCa_{pub}). Nowadays, it would be relatively simple to analyse the association of iCa_{raw} with sample pH and provide an iCa_{regr} that is specific for the model of IEP used and site of testing.

The findings of this study do not support the utilization of iCa adjusted to a mean serum pH of 7.4. Moreover, utilization of published normative equations describing the correlation of serum iCa with serum pH is a historic practice that may lead to significant confounders when considering research reports. Thode *et al.*[14] suggested otherwise and found consistent agreement between iCa adjusted based on published algorithms and actual values with relatively careful sampling techniques. While a comparison of iCa determined based on regression analysis versus sample pH was not reported by Thode *et al.*, the difference in findings emphasizes the uncertainty of normalization between alternate IEP devices, utilizing the same algorithm. Limitations of this study include only a single measure per subject. However, the coefficient of variation for single measures was found to be <2% per sample (data not shown). The Australian population cohort was multi-ethnic and this may have contributed to the variability in serum pH reported.

DECLARATIONS

Competing interests: None to declare.
Funding: This research was funded by the National Health and Medical Research Council of Australia.
Ethical approval: The ethics committee of Curtin University approved this study (REC number: HR97/2011).
Guarantor: JM.
Contributorship: VL and JM researched literature and conceived the study. VL and JM were involved in protocol development, gaining ethical approval and patient recruitment. VL and JM wrote the first draft of the manuscript. VL, JM and SD were responsible for data and statistical analysis. All authors reviewed and edited the manuscript and approved the final version of the manuscript.
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Supplementary methodology

Additional information for Publications 2 to 5 is provided on the processes undertaken for the recruitment of volunteers, sample collection and finally, comprehensive details of each neuropsychological test component.

Participant Recruitment

Participants were recruited through a newspaper article advertised in the West Australian in December 2011. 312 persons registered interest and were subsequently screened via phone or email and excluded based on the following criteria: self-reported or documented malignancies, active or previous cancers, active chemotherapy, severe renal or gastrointestinal disease, liver dysfunction, diagnosed psychiatric disorder or currently taking psychotropic drugs, diagnosed memory disorder, haemophilia, head injury within the last 5 years, individuals with HIV and those who were unable to complete the neuropsychological test battery.

A participant information form and consent sheet was given to all participants describing in detail the aim of the study, tasks expected of the volunteer and potential risks associated (see forms in **Appendix II**). Once consent was obtained, a comprehensive questionnaire was circulated among all participants to collect information on age, gender, education history, current or previous medical conditions, current medications/supplements, and history of head trauma. Two study visits were required; blood sampling and neurocognitive assessments. All participant data was collected between December 2011 and August 2012.

Sample Collection

A fasted blood sample was collected from each participant (approximately 5ml) by a trained phlebotomist for analysis of a number of serum biomarkers including 25-hydroxyvitamin D levels, total and ionised calcium, PTH, liver function tests, kidney function tests, S100B, albumin, leucocyte count and C-reactive protein (specific assay details are provided in each individual publication). Blood samples were thereafter centrifuged at 3000g for 10 minutes and kept frozen at -80°C until further analysis.

Neuropsychological cognitive assessments

The comprehensive panel of neuropsychological cognitive assessments administered to all participants was chosen on the basis on their widespread use within the field and ability to cover the principle domains of cognitive performance typically affected in age-related cognitive decline. All cognitive assessments were conducted by staffed trained by a registered clinical neuropsychologist. The following section describes in detail the test procedure for each of the measures and which cognitive domain is assessed.

Rey Auditory and Verbal Learning Test

The Rey Auditory and Verbal Learning Test (RAVLT) is essentially a neuropsychological assessment designed to evaluate verbal episodic learning and memory of an individual, in which the nature and severity of memory dysfunction can be tracked over time (Bean, 2011; Lezak, Howieson, & Loring, 2004).

The RAVLT test is presented orally over five consecutive learning trials. The participant is read a list of 15 unrelated nouns (list A) and immediately asked to recall as many words as possible over five trials (T1 to T5) and referred to as short delay free recall (SDFR). This assesses short-term memory and incremental learning, and an increase in the number of words recalled per consecutive trial is expected in cognitively sound individuals. Following the five trials, an interference list (list B), which is a new list of 15 unrelated words, is read out to the participants presented in the same manner where they are again asked to recall as many words as possible. Trial 6 (T6) is then administered immediately after recall of interference list where the participants are asked to recall words from list A. This essentially examines 'working memory' and how well the participant has consolidated List A to memory. Trial 7 (T7) is conducted 20 minutes after trial 6 and involves recall of words from the original list A without immediate prior exposure to the list as per previous trials, in aim to assess long-term memory (long delayed recall (LDFR)). Following trial 7, participants were required to complete a recognition trial involving the recognition of words from a list of 30 items, comprised of 15 words from the original list A and 15 filler items. Each of the 30 words was read out

in random order and the participant was asked to identify whether the word was from the original list. Recognition memory (TR) is assessed as opposed to long delay free recall.

The multiple recall trials for list A can be represented as a learning curve as seen in Publication 2, demonstrating relative memory capacity (Bean, 2011). Interference and retention is also assessed by the recall of words from list B and the immediate-recall of list A (Schmidt, 1996).

All scores were standardised and converted to a z-score based on standard normative data (Schmidt, 1996). The main variables of RAVLT include: T5, T6, T7 and TR, which reflects short term memory, short-delay working memory, long-delay memory, and recognition memory, respectively. Trial 5 was used as opposed to T1 – T4 due to saturation of results at T5. The differences between T6 and T5 (T6 - T5) and T7 and T5 (T7 - T5) were also determined to determine a short delay forgetting score and long delay forgetting score, respectively. Z-scores 1.5 standard deviations below the mean in any of these scores were regarded as cognitively impaired in their respective domains.

Mini-Mental State Exam

Mini-Mental State Exam (MMSE) is a cognitive measure used to screen mental functioning in older adults by administering a 30-item examination over a 5 to 10 minute period, typically used to screen for dementia and schizophrenia (Folstein, Folstein, & McHugh, 1975; Schatz, 2011). A total of 30 questions consisting of 10 questions related to time/place orientation, 3 questions for registration – object memory, 5 questions related to attention and calculation, 3 questions for short-term recall and 9 questions related to language and such as naming of objects, repetition of words, comprehension, reading and writing. Thus, the MMSE covers a variety of cognitive domains and generally used a screening tool rather than specific domain measure (Molley & Standish, 1997). A test score of less than 23 correctly answered questions is clinically interpreted as mental impairment, although the sensitivity of MMSE scoring remains controversial due to the variability of age and education (Crum, Anthony, Bassett, & Folstein, 1993).

Depression, Anxiety and Stress Scale

The Depression, Anxiety and Stress Scale (DASS) is a 21-item self-report instrument designed to measure current negative emotional states of depression, anxiety (symptoms of psychological arousal) and stress (enhanced cognitive and subjective symptoms of anxiety). These scales are merely a quantitative measure of distress in relation to depression, anxiety and stress, and not independently used for clinical diagnosis. High scores reported on the DASS is indicative of a high level of distress in the individual and were taken into consideration.

National Adult Reading Test

The National Adult Reading Test (NART) is an untimed test that assesses the ability of an individual to correctly read and pronounce 30 unrelated words and utilised as a means of assessing general intelligence to adjust for potential confounders from the other cognitive test components. The scores from the test evaluate premorbid levels of intellectual functioning (Nelson & Willison, 1991).

Digit Span Test

The Digit Span tests are a set of verbal subtests derived from the Wechsler Adult Intelligence Scale version 3 (WAIS-III) used to primarily assess an individual's working memory, mental capacity, attention and their ability to retain a circumscribed amount of information for a short period of time (Wechsler & Scale, 1997).

A random sequence of numbers was read to the participant for the digit span forward assessment. Thereafter, the participant was asked to recall the numbers in the exact order they were presented, assessing short-term memory (forward digit span). Following this, the participant is asked to attempt to recall a set numbers in reverse order, which accounts for both short-term and working memory (backward digit span). The raw score of the test is the sum of the trials completed correctly and thereafter converted to an age-corrected scale score where particular cut-off scores are used for screening various types of cognitive impairments (Wambach et al., 2011).

Digit Symbol Coding

The Digit Symbol Coding test is a timed performance subtest derived from the WAIS-III that evaluates an individual's perpetual speed and memory by completing a coding task, which involves the transcription of a geometric symbol with its corresponding numerical digit (Bettcher, Libon, Kaplan, Swenson, & Penney, 2011; Joy, Kaplan, & Fein, 2004). The score attained is the number of correctly transcribed items in the prescribed time limit and is sensitive to age-related changes, dementia and brain damage (Glosser, Butters, & Kaplan, 1977; Joy, Fein, Kaplan, & Freedman, 2001; Kluger et al., 1997).

Stroop Test

The Stroop test is used for the assessment of selective attention and executive function of individuals (Sbordone, Saul, & Purisch, 2007). The Victorian version of the Stroop test used is a shortened version of the original Stroop task. Stroop Colour and Word tests is comprised of a series of 3 test papers that consists of the following (1) Dots - the participants were asked to simply identify the colour of each dot on the page (red, green, yellow or blue) for each row from left to right; (2) Words – participants were required to name the ink colours in which colour words are printed and to disregard their verbal content; (3) Colours – participants were subjected to an interference effect where non-corresponding coloured stimuli and colour names were presented, requiring the participant to name the colours in the task as requested (Strauss, Sherman, & Spreen, 2006). A cognitively impaired individual would respond slower and demonstrate difficulties in the completion of each of the tasks (Koss, Ober, Delis, & Friedland, 1984; Ponsford & Kinsella, 1992).

Delis-Kaplan Executive Function System and Boston Naming Test

The Delis-Kaplan Execute Function System (D-KEFS) and the 60-item Boston Naming Test (BNT) were used to assess verbal ability of the study cohort. Deficits in verbal fluency ability is found associated in patients with mild-cognitive impairment (MCI) and AD when compared to age-matched controls (Nutter-Upham et al., 2008; Randolph, Braun, Goldberg, & Chase, 1993).

D-KEFS is a battery of psychometric tests used to assess a wide array of higher-level executive functions such as 'cognitive flexibility, ability to problem-solve, conceptual reasoning, inhibition, multi-tasking and non-verbal and verbal creativity' (Fine & Delis, 2011). D-KEFS verbal fluency, category fluency and switching tests were used in our modified test battery to assess primarily for milder forms of dementia-related cognitive deficits.

The D-KEFS subtest evaluates the fluent productivity of the verbal domain. The participant is requested to say words beginning with a letter specified by the examiner, present words that belong to a specific semantic category and alternate between saying words from two different semantic categories, which ultimately assesses letter fluency, category fluency and categorical switching, respectively. Raw scores derived from each of the subtests are converted to age-scaled scores.

The 60-item BNT is a confrontation naming-assessment where the participant is shown 60 line-drawn pictures and asked to name each item (Mitrushina, Boone, Razani, & D'Elia, 2005). If the participant misperceives the picture, an appropriate semantic cue is given. If more than 20 seconds was required to name an item, a standard phonemic cue was given. The number of correct responses scores the test.

Chapter 4

Chapter 4: Serum S100B and neurocognitive performance

The following article covers the content of this chapter:

Lam V., Albrecht M. A., Takechi R., Giles C., James A. P., Foster J. K., & Mamo J. C. (2013). The serum concentration of the calcium-binding S100B is positively associated with cognitive performance in older adults. *Frontiers in Aging Neuroscience,* 5, 61.

Publication 4:

Lam V., Albrecht M. A., Takechi R., Giles C., James A. P., Foster J. K., & Mamo J. C. (2013). The serum concentration of the calcium-binding S100B is positively associated with cognitive performance in older adults. *Frontiers in Aging Neuroscience,* 5, 61.

Synopsis:

Background

S100B is a low-molecular weight (21kDa) calcium-binding, neurotrophic protein produced predominantly by astroglial cells. Typically regarded as a CNS-specific protein, S100B is typically present in low concentrations in the systemic circulation when compared to CSF-S100B levels (Grocott & Arrowsmith, 2001; Reiber, 2001). Experimental and clinical data show serum S100B levels are markedly increased after head trauma/brain injury, commensurate with increased permeability of cerebral capillary endothelium (Kapural et al., 2002; Marchi et al., 2003). Individuals with mild traumatic injury are often cognitively impaired post-insult where changes in memory recall, attention, executive functioning and behavioural changes are reported (Smith, Johnson, & Stewart, 2013; Williams, 2013). Therefore, the aim of this study was to investigate the association between serum S100B, a putative marker of cerebral capillary dysfunction, and neurocognitive function.

Methods in brief

A cohort of 219 participants were included in this study where serum 100B was measured and considered against a comprehensive neurocognitive test battery including: MMSE, RAVLT, Delis-Kaplan Executive Function System (D-KEFS) verbal fluency subsets, Boston Naming Test (BNT), NART, Digit Span and Digit-Symbol Coding subtests from the Wechsler Adult Intelligence Scale (WAIS-III), and the Stroop test. A nested domain Bayesian mixed-model was used to analyse the

relationship between S100B and cognitive performance, where age, gender and NART were used as covariates.

Results in brief

No participant had serum S100B concentrations indicative of head trauma or injury. Serum S100B was found to positively correlate across all cognitive domains, in particular perpetual speed, Stroop test and verbal fluency.

Discussion and conclusion in brief

Overall, the results from this study demonstrate that serum S100B is positively associated with better global cognitive performance in healthy older adults. As the study cohort was functioning within 'healthy' range and no CNS dysfunction was reported, the findings are supportive of S100B's neurotrophic effects and pro-oxidant properties.

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The serum concentration of the calcium binding protein S100B is positively associated with cognitive performance in older adults

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S100B is a calcium binding peptide produced predominantly by astroglial cells in the central nervous system. S100B paradoxically has neurotrophic and apoptotic effects, dependent on extracellular concentration. This study investigated the relationship between serum S100B levels and neuropsychological performance across a range of cognitive domains in healthy older aged adults. A cohort of 219 participants between the ages of 43 and 84 years (141 female) were recruited. Subjects provided a fasting blood sample for S100B measurement (Mean = 0.24 ng/mL, SD = 0.14) and completed a battery of neuropsychological tests. S100B concentrations (both with and without the covariates of age and sex) were positively associated with the following measures of cognitive performance: digit-symbol coding, Stroop test, and measures of verbal ability. The results from this study show that serum S100B is positively associated with better cognitive performance in healthy older adults.

Keywords: S100B, neuropsychological measures, Alzheimer's disease, Bayesian analysis, neuro-inflammation

INTRODUCTION

In 1965, Moore described a mixture of low molecular weight proteins belonging to the calcium-sensor-binding-proteins superfamily (Moore, 1965). Indicative of their solubility in saturated ammonium sulfate solution, S100A is a heterodimer synthesized in muscles and neurons, whereas S100B is homodimer produced by neural astroglial and Schwann cells (Heizmann, 2002). The biological effects of S100 proteins are functional following binding of ionized calcium, which induces a conformational change and exposure of functional hydrophobic residues (Smith et al., 1996).

Key functional intracellular effects of S100B are purported to be in regulation of cell proliferation and cytoskeletal structure (Sorci et al., 1998). Extracellular effects at nanomolar concentrations of S100B have potent neurotrophic and gliotrophic actions, principally by ameliorating the effects of altered redox states that occur as a consequence of mitochondrial dysfunction (Selinfreund et al., 1991; Donato, 2001). In addition to its significance in central nervous system (CNS) development, S100B may be restorative following brain injury (Ellis et al., 2007). However, paradoxical biological effects of S100B are reported at micromolar levels in extracellular fluids such as plasma or cerebrospinal fluid (CSF). *In vitro* studies have identified that at these higher concentrations extracellular S100B binds to the receptor for advanced glycation end products, stimulating the caspase pathway (a pivotal mediator for programed cell death, necrosis, and inflammation) (Huttunen et al., 1999). At these concentrations, cytokine production is enhanced resulting in increased production of potentially cytotoxic amounts of reactive oxygen species and nitric oxide (Bianchi et al., 2007). It is these properties of S100B that have led to the hypothesis that elevated S100B contributes to neurovascular inflammatory disorders.

The demonstrated paracrine and autocrine effects of S100B on neurons and glia and the neural-to-plasma kinetic gradient of S100B has raised the possibility that S100B could be a useful blood biomarker for disorders of the CNS. The relatively short half-life of plasma S100B (~30 min) and renal clearance (2 h) would support the contention that a chronic change in serum S100B concentration may reflect homeostatic alterations of neural integrity (Jonsson et al., 2000). Yardan et al. (2011) provide an elegant review of S100B in the context of its putative function in individuals who have experienced head trauma, neurovascular degenerative conditions, or psychological disorders.

Alzheimer's disease (AD), the most common neurodegenerative disorder, is clinically characterized by a progressive loss of cognitive functioning that typically starts with a decline in episodic memory (Backman et al., 2004). In AD and in subjects with frontotemporal lobe dementia, S100B levels were reported to be significantly increased compared to healthy controls (Green et al., 1997; Peskind et al., 2001). However, Yardan et al. (2011) suggested that S100B's abundance and distribution and its putative role in the onset and progression of AD could notionally change over the time course of the disease. Chronically suppressed levels of extracellular S100B may be detrimental to neuronal function and be implicated in AD onset. However, during periods of heightened inflammation

or more active plaque formation, greater S100B concentration in serum might be expected. The latter could be considered an injury response that provides benefit (at lower concentration) or exacerbates inflammatory sequelae (at higher concentrations). Thereafter, at the end-stages of AD, normal or perhaps decreased synthesis and secretion of S100B may occur. This was reported by Peskind et al. (2001) who found that S100B concentrations were increased in mild-to-moderate AD subjects but not in the advanced stage of the disease. Consistent with a transient functional role of S100B in neurodegenerative disease, Schaf et al. (2005) reported a correlation of S100B with the Hoehn and Yahr stage of Parkinson's disease, but no difference *per se* when S100B was measured in Parkinson's disease subjects versus controls. Furthermore, Nooijen et al. (1997) found no significant difference in concentration of S100B in various types of dementia apart from in subjects with Creutzfeldt–Jakob disease. However, an association with disease onset or progression was not considered in this study.

Cognition (as per complex biological phenomena in general) is a multifactorial entity that reflects the composite domains of perceptual speed, primary memory, secondary memory, verbal ability, linguistic abilities, and executive functioning. In subjects with mild-cognitive impairment, secondary memory (episodic memory) deficits and (to a lesser extent) perceptual speed and executive functioning deficits appear to be most indicative of subjects who will progress to AD (Weintraub et al., 2012). The fractionation of different elements of cognition assessed against biomarkers could provide a powerful approach to modeling the putative relationship(s) between cognitive outcomes and regulatory biological factors in the aging process (specifically concerning biological risk factors for pro-dromal AD). In this study, we therefore used a battery of tests that specifically evaluated each of the aforementioned cognitive domains, and we explored if there was a statistical association between cognitive test performance and serum S100B concentration. The hypothesis was that, in generally healthy subjects, the neurotrophic properties of S100B would generally correlate positively with cognitive capacity.

MATERIALS AND METHODS

PARTICIPANTS

The study was approved by the Curtin University Human Research Ethics Committee (HR97/2011). A total of 250 participants (96 males, 154 females) over the age of 40 (range = 43–84 years) were recruited. All participants provided written consent and completed a medical history and medications questionnaire and were interviewed to confirm the information provided. Exclusion criteria for the study were: major surgery or a clinical event within 6 months; current diagnosis with a psychiatric disorder or taking psychotropic medications; hemophilia; cancer/chemotherapy; head injury within 5 years; diagnosis with HIV. Furthermore, participants were excluded from the statistical analyses if any of the following obtained: renal impairment; liver dysfunction; Mini Mental State Examination (MMSE) score <24.

SERUM S100B

Peripheral venous samples were collected into serum separator Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA) following an overnight fast for at least 8 h. Samples were allowed to clot for 30 min and serum was isolated and stored at −80°C following low-speed centrifugation. Serum S100B was measured using a commercially available ELISA kit (Cosmo Bio, Japan) with an interassay coefficient of variance of 4.82–9.20%. The sensitivity and dynamic range of the S100B assay is 0.078–5 ng/mL (7–470 pmol/L).

NEUROPSYCHOLOGICAL MEASURES

The cognitive tests were administered by trained staff under supervision of a registered clinical neuropsychologist. Tests were chosen based upon their widespread use, reliability, and validity, and to cover the principal domains of cognitive performance affected in age-related cognitive decline. The performance tasks included the MMSE (Folstein et al., 1975), Rey Auditory Verbal Learning Test (RAVLT) (Lezak et al., 2004), Delis–Kaplan Executive Function System (D–KEFS) verbal fluency subtests (Delis et al., 2001), 60-item Boston Naming Test (BNT) (Saxton et al., 2000), National Adult Reading Test (NART) (Nelson and Willison, 1991), Digit Span and Digit-Symbol Coding subtests from the Wechsler Adult Intelligence Scale-Third edition (WAIS-III) (Wechsler and Scale, 1997; Strauss et al., 2006), and the Stroop test (Victoria version) (Strauss et al., 2006).

STATISTICAL ANALYSIS

The relationship between serum S100B and cognitive performance was considered using a nested domain Bayesian mixed-model (Thurston et al., 2009). The nested domain model increases power by pooling outcome estimates from within a cognitive domain toward each other, reducing Type S (sign) and Type M (magnitude) errors through shrinkage toward common estimates (Gelman et al., 2012).

The principal domains assessed were D1 – verbal ability [BNT and D–KEFS fluency (letter fluency, category fluency, and category switching)]; D2 – Stroop [Dots, Words, and Colors response time, and interference (Colors/Dots) ratio]; D3 – secondary memory [total items recalled across learning trial, items recalled from interference list, short delay free recall, long delay free recall, recognition "hits," short delay forgetting score (learning trial 5 – short delay), and long delay forgetting score (learning trial 5 – long delay)]; D4 – primary memory (digits forward and digits backwards); and D5 – perceptual speed (digit-symbol coding). The outcome measurements to be nested within each domain were chosen *a priori*. An objective Bayesian approach to setting the priors was used, that is all priors could be described as being weakly informative, or uninformative for the scale of the data. Priors for the overarching coefficients were described by a normal distribution with a mean = 0 and SD = 100. The remaining coefficients for the outcomes and domains were described as being derived from a normal distribution centered on 0 and a SD estimated from a half-Cauchy distribution centered on 0, and scale set to 25 (Gelman, 2006). Outcome level errors were modeled as being derived from a t-distribution to render the analysis robust (Lange et al., 1989; Kruschke, 2012). The prior for the SD for each outcome was described by a uniform distribution between 0 and 100 and the degrees of freedom parameter was estimated from the inverse of a uniform distribution with lower and upper limits of 0.001 and 0.5. A large estimate for the degrees of freedom parameter indicates

that the residuals can be described by normal distribution, while a smaller degrees of freedom parameter indicates that the data have fatter tails and data points in this region are appropriately down-weighted. Each variable was scaled to a mean of 0 and a SD of 1. If a smaller score on any neuropsychological measure indicated "better" performance, the score was inverted. After 5000 adaptation steps and 50,000 burn-in steps, a total of 50,000 Markov Chain Monte Carlo (MCMC) samples (thinned every tenth step) were saved across three chains for the final parameter estimates. Convergence was confirmed by examining plots of the posterior and using the Gelman–Rubin diagnostic (Gelman and Rubin, 1992). All posterior distributions used for inference had a minimal effective sample size of at least 1000 (usually ∼10,000). The means ±95% highest density intervals (HDI) of the posterior distribution were used to describe the credibility interval for each of the parameter estimates (Kruschke, 2012). All statistical analyses were conducted in R version 3.0.0 using the "rjags" package (Plummer, 2013).

RESULTS

RELATIONSHIP BETWEEN S100B AND COVARIATES AGE AND SEX

A total of 219 participants met the key inclusion criteria for this study. The mean age of the cohort was ∼65 years. All included participants had an MMSE scores >25. The mean serum S100B concentration was 0.24 ng/mL and normally distributed for the cohort; however, the range was substantial (**Table 1**). The covariates of age (slope = −0.001 ng/mL/year, HDI = −0.004, 0.002) and sex (contrast F-M = −0.02 ng/mL, HDI = −0.06, 0.02) were not substantially associated with S100B. By contrast, NART errors were negatively associated with S100B concentrations (slope = −0.004 ng/mL/error, 95% HDI = −0.007, −0.0008).

Table 1 | Measures of age, S100B concentration and MMSE scores and neuropsychological measures for the study cohort of 219 participants.

		Mean	SD	Range
Sex (F | M)	141 | 78			
Age		64.9	7.3	43.6–84.2
S100B (ng/mL)		0.24	0.14	0.08–0.62
MMSE		28.7	1.3	25–30
NART error score		14.1	6.6	3–39
NEUROPSYCHOLOGICAL MEASURES				
Perceptual speed				
Digit-symbol coding		64.6	13.9	23–95
Primary memory				
Digits forward		6.8	2.0	6–16
Digits backward		10.4	2.2	3–18
Secondary memory				
RAVLT sum of trials 1–5		44.5	9.6	16–64
RAVLT interference list		5.1	1.9	1–11
RAVLT short delay recall		9.1	3.1	0–15
RAVLT long delay recall		9.1	3.1	0–15
RAVLT recognition 'Hits'		13.4	1.9	0–15
RAVLT decay T5 – short delay		2.1	2.0	−3–11
RAVLT 'decay' T7 – short delay		2.2	2.0	−3–8
Stroop				
Stroop words time		18.5	7.3	11–101
Stroop dots time		13.9	3.5	7–36
Stroop colors time		29.9	10.1	14–80
Stroop interference (C/D)		2.2	0.66	0.8–4.8
Verbal ability				
D–KEFS letter fluency		40.9	11.8	7–71
D–KEFS category fluency		44.5	8.9	23–69
D–KEFS switching total		13.6	2.9	4–21
Boston naming test		56.3	4.2	26–60

RELATIONSHIP BETWEEN S100B AND NEUROPSYCHOLOGICAL PERFORMANCE

Figure 1 presents the standardized slope coefficients (mean ±80%, 95% HDI) for the association between S100B and neuropsychological performance both with and without the covariates of age, sex, and NART. S100B both with and without the covariates of age and sex was positively associated with performance on a range of neuropsychological measures. In the models that excluded covariates, S100B was positively associated with perceptual speed (D5), Stroop (D2), and verbal ability (D1). While the credible intervals for the primary memory (D4) and secondary memory (D3) domains included 0, their mean effect sizes were similar to the Stroop (D2) and verbal ability (D1) domains. Including the covariates age and sex did not appreciably alter the relationship between S100B and cognitive performance. By contrast, the addition of NART scores modestly reduced the mean estimates and associated confidence intervals across the range of neuropsychological measures used.

DISCUSSION

Serum S100B has been suggested to be a candidate marker of CNS injury because its concentration is increased after head trauma and in several neurodegenerative conditions (Sen and Belli, 2007). In these circumstances, heightened overproduction of S100B may amplify inflammatory processes and exacerbate cell stress as a consequence of altered redox state. An alternative interpretation is that elevated serum S100B is a consequence of amplified inflammation and cell stress, as several lines of evidence show significant positive trophic effects of S100B consistent with a neuroprotective function. This study explored for the first time the relationship between serum S100B and neuropsychological test performance across a range of cognitive domains in a relatively health cohort of older aged participants. Our analyses indicated that this study cohort manifested serum S100B concentrations which have been reported to support cell function and integrity in cell culture studies (i.e., <nanomolar range). No participant had serum S100B concentrations considered potentially indicative of CNS injury (i.e., micromolar range). Utilizing a nested cognitive domain model to explore the hypothesis of an association between cognitive capacity and serum S100B, the data indicates a positive association with all cognitive domains and in particular for perceptual speed, Stroop, and verbal ability. Adjustments for NART, but not age and gender, had a modest attenuating effect on the associations observed.

The largest effect size with respect to the association between cognitive performance and S100B was on the digit-symbol coding measure, reflecting perceptual, and motor speed. Similarly,

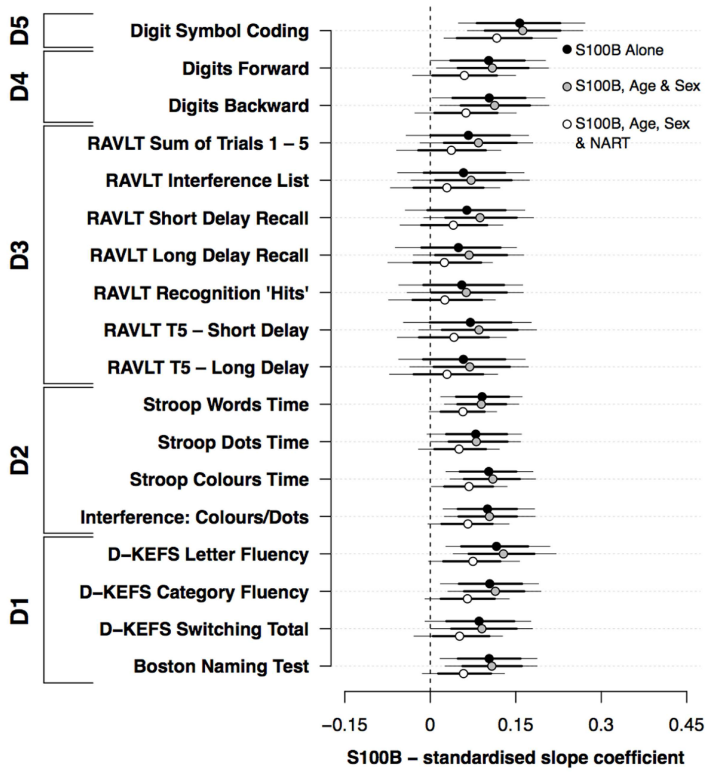

FIGURE 1 | Standardized slope coefficients (mean ±80%, 95% HDI) for the association between S100B and neuropsychological performance; both with and without the covariates of age, sex, and premorbid functioning.

performances on other speeded tasks (Stroop and verbal ability) were positively associated with serum S100B. The finding may indicate a beneficial association between S100B and "on line" speeded tasks. This is a potentially important finding, as "speed" has been considered a fundamental component of age-related CNS functional integrity (Salthouse, 1996). However, given that there are only very modest differences between the mean parameter estimates for the different cognitive domains that were evaluated in this study, the apparent selectivity of S100B for the speeded tasks may be more reflective of the psychometric properties of the tasks. For example, many of the secondary memory measures (which are derived from the RAVLT) only have a range of 15 discrete possibility outcomes, compared to the more continuous nature of the Stroop test and the larger range of possible scores on the Digit-Symbol Coding test. Therefore, an alternative hypothesis to be explored is whether S100B is selectively associated with particular cognitive domain(s) (for example, if the functional capacity of specific brain regions are associated with levels of S100B), or whether these concentrations are associated with a global enhancement of cognitive performance (as might be expected if cognitive processing were generally "faster" when higher concentrations of S100B are present within the healthy physiological range).

There has been significant interest in S100B as a potential marker of CNS trauma, distress, or pathological disturbances, and in this context S100B is described as an acute-phase response protein (Sen and Belli, 2007). In contrast, non-injurious and chronic levels of serum S100B would presumably represent constitutive rates of biosynthesis and metabolism. For this study, participants were currently functioning within the "healthy" range and individuals with head trauma, or other conditions known to significantly influence serum S100B concentration were excluded. Therefore, it appears reasonable to assume that S100B was not indexing CNS dysfunction.

Several lines of evidence support an important role for S100B in CNS development. Heightened S100B in response to stressors may be indicative of insult severity, although in many instances it has been suggested to be causally related to pathological sequelae. Intraventricular infusion of low concentrations of S100B induces

neurogenesis within the hippocampus in a traumatic brain injury model, and this was associated with enhanced cognitive function (Kleindienst et al., 2005).

S100B is mainly found in astroglial and Schwann cells and is enriched in CSF relative to blood. Many studies have therefore suggested that elevated serum S100B could be a useful surrogate marker of blood-brain leakage (Marchi et al., 2003); indeed, this proposal is supported in clinical and animal head trauma findings. However, other sources of serum S100B could include adipocytes, chondrocytes, lymphocytes, bone marrow cells, and melanocytes, with clearance occurring predominantly via renal excretion. Based on the exclusion criteria indicated in this study (no recent head trauma, frank neurological disorder, or renal dysfunction), serum S100B homeostasis was evaluated in a "healthy" physiological context rather than in a neuropathological context, with the evidence suggesting positive associations with cognition in healthy participants.

The cross-sectional study design that was used unfortunately does not permit delineation of possible casual mechanisms with respect to the association between serum S100B and cognitive performance. Possible effects of S100B relevant to cognition may include improved redox state and cell function; suppression of neurovascular inflammation; enhanced conduction and transmission of nerve impulses (putatively of marked relevance given the findings on "speeded" cognitive tasks noted in this study); promotion of cell growth and/or differentiation or enhanced cytoskeletal structure.

Future studies to investigate the relationship of serum S100B levels and cognitive/neurological functions under stress/pathological conditions may be particularly warranted. The body of evidence to date suggests that S100B may be an acute-phase response-to-injury protein that confers positive trophic and functional effects of the neurovascular unit. However, S100B is not normally considered as a chronic modulator of neurovascular function. Evidence consistent with the latter may provide therapeutic opportunities particularly in the pre- and pro-dromal phase of neurodegenerative conditions such as AD. Realization of the clinical translatability will however require a comprehensive understanding of S100B metabolism, kinetics, and molecular mechanisms. Common to these proposed functional effects is binding of S100B to ionized calcium, which as a consequence exposes hydrophobic residues (thereby enabling S100B to interact with other proteins and thus exert a biological effect). Possible synergistic effects of S100B and calcium metabolism on cognition may therefore be of particular interest in future studies.

AUTHORS CONTRIBUTION

Virginie Lam and John C. L. Mamo researched literature and conceived the study. Virginie Lam, John C. L. Mamo, Ryusuke Takechi, Corey Giles, Anthony P. James, and Jonathan K. Foster were involved in protocol development, gaining ethical approval, and patient recruitment. Virginie Lam, Matthew A. Albrecht, Jonathan K. Foster, and John C. L. Mamo were responsible for data and statistical analysis. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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Chapter 5

Chapter 5: Serum albumin and neurocognitive performance

The following article covers the content of this chapter:

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Lam V., Albrecht M. A., Takechi R., Heidari-Nejad S., Foster J. K., & Mamo J. C. (2014). Neuropsychological performance is positively associated with plasma albumin in healthy adults. *Neuropsychobiology, 69*(1), 31-38.

Synopsis:

Background

Albumin is a major plasma protein important in a number of physiological functions including regulation of peripheral microvascular permeability, inflammation, oncotic pressure, and oxidant status (Burtis & Ashwood, 2001; Demling, 1986; Oettl & Stauber, 2007). Approximately 42% of total albumin is found plasma and the remaining fraction is found in extravascular compartments. Reduced concentrations of plasma albumin are related to an increase in vascular permeability, which results in the leakage of proteins, inflammatory mediators and large volume of extracellular fluid into interstitial space (Fleck et al., 1985; Nicholson, Wolmarans, & Park, 2000). Moreover, protective effects of albumin on capillary integrity were demonstrated by the prevention of apoptosis of cultured endothelial cells (Zoellner et al., 1996). Low levels of plasma albumin due to altered distribution between intra - and - extra -vascular compartments or reduced reabsorption relative to kidney dysfunction, have been associated with critical illness, increased morbidity and mortality, and poorer mental health (Goldwasser & Feldman, 1997; Nicholson et al., 2000; Spiegel & Breyer, 1994). Based on its important physiological functions, circulating plasma albumin may be related with cognitive performance. Indeed, positive associations between plasma albumin and cognitive performance have been reported, however these findings are generally derived from subjects with impaired albumin homeostasis due to liver or renal dysfunction (Griva et al., 2004; Ortiz et al., 2006). The aim of this study was to determine whether an association exists between plasma albumin and global cognitive performance in a cohort of healthy adults free of liver or renal dysfunction.

Methods in brief

A wide-range neuropsychological test battery was used to explore the relationship between global cognitive performance and plasma albumin homeostasis in 222 healthy participants between the ages of 43 and 84, free of liver or renal impairment. A nested-domain Bayesian mixed-model approach was used to analyse the data, where age, gender and acute-phase proteins, were used a covariates.

Results in brief

Serum albumin was positively correlated with better performance across a range of cognitive domains range assessed by our comprehensive test battery. Measurement of perceptual speed, Stroop and verbal ability domains showed the greatest positive association with plasma albumin.

Discussion and conclusion in brief

Our findings extend the findings of previous studies conducted in hypo-albuminaemic individuals and to our knowledge, present for the first time, a positive association between plasma albumin homeostasis and global cognitive performance in a cohort of healthy individuals with normal renal and liver function. The relationship reported between albumin and cognitive function suggests cognitive decline may result from a state of heightened inflammation due to compromised vascular permeability mediated by plasma albumin.

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Neuropsychological Performance Is Positively Associated with Plasma Albumin in Healthy Adults

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Key Words
Plasma albumin · Neuropsychological performance · Neurovascular inflammation · Bayesian analysis

Abstract
Background: Albumin serves a range of physiological functions that are vital to overall brain and cognitive health. Indeed, associations between cognitive performance and albumin have been demonstrated in individuals with chronic liver or kidney disease and in patients with a high urinary excretion of albumin. However, an association of plasma albumin with cognitive performance has not been reported in otherwise healthy participants with clinically acceptable plasma albumin concentrations. ***Method:*** This study utilized a wide-ranging neuropsychological test battery to investigate the relationship between cognitive performance and plasma albumin homeostasis in 222 healthy participants (143 females) between the ages of 43 and 84 years (mean 65 years). ***Results:*** Albumin both with and without the covariates of age, sex and acute-phase proteins was positively associated with enhanced performance on a range of neuropsychological domains including perceptual speed, Stroop and verbal ability. Albumin manifested generally positive but less robust associations with secondary and primary memory. ***Conclusion:*** The results indicate that there is a positive association between albumin and cognitive performance in physiologically healthy participants free of chronic renal or liver disease.

Introduction

Albumin is a major component in blood, accounting for 50–60% of the total protein [1]. The abundance of albumin, as well as its relatively small size and negative charge, makes it the pivotal protein for maintaining oncotic pressure homeostasis and microvascular permeability [1–3]. Albumin serves as a chaperone-transporter for a range of exogenous and endogenous substances, particularly those with hydrophobic properties [4]. The chaperone function of albumin provides reservoir functionality for important active biological compounds and facilitates its role as a scavenger for potentially toxic compounds [4, 5]. Through mechanisms that may involve heightened inflammation and aberrant capillary vascular permeability [6], low levels of plasma albumin have been associated with frailty [7, 8], depression and apathy [9], increased morbidity [10], mortality [11] and poorer recovery after surgery [12].

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An association of plasma albumin with cognitive capacity is a reasonable proposition given its critical physiological function. On clinical diagnostic status and brief measures of global neuropsychological performance such as the Mini-Mental State Exam (MMSE), a number of studies have reported a positive association with plasma albumin (or a negative association with albuminuria) [6, 13–21]. Clinical findings in patients with cirrhotic or alcoholic liver disease [22–29] and in patients with renal failure [30–32] suggest a positive association between plasma albumin and performance on the Digit Symbol Coding test, reflecting potential enhancements of albumin on perceptual speed. In other cognitive domains, the association between albumin and cognitive function has been less robust but also generally positive. Performance on secondary memory [15, 19, 22, 25, 30], trails A and B [15, 18, 22–24, 27, 30, 33], the Rey Copy Figure test [15], animal and category fluency [18, 19, 23, 26], primary memory [19, 23, 28, 30] and the Stroop test [26] support positive associations between plasma albumin and cognitive functioning. However, further studies are needed, as several papers have failed to find evidence for a statistically significant association between plasma/urinary albumin and cognitive functioning [27, 29, 34–38]. Moreover, a limitation concerning the interpretation of extant findings is that the majority of studies reported to date may have been confounded by comorbidities, including kidney or liver diseases (e.g. Fontana et al. [29], Griva et al. [27] and Radic et al. [28]). Other relevant confounders include the age and sex of the participant, both of which are known to influence cognition and normal physiology. Therefore, it is important to take into account the influence of these factors on any relationship found between albumin and neuropsychological performance.

As with other complex biological phenomena, cognition is a multifactorial entity that reflects the composite of many different elements, including the domains of perceptual speed, primary memory, secondary memory, verbal ability, linguistic ability and executive functioning. In measuring cognitive capacity in healthy subjects with plasma albumin in the normal reference range, we used in this study a battery of tests that specifically evaluated each of the aforementioned domains. Age and sex were used as covariates to avoid potential confounding effects on cognitive performance. The fractionation of different elements of cognition assessed against important biomarkers such as plasma albumin provides a powerful approach to modelling the putative relationship between cognitive outcomes and regulatory biological factors.

Methods

Participants

A total of 250 participants (96 males and 154 females) over the age of 40 years (range 43–84 years) were recruited for this study. This study was approved by the Curtin University Human Research Ethics Committee (approval No. HR97/2011). Potential participants completed a medical history and medication questionnaire and were interviewed to confirm the information provided. Participants were excluded from this study if any of the following criteria applied: major surgery or a clinical event 'in the last 6 months', currently diagnosed with a psychiatric disorder or taking psychotropic medications, haemophilia, cancer/chemotherapy, head injury within the last 5 years, or diagnosed with HIV. In addition, data from participants suggesting renal impairment (estimated glomerular filtration rate <55 ml/min/1.73 m^2) or liver dysfunction (abnormal alanine aminotransaminase, bilirubin, alkaline phosphatase or gamma glutamyl transferase) were not included in the statistical analyses.

Serum Albumin

Participants arrived for the provision of fasting blood samples between 8.00 and 10.00 a.m. The samples were taken in collection rooms adjacent to an accredited diagnostic centre following an overnight fast (minimum 8 h). Participants were asked to avoid prolonged strenuous exercise and to limit their alcohol intake to 2 standard drinks over the 24-hour period prior to blood sampling. A venous sample was drawn from each subject (after being seated for 10 min) into lithium heparin-coated Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, N.J., USA) and centrifuged at 3,800 rpm for 7 min at room temperature for analysis. Blood albumin was measured using an Abbott Diagnostics c16000 analyser (Abbott Diagnostics, Abbott Laboratories, Abbott Park, Ill., USA) via the Bromcresol Green dye-binding method (CV 0.48–0.55%).

Neuropsychological Measures

Trained staff under the supervision of a registered clinical neuropsychologist administered the cognitive battery. Tests were selected based upon their widespread use within the field and their reliability and validity, and to cover the main domains of cognitive performance affected by age-related cognitive decline. The battery of cognitive tests included the MMSE [39], the Rey Auditory Verbal Learning Test (RAVLT) [40], Delis-Kaplan Executive Function System (D-KEFS) verbal fluency subtests [41], the 60-item Boston Naming Test (BNT) [42], the National Adult Reading Test (NART) [43], the Digit Span and Digit Symbol Coding subtests of the Wechsler Adult Intelligence Scale, third edition (WAIS-III) [44, 45], and the Stroop test (Victoria version) [44].

Statistical Analysis

We used a nested-domain bayesian mixed-model approach, similar to that described by Thurston et al. [46], to estimate the relationship between plasma albumin and cognitive performance. The method accounts for multiple correlated outcomes that are nested within cognitive domains, e.g. the domain secondary memory in this study is measured by the RAVLT which is composed of several correlated outcome measurements, including short-delay and long-delay recall, that are moderately correlated. The hierarchical nature of the nested-domain model significantly increases power by partially pooling outcome estimates from within a cognitive domain

towards each other, i.e. the coefficients for each individual outcome level coefficient take into account the information that is present within each domain and the overall influence of the variable. The property of hierarchical models also aids in reducing type S (sign) and type M (magnitude) errors through shrinkage towards common estimates [47], e.g. by partially shrinking the coefficient obtained for RAVLT short-delay recall towards a common overall estimate for episodic memory. We included the covariates of age, sex, and acute-phase proteins within a series of models to account for possible shared variance of these variables on cognitive performance with albumin. Most importantly, age and sex are two factors that are known to influence both cognitive performance and a range of physiological attributes that may offer an alternative explanation for the relationship between serum albumin concentration and cognitive performance. In addition, two relevant acute phase markers, plasma C-Reactive Protein (CRP) and white blood cell count (WBC) were also evaluated to determine if these markers significantly impact upon the relationship between albumin and cognitive performance.

The domains (and associated outcome measures) assessed in the present study were: D1 – verbal ability [BNT and D-KEFS fluency (letter fluency, category fluency and switching fluency)]; D2 – Stroop [dots, words, colours and interference (colours/dots) response time]; D3 – secondary memory [total items recalled across a learning trial, interference list, short-delay free recall, long-delay free recall, recognition 'hits', short-delay decay (learning trial 5 – short delay) and long-delay decay (learning trial 5 – long delay)]; D4 – primary memory (digits forward, digits backward, and the sum of the digits recalled both backward and forward), and D5 – perceptual speed (digit symbol coding). The selection of outcome measurements to be nested within each domain was chosen a priori based upon the instrument used and the cognitive domain that the measure tested.

An objective bayesian approach to setting the priors was used, i.e. all priors could be described as being weakly informative or uninformative for the scale of the data. Priors for the overarching coefficients were described by a normal distribution with a mean of 0 and a standard deviation (SD) of 100 (relatively flat for standardized data). The remaining coefficients for the outcomes and domains were described as being derived from a normal distribution centred on 0 and an SD estimated from a half-Cauchy distribution centred on 0, and a scale set to 25 (again, diffuse for the scale) [48]. Outcome level errors were modelled as being derived from a t distribution to render the analysis robust [49, 50]. The prior for the SD for each outcome was described by a uniform distribution between 0 and 100 and the degrees-of-freedom parameter was estimated from the inverse of a uniform distribution with lower and upper limits of 0.001 and 0.5. A large estimate for the degrees-of-freedom parameter indicates that the residuals can be described by a normal distribution, while a smaller degrees-of-freedom parameter indicates that the data have fatter tails and data points in this region are appropriately down-weighted.

Before entering the data into the analysis, each dependent variable was scaled to a mean of 0 and a SD of 1. If better performance was indicated by a smaller score, the variable was inverted by subtracting each score from the maximum. Similarly, albumin and the covariates age and NART error score were also centred on 0 and scaled.

In total, 5,000 steps were used to tune the samplers, 50,000 steps were used to burn in the chains and a total of 50,000 Markov chain Monte Carlo samples thinned every 10th step were saved across 3 chains for the final parameter estimates. Convergence was confirmed by examining plots of the posterior and using the Gelman-Rubin convergence diagnostic [51]. All posterior distributions used for inference had a minimal effective sample size of at least 1,000 (usually ~10,000). The means ± 95% highest density intervals (HDI) of the posterior distribution were used to describe the credibility interval for each of the estimates and contrasts [49].

All statistical analyses were conducted with the open-source statistical package R version 2.15.3 (R Development Core Team, 2013) using the 'rjags' package to link with the Gibbs sampler 'JAGS' [52].

Table 1. Mean and range of the age, albumin concentration and gender of the study population of 222 otherwise healthy participants

Biological measures	Mean ± SD	Range
Age, years	64.9±7.3	43.6–84.2
Albumin, g/l	42.1±2.3	36–49
Sex (F/M)	143/79	

Results

A total of 222 individuals (79 men and 143 women) met the key inclusion criteria for participation in this study (table 1) out of 250 individuals in the full cohort. Nine participants were excluded because they had an estimated glomerular filtration rate below 55 ml/min/1.73 m^2, suggesting impaired renal function. All participants had plasma albumin concentrations within the normal reference range (35–50 g/l). Sixteen participants were excluded because their score on the MMSE was less than 24, suggesting significant cognitive impairment. Three individuals were excluded due to liver dysfunction (abnormal alanine aminotransaminase, bilirubin, alkaline phosphatase or gamma glutamyl transferase).

Relationship between Albumin and the Covariates Age and Sex

For the selected cohort, there was a negative relationship between age and plasma albumin concentration, with a mean reduction of 0.079 g/ml/year (HDI = –0.12 to –0.04) (table 2). However, albumin concentrations were not credibly different between males and females.

Relationship between Albumin and Neuropsychological Performance

Figure 1 presents the standardized slope coefficients (mean ± 80%, 95% HDI) for the association between albumin and neuropsychological performance both with

Table 2. Neuropsychological measure scores (MMSE, RAVLT, D-KEFS letter Fluency, category fluency and switching, BNT, NART, digit span, digit symbol coding and the Stroop test) and the relationship between age, sex and acute-phase proteins NART error scores as covariates for the entire study cohort of 222 patients

Neuropsychological measures		Mean ± SD	Range
	MMSE	28.7±1.4	24 to 30
	NART errors	14.6±7.5	3 to 50
D5 – perceptual speed	Digit symbol coding	64.6±13.8	23 to 95
D4 – primary memory	Digits forward	10.4±2.0	6 to 16
	Digits backward	6.8±2.2	3 to 18
	Digits total	16.8±3.8	9 to 29
D3 – RAVLT	Learning 1–5	44.5±9.6	16 to 64
	List B	5.1±1.9	1 to 11
	Short delay	9.1±3.1	0 to 15
	Long delay	9.1±3.1	0 to 15
	Recognition	13.4±1.9	0 to 15
	T5 – short delay	2.2±2.0	–3 to 11
	T5 – long delay	2.2±2.0	–3 to 8
D2 – Stroop	Dots	13.9±3.4	7 to 36
	Words	18.5±7.2	11 to 101
	Colours	29.9±10.1	14 to 80
	Interference C/D	2.2±0.7	0.83 to 4.8
D1 – verbal ability	BNT	56.3±4.2	26 to 60
	D-KEFS letter fluency	40.9±11.9	7 to 71
	D-KEFS category fluency	44.5±8.9	23 to 69
	D-KEFS switching	13.6±2.9	4 to 21
Albumin and covariate relationships		β	95% HDI
	Albumin-age	–0.08	–0.12 to –0.04
	Albumin-sex (F, M)	–0.07	–0.74 to 0.57
	NART-albumin	–0.04	–0.09 to 0.004
	Albumin-CRP	–0.11	–0.24 to 0.02
	Albumin-WBC	0.01	–0.23 to 0.25

and without the covariates of age, sex and acute-phase proteins (APP) as a marker of significant inflammation (APP are indicated by plasma C-reactive protein and white blood cells). Albumin both with and without these covariates was positively associated with enhanced performance on a range of neuropsychological domains and their outcome measures. In the models that excluded covariates, albumin was positively associated with all domains and their outcome measurements, with the exception of the primary memory domain (D4) and some measures within the secondary memory domain (D3). After relevant covariates were included in the model, the relationship between albumin and cognitive performance was modestly reduced. However, the relationships between albumin and the perceptual speed (D5), Stroop (D2) and verbal ability (D1) domains all remained credibly larger than 0 when age and sex were included as covariates, with the perceptual speed measure digit symbol coding (D5) demonstrating the most robust association. While all 95% credible intervals for secondary memory (D3) and primary memory (D4) covered 0 after including the covariates, the relationship with albumin on each domain was predominantly positive and not substantially different from the Stroop (D2) and verbal ability (D1) domains.

Discussion

Albumin was positively associated with neuropsychological performance across a range of cognitive domains. In particular, the perceptual speed measure digit symbol

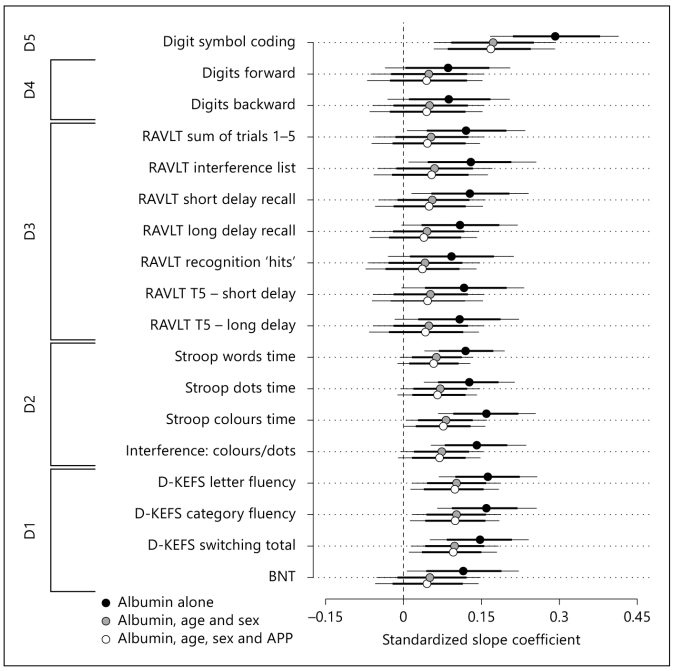

Fig. 1. Standardized slope coefficients (±80, 95% credible intervals) for the association between albumin and neuropsychological performance. The domains (D1 to D5) indicate the nesting structure of the bayesian nested-domain model. Black circles indicate the effect of albumin without covariates (n = 222); grey circles indicate the effect of albumin with the covariates age and sex; white circles indicate the effect of albumin with the covariates age, sex and acute phase proteins.

coding showed the largest association with plasma albumin. Positive associations were also found with respect to performance on the Stroop and verbal ability domains, while weaker evidence was found for the secondary memory and primary memory domains. The results indicate a wide-ranging positive association between albumin and cognitive performance that was most evident on the perceptual speed domain.

D5: Perceptual Speed

The largest association between albumin and cognitive performance was found with the perceptual speed measure digit symbol coding. Mean estimates from the hierarchical models indicated a correlation between 0.2 and 0.3 (depending on the covariates). The magnitude of this positive association is consistent with the literature showing correlations ranging from 0.07 to 0.36 between albumin and digit symbol coding performance in cirrhotic and kidney patients [22–25, 27, 29]. More specifically, this finding suggests a positive association with psychomotor speed and ability. For example, Joy et al. [53] reported that 50% of the variance in performance on digit symbol coding can be attributed to psychomotor speed and coordination, while only approximately 7–15% is attributable to memory mechanisms. Digit symbol coding is also sensitive to age-related changes [54], dementia [55] and brain damage [56].

D1 and D2: Verbal Ability and Stroop

Increased plasma albumin was also associated with better performance on the two other predominantly speed-oriented task domains: verbal ability (which comprised D-KEFS fluency measures and the BNT) and the Stroop test. A positive association between the D-KEFS measures and albumin is consistent with Tarter et al. [23] who found a statistically significant correlation of 0.43 for category fluency in participants with cirrhotic liver disease. Our reported correlation of 0.10–0.15 (depending on the inclusion of covariates) was substantially lower than their estimate. The differences in strength of association may be because the positive association between plasma albumin and cognitive function is accentuated in

patients with cirrhosis and the use of relevant covariates that share common variance between albumin and cognitive function (e.g. age) was not included in previous studies. Additionally, the hierarchical model used for our analysis partially shrinks estimates towards each other, which results in smaller effect differences between individual outcome measures.

The verbal ability and Stroop measures represent tests that are sensitive to damage to the frontal and temporal brain regions. Better performance on these verbal fluency measurements indicates enhancements in retrieval strategies underpinning both lexical and semantic memories [57]. Better performance on the Stroop test indicates enhancements in attention and executive functioning abilities [58]. Therefore, these findings suggest that albumin is a positive modulator of memory retrieval and elements of executive functioning and attention.

D3 and D4: Secondary and Primary Memory

A less robust association was found between albumin and the two memory domains, i.e. secondary memory and primary memory. Nevertheless, the mean parameter estimates were positive and the majority of the posterior densities were above 0 and small. Positive associations are consistent with previous studies [22, 25, 29]. Furthermore, the influence of albumin on these two cognitive domains could plausibly be as large as the influence of albumin on the Stroop and verbal ability domains (given the overlap of the 95% credible intervals). Secondary memory and primary memory rely on distinct neural substrates, with the former being dependent on hippocampal and medial temporal lobe regions [59] while the latter is more dependent on left temporo-parietal and frontal regions [60].

Putative Mechanisms for the Relationship between Albumin and Cognitive Performance

Moderate plasma hypoalbuminaemia has few, if any, associated clinical consequences. Hence, in an otherwise healthy cohort with plasma albumin within an accepted reference range, a positive association with measures of cognitive function may be indicative of other genetic, environmental or lifestyle factors that influence both cognition and albumin separately. For example, people with a better nutritional status or a greater dietary protein intake or those who exercise more may manifest better cognitive performance and also higher plasma albumin. However, the dietary intake of protein and other macronutrients has little effect on plasma albumin in subjects with a good nutritional status [61]. Rather, hepatocytes have a substantial capacity to increase albumin biogenesis (threefold), reflecting its critical function in maintaining oncotic pressure [3]. A positive association of plasma albumin with exercise (and, by extension, exercise-enhanced cognitive performance) is also unlikely given that albumin degradation is increased in skeletal muscle and skin as a consequence of increased physical activity [62].

Albumin is a critical transporter of a range of biochemical elements and compounds, including calcium, copper, cysteine, fat-soluble vitamins, fatty acids, free radicals, glucocorticoids, thyroxine, tryptophan and bilirubin [63]. Cognitive deficits associated with reduced plasma albumin may therefore be a consequence of greater exposure to unconjugated bioactive proteins that influence cognition. However, this is unlikely in the cohort studied here because the plasma albumin is within a normal reference range (36–49 g/l).

Heightened inflammation exerts a negative effect on albumin synthesis [64–66], and many studies have reported an association between inflammation and cognitive decline [67, 68]. Conversely, a change in microvascular permeability, which is principally modulated by plasma albumin (a negative acute-phase marker), promotes cytokine production and the activation of inflammatory cells. A variety of inflammatory cytokines have been found to impair long-term potentiation [69], a fundamental neuronal learning mechanism that is strongly linked to learning and memory. There is, therefore, a plausible link between albumin and the physiological mechanisms underlying a central component of cognitive functioning, i.e. secondary memory. However, in this study group, the acute-phase inflammatory cytokines C-reactive protein and interleukin-6 were not associated with cognitive performance testing. Moreover, subjects with aberrant white blood cell counts were also excluded from analysis.

A relationship between albumin and cognitive functioning may also be associated with vascular sequelae, specifically with respect to neurovascular inflammation. Modest reductions in colloid oncotic pressure can result in extravascular fluid accumulation [3, 61]. Of potential relevance for cognitive outcomes, the integrity of the blood-brain barrier may be compromised resulting in the parenchymal extravasation of plasma proteins and macromolecules, the activation of astroglial cells and a heightened redox state [70–73]. Therefore, a subtle but heightened state of neurovascular inflammation could be a relevant mechanism underlying the relationship between albumin and cognitive performance observed in this study.

Caveats

From this research, it cannot be determined whether plasma albumin is causally associated with cognitive performance in healthy adult men and women. Plasma albumin only measures the intravascular pool (40% of the total endogenous pool) and may not accurately reflect the extravascular concentration of albumin. However, there is flux between the two pools at approximately 4%/h and, generally, a positive association between the two compartments is indicated. Consideration should also be given to the substantial disulphide binding heterogeneity of plasma albumin that exists among individuals and which may be of functional significance. Though less well characterized, disulphide-binding domains have the potential to modulate the chaperone transporter function and microvascular regulatory properties of albumin. The latter is a consequence of altered albumin kinetics between intravascular and extravascular pools.

Conclusion

The findings of this study extend the findings of previous studies conducted in participants with plasma hypoalbuminaemia and demonstrate for the first time a positive association of plasma albumin with global measures of cognitive performance in healthy participants with unimpaired renal and liver function.

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Disclosure Statement

There are no conflicts of interest to our knowledge.

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Chapter 6

Chapter 6: General discussion, limitations and future directions

The final chapter of this thesis includes a general discussion to deliberate the major results reported in the animal model studies presented in Chapter 2 and the cross-sectional clinical study presented in Chapter 3. This chapter discusses potential mechanisms and significance of the findings in which VD and its homeostatic counterparts may modulate cerebral capillary integrity and in a clinical context, the potential effects of VD on neurocognitive function. In addition, the limitations relevant to each of the studies presented as part of this thesis are addressed followed by the consideration of future study directions.

6.1 General discussion

The fundamental findings of Chapter 2 and Chapter 3 support the broad thesis hypothesis that the 'Vitamin D - Calcium - Parathyroid Hormone' endocrinal axis has vascular-modulating effects on cerebral capillary integrity. The key results derived from the *in vivo* animal model studies demonstrated for the first time the potential detrimental effects of hypervitaminosis D on cerebral capillary integrity. Experimental and clinical evidence suggests compromised integrity of the cerebral capillary endothelium precedes cognitive impairment and the onset of AD and VaD, however the potential effects of BBB dysfunction on cognitive performance remain poorly understood (Bell & Zlokovic, 2009; Ujiie, Dickstein, Carlow, & Jefferies, 2003). The second principal finding presented was the observation that verbal episodic memory performance was negatively associated with serum VD homeostasis in otherwise healthy, older aged adults. The findings were considered in the context of iCa and PTH homeostasis and in a broader context are consistent with the notion that this may occur via modulation of cerebral capillary function. The overall findings suggest that clinically, supplementary use of VD should only be considered if there evidence of VD deficiency.

6.1.1 Cerebral capillary dysfunction and cognitive performance in late-onset Alzheimer's disease and vascular dementia

The cerebral capillary endothelium representing the main interface between the systemic circulation and brain parenchyme is crucial in maintaining CNS homeostasis (Banks, 2012; Saunders, Ek, Habgood, & Dziegielewska, 2008). Cerebral capillary dysfunction is characterised by morphological changes in capillary structure and proliferative changes in smooth muscle cells which are pseudo-pathologies often reported in a number of neurodegenerative diseases including AD, VaD, Parkinson disease and multiple sclerosis (Chung et al., 2010; Nicolakakis & Hamel, 2011; Tourdias & Dousset, 2013; Zlokovic, 2008). Loss of BBB functionality may result in the 'leakage' of peripherally-derived neurotoxic substances into the CNS; deranged protein-transporter systems leading to inadequate nutrient supply; a potential accumulation of neurotoxic substances in the CNS and entry of inflammatory compounds that are normally excluded (Erickson & Banks, 2013; Serlin, Levy, & Shalev, 2011). A consequence of these perturbations include altered protein expression and secretions of cytokines and vasoactive substances by endothelial cells and cells of the neurovascular unit that can initiate an inflammatory cascade, promote oxidative stress and exacerbate neuronal damage (Erickson & Banks, 2013; Serlin et al., 2011).

Accumulating evidence suggests compromised integrity of the cerebral capillary endothelium may precede amyloidosis and neurodegeneration (Grammas, 2011). A number of animal model studies have reported progressive behavioural and cognitive changes, hippocampal and cortical atrophy, and neuronal dysfunction, as a result of prolonged cerebral capillary dysfunction (Bell et al., 2010; Bell et al., 2012; Grammas, 2011; Winkler et al., 2014). Consistent with a causal role, pathological alterations of the BBB coincide with progressive decline of cognitive performance (Frisoni, Galluzzi, Pantoni, & Filippi, 2007; Popescu et al., 2009) and extensive capillary damage has been reported in brain regions specifically involved in cognition (Hosokawa & Ueno, 1999; Montagne et al., 2015). Indeed, a progressive increase in cerebral capillary permeability may precede hippocampal atrophy as demonstrated in older-aged mildly cognitively impaired and AD-individuals (Apostolova et al., 2010; Montagne et al., 2015; Whitwell et al., 2012). Both

experimental and clinical data have suggested that the progression of AD may be halted upon restoration of cerebrovascular function through dietary and/or pharmacological interventions (Dickstein et al., 2006; Gorelick et al., 2011; Takechi et al., 2014; Takeda et al., 2009).

6.1.2 Vitamin D - Calcium - Parathyroid hormone homeostasis and cerebral capillary permeability and neurocognitive performance

6.1.2.1 Putative effects of vitamin D on cerebral capillary endothelia and neurocognitive performance

The extra-skeletal roles of VD, particularly in neurocognitive performance, have been an area of considerable interest in recent years (McCann & Ames, 2008). The major components of the VD system, the VD receptor and its key activation enzymes, have been identified in brain regions commonly affected in neurodegenerative diseases (Eyles et al., 2013; Eyles et al., 2005; Fernandes de Abreu, Eyles, & Feron, 2009). As discussed in Chapter 1, the vast majority of the VD literature relative to its neuroprotective properties has been studied extensively in context of VD deficiency. A number of experimental studies have reported an association between VD deficient rodent models and modulation of specific brain biomarkers, glucose metabolism, brain protein expression and behavioural changes (Almeras et al., 2007; Altemus, Finger, Wolf, & Birge, 1987; Durk et al., 2014; Feron et al., 2005; Groves et al., 2013; Keeney et al., 2013b). A number of cross-sectional and epidemiological studies have defined a positive association between serum VD concentration and better cognitive performance (Littlejohns et al., 2014). Upon correction of VD deficient status, both experimental and clinical studies have reported attenuation of various brain biochemical stressors and improvement in behavioural/cognitive performance (Latimer et al., 2014; Przybelski & Binkley, 2007; Wang et al., 2001). Contrasting data found no marked differences in experimental models of VD deficiency and their relevant controls (Brouwer-Brolsma et al., 2014; Byrne et al., 2013). Different experimental design including dietary composition and concentrations of VD among the studies may have led to

inconsistent results among the literature. In addition, gradual modification of dietary VD consumption rather than abrupt changes in VD content would closely represent a better, physiologically relevant model.

Brown et al. (2003) showed paradoxical results in which VD treatment of cultured hippocampal cells led to significant cellular apoptosis (Brown, Bianco, McGrath, & Eyles, 2003). Apoptotic effects of high levels of VD were also demonstrated in studies in cancerous prostate and breast cell lines (Johnson, Hershberger, & Trump, 2002; Narvaez, Zinser, & Welsh, 2001). Parallel to the findings of Brown and colleagues (2003), Granic et al. (2015) and others have demonstrated a biphasic relationship between VD status and global cognition in the elderly in which both low and high VD status impose adverse effects on the CNS (Brown et al., 2003; Granic et al., 2015b; McGrath et al., 2007). Clearly, there is a paucity of studies investigating the adverse effects of VD in an excess context and the potential mechanisms underlying the physiological effects of VD in the CNS warrant further investigation.

The results presented in Chapter 2 and Chapter 3 highlight the importance of considering the adverse effects of hypervitaminosis D in association with the CNS. Central to the thesis hypothesis, Chapter 2 introduced for the first time, the causal role of VD in modulating the permeability of the BBB in context of dietary-induced hypervitaminosis D. Marked disturbances in barrier properties of cerebral capillary vessels associated with exogenous supplementation of dietary VD was demonstrated in 2 rodent species, concomitant to serum hypercalcemia and suppression of circulating PTH. The mechanisms underlying hypervitaminosis D-mediated cerebral capillary dysfunction are unclear. Durk and colleagues recently identified abundant VDR expression on rat and mouse brain capillary endothelia in both *in vivo* and *in vitro* studies (Chow, Sondervan, Jin, Groothuis, & Pang, 2011; Durk et al., 2012; Durk et al., 2015). Moreover, activation of neural VDR by administration of its bioactive ligand was found to alter the pharmacokinetics of endothelial p-glycoprotein expression and its substrates (Durk et al., 2015). Whilst these findings do not directly implicate VD and the regulation of cerebral capillary function and permeability, they certainly support this notion relative to the study findings. In

addition, increased transcytosis of plasma proteins into the brain parenchyme may be due to upstream effects of VD on intracellular calcium homeostasis as seen in neuronal ageing (Alexianu, Robbins, Carswell, & Appel, 1998; Gascon-Barré et al., 1994). Indeed, pericyte and astrocytic function may be modulated based on calcium regulatory mechanisms and further influence capillary permeability (Abbott et al., 2006; Hughes et al., 2006; Peppiatt, Howarth, Mobbs, & Attwell, 2006).

Interestingly, the results from Chapter 2 do not suggest that exaggerated provision of supplemental VD significantly promote neurovascular inflammation per se. Substantive neuro-inflammation commonly co-exists with compromised cerebral capillary endothelium induced by diets enriched in pro-atherogenic lipids (Freeman & Granholm, 2012; Grammas, 2011; Takechi et al., 2012). The immuno-modulating properties of VD have been well documented and *in vitro* studies have demonstrated direct effects of VD in modulating pro-inflammatory cytokine production in the brain (Lefebvre, Montero-Menei, Bernard, & Couez, 2003; Moore et al., 2005). Whilst the experimental design does not investigate if VD will attenuate neurovascular inflammation per se, it is possible the pro-inflammatory nature of the vitamin may have modulated the degree of neuro-inflammation in relation to dysfunction of cerebral capillary vessels.

Based on the experimental findings of Chapter 2, Chapter 3 extends on the putative regulatory properties of VD influencing cognition. Cross-sectional data from a group of healthy middle aged to older aged individuals demonstrated that greater serum VD status was associated with poorer verbal episodic memory, a sensitive and specific marker of age-related cognitive changes. Indeed, a multitude of factors are involved in cognitive performance however as previously discussed, compromised cerebral capillary endothelium is considered a major determinant of declined cognitive performance. The findings from this study are in concert with those of Granic et al. (2015) and McGrath et al. (2007); higher serum VD concentrations coincide with decline in global cognitive performance (Granic et al., 2015b; McGrath et al., 2007). Moreover, the positive effects of VD on brain development and cognition as postulated by Annweiler et al. (2009) and others are not supported by a number of studies (Annweiler et al., 2009; Dean et al., 2011; Michos et al.,

2014; Schneider et al., 2014). It remains unclear whether the neuroprotective actions of VD are active only in VD deficient individuals or if it exerts specific pharmacological effects in those with adequate concentrations of VD.

6.1.2.2 Putative calcium-related implications of dysfunctional blood-brain barrier and cognitive function

Vitamin D is a potent regulator of extracellular and intracellular calcium homeostasis. Cellular control of calcium homeostasis supports brain physiology and the maintenance of cognitive health. Complex integrative mechanisms of intracellular calcium signalling regulate neuronal plasticity underlying learning and memory and neuronal survival (Zündorf & Reiser, 2011).

In Chapter 2, elevated serum iCa markedly correlated with substantial increases of cerebral capillary permeability in VD supplemented rats and mice. In contrast, mildly increased BBB permeability concomitant to reduced serum iCa was reported in the PTX rat model whereas exogenous administration of PTH fragment did not increase capillary permeability despite a considerable increase in serum iCa. These findings suggest deranged calcium regulation is unlikely the causative factor in the breakdown of capillary endothelium but may exacerbate cellular dysfunctioning via promotion of neurotoxic signalling cascades.

In neurodegenerative disorders such as AD, neuronal calcium-regulating systems are compromised (Foster, 2006). Talmor-Barkan et al. (2009) demonstrated hypocalcemia exacerbated vascular endothelial cell inflammation and thereby cellular function (Talmor-Barkan et al., 2009b). Recent *in vivo* studies by Hopp et al. (2015) reported chronic neuro-inflammation and neuronal calcium dysregulation synergistically modulate memory deficits and synaptic dysfunction (Hopp et al., 2015). Although not investigated in this study, loss of calcium-phosphate regulatory mechanisms may cause vascular calcification of the capillary endothelia and exacerbate the loss of BBB functionality. Indeed, Payne and colleagues have published reports on exaggerated serum iCa in association with cerebral white matter

lesions, greater brain lesion volumes and initiation of the apoptotic cascade (Payne et al., 2008; Payne et al., 2014).

Vitamin D has been reported to modulate intra-neuronal calcium homeostasis by controlling voltage-gated calcium channels (Annweiler et al., 2010; Garcion, Wion-Barbot, Monetero-Menei, Berger, & Wion, 2002). Higher serum iCa (upper end of normal range) levels was found correlated with better cognitive performance in Chapter 3. These results are in disagreement with the findings of Schram et al. (2007) and others who reported greater cognitive decline in older adults with higher serum iCa concentrations (Schram et al., 2007; Tilvis et al., 2004; van Vliet et al., 2009). It is possible calcium may exert a protective effect on cognitive function through its feedback regulation of VD homeostasis.

6.1.2.3 Potential neurovascular-beneficial effects of parathyroid hormone

Emerging evidence has implicated PTH in directly affecting neurovascular integrity independent of VD homeostasis. Significant association between serum concentrations of PTH and cerebral capillary integrity was reported in Chapter 2. In both intervention models of VD supplementation and parathyroid gland-ablation where serum levels of PTH were markedly suppressed, a direct neurovascular protective effect was purported. However these effects should not be considered alone as VD and PTH act as regulatory counterparts of one another. Indeed, many cases of hyperparathyroidism arise from VD deficiency. *In vitro* studies report that endothelial cells abundantly express PTH-receptor 1 and that PTH stimulates the modulation of endothelial cell proliferation by promoting the expression of vascular endothelial growth factor (VEGF) (Rashid, Bernheim, Green, & Benchetrit, 2007; Rashid et al., 2008; Throckmorton et al., 2002). Moreover, PTH has been implicated as a modulator of the functional activity of neurons (Khudaverdyan & Ter-Markosyan, 2000). Conversely, Hirasawa et al. (2000) demonstrated exaggerated concentrations of CSF-PTH led to cellular-iCa overload and consequently, neuronal cell death (Hirasawa et al., 2000).

Clinical primary hyperparathyroidism has been implicated as a primary risk factor of age-related cognitive deterioration and cognitive performance was found to improve upon correcting the cause of excessive PTH synthesis and secretion (Bjorkman et al., 2008; Roman et al., 2005). Serum PTH levels were not correlated with better cognitive performance in Chapter 3 and thus not assimilated to the findings of Chapter 2. Due to the nature of our study cohort with relatively normal VD - iCa - PTH homeostasis, the association between cognitive performance and serum PTH must not be excluded on this basis.

6.1.2.4 Conclusion

Cerebral capillary dysfunction is frequently observed in neurodegenerative disorders including AD and VaD. Reduced functionality of the BBB is associated with neuro-inflammatory changes that ultimately cause tissue damage and negatively impact specific domains of neurocognition. Indeed, increasing dementia research is focused upon vascular-related implications in delaying disease development and clinical progression.

As with all vitamin and nutrients, the complex interplay of many factors may contribute to hypervitaminosis D and compromised cerebral capillary permeability and neurocognitive performance. The major findings presented in this thesis provide an important platform in understanding potential mechanisms and possible treatment of AD/VaD relative to VD homeostasis and its hormonal counterparts. The main outcomes of this thesis were that hypervitaminosis D causes cerebral capillary aberrations in genetically un-manipulated rodents that may be associated with poorer cognitive performance. The findings reinforce the importance of establishing an optimal concentration for serum VD, particularly in older aged adults, as higher concentrations of VD are potentially detrimental. Thus based on the findings from the studies presented, the administration of VD supplementation should be considered with caution based on the paradoxical damaging effects that can occur when orally ingested in high doses. Further studies are required to elucidate the specific mechanisms involved in VD homeostasis and cerebral capillary dysfunction and cognitive performance.

6.2 Limitations of the present study

The genetically un-manipulated mouse and rats models used in this study are commonly used rodent models in the context of VD experimental research including dietary interventions and behavioural/cognitive studies. Whilst the distribution of the VD receptor and its regulatory enzyme, 1,α-hydroxylase, are closely distributed in the human and rodent adult brain, studies have indicated species differences in VD metabolism and the binding kinetics of 25(OH)D to VD binding protein (Eyles et al., 2005; Luine, Sonnenberg, & Christakos, 1987; McCann & Ames, 2008; Vieth et al., 1990). A limitation of the animal model studies was the requirement to dose rodent models with substantially greater VD dosage than what occurs in a clinical context. Thus the translation of study findings from animal to clinical studies must be carefully considered. Cognitive assessment was also not considered appropriate in the animal model studies because of confounders in extrapolation to human cognitive performance measures. The young age of rodents following dietary/surgical intervention and the uncertainty as to whether rodent cognitive measures truly assess behaviour, learning or explicit memory would limit consideration of whether hypervitaminosis D compromises cognitive performance as a consequence of cerebral capillary dysfunction.

The parenchymal abundance of plasma protein IgG was used as a surrogate marker of cerebral capillary permeability and GFAP was used to measure the extent of neurovascular inflammation/astroglial activation. As the semi-quantitative measurement of IgG extravasation is indicative only of cerebral capillary dysfunction, the microstructure of the capillary endothelia and astroglial end-feet should be further investigated to corroborate the study findings. Additionally, further exploration of the structural features of the neurovascular unit may be insightful.

The findings from the clinical study are valuable in context of the expanding VD research in the dementia field. The primary limitation of this study is that presently, there is no sensitive marker of cerebral capillary function (permeability) that can be readily measured and thus the results cannot be directly correlated with cognitive performance of the study population. A larger sample size and a longitudinal study design would be more informative in terms of blood sampling and

cognitive measurements. However, single samples and similar cross-sectional study designs have been reported previously (Buell et al., 2010; McGrath et al., 2007). As our cross-sectional cohort sample consisted of subjects free of major psychiatric illness and cognitive impairment, our findings may not truly represent general clinical populations at risk of cognitive decline or who already exhibit mild cognitive impairment, diagnosed memory or emotional disorders.

6.3 Future directions and clinical implications

The principle and novel findings presented in this thesis suggest a causal role of hypervitaminosis D and its hormonal counterparts in the modulation of cerebral capillary permeability and in a clinical context, poorer cognitive performance. The data derived from the integrated studies suggest that the use of supplementary VD should only be considered in context of VD deficiency. It is crucial the critical regulatory effects of VD on cerebral capillary structure and function are further investigated as this may enable the recommendation for the use of VD supplements in the context of cerebrovascular integrity.

The effects of VD and its metabolite concentrations vary among individuals based on the genetic variation of the VD receptor, which has been implicated with susceptibility of age-related changes in cognitive function and depressive symptoms in the elderly (Kuningas et al., 2009). The measurement of the pro-hormone, 25-hydroxyvitamin D (25(OH)D) is universally accepted as the marker of vitamin D status due to its bioavailability and stable half-life when compared to its' active metabolites. However, whilst circulating levels of the prohormone may reflect a longer half-life, it does not represent the circulating levels of the physiologically relevant vitamin, calcitriol, responsible for transcription of the genomic and non-genomic actions of VD. Indeed, may be marker of poor health (reverse causation) rather than a risk factor of adverse cognitive outcomes. Further studies need to identify and validate the most suitable VD 'analyte' in a neurocognitive context to allow for recommendations of 'optimal' VD concentrations.

To further expand on the findings from the animal studies, trans-endothelial blood-to-brain protein kinetics need to be considered in detail to determine the mechanisms underlying VD-mediated BBB dysfunction. From a translational perspective, randomised controlled clinical trials involving VD and cognition are lacking in the literature and warrants further investigation. Findings from randomised controlled trials may provide valuable insight on the paradoxical mechanisms of VD involved in cognitive performance and contribute to development of treatment strategies.

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Author and co-author contributions for each paper have been stated accordingly and indicated in the section below. All authors read and approved the final version of each manuscript prior submission and publication.

Publication 1:

Lam V., Takechi R., Pallebage-Gamarallage M., Giles C., & Mamo J. C. (2015). The vitamin D, ionised calcium and parathyroid hormone axis of cerebral capillary function: therapeutic considerations for vascular-based neurodegenerative disorders. *PLoS One, 10*(4), e0125504.

To Whom It May Concern:

I, Virginie Lam, was responsible for generating the study design, experimental procedures, data collection, data analysis and interpretation, and manuscript preparation for the publication entitled **'The vitamin D, ionised calcium and parathyroid hormone axis of cerebral capillary function: therapeutic considerations for vascular-based neurodegenerative disorders.** ***PLoS One, 10*****(4), e0125504'**.

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Publication 2:

Lam V., Albrecht M. A., Takechi R., Prasopsang P., Lee Y. P., Foster J. K., & Mamo J. C. L. (2016). Serum 25-hydroxyvitamin D is associated with reduced cognitive performance in healthy, middle-aged and older adults. *European Journal of Nutrition*, *55*(4), 1503-1513.

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Licensed content title	Serum 25-hydroxyvitamin D is associated with reduced verbal episodic memory in healthy, middle-aged and older adults
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Licensed content date	Jan 1, 2015
Type of Use	Thesis/Dissertation
Portion	Full text
Number of copies	1
Author of this Springer article	Yes and you are a contributor of the new work
Order reference number	None
Title of your thesis / dissertation	An investigation of a vitamin D, ionised calcium and parathyroid hormone regulatory axis of cerebral capillary function: Implications for cognitive performance in ageing
Expected completion date	Aug 2015
Estimated size(pages)	200
Total	0.00 USD
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I, Virginie Lam, contributed to the conception and design of the project, experimental procedures, ethical approval, patient recruitment, data collection, data analysis and interpretation, and prepared the manuscript for the publication entitled **'Serum 25-hydroxyvitamin D is associated with reduced cognitive performance in healthy, middle-aged and older adults.** ***European Journal of Nutrition,*** **55(4), 1503-1513'**.

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Publication 3:

Lam V., Dhaliwal S. S. & Mamo J. C. (2013). Adjustment of ionized calcium concentration for serum pH is not a valid marker of calcium homeostasis: implications for identifying individuals at risk of calcium metabolic disorders. *Annals of Clinical Biochemistry, 50*(Pt 3), 224-229.

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Lam V., Albrecht M. A., Takechi R., Giles C., James A. P., Foster J. K., & Mamo J. C. (2013). The serum concentration of the calcium-binding S100B is positively associated with cognitive performance in older adults. *Frontiers in Aging Neuroscience, 5*, 61.

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Lam V., Albrecht M. A., Takechi R., Heidari-Nejad S., Foster J. K., & Mamo J.C. (2014). Neuropsychological performance is positively associated with plasma albumin in healthy adults. *Neuropsychobiology, 69*(1), 31-38.

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Appendix II: Participant Information and Consent Form

Novel plasma biomarkers that may identify subjects at risk of developing Alzheimer's disease

Participant Information Sheet/Consent Form

You have been asked to participate in this study because you are either aged between 20 and 80 years old, or concerned about your memory, or have been diagnosed with mild cognitive impairment. Please read this document carefully and ask any questions that you wish. Do not sign this consent form unless you fully understand the study and any possible side effects involved.

Background information

With the increased life expectancy of the aging population, the occurrence of Alzheimer's disease (AD) and dementia are expected to increase substantially. There are currently more than 35 million people living with dementia and this number is expected to increase to 115 million by 2050. Strategies to reduce the incidence of dementia are therefore urgently required.

Early detection may be helpful to slow and perhaps one day, effectively treat Alzheimer's disease and dementia. Our laboratory recently developed a blood-test which may be helpful in identifying individuals at risk of developing Alzheimer's disease and in this study, we wish to investigate this possibility further, by sampling a larger number of individuals.

What does the study involve?

If you are below the age of 50 years, your participation involves one visit to Curtin University. Participants older or equal to 50 years, may need to visit Curtin University on two separate occasions. On the first visit, we will collect one fasting blood sample between 8:00 -10:00am (20ml, or approximately 1.5 tablespoons). We will also record your weight, height, waist circumference and blood pressure.

All participants will be required to complete a questionnaire principally on diet and exercise, but we will also collect some lifestyle, medical history and medications that are relevant to the research objectives. This questionnaire takes approximately 30min to complete and can be done online, submitted by email, or if you prefer by regular mail.

For participants over 50 years of age, you will also be asked to complete questionnaires that will give researchers an indication of your ability to think and remember. The questionnaires take approximately one hour to complete. If you are in this category of participants, your total time commitment at Curtin University (including taking of blood) will be approximately 2 hours.

On the day prior to your visit you will need to consume your dinner before 8pm and fast from that time onwards. Please continue drinking water to stay well hydrated.

Possible adverse effects

There may be slight discomfort associated with blood collection. In some people, slight bruising and tenderness may appear at the site of blood collection but will disappear within several days.

Ability to withdraw from study

Your participation in this study is entirely voluntary; you are free to withdraw from the study at any stage. It is important that you do not feel any pressure to complete the study particularly if it is not what you had originally anticipated.

Benefits to the participant

By taking part in this study, you will gain useful information about your current lipid and cholesterol levels. By agreeing to participate in the study, you are giving us permission to notify your General Practitioner if we identify abnormal levels of lipids or cholesterol in your blood.

We don't yet know whether our new blood-test truly identifies people at risk of developing Alzheimer's disease, so this information will not be disclosed to you, or to your doctor. Rather, only the researchers involved in this study will have access to this new data and your identification will never be disclosed.

Your participation in this study will provide us with important data enabling us to possibly identify individuals at risk of developing Alzheimer's disease.

All information will be strictly confidential and any publications arising from this work will not include your name or any identifying feature.

If you have any queries or require further clarification, please contact the lead investigator:

Professor John Mamo

School of Public Health, Curtin University.

Phone: 92667232

Email: J.Mamo@Curtin.edu.au

Urgent contact: Reception at School of Public Health, 92667819

This study has been approved by the Curtin University Human Research Ethics Committee (Approval number: HR97/2011). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. Its main role is to protect participants. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University of Technology, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au

I,..... agree to participate in the above study. I have read and understood the Study Information and I have been given a copy of it. I understand the risks associated with participation in this study. I have been given the opportunity to ask questions about the study. I understand that I may withdraw from the study at any time.

Signed.....
Date.....

Signature of Investigator.....
Date.....