Plasma cyclic-glycine-proline/IGF-1, a potential biomarker for circulating IGF-1 function and its applications in neurological conditions

New insights into the regulation of IGF-1 function in neurological conditions

Dawei Fan

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science, The University of Auckland, 2019.

Abstract

Circulating insulin-like growth factor-1 (IGF-1) has a role in stroke and maintenance of cognition. Therefore, biomarkers for circulating IGF-1 function may assist the monitoring and prognosis of stroke recovery and progression of cognitive impairment in diseases, such as Parkinson's disease (PD). Current biomarkers for IGF-1 function are not reliable and an alternative is cyclic-glycine-proline (cGP)/IGF-1 molar ratio, which potentially could be applied to neurological conditions. This thesis investigates the relationship of plasma cGP/IGF-1 with stroke recovery and cognitive impairment in PD. The oral bioavailability of cGP-rich blackcurrant anthocyanin (BCA) is also investigated.

The concentration of cGP, IGF-1 and insulin growth factor binding protein-3 (IGFBP-3) were measured using ELISA and HPLC-MS in: 1) plasma collected from stroke patients (n = 34) within 3 days of stroke onset (baseline), 7 days and 90 days after stroke and controls. 2) plasma from PD patients across the range of cognitive function, normal cognitive function (PD-N, n = 74), mild cognitive impairment (PD-MCI, n = 71) and dementia (PD-D, n = 33), and controls (n = 23). And 3) plasma and cerebrospinal fluid (CSF) from idiopathic PD patients with normal cognition (n = 10) before and after a 28-day supplementation with BCA.

In stroke patients, baseline cGP/IGF-1 was lower compared to the controls, then gradually increased over the initial 90 days of stroke recovery, paralleled with improved neurological scores. High baseline cGP/IGF-1 predicted improved NIHSS score. In PD, plasma cGP/IGF-1 was positively associated with age in PD-N and negatively associated with age in PD-D. Plasma IGF-1 or IGF-1/IGFBP-3 did not change during stroke recovery and had no altered relationship with age in PD. Following the BCA supplementation, CSF cGP increased by around 20 %.

These data suggest plasma cGP/IGF-1 molar ratio as a new biomarker for circulating IGF-1 function and its potential application to stroke and cognitive status in PD. Given that stroke and cognition are closely related to cerebrovascular function and integrity, this biomarker may be applicable to other neurological conditions with changes in cerebral vascular function. cGP is oral bioavailable. These findings would benefit from replication in larger cohorts with longer follow-up.

List of publications generated from this thesis

Journal articles:

1. Fan, D., Alamri, Y., Liu, K., MacAskill, M., Harris, P., Brimble, M., … Guan, J. (2018) Supplementation of Blackcurrant Anthocyanins Increased Cyclic Glycine-Proline in the Cerebrospinal Fluid of Parkinson Patients: Potential Treatment to Improve Insulin-Like Growth Factor-1 Function. Nutrients 10(6), 714. (impact factor 4.17)

2. Fan, D., Krishnamurthi, R., Harris, P., Barber, P. A. and Guan, J. (2019) Plasma cyclic glycine proline/IGF-1 ratio predicts clinical outcome and recovery in stroke patients. Ann Clin Transl Neurol, 6: 669-677. (impact factor 4.66)

3. Fan, D., Pitcher, T., Dalrymple-Alford, J.C., MacAskill, M., Anderson, T., Guan, J. (2020) Changes of plasma cGP/IGF-1 molar ratio with age is associated with cognitive status of Parkinson's disease. Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring 12(1), e12025. (impact factor 5.2)

Conference presentations:

1. Fan, D. et al (2017) Cyclic glycine-proline (cGP) increased in the cerebrospinal fluid of Parkinson patients after supplementation of blackcurrant anthocyanins: a potential biomarker for treatment. In 'Australasian Winter Conference on Brain Research, Queenstown, New Zealand;

2. Fan, D., Krishnamurthi, R., Harris, P., Barber, P. A., & Guan, J. (2018). The changes of Cyclic Glycine Proline, a biomarker for IGF-1 function, may predict stroke recovery. In Biomarker Summit, San Diego, CA.

Acknowledgements

I would like to express my thanks and appreciation to my supervisors Dr. Jian Guan and Dr. Toni Pitcher. It is under their guidance that I have completed the thesis. Your continual feedback, encouragement and support make the completion of this thesis possible. Dr. Toni Pitcher spent a lot of time checking the database and organising the shipment of samples from PD patients. A special thanks to my primary supervisor, Dr Jian Guan, who has been a tremendous mentor to me since the time I transferred to her supervision and initiated a new PhD project. Without her considering me for this PhD project, I could not stay at the University of Auckland to continue my research career. Moreover, your skills in analysis and interpretation of data I have learnt is critical and useful for my future career.

This research would not have been possible without the generous contribution of many. I would like to express my deep appreciation for the support of the Brain Research Institute in the collection of samples and clinical information from PD patients, and the platform of the Biomarkers and Recovery in Stroke (BARISTa), Brain Research New Zealand-A Centre of Research Excellence in the collection of samples and clinical information from stroke patients. I also appreciate funders including the Brain Research New Zealand, the Vitality New Zealand and the University of Auckland. My thanks are extended to Professor Cathy Stinear, Professor John Dalrymple-Alford, Dr Michael MacAskill, Ying Huang, Bert van der Werf for advice on data analysis.

I thank my friends and colleagues at the Liggins Institute. They provided me with much-needed support, advice and technical expertise. In particular, I am grateful for Eric and Karen, who not only trained me when I first started this programme but has also been a constant source of encouragement and support. I thank colleagues from the Pharmacology Department for the needed support and advice. In particular, Angela supported me for the collection of samples

from stroke patients and controls. I also thank my friends and colleagues at the Discipline of Nutrients, who helped me go through a tough time before I transferred to the new team and project.

I probably cannot thank my family enough; without them, I would have not been able to complete this thesis. Big thanks to my lovely wife, Meimei, who encouraged and supported me, and tolerated my bad-mood days. I am sorry for worrying you when I was depressed. I am wholeheartedly grateful to Mom and Dad, who supported me both financially and emotionally throughout my studies in New Zealand.

I would also like to acknowledge the financial support of the Chinese Scholarship Council (CSC) and the School of Medical Sciences for providing the scholarships, and the Brain Research New Zealand, Health Research Council New Zealand, Auckland Medical Research Foundation and the Vitality New Zealand for providing funds for the research program.

Table of Contents

List of Figures

List of Tables

Co-authorship forms

School of Graduate Studies
AskAuckland Central
Alfred Nathan House
The University of Auckland
Tel: +64 9 373 7599 ext 81321
Email: postgradinfo@auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work**. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. Chapter 3:Plasma cyclic Glycine Proline/IGF-1 ratio predicts clinical outcome and recovery in stroke patients Fan D, Krishnamurthi R, Harris P, Barber PA, Guan J. Plasma cyclic glycine proline/IGF-1 ratio predicts clinical outcome and recovery in stroke patients. Annals of Clinical and Translational Neurology 2019;0. doi:10.1002/acn3.743.

Nature of contribution by PhD candidate	conducted the experiment, performed the laboratory experiments, analysed the data and wrote the manusript
Extent of contribution by PhD candidate (%)	80%

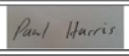
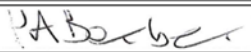
CO-AUTHORS

Name	Nature of Contribution
Rita Krishnamurthi	helped revision of the manuscript
Paul Harris	helped revision of the manuscript
Alan Barber	helped in experimental design and revision of the manuscript
Jian Guan	designed the experiment and assisst in writing and revision of the manusript

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Rita Krishnamurthi		08/06/2019
Paul Harris		01/07/2019
Alan Barber		19/03/2019
Jian Guan		01/07/2019

Last updated: 28 November 2017

THE UNIVERSITY OF AUCKLAND
Te Whare Wānanga o Tāmaki Makaurau
NEW ZEALAND

School of Graduate Studies
AskAuckland Central
Alfred Nathan House
The University of Auckland
Tel: +64 9 373 7599 ext 81321
Email: postgradinfo@auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work**. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. Chapter 4: Changes of plasma cGP/IGF-1 molar ratio with age is associated with the cognitive status of Parkinson's disease Fan, D., Pitcher, T., Dalrymple-Alford, J.C., MacAskill, M., Anderson, T., Guan, J. (2020) Changes of plasma cGP/IGF-1 molar ratio with age is associated with cognitive status of Parkinson's disease. Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring 12(1), e12025.

Nature of contribution by PhD candidate	conducted the experiment, performed the laboratory experiments, analysed the data and wrote the manusript
Extent of contribution by PhD candidate (%)	90%

CO-AUTHORS

Name	Nature of Contribution
Toni Pitcher	helped data collection and revision of the manuscript
John Dalrymple-Alford	helped revision of the manuscript
Michael MacAskill	helped in experimental design
Tim Anderson	helped in experimental design and revision of the manuscript
Jian Guan	designed the experiment and assisst in writing and revision of the manusript

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Toni Pitcher		22 Jul 2020
John Dalrymple-Alford		12 Jul 2020
Michael MacAskill		15 Jul 2020
Tim Anderson		12 Jul 2020

Last updated: 28 November 2017

Jian Guan	Jian Guan	19 Jul 2020

THE UNIVERSITY OF AUCKLAND
Te Whare Wānanga o Tāmaki Makaurau
NEW ZEALAND

School of Graduate Studies
AskAuckland Central
Alfred Nathan House
The University of Auckland
Tel: +64 9 373 7599 ext 81321
Email: postgradinfo@auckland.ac.nz

Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work**. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work. Chapter 5 Fan D, Alamri Y, Liu K, MacAskill M, Harris P, Brimble M, et al. Supplementation of Blackcurrant Anthocyanins Increased Cyclic Glycine-Proline in the Cerebrospinal Fluid of Parkinson Patients: Potential Treatment to Improve Insulin-Like Growth Factor-1 Function. Nutrients 2018;10:714. doi:10.3390/nu10060714.

Nature of contribution by PhD candidate	performed the laboratory experiments, analysed the data and wrote the manusript
Extent of contribution by PhD candidate (%)	90%

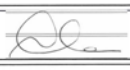
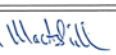
CO-AUTHORS

Name	Nature of Contribution
Yassar Alamri	conducted clinical trial, collected samples, and helped revision of the manuscript
Karen Liu	helped teach biological measurement
Tim Anderson, John Dalrymple-Alford and Michael MacAskill	designed the clinical trial and helped revision of the manuscript
Paul Harris and Margaret Brimble	designed and synthesized the internal standard for cGP assay
Tim Prickett, Oliver Menzies and Andrew Laurenson	assisted sample collections
Jian Guan	conceived and designed the biological experiment, and assissted in writing and revision of the manusript

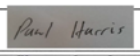
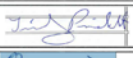
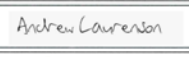
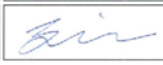
Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Yassar Alamri		01/07/2019
Karen Liu		1/7/2019
Tim Anderson		3/7/19
John Dalrymple-Alford		01 July 2019
Michael MacAskill		20 Aug 2019

Last updated: 28 November 2017

Paul Harris		1 July, 2019
Margaret Brimble		1 July, 2019
Tim Prickett		1-7-19
Oliver Menzies		1 July, 2019
Andrew Laurenson		1 July, 2019
Jian Guan		1st July 2019

List of Abbreviations

ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
BBB	Blood-brain barrier
BCA	Blackcurrant anthocyanin
cGP	Cyclic-glycine-proline
CNS	Central nervous system
CSF	Cerebrospinal fluid
ELISA	Enzyme-linked immunosorbent assay
FM-UL	Fugl-Meyer Upper Limb Assessment Scale
GPE	Glycine-proline-glutamate
HI	Hypoxia-ischemia
HPLC-MS	High-performance liquid chromatography-mass spectrometry
H&Y	Hoehn and Yahr scale
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IGFBP	Insulin-like growth factor binding protein
LED	Levodopa equivalent dose

MDS-UPDRS III	Movement disorder society-Unified Parkinson's disease rating scale part III
MoCA	Montreal Cognitive Assessment
mRS	Modified Rankin Scale
mTorr	Millitorr
NIHSS	National Institutes of Health Stroke Scale
PD	Parkinson's disease
PD-D	Parkinson's disease with dementia
PD-MCI	Parkinson's disease with mild cognitive impairment
PD-N	Parkinson's disease with normal cognition

1 Introduction

1.1 Biomarker

A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of physiological processes, pathological processes or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). The very common clinical biomarkers are blood pressure and glucose levels. Brain images have also been used as a biomarker showing pathological changes in the brain without an autopsy to assist the diagnosis of neurodegenerative diseases (Shimizu et al., 2018). In the era of molecular biology, biomarkers usually are referred to as molecular biomarkers, measurable alterations on DNA, RNA, protein and metabolite levels.

1.1.1 Sources of biomarkers for neurological conditions

Several sources can provide information on alterations of molecular levels, such as body fluid, tissues and brain images. Body fluids such as plasma, serum, cerebrospinal fluid (CSF) and urine are commonly used for the identification of biomarkers. Compared with the collection of CSF, it is easier and less invasive to collect blood and it is more acceptable for patients to collect blood than CSF. The collection of urine samples has no risk and is non-invasive. Moreover, patients with a good motor and cognitive function can collect urine themselves. Nevertheless, many patients with neurological conditions such as stroke and dementia suffer from seriously impaired motor and cognitive function. Therefore, it is not convenient for these patients to provide urine samples and for researchers to study urine biomarkers. Additionally,

saliva and peripheral tissue have also been used to find biomarkers (Beach et al., 2016; Walton, 2018), but they are not as common as blood in neurological conditions.

1.1.2 Characteristics of an ideal biomarker

The characteristics of an ideal biomarker for reflecting a change in pathological or clinical trait X (Michell et al., 2004):

- should be specific to a change in X
- must be sensitive so that even small changes in X can be reflected
- changes linearly responding to a change in X
- its measurements should be reproducible at a different time or in a different center

An ideal biomarker also should be easy to measure and give a quick and reliable readout.

1.1.3 Application of biomarkers

In clinical practice, reliable biomarkers would contribute to diagnosis and prognosis of diseases, monitoring disease progression and treatment, and thus guiding subsequent therapy or clinical care. For example, the availability of molecular biomarkers would facilitate the diagnosis of psychiatric disorders which is currently based on symptoms and interview-based communications (Filiou & Turck, 2011). For another example, the evaluation of stroke prognosis is usually based on the judgment of the assessing doctor. However, evidence has shown that clinicians are often over-optimistic about recovery, with only 65% of patients who are predicted to recover

gaining independence by one year after stroke (Counsell et al., 2004). Therefore, it is necessary to exploit other tools, such as biomarkers, to assist the doctors in the prediction of stroke recovery. Reliable predictions about stroke outcomes can provide clinicians with guidance to make decisions about medical treatment and patient management as well as allowing assessment of the performance of stroke services.

Cognitive impairment in Parkinson's disease (PD) patients is now a recognized primary problem affecting patient and carer well-being (Jones et al., 2017; Lawson et al., 2014). A reliable plasma biomarker for predicting and tracking the changes of cognitive status may assist to identify those at greater risk of developing cognitive impairment, and thereby the window of opportunity for suitable intervention.

For intervention study, changes in biomarkers can give us a hint about the effects of certain treatment or supplementation. For example, a recent clinical study in Alzheimer's disease (AD) patients measured changes of biomarkers of oxidative stress and inflammation such as nitric oxide and C-reactive protein to indicate the effects of 12-weeks supplementation of probiotic (Akbari et al., 2016). In addition, biomarkers can predict the likelihood that a patient will benefit from an intervention. For example, a RAS mutation in the tumor can predict lack of benefit from anti-epidermal growth factor receptor (EGFR) therapy in colorectal cancer (Allegra et al., 2009; Douillard et al., 2013). Therefore, patients with this mutation do not have a response to anti-EGFR therapy. Identifying the patients who are lack of benefit from intervention can assist physicians to choose different treatment, improve outcomes and decrease medical costs.

1.2 The IGF family

The family of IGF consists of two polypeptides, IGF-1, the adult form, and IGF-2, the major fetal form (Bergman et al., 2013). This study focuses on evaluating a biomarker for IGF-1 function due to the interaction of cGP with IGFBP-3. cGP is a metabolite of IGF-1 and is not associated with IGF-2. Thus, IGF-2 is not reviewed. IGF-1 binds to IGF receptors (IGFRs) and insulin receptor, but only to IGF-1R with the highest affinity, to activate signalling pathways (Philippou, Maridaki, Pneumaticos, & Koutsilieris, 2014; Sherbet, 2011). To date, at least six distinct homologous IGF binding proteins (IGFBPs) classified as IGFBP 1-6 are identified and bind IGFs with higher affinities than that of IGFs to receptors (Duan & Xu, 2005; Fernandez & Torres-Alemán, 2012; Kelley et al., 2002). They have been detected in various tissues including the brain (Fernandez & Torres-Alemán, 2012) and biological fluids, like plasma and CSF (Juul, 2003). IGFBP-3 is the most abundant IGFBP in blood, carrying 75% or more of serum IGF-1 and IGF-2 (Binoux, 1995; Firth & Baxter, 2002). These binding proteins translocate IGF-1 to targeting tissues and protect IGF-1 from being metabolized (Duan & Xu, 2005; Kelley et al., 2002). However, IGFBP-3 prevents IGF-1 from activating its receptor due to a higher affinity with IGF-1 than IGF-1R (Fernandez & Torres-Alemán, 2012). Whether other IGFBPs are involved in regulating circulating IGF-1 bioavailability is not clear. The regulation of IGF-1 function has been mainly described in 1.5.1.

IGF-1 function is mediated through activating IGF-1 receptors, which is widely distributed in the CNS, with the highest levels in cerebral vessels and choroid plexus of the adult brain (Fernandez & Torres-Alemán, 2012). The binding to α sub-units of IGF-1R phosphorylates the β sub-units, which triggers its downstream signal cascades leading to IGF-1 induced function (Laviola et al., 2007). For example, the activation

of IGF-1R in vessels can modulate cerebral vascular remodeling, maintain cerebral vascular function and offer vascular protection from injury (Bach, 2015; Guan et al., 2014; Lopez-Lopez et al., 2004). The vascular protection of IGF-1 has been suggested to be the underlying mechanism of stroke recovery (Zhu et al., 2008). The receptor in vessels and choroid plexus is also related to the transportation of unbound IGF-1 released from binding proteins in plasma into the brain (Fernandez & Torres-Alemán, 2012; Nishijima et al., 2010).

In blood and the brain, unbound IGF-1 enzymatically breaks down into des (1-3) N-IGF-1 and N-terminal tripeptide GPE, which is the key binding site for interaction between IGF-1 and IGFBPs (Guan et al., 2015; Yamamoto & Murphy, 1995). GPE does not interact with the IGF-1 receptor (Sara et al., 1989). Although GPE was initially considered a non-bioactive by-product of IGF-1, exogenous application of GPE has shown a neuroprotective effect in the rat model of PD and ischemic stroke (Guan et al., 1999; Guan, Krishnamurthi, et al., 2000; Guan & Gluckman, 2009). However, its clinical practice is limited since GPE has a very short half-life time due to the enzymatic instability (Batchelor et al., 2003). GPE exists as an 80:20 *trans: cis* isomeric mixture (Figure 1.1) and the *cis* isoform of GPE is further rapidly metabolized into single amino acids and dipeptides including cGP (Guan et al., 2015; Guan & Gluckman, 2009).

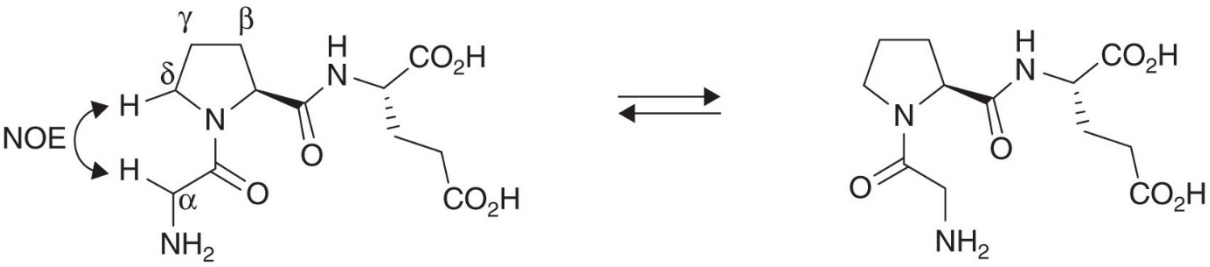


Figure 1.1 The *trans* and *cis* isoform of GPE

cGP has been isolated from adult rat brain tissue (Gudasheva et al., 1996) and is also present in rat milk and plasma (Singh-Mallah et al., 2016). In contrast to IGF-1 and GPE, cGP is a relatively small peptide with cyclic structure (Figure 1.2) and thus has improved paracellular permeability and resistance to enzymatic breakdown (Guan et al., 2015).

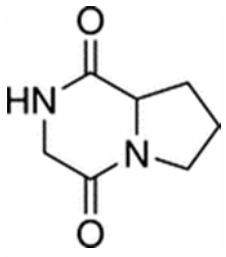


Figure 1.2 The chemical structure of cGP

Moreover, the concentrations of cGP analog, cGly-2allyl-Pro, in CSF from normal rats were found similar to those in plasma after single intravenous injection, suggesting the central uptake of cGly-2allyl-Pro is independent of injury and easy to access to the CNS (Guan et al., 2007). It is recently reported that the bioactivity of cGP is mediated through regulating IGF-1 bioavailability by interfering with IGF-1 binding to the IGFBP-3 (Guan et al., 2014).

1.3 The role of IGF-1 in stoke and cognitive impairment

1.3.1 IGF-1 in stroke

1.3.1.1 Stroke and its recovery

Traditionally, stroke is defined based on the acute focal neurological dysfunction by a vascular cause including infarction or hemorrhage in the CNS (Hankey, 2017). An updated definition is an acute episode of focal dysfunction in the CNS with more than 24 hours, or any duration if imaging or autopsy show focal infarction or hemorrhage related to the symptoms (Sacco et al., 2013). According to pathology, there are two subtypes of stroke including ischemic stroke and hemorrhagic stroke (Hankey, 2017). The majority of strokes are ischemic and have a multitude of possible causes and 60% of ischemic strokes result from obstruction of a blood vessel caused by a locally-formed blood clot and about 25% are attributed to obstruction by an embolus from elsewhere in the body (Mohr et al., 2011). The etiological subtypes classification of ischemic stroke is generally according to the Trial of Org 10172 (danaparoid sodium, low molecular weight heparinoid) in Acute Stroke Treatment (TOAST) criteria, the ASCOD phenotyping system (A: atherosclerosis; S: small-vessel disease; C: cardiac pathology; O: other cause; D: dissection), and the Causative Classification System (Hankey, 2017). The most effective early treatment for acute ischemic stroke is recombinant tissue plasminogen activator (tPA), which aims to timely restore blood flow in patients so that long-term morbidity is reduced (Powers et al., 2015). However, tPA intervention has a short window opportunity of a few hours after the onset of stroke. Mechanical thrombectomy has also been adopted to remove a blood clot from a blood vessel. When patients are clinically stable after stroke, they are often transferred for rehabilitation to experience a progressive, dynamic, goal-oriented

process aimed at enabling a person with impairment to reach their optimal functional level (Richards et al., 2015). A common impairment after stroke is motor impairment and the recovery of motor function after stroke is crucial for the patient to regain independence (Langhorne et al., 2009).

Stroke recovery is a complex process that can last for months or years after stroke (Langhorne et al., 2011). Many surviving patients make at least a partial recovery in function over the first 3 months, a critical period for the recovery (Lee et al., 2015). Spontaneous recovery referring to the 'self-made' recovery particularly during the first 3 months after stroke is an important process involved in stroke recovery and begins hours after stroke onset (Langhorne et al., 2011). Several cohort studies suggest that recovery of motor functions is predictable in the first days after stroke (Mattlage et al., 2016; Nijland et al., 2010; Stinear, 2017; Tang et al., 2014). It is likely to involve the ability of spontaneous recovery, which is partly, if not all, dependent on neurotrophic and neuroprotective factors, such as IGF-1 (Carro et al., 2003; Guan et al., 2015; Larpthaveesarp et al., 2015). The cellular events underlying spontaneous recovery including structural changes in axons, dendrites and synapses, angiogenesis, neuronal sprouting (Cramer, 2008). IGF-1 has been shown to be involved in these events (Dyer et al., 2016). After a three-month spontaneous recovery period, recovery slows down and reaches a plateau in three to six months after stroke (Richards et al., 2015).

1.3.1.2 Evidence for the role of IGF-1 in stroke recovery

Administration of IGF-1 protects the brain from ischemic injury and improves sensory and motor functions in various stroke models (Guan et al., 2003). IGF-1 accumulates around the blood vessels of damaged brain regions within a few hours after hypoxic-ischemic injury (Beilharz et al., 1998). The expression of IGF-1 is also

found increased in the brain tissue 1-5 days after hypoxic-ischemic (HI) brain injury in rats (Gluckman et al., 1992) and this may contribute to a partial recovery of sensory-motor function (Guan et al., 2001). Timely administration of human recombinant IGF-1 within 6 hours after hypoxic-ischemic injury reduces brain damage and improves long-term sensory-motor function (Gluckman et al., 1992; Guan et al., 1993, 2001; Guan, Gunn, et al., 2000).

The precise mechanisms underlying the role of IGF-1 in stroke recovery are not totally clarified. IGF-1 in the blood is binding to receptors in the endothelial cells of brain vessels and could be internalized into the brain to protect neurons (Fernandez & Torres-Alemán, 2012). Alternatively, peripheral IGF-1 can act on brain vessels to protect neurons without entering the parenchyma (Bake et al., 2016).

1.3.2 IGF-1 and cognitive function

1.3.2.1 Cognitive impairment in PD

Cognitive functions are mental processes related to gathering and processing information and it is well-known that normal people tend to have a decline in cognitive function during aging (Harada et al., 2013). Cognitive impairment is common in a range of neurological disorders, including stroke and PD. Although the cellular changes are thought to relate to cognitive impairment, increasing evidence shows that cerebrovascular changes have an influence on cognitive function in the general population and PD patients (Gorelick et al., 2011; Kapasi & Schneider, 2016; Malek et al., 2016; Pilotto et al., 2016). The main pathological features of PD are progressive loss of neurons within the substantia nigra and the presence of Lewy bodies and Lewy neurites (Kalia & Lang, 2015). The majority of PD cases are sporadic, having no specific known cause and only less than 10% of PD cases are familial and caused by

mutations in certain genes (Thomas & Beal, 2007). Recent studies also suggest a contribution of fungal infections to PD (Pisa et al., 2016, 2020). The clinical features during PD progression include both motor symptoms and diverse non-motor symptoms (Kalia & Lang, 2015). The diagnosis of PD is primarily based on the presence of motor symptoms in PD patients.

Although the dominant features early in the disorder for most PD patients are motor symptoms, there is an increasing concern about non-motor symptoms in PD. Cognitive impairment in PD patients is now recognized as a primary problem affecting the well-being of both patients and caregivers (Jones et al., 2017; Lawson et al., 2014). Mild cognitive impairment (MCI) is the stage between the expected cognitive decline in normal aging and the more serious cognitive impairment, dementia. In PD, mild cognitive impairment often occurs since the early stage of the disease with impairment of multiple cognitive domains (Santangelo et al., 2015). PD patients with MCI (PD-MCI) are at increased risk of developing PD dementia (PD-D) (Wood et al., 2016). A large proportion of patients with MCI develop dementia in a relatively short period of time (Litvan et al., 2011).

1.3.2.2 Evidence for the role of IGF-1 in the cognitive function

Although the underlying mechanisms are not clear in cognitive dysfunction, studies have shown the actions of IGF-1 on cognitive function. Intracerebroventricular administration of IGF-1 to normal aged rats improved or restored age-related cognitive deficit (Markowska et al., 1998; Morel et al., 2017; Pardo et al., 2016). Intracerebroventricular administration of IGF-1 also increases the levels of neurogenesis in the hippocampal dentate gyrus where is related to learning and memory performance (Morel et al., 2017). Plasma unbound IGF-1 released from

binding proteins can enter CSF by a transcytosis process after binding to the IGF-1 receptor in the choroid plexus (Fernandez & Torres-Alemán, 2012). It can also enter parenchyma through blood-brain barrier (Fernandez & Torres-Alemán, 2012). It has been found that the entry of IGF-1 into the brain parenchyma is through the interaction of IGF-1 receptors with low-density lipoprotein receptor-related protein-1 (Nishijima et al., 2010). Prolonged systematic administration of IGF-1 can also ameliorate cognitive impairment in endocrine IGF-1-deficient mice (Trejo et al., 2007) and improved spatial memory (Sonntag et al., 2005). On the contrary, inhibition of blood-borne endocrine IGF-1 in adult and aged mice by inactivating IGF-1 production in the liver can lead to a decline of cognitive function (Svensson et al., 2006).

Human clinical intervention studies show more direct IGF-1 effects on cognitive function in normal aging. People treated with daily growth hormone have increased circulating IGF-1 concentrations and improved cognitive functions compared to those in the placebo group (Vitiello et al., 2006). Given PD is an age-related neurological condition, the decline of IGF-1 with age might contribute to cognitive impairment in PD.

1.4 Current biomarkers under investigation for IGF-1 function in stroke and cognitive impairment

Given the association of IGF-1 with the neurological conditions, biomarkers for IGF-1 function could be applied to these neurological conditions. Thus, it is interesting to find reliable biomarkers for IGF-1 function.

1.4.1 Plasma total IGF-1

As mentioned above, only unbound IGF-1 is bioactive and associated with IGF-1 function. Moreover, concentrations of circulating unbound IGF-1 cannot be measured

due to swiftly enzymatic breakdown and internalization mediated by receptors (Fernandez & Torres-Alemán, 2012; Fowlkes et al., 2004; Nishijima et al., 2010). Thus, circulating total IGF-1 has been investigated as a biomarker for IGF-1 function.

Circulating total IGF-1 has been studied to predict recovery of stroke. A study of 42 patients during rehabilitation after acute stroke found that circulating IGF-1 might predict functional performance during rehabilitation and ischemic stroke outcome due to a relationship between higher levels of IGF-1 and improvement in functional and cognitive scores (Bondanelli et al., 2006). Similarly, by measuring serum samples from 255 patients within six hours after stroke onset, another study showed that subjects with high IGF-1 levels had better neurological and functional outcomes at three months (De Smedt et al., 2011). Furthermore, a relationship between unfavorable outcomes and significantly decreased serum IGF-1 levels on admission was found in a study of 168 patients (Tang et al., 2014). Nevertheless, a study assessing functional outcome one year after ischemic stroke suggested that circulating IGFBP-3 but not IGF-1 levels had an association with functional outcome (Ebinger et al., 2015).

Studies on the relationship between circulating IGF-1 and cognition in PD patients are just at the beginning stage. Although these studies show a negative correlation of circulating IGF-1 with cognitive function in PD (Ma et al., 2015; Picillo et al., 2017), most human observational studies measuring circulating IGF-1 concentrations have mixed results on the correlation of circulating IGF-1 with cognitive function in general population (Frater et al., 2017). Thus, it is still doubtable regarding the relationship between circulating IGF-1 and cognitive function in PD. New biomarkers could better reflect the change of circulating IGF-1 function.

1.4.2 Plasma IGF-1/IGFBP-3

The bioactivity of IGF-1 in blood is related to the amount of free IGF-1 and displacement of endogenous IGF from the binding proteins can elevate levels of free IGF-1 and is biologically active in both *in vitro* and *in vivo* studies (Liu et al., 2001). Moreover, it is well known that the bioavailability of IGF-1 is regulated by IGFBPs (Duan & Xu, 2005; Salehi et al., 2008). Thus, the total IGF-1/IGFBP-3 molar ratio may be a measure of bioactive IGF-1. Indeed, this ratio has been analysed in clinical studies to evaluate the bioavailability of IGF-1 (Huang et al., 2015; Vardy et al., 2007). In stroke, patients with a high total IGF-1/IGFBP-3 molar ratio within six hours after stroke onset also shows a better neurological and functional outcomes at three months (De Smedt et al., 2011). However, this ratio does not consistently represent IGF-1 function because the majority of IGFBP-3 is not associated with plasma IGF-1. Using a mathematical analysis, Guan et al. (2018) estimate the bound and unbound forms of IGF-1, cGP and IGFBP-3 in the plasma (Guan et al., 2018). They found the majority of IGF-1 and cGP are in the bound form suggesting most plasma IGFBP-3 is unrelated to plasma IGF-1. Indeed, IGFBP-3 also transports IGF-2 and has IGF-independent actions (Ranke, 2015).

Taken together, current biomarkers for IGF function are not reliable and new biomarkers of its function are needed for the advancement of our understanding of the role of IGF-1 in neurological conditions.

1.5 Plasma cGP/IGF-1 ratio could be a potential biomarker for IGF-1 function

1.5.1 Regulation of IGF-1 function

The IGF-1 function depends on IGF-1 bioavailability and is regulated through endocrine, paracrine and autocrine regulation to maintain the homeostasis of IGF-1 function.

1.5.1.1 Endocrine and paracrine regulation

IGF-1 production is regulated through the endocrine and paracrine regulation. In plasma, growth hormone (GH) regulates the IGF-1 production in liver through GH-IGF-1 axis that promotes or inhibits IGF-1 liver production through JAK-STAT pathway. In brain, GH also regulated IGF-1 production in all types of brain cells, known as paracrine IGF-1 (Fernandez & Torres-Alemán, 2012). Local IGF-1 in the brain promotes neural precursors, neurogenesis and gliogenesis during brain development (Ajo et al., 2003). Prolactin is also involved in the expression of IGF-1 demonstrated in the brain tissues of human foetuses (Pathipati et al., 2011).

GH concentration declines from adulthood. IGF-1 production mediated through endocrine and paracrine regulation decreases and results in the decline of plasma concentration of IGF-1. The following section describes the role of autocrine regulation by cGP and IGFBP-3 on improving the amount of bioavailable IGF-1, thus IGF-1 function in circulation.

1.5.1.2 Autocrine regulation through cGP and IGFBP-3

Autocrine regulation of IGF-1 determines the amount of bioavailable IGF-1 in plasma through the reversible binding, mainly to IGFBP-3. GH is also the main regulator of

the hepatic synthesis of IGFBP-3. Increased GH can increase the production of liver-derived IGFBP-3, the main source of plasma IGBFP-3. A reduction of IGFBP-3 levels is coupled with the decline of GH with aging process in healthy individuals (Corpas et al., 1993).

In blood, the majority of IGF-1 is bound to IGFBP-3 leaving a small portion of IGF-1 present in the free form with biological activity (Duan & Xu, 2005; Kelley et al., 2002; Sara & Hall, 1990). More unbound bioactive IGF-1 can be available after IGFBP-3 is cleaved by certain proteases (Collett-Solberg & Cohen, 1996; Fowlkes et al., 1995, 2004; Nishijima et al., 2010; Sadowski et al., 2003). A decrease in IGFBP-3 corresponding to a decline of IGF-1 was observed in rats plasma, suggesting a role of plasma IGFBP-3 in regulating IGF-1 function (Singh-Mallah et al., 2016)

Recently, cGP has also been found to be involved in the autoregulation of IGF-1 function by altering the binding of IGFBP-3 to IGF-1 (Guan et al., 2014). As the major binding site of IGF-1 to IGFBP-3, cGP retains its binding affinity to IGFBP-3 and competes with IGF-1 to IGFBP-3 binding in a concentration-dependent manner (Guan et al., 2014). Thus, cGP further regulates the binding of IGF-1 to IGFBP-3 and collectively determine the amount of unbound, bioavailable IGF-1 in plasma. Only unbound IGF-1 can activate IGF-1 receptors, leading to IGF-1 specific functions. In the preclinical study, cGP was first found to restore IGF-1 receptor-associated angiogenic capillaries following ischemic injury in the rats. Then in vitro, cGP was shown to modulate IGF-1-induced cell viability in human endothelial cells when IGF-1 and IGF-1 receptor exist. However, the effects of cGP diminished after either serum withdrawal or IGF-1 receptors knockdown. These results confirmed that cGP modulated IGF-1 function. They also found that blockade of IGFBP-3 changed the effects of cGP on IGF-1-induced cell viability, suggesting cGP may have an

interaction with IGFBP-3 to modulate IGF-1 function. Furthermore, they showed that high cGP/IGF-1 molar ratio enhanced the release of IGF-1 from IGFBP-3, while equimolar ratio maintained and the low molar ratio reduced the release. These data demonstrated that cGP/IGF-1 molar ratio may modulate bioavailable IGF-1 levels through interaction with IGFBP-3, although the complicated interactions between cGP, IGF-1 and IGFBP-3 could not be fully explained.

Later, in an animal study, an increase in plasma endogenous cGP and cGP/IGF-1 molar ratio corresponding to a decline of plasma IGF-1 was observed, suggesting that the role of plasma cGP in regulating IGF-1 function, although the underlying mechanisms associated with the increase in endogenous cGP are not clear (Singh-Mallah et al., 2016). In support, a cross-sectional study in pregnant women showed that women with obesity had lower plasma IGF-1 but higher cGP/IGF-1 ratio and a trend towards an increase in cGP (Guan et al., 2018). These results indicate that plasma cGP plays a pivotal role in the regulation of IGF-1 bioavailability when plasma total IGF-1 is deficient.

Taken together, these studies suggest that plasma cGP contributes to the regulation of IGF-1 bioavailability in circulation and the molar ratio of cGP/IGF-1 could be a measure of bioavailability of IGF-1. Figure 1.3 shows the autocrine regulation of IGF-1 function by cGP.

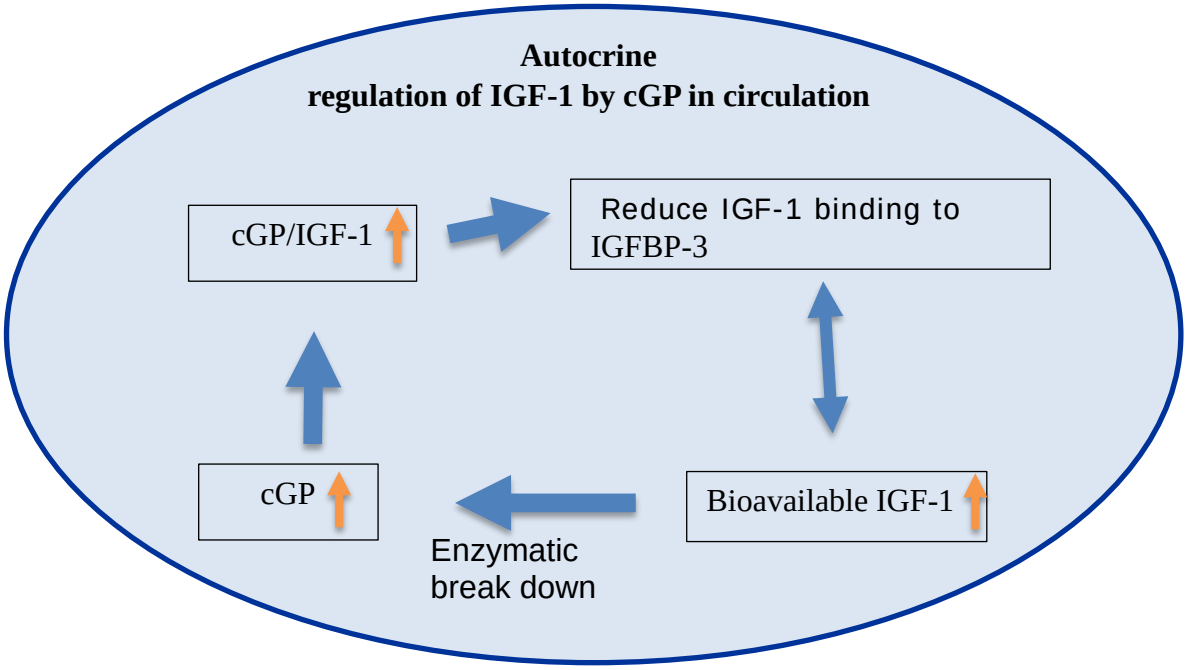


Figure 1.3 Autocrine regulation of endogenous IGF-1 function through cGP and IGFBP-3 under physiological and pathophysiological conditions of diminished IGF-1 function.

A balanced regulatory axis exists under normal physiological conditions, in which cGP/IGF-1 molar ratio dynamically regulates the amount of free, bioavailable IGF-1 (blue arrow). Under both physiological and pathophysiological conditions of IGF-1 deficiency, cGP increases (orange arrow) and competes with IGF-1 to bind IGFBP-3 and thus increase the release of bioavailable IGF-1.

1.5.2 Evidence for the relationship between plasma cGP/IGF-1 ratio and circulating IGF-1 function

It has been demonstrated that the changes in endogenous cGP in responding to the concentration of circulation IGF-1 in developing rats. The concentration of cGP increased in infant rats with low plasma IGF-1 and decreased during adolescent rats with high IGF-1 concentration (Singh-Mallah et al., 2016). Given that cGP contributes to the regulation of IGF-1 function and the molar ratio of cGP/IGF-1 determines the

bioavailability of IGF-1 (Figure 1.3), the molar ratio of cGP/IGF-1 may be a more reliable biomarker for IGF-1 function in neurological conditions. Subsequently, a longitudinal clinical observation study shows that increased plasma cGP/IGF-1 ratio was observed in women with loss of weight from 15-week pregnancy to 6 years postpartum (Guan et al., 2018). These results suggest the changes in plasma cGP/IGF-1 molar ratio could be a novel biomarker for IGF-1 function.

In addition, because of enzymatic resistance of cGP, its clearance from plasma is large, if not all, through urine. There is a possibility that the urine concentration of cGP may be a surrogate marker for its plasma concentration, which would be a more accessible and economical option.

1.6 Summary

This section summarises the Chapter 1.

- IGF-1 family and regulation of IGF-1function, with a focus on cGP/IGF-1 molar ratio determining bioavailable IGF-1 levels, are reviewed
- IGF-1 has a role in stroke recovery and maintenance of cognitive function
- Current biomarkers under investigation for IGF-1 function, plasma IGF-1 and IGF-1/IGFBP-3 ratio, are not reliable in the neurological conditions. Therefore, additional biomarkers for IGF-1 function are needed
- Plasma cGP/IGF-1 molar ratio could be a novel biomarker for circulating IGF-1 function and applied to neurological conditions

1.7 Hypothesis and aims

1.7.1 Hypothesis

As a biomarker for the function of circulating IGF-1, the changes of cGP/IGF-1 molar ratio in plasma may be applied to stroke and cognitive impairment in PD.

1.7.2 Aims

1. Examining changes in plasma cGP, IGF-1, IGFBP-3 and cGP/IGF-1 molar ratio during 90-day stroke recovery, and their associations with clinical outcomes and recovery.

2. Investigate associations of cGP, IGF-1, IGFBP-3 and cGP/IGF-1 with cognitive status in PD.

3. Examining changes in plasma and CSF cGP, IGF-1, IGFBP-3 after the BCA supplementation in PD.

2 General materials and methodology

This chapter includes a description of the general materials and methods used in more than two studies. The methods used only once in the thesis are briefly mentioned here and details are given in the specific chapter.

2.1 Materials

Manufacturers of brand products are mentioned specifically within the methods section of Chapter 3, 4 and 5. All reagents used for HPLC-MS assay were of analytical grade unless stated otherwise.

2.2 Methods

2.2.1 Study design and population

The detailed designs are described in the methods section of each corresponding chapter.

2.2.2 Sample collection and preparation

The biological samples were provided by different collaborators and sample preparations are described in the methods of specific chapters.

2.2.3 Enzyme-Linked Immunosorbent Assays

The concentration of total IGF-1 in plasma and CSF samples were analysed by ELISA employing a commercially available ELISA kit specific for human IGF-1 according to manufacturer's instructions. Firstly, all reagents were brought to room temperature for 30 minutes and then after ten-second centrifuge, standards and controls were reconstituted with the dilution buffer followed by sitting for 15 minutes at room

temperature. After, the standards and controls were vortexed thoroughly but gently using a vortex mixer. Samples and controls (10 µl per sample and control) were then diluted by an acid buffer to dissociate IGF-1 from endogenous binding proteins. The dilution ratio was adjusted as appropriate. Next, 80 µl of antibody conjugate was added to all wells to be used in a 96-well microtiter plate to coat the first antibody on the plate. Within 2 hours since dilution, 20 µl of the diluted sample or 20 µl of standard or 20 µl of diluted control was then aliquoted into each well. The wells were covered with sealing tape and shaken at 350 rpm for 1 hour at room temperature. After incubation, wells were washed five times with wash buffer prior to the addition of 100 µl enzyme conjugate. After enzyme-conjugated specific anti-IGF-1 antibody binding to the immobilized IGF-1, another 30-minute incubation and washing were performed. Following washing, a substrate solution was added to each well before a 15-minute incubation in a dark room without a shake. The reaction was ceased by adding 100 µl stop solution into each well and absorbance was measured within 30 minutes using the Synergy 2 plate reader (BioTek® Synergy™ 2 Multi-detection Microplate Reader, Gen5 software, Winooski, VT, USA) at 450 nm with a reference filter of 630 nm. To determine the IGF-1 concentration, the IGF-1 calibration curve was constructed by plotting the mean change in absorbance value for each calibrator on the Y-axis versus the corresponding IGF-1 concentration on the X-axis using the BioTek's Gen5 Reader Control and Data Analysis Software (BioTek® Synergy™ 2 Multi-detection Microplate Reader, Gen5 software, Winooski, VT, USA). The final IGF-1 concentration was obtained by using the calibration curve and multiplying the dilution factor. Concentrations were reported as ng/ml. The assay was fit for detecting the IGF-1 concentration range from 2-50 ng/ml with a within-run and total precision of CV< 10%.

The concentrations of IGFBP-3 were measured by commercially available ELISA kits specific for human IGFBP-3 (Crystal Chem, Inc., Chicago, IL, USA) according to the manufacturer's protocol. Briefly, all reagents were brought to room temperature for 30 minutes and then after ten-second centrifuge, standards and controls were diluted with the dilution buffer followed by sitting for 15 mins at room temperature. After, the standards and controls were vortexed thoroughly but gently using a vortex mixer. Samples and controls (at least 10 µl) were then diluted in the buffer. Antibody conjugate was added to all wells to be used in a 96-well microtiter plate to coat the first antibody on the plate. Within 1 hour since dilution, diluted samples or standards or controls were then aliquoted into each well. The wells were covered with sealing tape and shaken at 350 rpm for 1 hour at room temperature. After incubation, wells were washed five times with wash buffer before the addition of enzyme conjugate. After enzyme-conjugated specific antibody binding to the specific binding protein, another 1-hour incubation and washing were performed. Following washing, a substrate solution was added to each well prior to 30-minute incubation in a dark room without a shake. The reaction was stopped by adding 100 µl stop solution into each well and absorbance was measured within 30 minutes using the same method to that for IGF-1. Concentrations were reported as ng/ml. The assay was fit for detecting IGFBP-3 from 0.4-30 ng/ml. All assays had a within-run and total precision of CV<10%.

2.2.4 Plasma extraction of cGP

The cGP-1,5,6,7,8-^{13}C, 4-^{15}N (cGP-5x^{13}C, 1x^{15}N, 50 µL of 500 ng/ml) (internal standard for the assay, Chemistry Laboratory, Auckland, New Zealand) was added to 100 µL of plasma, urine or CSF and vortex mixed. The resulting solution was

transferred to a 1mL Phree phospholipid removal cartridge (Phenomenex, Auckland, New Zealand) contained in a 12 mL Nunc CryoTubeTM vial (NuncTM Brand Products, Nalge Nunc International). 500 µL of 1% formic acid in acetonitrile was then added to the cartridge and centrifuged at 1000 rpm (284 g) for 5 min at 4 °C to accomplish the collection of the filtrate. Another 500 µl of 1 % formic acid in acetonitrile was loaded and centrifuged as before. The filtrate was dried using Thermo Scientific Savant SC250 EXP SpeedVac concentrator (Thermo Fisher Scientific, Asheville, NC, USA) at room temperature with vacuum pressure initially set at 1.5 mTorr for one hour and then reduced to 0.7 mTorr for one hour. To thoroughly reconstitute the dried extract, 100µL of water/methanol (90/10 vol/vol) was added into the tube, vortexed gently for 30 seconds and then transferred to HPLC vials for quantitation. After another 500-rpm (142g) centrifuge at 4 °C, the samples were ready for the next step.

2.2.5 High-Performance Liquid Chromatography mass spectrometry (HPLC-MS) assay

Prepared samples were subsequently injected into an HPLC-MS system consisting of an Accela MS pump and autosampler with an Ion Max API source on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer all controlled by Finnigan Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA). The chromatography conditions consisted of a Synergy Hydro 2.5µm column (Phenomenex, Auckland, New Zealand) 100 × 2 mm with an initial mobile phase composition of 10% methanol / 90% water flowing at 200 µl per minute with a column temperature of 35°C. The mass spectrometry conditions comprised electrospray ionization in positive mode with a voltage of 4000 V, a sheath gas flow of 30 psi, an auxiliary gas flow of 2 psi, and a capillary temperature of 250 °C. Fragmentation was

achieved with argon at 1.2 mTorr as the collision gas and a dissociation voltage of 35 V. The mass spectrometer ran in selective reaction monitoring (SRM) mode with the following two transitions 155.1 → 70.2 m/z and 161 → 75.1 m/z utilized for cGP and cGP- cGP-1,5,6,7,8-^{13}C, 4-^{15}N, respectively. The retention time for both peaks was 3.6 minutes. Unknown samples were quantitated using the peak area ratio of cGP/cGP-5x^{13}C, 1x^{15}N compared with the standard curve of known concentrations. The retention time for both peaks was 3.6 min. Unknown samples were quantitated using the peak area ratio of c-GP/cGP-5x^{13}C, 1x^{15}N compared with the standard curve of known concentrations.

2.2.6 Measurement of concentrations of urine creatinine

The details are described in section 3.3.

2.2.7 Statistical analysis

A commercially available software SPSS (IBM SPSS Statistics 24, Chicago, IL, USA) was used for the statistical analysis and GraphPad Prism 7 (GraphPad Software, Inc, USA) for making figures. The details were described in the methods sections of Chapters 3, 4 and 5. A p-value of less than 0.05 was considered to be significant.

A p-value of less than 0.05 was considered to be significant.

3 Plasma cyclic glycine proline/IGF-1 ratio predicts clinical outcome and recovery in stroke patients

3.1 Summary of chapter contents

This chapter investigates the changes in concentrations of cGP, cGP/IGF-1 molar ratio, IGF-1 and IGFBP-3 in plasma during the 90-day period after stroke onset. The differences in these biological measures between stroke and controls are also investigated. In addition, the relationships between these biological measures and clinical outcomes are investigated. This chapter has been published recently as an original research article in Annals of Clinical and Translational Neurology.

3.2 Introduction

Stroke remains to be a major cause of mortality and disability burden (Feigin et al., 2014). Many surviving patients make at least a partial recovery in function over the first 3 months, a critical period for the recovery (Lee et al., 2015). A biomarker that is associated with recovery would be clinically useful for predicting outcome and assist clinical management (Biomarkers Definitions Working Group, 2001). The expression of IGF-1 increases in the brain tissues 1-5 days after hypoxic-ischemic brain injury in rats (Gluckman et al., 1992), and this may contribute to a partial recovery of sensory-motor function (Guan et al., 2001). Timely administration of human recombinant IGF-1 within 6 hours after hypoxic-ischemic injury reduces brain damage and improves long-term sensory-motor function (Gluckman et al., 1992; Guan et al., 1993, 2001; Guan, Gunn, et al., 2000). Changes in plasma concentrations of IGF-1 have been studied as a prognostic biomarker in stroke but the results have been inconsistent (Gandolfi et al., 2017).

The majority of circulating IGF-1 is inactive due to high-affinity binding, mainly to IGFBP-3, which prevents IGF-1 to activate its functional receptors. Only unbound IGF-1 is bioactive (Duan & Xu, 2005; Kelley et al., 2002; V. R. Sara & Hall, 1990) and thus the plasma concentrations of IGF-1 do not represent IGF-1 function. However, unbound IGF-1 is either swiftly metabolized or internalized after activating IGF receptors (Fernandez & Torres-Alemán, 2012; Fowlkes et al., 2004; Nishijima et al., 2010) and impossible to measure directly. Binding of IGF-1 to IGFBP-3 is reversible and this regulates the amount of bioavailable IGF-1 in plasma, a process namely autocrine regulation of IGF-1. The molar ratio of IGF-1 to IGFBP-3 has been used to characterize IGF-1 function, but this does not consistently represent IGF-1 function because the majority of IGFBP-3 is not associated with plasma IGF-1 (Guan et al., 2018).

Our previous publication suggests that the changes in the plasma concentration of cGP and its molar ratio to plasma IGF-1 may be a better representation of bioavailable IGF-1 (Guan et al., 2018; Singh-Mallah et al., 2016). cGP is naturally cleaved from the N-terminus of unbound IGF-1 (Guan et al., 2015) by an acid enzyme (Yamamoto & Murphy, 1994, 1995). The N-terminal region of IGF-1 is a major binding site to IGFBP-3 (Sara et al., 1993) and cGP retains the binding affinity to the IGFBP-3, leading to a concentration-dependent competitive binding between IGF-1 and cGP (Guan et al., 2014). The relative concentration of cGP to IGF-1 may be used to determine the bioavailability of circulating IGF-1 as a higher cGP/IGF-1 ratio is associated with a greater concentration of unbound, bioavailable IGF-1 (Guan et al., 2018; Guan et al., 2014). The aim of this study was to evaluate whether the changes in plasma concentrations of cGP and cGP/IGF-1 molar ratio would be associated with the recovery during the first 3 months after stroke. Urine cGP was also measured to explore the relationship between urine and plasma cGP.

3.3 Methods:

3.3.1 Study design and population

The clinical information, assessment scores and detailed methods used for clinical evaluations were provided by the BARISTa, Brain Research New Zealand. The study was approved by Auckland District Health Board (A+: 6627), the University of Auckland Human Participants Ethics Committee (UAHPEC: 015655) and Health and Disability Ethics Committees (HDEC: 15STH73) and written informed consent was obtained from all enrolled patients and control participants. The inclusion criteria of stroke patients were: 1) less than 3 days after the onset of stroke at the time of hospital admission; 2) 18 to 90 years of age and 3) able to understand English, with help from a friend or family member if required. Exclusion criteria for stroke patients are: 1) current cancer diagnosis; 2) pregnancy or childbirth within the previous 90 days; 3) blood disorders rendering additional blood sampling inappropriate, as assessed by the patient's clinical team. For control subjects, the inclusion criteria include 1) no history of stroke; 2) 18 to 90 years of age; 3) capable of understanding English, with help if needed. Exclusion criteria include: 1) being pregnant or giving birth in the last 3 months; 2) having a blood disorder which makes the blood samples inappropriate; 3) currently diagnosed with cancer.

Forty-four patients were screened within 3 days of stroke. Thirteen patients withdrew, one patient died and two did not complete the follow up over the 90-day course of the study. All stroke patients have completed standard in-patient rehabilitation.

Plasma samples were collected from 34 stroke patients at baseline, from 21 patients at day 7 and from 26 patients at day 90 after stroke. Twenty-eight patients had completed the follow-ups, 21 of whom provided plasma samples at day 7, and 26 who provided plasma samples at day 90. A total of 21 patients had completed clinical scores and provided plasma samples at

all-time points. Twenty-eight patients provided plasma samples at baseline and had clinical assessments at day 90. Fifty age-matched control participants (35 women and 15 men) with no history of stroke were also recruited and all provided plasma samples.

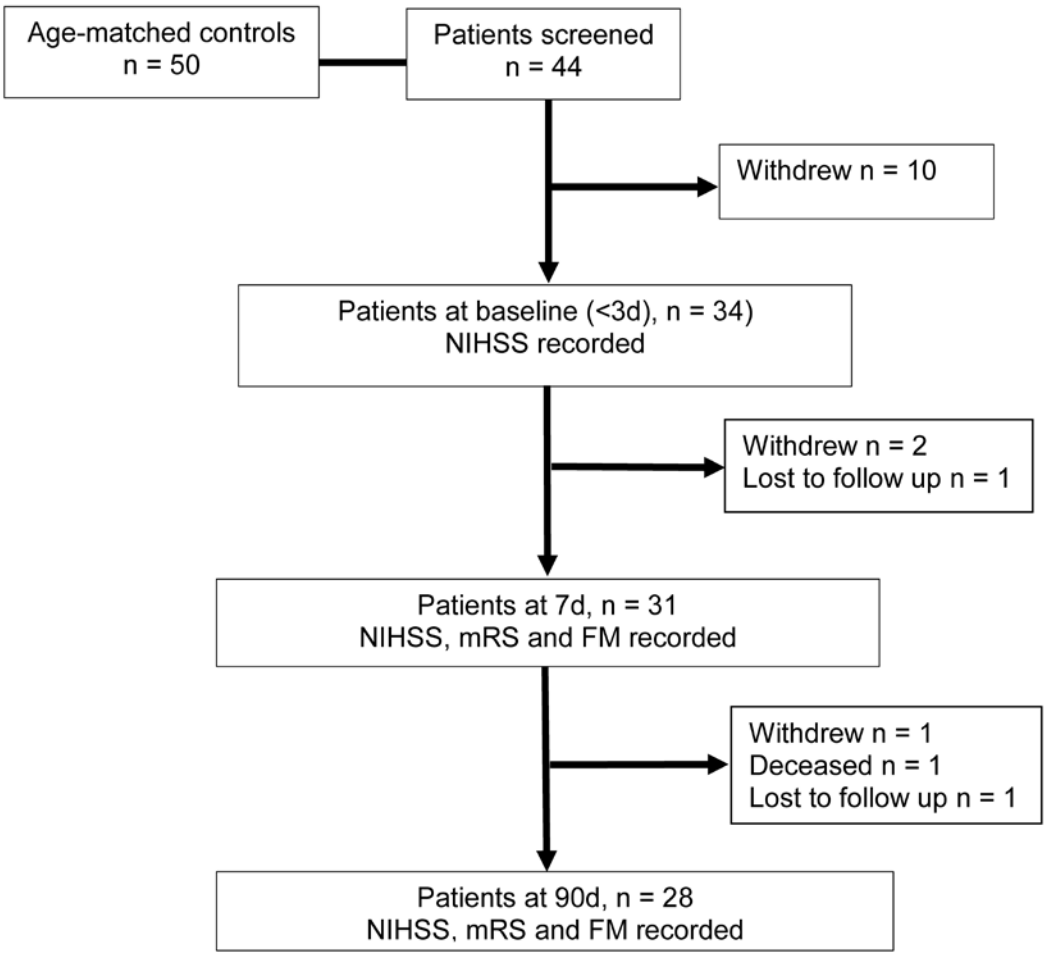


Figure 3.1 The flowchart of the study population

3.3.2 Sample collection and storage

Plasma samples were collected from age-matched controls and from stroke patients at baseline (within 3 days), day 7 and day 90 after stroke onset. The samples were collected and prepared

by the Biomarkers and Recovery in Stroke (BARISTa), Brain Research New Zealand. Blood samples obtained via venepuncture of the antecubital fossa were collected in ethylene di-amino tetra-acetic acid (EDTA) tubes and immediately transferred to the processing laboratory. The samples were left for at least 1 hour and centrifuged at 1300g for 10 minutes at room temperature. The plasma samples obtained by centrifugation were aliquot into 500µl volumes and immediately frozen at -80 °C until required.

3.3.3 Measurement of clinical outcomes

Clinical assessments were performed by the members of the independent clinical team who were trained in their administration. Clinical assessments included the National Institutes of Health Stroke Scale (NIHSS) at baseline (within 3 days), then again at days 7 and 90. The modified Rankin Scale (mRS) and Fugl-Meyer Upper Limb Assessment Scale (FM-UL) were performed at days 7 and 90. Clinical recovery after stroke was evaluated using the difference in NIHSS between the baseline and 90 days after stroke (ΔNIHSS).

3.3.4 Measurement of concentrations of cGP in plasma and urine using HPLC-MS

An HPLC-MS was utilized to measure the concentration of cGP in the samples. The protocol for plasma cGP extraction and HPLC-MS has been described in section 2.2.4 and 2.2.5. The urine cGP concentration is normalized by urine creatinine and calculated by the ratio of cGP/creatinine. The procedure of extraction and HPLC-MS for urine cGP is the same as that of plasma cGP.

Urine creatinine concentrations were determined by the kinetic colorimetric assay based on the Jaffé method (Roche, Mannheim, Germany) on a Cobas C311 automated analyzer (Hitachi High Technologies Corporation, Tokyo, Japan). 60 µl of urine sample was pipetted into a specific sample cup for the analyzer. The samples were mixed by pipetting them and bubbles

removed before analysis began. Normal pooled urine control selected for two distinct levels of concentration were used as quality controls.

3.3.5 Measurement of plasma concentration of IGF-1 and IGFBP-3 using ELISA

The concentrations of IGF-1 and IGFBP-3 were measured by ELISA. The methodology has been described in section 2.2.3.

3.3.6 Statistical analysis

The differences between the stroke (baseline) and the control groups were analysed using independent-samples t-test, Chi-Square tests or Fisher's exact test as appropriate. The difference between the stroke (baseline) and the control group was analysed using the independent-samples t-test for continuous variables and the Chi-square test for categorical variables. The Fisher's exact test was used instead of the Chi-square test when expected frequencies in each cell for some categorical variables are less than 5 due to missing data. Changes over time in stroke patients were analysed using the one-way repeated ANOVA (parametric test) or Friedman test (non-parametric test) with *post hoc* tests using the Bonferroni correction. The correlation between clinical outcomes and biological measurements or age were firstly analysed by Pearson or Spearman correlation analysis. Then multiple linear regression analysis was used to rule out the effects of potential confounders on the correlation. Only the variables that are associated with the clinical outcomes were entered into the regression model. Together with prior knowledge (Rost et al., 2016), age and baseline NIHSS was included in the model. Due to the small sample size of our study, other variables that could likely account for variability in outcomes such as gender and hypertension were not included in the linear model to decrease the risk of statistical errors (Babyak, 2004; Hawkins, 2004). In the final regression model, the associations between biological changes and clinical scores were

analysed after adjustment for age and baseline NIHSS. The significance level was set at $p < 0.05$. Data were presented as mean ± standard error of the mean (SEM) unless otherwise stated.

3.4 Results

3.4.1 Demographic characteristics of patients at baseline and controls

The demographic details of the stroke patients at baseline and control participants are presented in Table 3.1. There was no significant difference between patients and controls in the average age (64.8 ± 10.03 vs 66.79 ± 14.64, $p = 0.49$). Compared with controls, there were fewer females ($p = 0.018$) and more individuals with atrial fibrillation in stroke patients. The stroke patients also tended to have more individuals with diabetes, hypertension and dyslipidemia.

Table 3.1 Baseline characteristics of acute stroke patients and controls

	Control	Patients	p Value
Number of participants	50	34	
Age, years (mean ± SD)	64.8±10.03	66.79±14.64	0.49[a]
F/M	35/15	15/19	0.018[b]
Diabetes (%)	2 (4)	6 (17.6)	0.057[c]
Current smoker (%)	1 (2)	4 (11.8)	0.153[c]
Ex-smoker (%)	16 (32)	9 (26.5)	0.586[c]
Hypertension (%)	13 (26)	15 (44.1)	0.084[b]
Dyslipidaemia (%)	9 (18)	12 (35.3)	0.072[b]
Atrial fibrillation (%)	4 (8)	9 (26.5)	0.022[b]

a: t-test; b: Chi-Square tests; c: Fisher's exact test;

3.4.2 Changes in clinical scores

In stroke patients, NIHSS scores improved over time (F (2, 20) = 27.48, p < .001, n = 21, A). Compared to baseline, the NIHSS score reduced by day 7 (median: 2 vs 4, p = 0.01) and day 90 (median: 1 vs 4, p < 0.001). The mRS score also improved (median: 3 vs 2, p = 0.003, n = 21, Fig 2B) as did the FM-UL scores (median: 64 vs 65, p = 0.001, n = 21, Fig 2C). The control participants had no significant neurological deficits (NIHSS median (range): 0 (0 - 2)) nor global disability (mRS median (range) 0 (0 - 1)) with normal up limb functions (FM-UL median (range): 66 (65 - 66)).

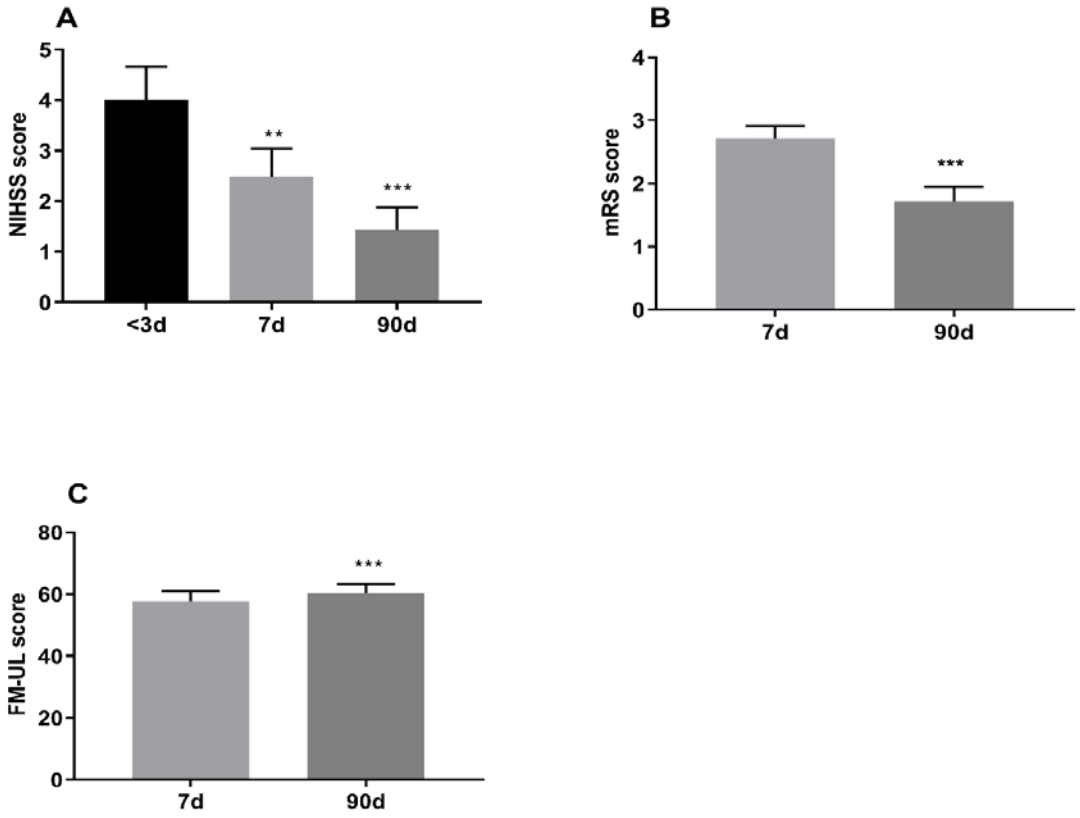


Figure 3.2 The changes in clinical scores over time after stroke

Compared to the baseline, NIHSS significantly decreased in 7 and 90 days after stroke (A). The modified Rankin Scale (mRS) score reduced from days 7 to 90 (B) and Fugl-Meyer Upper Limb Assessment Scale (FM-UL) score was increased from 7 to 90 days after stroke (C). Data are presented as mean ± SEM. **p < 0.01, ***p < 0.001, n = 21.

3.4.3 Changes in biological measurement of plasma of patients within 3 days after stroke

There was no difference in IGF-1 concentration (Figure 3.3A) between stroke and control groups. Compared to the control group, stroke patients had lower IGFBP-3 concentrations ($p = 0.002$, Figure 3.3B), cGP concentrations ($p = 0.04$, Figure 3.3C) and cGP/IGF-1 molar ratio ($p = 0.04$, Figure 3.3B) at the baseline. IGF-1/IGFBP-3 molar ratio was also not significantly different ($p > 0.05$).

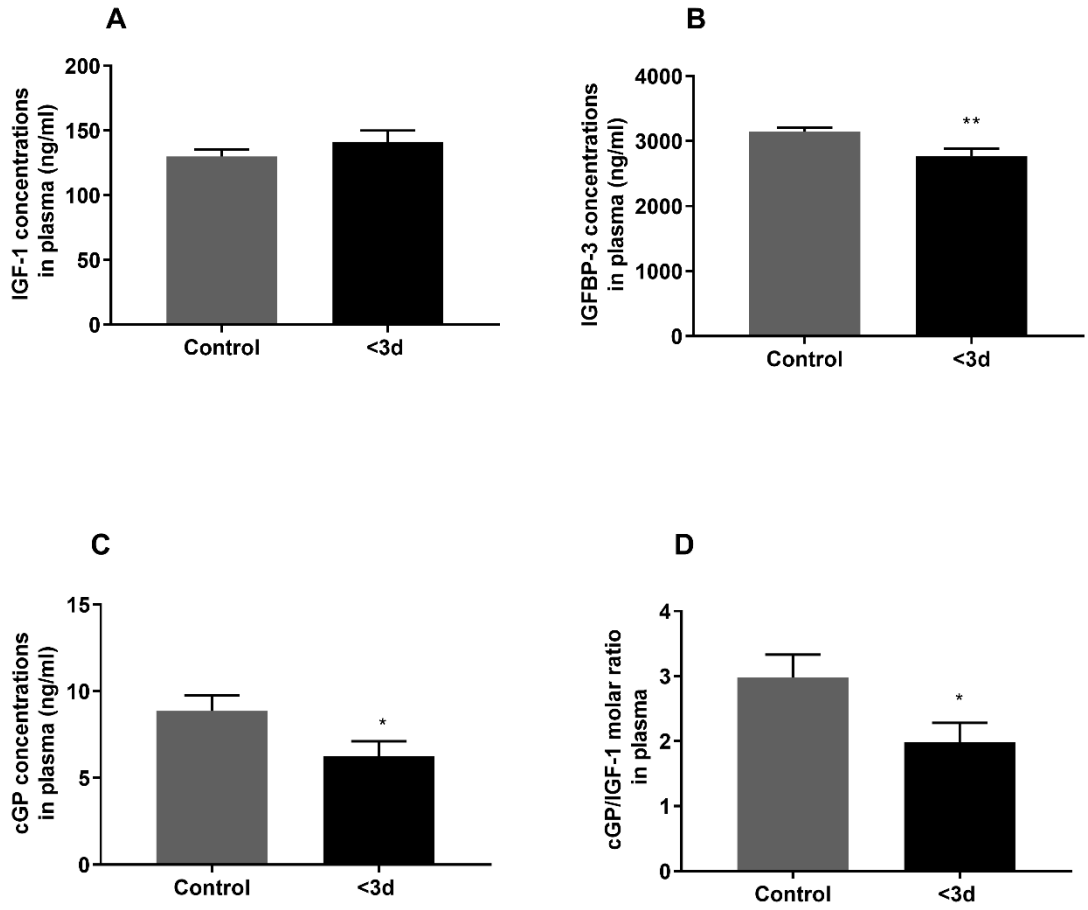


Figure 3.3 The differences in biological measures between the control and stroke group

IGF-1 concentration did not change after stroke (A). Compared to the control group, the baseline concentrations of IGFBP-3 (B), cGP (C) and cGP/IGF-1 ratio (D) were lower in stroke patients. Data presented as Mean ± SEM, *$p < 0.05$, **$p < 0.01$

The concentrations of IGF-1 and IGFBP-3 remained stable over time after stroke (Figure 3.4A and B). Their molar ratio was thus also stable (p = 0.28). There was a significant increase in cGP concentrations (F (2, 20) = 5.34, p = 0.01, n = 21, Figure 3.4C) and cGP/IGF-1 molar ratio (F (2, 20) = 3.94, p = 0.02, n = 21, Figure 3.4D) over time by ANOVA repeated analysis. Compared to the baseline, the concentration of cGP (p = 0.01) and cGP/IGF-1 ratio (p = 0.03) was significantly increased by 90 days in the stroke patients.

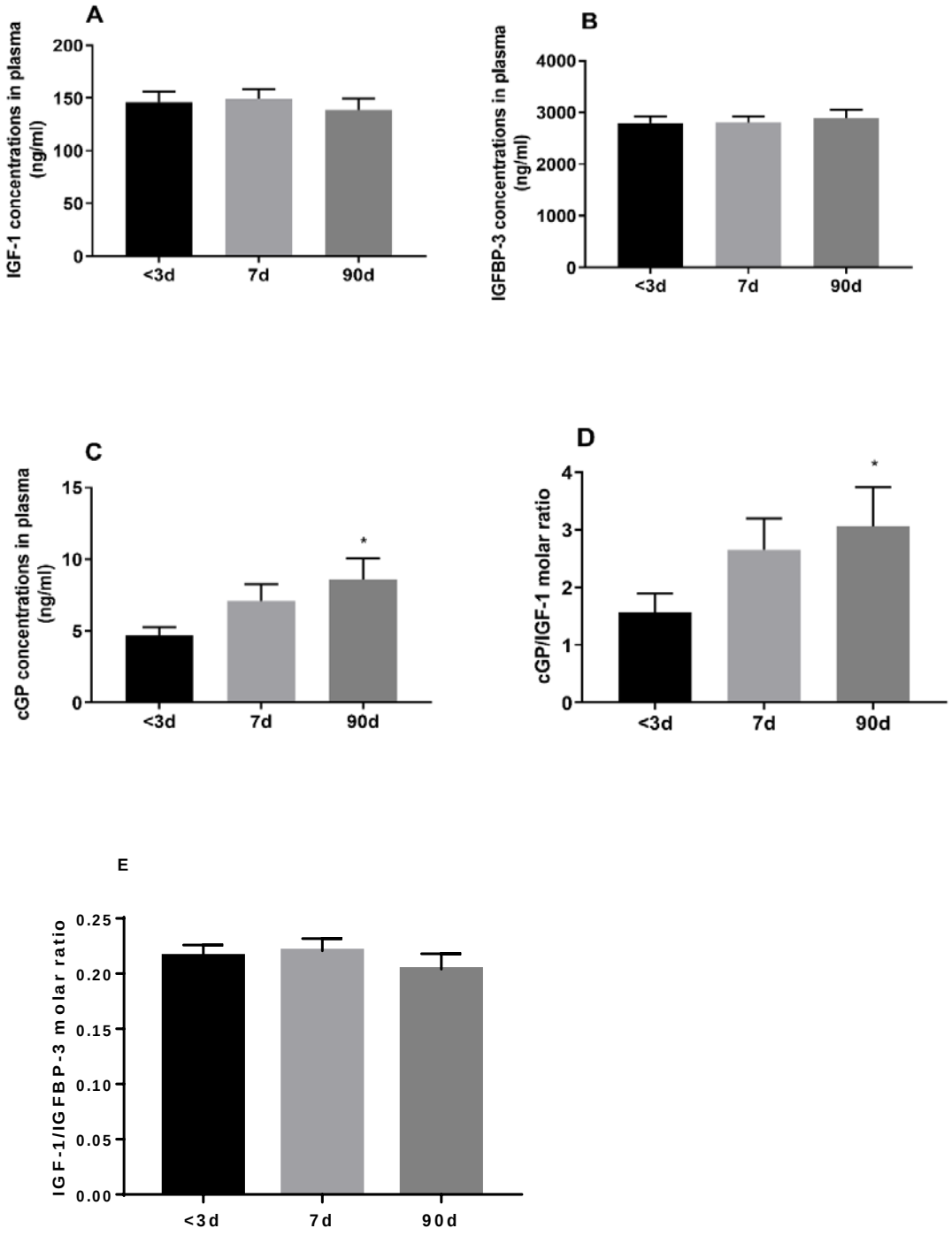


Figure 3.4 The biological changes over time in stroke patients.

The plasma concentration of IGF-1 (A) and IGFBP-3 (B) did not change over time. cGP concentration (C) and cGP/IGF-1 ratio (D) increased over time. Compared to the baseline, cGP concentration and cGP/IGF-1 ratio were significantly increased 90 days after stroke. IGF-1/IGFBP-3 molar ratio also did not change over time (E) Data presented as Mean ± SEM, *$p < 0.05$, $n = 21$

3.4.4 Correlation analysis in plasma

Pearson correlation showed no significant correlation between NIHSS and plasma cGP (r = -0.14, p = 0.21) or cGP/IGF-1 molar ratio (r = -0.11, p = 0.34). Table 3.1 shows the results from correlation analysis between biological changes at the baseline and clinical scores either at day 90 or the changes from the baseline to day 90 in the stroke patients after adjustment for age and baseline NIHSS. The cGP/IGF-1 molar ratio was negatively correlated to the NIHSS scores at day 90 (B = - 0.30; 95%CI = -0.59 −-0.02; p = 0.03; n = 28) and positively correlated to ΔNIHSS (B = 0.30; 95%CI = 0.02 − 0.59; p = 0.03; n = 28). The baseline cGP/IGF-1 molar ratio showed a trend towards a positive correlation with FM-UL scores of day 90 (B = 2.33, 95%CI = -0.07 − 4.73; p = 0.05; n = 27), but not with the mRS.

Table 3.2 Relationships between clinical outcomes and recovery at 90 day and baseline biological concentrations in plasma

Baseline / 90 day		IGF-1	IGFBP-3	cGP	cGP/IGF-1 ratio
NIHSS (n=28)	B	0.006	0.00	-0.06	-0.30
	p	0.11	0.09	0.36	0 .03
	95%CI	(-0.002, 0.01)	(0, 0.001)	(-0.21, 0.08)	(-0.59, -0.02)
mRS (n=28)	B	-0.001	0.00	0.09	0.19
	p	0.72	0.52	0.10	0.08
	95%CI	(-0.008, 0.005)	(-0.001, 0)	(-0.02, 0.20)	(-0.03, 0.42)
FM (n=27)	B	-0.01	-0.002	0.84	2.33
	p	0.64	0.38	0.16	0.05
	95%CI	(-0.08, 0.05)	(-0.007, 0.003)	(-0.35, 2.04)	(-0.07, 4.73)
ΔNIHSS (n=28)	B	-0.006	0.00	0.06	0.30
	p	0.11	0.09	0.36	0.03
	95%CI	(-0.01,0.002)	(-0.001,0.00)	(-0.08, 0.21)	(0.02,0.59)

NIHSS, the National Institutes of Health Stroke Scale. ΔNIHSS, the change between baseline and 90 days in the National Institutes of Health Stroke Scale. mRS, the modified Rankin Scale score. FM, The Fugl-Meyer Upper Limb Assessment Scale score. B: beta coefficients; 95%CI: 95% confidence intervals. The analysis was adjusted for age and baseline NIHSS.

3.4.5 Analysis in urine

While there were no differences in IGF-1, IGFBP-3 and IGF-1/IGFBP-3, the cGP concentrations was lower in stroke patients than in controls (0.431±0.068 vs 0.894±0.095), t (44.55) = 3.95, p<0.001 (Figure 3.5). There were no statistically significant differences in the cGP concentration over time (Figure 3.5). It should be stressed that the sample size is small.

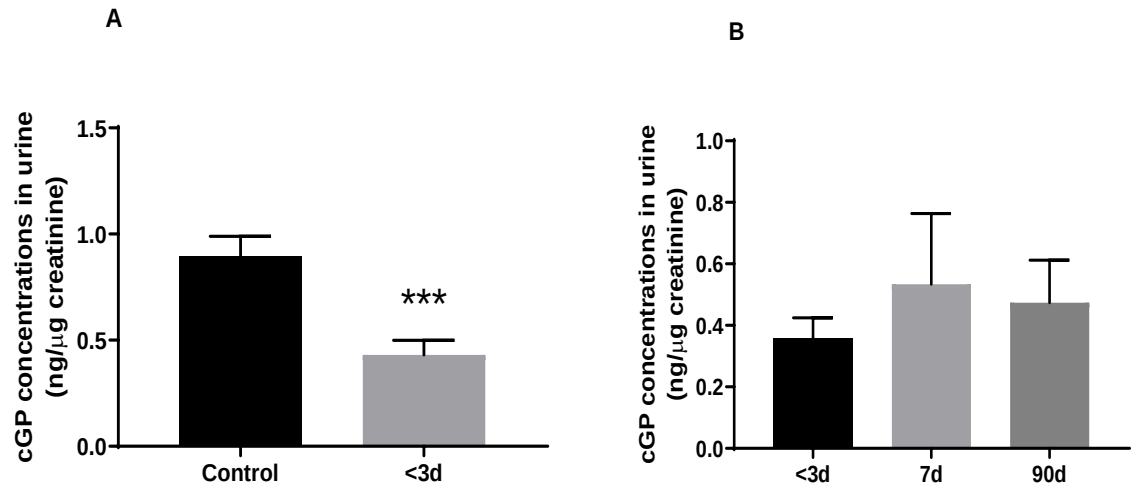


Figure 3.5 Changes in urine cGP concentrations

Concentrations of urine cGP were measured in normal controls and patients. (A) Comparisons between baseline patients and control group, n= 46 and 10 (age-matched controls and stroke patients). (B) Comparisons between follow-up time points and baseline patients, n=6. Data are presented as mean ± SEM, In A, significant differences compared with the control group are denoted by ***$p < 0.001$.

To assess a potential direct association between plasma and urine cGP, a Pearson correlation was performed. There was a strongly positive correlation between urine and plasma cGP concentrations in both controls and patients ($r = 0.54$, $p < 0.001$ and $r = 0.78$, $p < 0.001$, respectively, Figure 3.6).

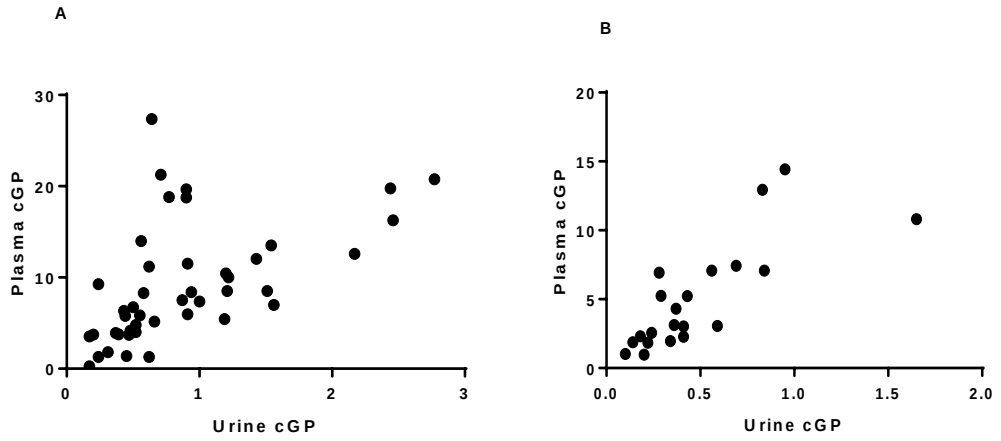


Figure 3.6 Correlation between plasma and urine cGP in controls and patients

(A) Correlation between urine and plasma cGP in controls (n = 44). (B) Correlation between urine and plasma cGP in patients (n = 7). Data include samples collected from all time points.

3.5 Discussion

This study has shown the lower plasma concentrations of cGP, IGFBP-3 and cGP/IGF-1 ratio in stroke patients within three days of stroke, suggesting an impairment of autocrine regulation by cGP. Over the initial 90 days of stroke recovery, the stroke patients showed an improvement in neurological function and global disability, which occurred in parallel with the gradual increases in plasma cGP and cGP/IGF-1 ratio. The stroke patients with higher baseline cGP/IGF-1 ratio had fewer neurological deficits at day 90 after stroke and made a better recovery by 90 days. The results of this study may suggest a role for autocrine regulation of IGF-1 in stroke recovery and that the molar ratio of cGP/IGF-1, if further confirmed through larger studies, may be a potential prognostic biomarker for predicting the ability of recovery in stroke patients.

The amount of bioavailable IGF-1 in plasma is collectively regulated by IGFBP-3 and cGP. The less IGFBP-3 and more cGP can increase the amount of bioavailable IGF-1 in plasma

(Guan et al., 2018; Guan et al., 2014; Singh-Mallah et al., 2016). Compared to the age-matched controls, the concentrations of IGFBP-3 and cGP were lower in stroke patients at the baseline. We have recently shown that hypertensive women also have lower plasma IGFBP-3 and cGP compared to normotensive women (Guan et al., 2018). These changes in hypertension and stroke patients suggest a role for autocrine regulation in cardiovascular functions. While the reduction of plasma IGFBP-3 is an autocrine response to IGF-1 deficiency to increase bioavailable IGF-1 in plasma, the lower plasma cGP may suggest an impairment of autocrine regulation by cGP in hypertension and stroke patients (Guan et al., 2018). This interpretation is also supported by the observations that cGP administration prevents neuronal and vascular damage after ischemic brain injury in rats (Guan et al., 2014). The function of cGP in vascular protection is mediated through improving IGF-1 function which has been described by *in vivo* and *in vitro* studies(Guan et al., 2014). Hypertension is a major risk factor of stroke (Owolabi & Agunloye, 2013) and the impairment of autocrine regulation of IGF-1 may be a pathophysiology process shared by hypertension and stroke.

Most patients in our study made a partial recovery over the 90-days of the study. These clinical improvements were in parallel with an increase in cGP concentrations and cGP/IGF-1 molar ratio over time. The molar ratio cGP/IGF-1 increases 90 days after a stroke due to increased cGP and stable IGF-1 levels in plasma. Given the relationship between cGP/IGF-1 molar ratio and IGF-1 bioavailability (Sara et al., 1993; Singh-Mallah et al., 2016), we speculate that the improvement of plasma IGF-1 bioavailability may contribute to the functional recovery, but a correlation analysis failed to show a significant association, possibly due to the limited sample size in this study. While there was no change in the plasma IGF-1 concentration during stroke recovery, the increase of plasma cGP over time is more likely a result of promoting the enzymatic formation of cGP in plasma. However, the mechanism that modulates the enzymatic activity is unknown and needs to be investigated.

Age and baseline neurological deficits are crucial factors that influence stroke recovery (Rost et al., 2016). The correlation analysis with the adjustment for age and baseline NIHSS scores showed that the patients with a higher molar ratio of cGP/IGF-1 at the baseline had fewer neurological deficits at day 90 of stroke and made a better recovery by 90 days. This hypothesis-generating study is limited by small sample size but the results merit further investigation.

Previous studies have reported inconsistent results in plasma IGF-1 concentrations in stroke patients. One study showed lower plasma IGF-1 concentrations (Schwab et al., 1997). On the contrary, another clinical study showed higher plasma IGF-1 concentrations 3 days after stroke compared to control groups (Åberg et al., 2011). Moreover, changes in plasma IGF-1 concentrations have been associated with mortality but not functional recovery (Dong et al., 2014). This study has also shown no changes in plasma IGF-1 concentrations during the first 90-days following stroke. This supports the suggestion that changes in plasma IGF-1 concentrations are not a reliable measure of IGF-1 function during stroke recovery.

Sensitivity and specificity are two critical elements for identifying potential clinical biomarkers. The cGP-related changes are specific to IGF-1 function, but not stroke. Their association with stroke was the IGF-1 function-mediated recovery. Thus, the biomarker may also have potential clinical applications for other medical conditions with IGF-1 deficiency. The cGP-related changes in plasma appeared to be sensitive, which has been demonstrated by other small clinical observations in obesity, hypertension and PD (Fan et al., 2018; Guan et al., 2018).

The neuroprotective function of IGF-1 has been well-demonstrated in stroke models (Guan et al., 2003). cGP protects the brain from ischemic injury by improving phosphorylation of IGF-1 receptors, with a well-defined mechanism (Guan et al., 2018; Guan et al., 2014; Singh-Mallah

et al., 2016). Given the oral availability and dynamic central uptake of cGP in humans (Fan et al., 2018), further development of this biomarker may provide guidance to assist their future clinical trial in stroke.

A comparison of cGP in urine samples showed similar results in plasma, although the sample size was small. More interestingly, a strongly positive correlation was shown between urine and plasma cGP concentrations in both controls and patients. These results suggest that the change in urine cGP could reflect the changes in plasma cGP.

In conclusion, these hypothesis-generating observations suggest that the increase in cGP/IGF-1 ratio, but not plasma IGF-1 within 3 days after stroke may be further developed as a prognostic biomarker for predicting the ability of functional recovery in stroke patients. The hypothesis that the progressive decline of cGP concentration in hypertensive patients can be a biomarker for stroke risk merits further investigation.

3.6 Summary

This study evaluated plasma concentrations of IGF-1, IGFBP-3 and cGP and their associations with clinical outcomes in stroke patients. The baseline concentrations of IGFBP-3, cGP and cGP/IGF-1 ratio were lower in stroke patients than the control group, although these may be affected by the difference in gender and other medication conditions. These results suggest an impaired autocrine regulation of circulating IGF-1 function by cGP in stroke. The neurological scores of stroke patients were improved and plasma cGP and cGP/IGF-1 ratio increased over time. The baseline cGP/IGF-1 ratio was correlated with the NIHSS scores at day 90 and the changes in NIHSS scores from the baseline to 90 days. On the contrary, plasma IGF-1 and IGF-1/IGFBP-3 molar ratio remained stable over 90 days and their baseline levels did not correlate with the clinical scores at day 90. The cGP/IGF-1 ratio at admission may be further

developed as a prognostic biomarker for stroke recovery if the results are replicated in the future.

4 Changes of plasma cGP/IGF-1 molar ratio with age is associated with the cognitive status of Parkinson's disease

4.1 Summary of chapter contents

The second aim of this thesis was to evaluate the relationship between plasma cGP/IGF-1 molar ratio and cognitive status of PD. This chapter measures the concentrations of cGP, IGF-1 and IGFBP-3 in plasma from PD patients with normal cognitive function (PD-N), PD patients with mild cognitive impairment (PD-MCI) and PD patients with dementia (PD-D). The relationships between them and clinical outcomes are also investigated. This chapter is published by Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring.

4.2 Introduction

Cognitive impairment in PD patients is now recognized as a primary problem affecting patient and carer well-being (Jones et al., 2017; Lawson et al., 2014). PD-MCI have an increased risk of developing PD-D compared to PD-N (Wood et al., 2016). A reliable biomarker for predicting cognitive status may assist in identifying those at greatest risk of developing cognitive impairment and thereby identifying a population and the window suitable for intervention.

IGF-1 is a neurotrophic factor and plays a critical role in cognitive function (Aleman & Torres-Alemán, 2009). The decline of IGF-1 function with age is a major contributor to age-related cognitive impairment (Muller et al., 2012; O. Okereke, Kang, Ma, Gaziano, & Grodstein, 2006; O. Okereke et al., 2007). Given aging plays a substantive role in cognitive impairment in PD (Aarsland & Kurz, 2010; Levy, 2007), the decline of IGF-1 function with age could be related to cognitive impairment in PD patients. Therefore, the biomarkers for IGF-1 function could be applied to the prediction of cognitive impairment in PD. The association between plasma IGF-

1, a biomarker under clinical evaluation for IGF-1 function, and cognitive function has been studied, however, the findings thus far have been mixed and difficult to interpret (Frater et al., 2017). One explanation for the inconsistencies is that the majority of plasma IGF-1 is inactive, due to its high-affinity binding to IGF binding proteins, particularly IGFBP-3, thereby preventing IGF-1 from activating its functional receptors (Duan & Xu, 2005). The ratio of IGF-1/IGFBP-3 has also been evaluated as a marker of the amount of bioactive IGF-1 (Duan & Xu, 2005), however, given a large amount of plasma IGFBP-3 does not associate with IGF-1 (Guan et al., 2018), other markers need to be explored.

An alternative is cyclic-glycine-proline (cGP)/IGF-1 molar ratio. cGP is a stable, natural metabolite of IGF-1 (Guan et al., 2015). As the fraction of IGF-1 that binds to IGFBP-3, cGP retains the binding affinity to IGFBP-3. Therefore, cGP competes against IGF-1 to bind with IGFBP-3, in a concentration-dependent manner (Fan et al., 2018; Guan et al., 2018; Singh-Mallah et al., 2016). A higher concentration of cGP would occupy a greater proportion of IGFBP-3, increasing the amount of free, bioavailable IGF-1 (Guan et al., 2014; Singh-Mallah et al., 2016). Our recent studies suggest that cGP/IGF-1 molar ratio could represent the amount of bioavailable IGF-1 (Guan et al., 2014) and plasma cGP/IGF-1 molar ratio is associated with IGF-1 function in metabolic disorders (Guan et al., 2018). Therefore, the plasma cGP/IGF-1 ratio could be applied to predict the cognitive status of PD. This study examined the relationship between cGP/IGF-1 molar ratio and cognitive status in PD. The concentrations of IGFBP-3 were also measured due to its involvement in the autocrine regulation of IGF-1 function.

4.3 Methods

4.3.1 Study design and population

The study was approved by the New Zealand Health and Disability Ethics Committee under approval number URB/09/08/037, with informed consent provided by all the participants. This is a cross-sectional trial with 204 PD patients, and 24 controls recruited for screening at the Van der Veer Movement Disorders clinic and the patient database of the New Zealand Brain Research Institute. Figure 4.1 shows the study population.

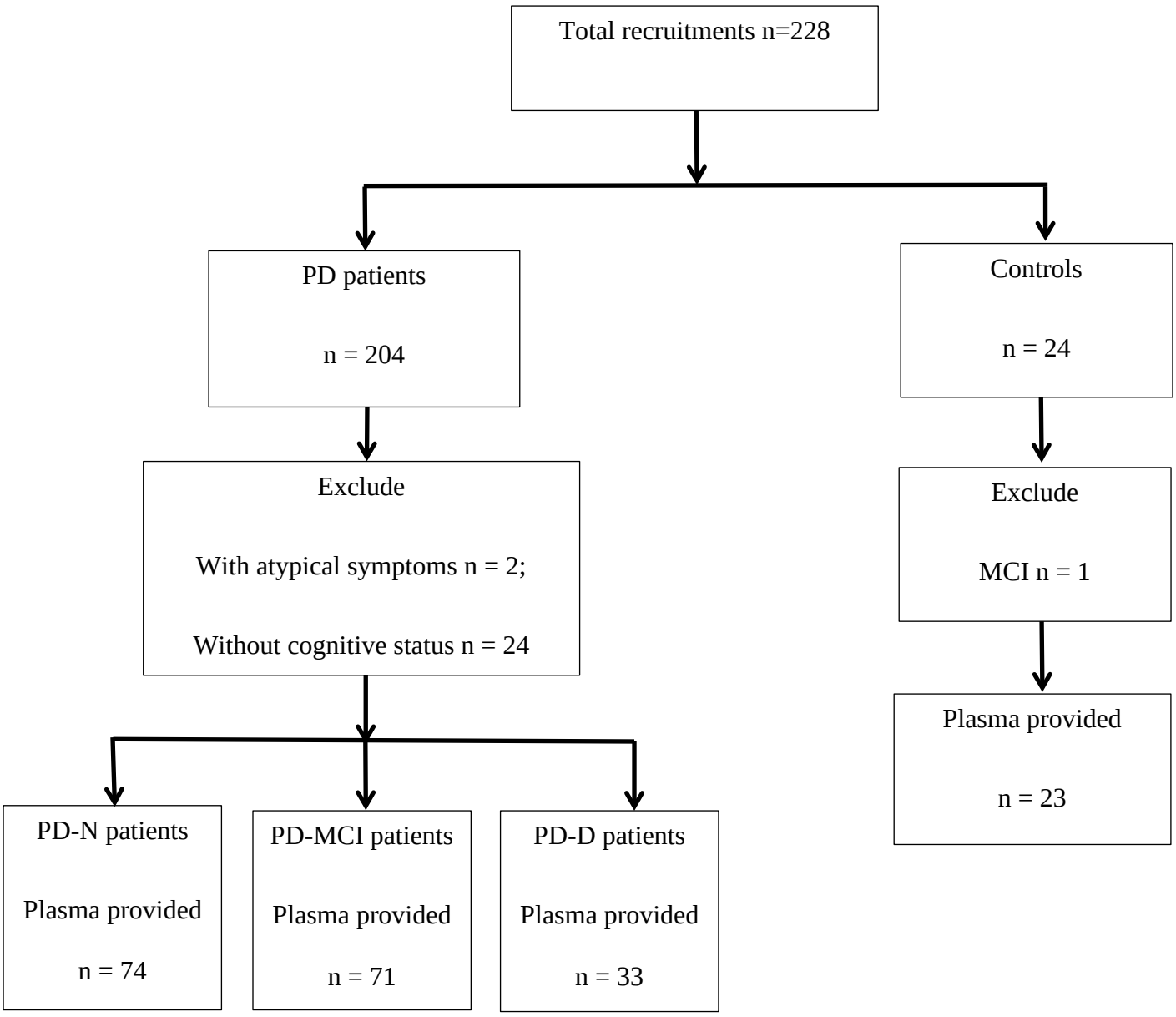


Figure 4.1 The flowchart of the study population

Two PD patients with atypical symptoms and 24 without cognitive status were excluded. One control with mild cognitive impairment was also excluded. Finally, plasma samples from 178 PD patients and 23 controls were measured. Within the PD patients, 74 belongs to PD-N, 71 belongs to PD-MCI and 33 belongs to PD-D.

The PD patients were diagnosed according to the UK Parkinson's Society criteria by movement disorders neurologists. Clinical assessment was performed by experts using a large battery of rating scales shown in the following section. The exclusion criteria included atypical parkinsonian disorder, other medical conditions (e.g., history of moderate/severe head injury, stroke, and early-life learning disability, major psychiatric or medical illness in the previous 6 months) and poor English (precluding testing). The PD-MCI criteria suggested by a recent study was adopted (Wood et al., 2016). The PD-MCI patients had at least two z scores (see below) with 1.5 standard deviations (SD) below normative data on the individual test but within a single domain.

A PD-D diagnosis required the presence of significant impairments (2 SD below normative data) in at least two of five cognitive domains and significant impairment in everyday functional activities, not caused by motor impairments. Patients not fulfilling the criteria for PD-MCI or PD-D were classified as PD-N. All the participants took their usual medications on the day of testing so that optimal performance was achieved during the morning test sessions.

The controls were recruited in response to community advertisements and did not report subjective cognitive complaints. All controls had undergone clinical and cognitive testing.

4.3.2 Sample collection and storage

The plasma samples were provided by the New Zealand Brain Research Institute, Christchurch, New Zealand. Venous blood was collected from an arm vein into lithium heparin tubes (Beckon Dickinson) and gently inverted as per manufacture directions. Filled tubes were placed on wet ice and immediately transferred to the processing laboratory. The samples were spun in a cooled centrifuge (4 °C) for 10 minutes at 2000 x g and plasma stored as 250 µl aliquots at -80°C until assayed. When needed, the samples were thawed at 4 °C and thoroughly vortexed.

4.3.3 Measurement of disease stage and cognitive function

The clinical information, assessment scores and detailed methods used for clinical evaluations were provided by the Brain Research Institute, Christchurch, New Zealand. Cognitive status was determined using a battery of assessments meeting the requirements of the Movement Disorder Society−Task Force (MDS-TF) Level II criteria (Emre et al., 2007; Litvan et al., 2012). Five cognitive domains were examined, with the tests conducted over two sessions (Dalrymple-Alford et al., 2011; Morris, 1993). Executive function was assessed using Stroop interference, letter fluency, category fluency and category switching (from the Delis-Kaplan Executive Function System 45), and action fluency and Trails B. Attention, working memory and processing speed was evaluated using digits forwards/backwards, digit ordering, map search task (from the Test of Everyday Attention), Stroop color reading, Stroop word reading and Trails A. Memory was measured with the California Verbal Language Test-II Short Form (acquisition, short and long delays), and the Rey Complex Figure Test (short and long delays); impairment in either or both delay components of each memory test counted as one impairment. Visuoperceptual/visuospatial performance was determined using the judgment of line orientation, fragmented letters test, the picture completion test and the Rey Complex Figure Test-Copy. Language was assessed using the Boston Naming Test, Dementia Rating Scale-2 similarities sub-test, and the language component of the Alzheimer's Dementia Assessment Cognitive Scale (object and finger naming, commands, comprehension, spoken language and word-finding difficulties). Scoring of the neuropsychological tests was conducted using age- and education-adjusted normative data. Participants also completed the Montreal Cognitive Assessment (MoCA). Global Z scores were derived from the average of each domain and averaged across domains. Classification of PD-MCI required two deficits at 1.5 standard deviations (SD) or more below age and education-adjusted norms but everyday function not significantly impaired; these criteria discriminate PD-N from those PD-MCI patients who are

at increased risk of PD-D over four years (Wood et al., 2016). Impairments in everyday cognitive function not attributed to motor impairments were necessary for a PD-D diagnosis, based on interviews with a significant other using the Clinical Dementia Rating, the Global Deterioration Scale and instrumental activities of daily function assessment (Morris, 1993; B. Reisberg et al., 1982; Barry Reisberg et al., 2001). Motor examination of the PD patients was performed using the Unified PD rating scale part III (UPDRS III) and Hoehn and Yahr staging (H&Y).

4.3.4 Measurement of concentrations of cGP in plasma using HPLC-MS

The HPLC-MS assay was utilized to measure the concentration of cGP in the samples. The protocol for plasma cGP extraction and HPLC-MS has been described in section 2.2.4 and 2.2.5.

4.3.5 Measurement of plasma concentration of IGF-1 and IGFBP-3 using ELISA

The concentrations of IGF-1 and IGFBP-3 were measured by ELISA. The protocol has been described in section 2.2.3.

4.3.6 Statistical analysis

SPSS (IBM SPSS Statistics 24, Chicago, IL, USA) was used for the statistical analysis and making figures. Parametrical data were analysed using independent-samples t-test and one-way ANOVA with Tukey post hoc tests for comparison between controls and PD-N and within PD groups respectively. Non-parametric data were analysed using the Mann-Whitney U test and Kruskal-Wallis test with Dunn-Bonferroni post hoc test accordingly and presented as median (range). The gender effects were analysed using the Chi-Square test. The Mann-Whitney U test was used to compare the education years between PD-N and control groups.

Kruskal-Wallis test with Dunn-Bonferroni post hoc test was used for comparing the education years, LED and H&Y score within PD groups. Data from the non-parametric analysis were presented as median (range). The data that were normally distributed were analysed using unpaired t-test or one-way ANOVA with Tukey post hoc tests and presented as mean (SEM).To find whether age-related changes in cGP/IGF-1 ratio is associated with cognitive status (normal, MCI and dementia) in PD, a multiple regression test with interaction terms was performed. Following a significant interaction effect, a simple slope analysis was performed to show the specific relationship of biological measures with age in the three PD groups with different cognitive status. The correlations between biological measures and global cognitive scores in controls were analysed using Pearson correlation analysis. The relationships after adjustment for age in controls were also analysed using multiple regression analysis. The significant level was set at $p < 0.05$. Data are presented as mean ± SEM unless otherwise stated.

4.4 Results

4.4.1 Demographic characteristics

Table 4.1 summarized the demographic data of the patients and healthy controls. Between the PD-N group and controls, there was no significant difference in gender and education, but the PD-N group was averagely younger than the control group ($p = 0.03$). Compared with the PD-N group, the PD-MCI but not the PD-D group had fewer years of education ($p = 0.004$ and 0.21 respectively).

Table 4.1 Demography of control participants and PD patients

	Control (n=23)	PD-N (n=74)	PD-MCI (n=71)	PD-D (n=33)	p Value[a]	p Value[b]
Age, y	74.53 (1.37)	70.91 (0.82)	72.89 (0.76)	76.17*# (0.93)	.03	.001
F/M	8/15	28/46	19/52	5/28	ns	
Education Median (Range)	13 (10-18)	13 (9-20)	11* (8-19)	11 (9-20)	ns	.005
Years since symptom duration		11.56 (.61)	11.60 (.57)	11.40 (1.19)		ns
Years since diagnosis		8.96 (.53)	10.04 (.56)	10.07 (1.17)		ns
LED Median (Range)		845.37 (0-3893)	914.38 (0-3162)	798 (0-3140)		ns

a, the comparison between PD-N and controls; b, the overall comparison between PD groups. * $p<0.05$, compared to PDN; #$p<0.05$ compared to PD-MCI. LED: Levodopa equivalent dose. Data were shown as mean (SEM) if not specifically stated.

4.4.2 Neuropsychological assessment and biological changes

Table 4.2 summarized the neurophysiological and biological data of the patients and healthy controls. Compared with the controls, the PD patients had more severe cognitive impairment shown by a lower cognitive rating scale. The concentrations of plasma cGP or cGP/IGF-1 molar ratio, IGF-1 was similar between the two groups. The PD groups had a higher IGF-1/IGFBP-3 ratio ($p = 0.03$). Compared with the PD-N group, the PD-MCI and PD-D group had lower UPDRS and higher cognitive function rating scores. Compared with the PD-N and PD-MCI group, the PD-D group had lower plasma IGF-1 concentrations. There was no difference in other biological measures between PD groups.

Table 4.2 Clinical and biological characteristics of the control participants and PD patients

(ng/ml)	Control (n=23)	PDN (n=74)	PDMCI (n=71)	PDD (n=33)	p Valuea	p Valueb
H&Y Median (Range)		2.50 (1-3)	2.50** (1-5)	3.00*** (2-5)		< 0.001
UPDRSIII		35.20 (1.68)	43.73* (1.84)	48.95* (2.95)		< 0.001
MoCA	27.57 (0.32)	26.51 (0.27)	22.53* (0.34)	16.85*# (0.86)	0.01	< 0.001
Global Z Score	.87 (0.08)	0.33 (0.06)	-0.85* (0.06)	-1.79*# (0.14)	< 0.001	< 0.001
IGF-1	146.27 (10.41)	159.50 (6.03)	160.30 (5.95)	135.20*# (8.15)	ns	0.03
IGFBP-3	3332.92 (154.22)	3128.31 (74.55)	3166.86 (85.41)	2865.40 (122.71)	ns	ns
cGP	7.44 (1.69)	7.41 (0.54)	7.56 (0.61)	6.24 (0.74)	ns	ns
cGP/IGF-1 ratio	2.32 (0.61)	2.07 (0.17)	2.11 (0.20)	2.10 (0.31)	ns	ns
IGF-1/IGFBP-3 ratio	0.18 (0.009)	.21 (0.007)	.21 (0.007)	.19 (0.008)	0.03	ns

H&Y: Hoehn and Yahr scale; MDS-UPDRS: Movement disorder society-Unified Parkinson's disease rating scale; MoCA: Montreal Cognitive Assessment; Global Z score: mean derived from executive function, attention, learning & memory, and visuoperceptual/visuospatial. a, comparison between PDN and controls; b, comparison in PD groups; Data were shown as mean (SEM), *p<0.05, ** p<0.01, ***p<0.001 compared to PD-N. #p<0.05 compared to PDMCI.

4.4.3 The relationship between cognitive scores and plasma cGP/IGF-1 and other IGF-1-related biological measures in controls

Pearson correlation analysis showed that the cGP/IGF-1 molar ratio was positively correlated to global Z score ($r = 0.52$, $p = 0.01$) and had a trend towards a positive correlation with the MoCA score ($r = 0.37$, $p = 0.08$) in the controls. Plasma cGP was positively correlated to the MoCA score ($r = 0.42$, $p = 0.048$) and global Z score ($r = 0.57$, $p = 0.005$) in the controls. Other IGF-1-related changes (plasma IGF-1, IGFBP-3 and IGF-1/IGFBP-3 molar ratio) were not significantly associated with the scores in controls. After adjustment for age, plasma cGP was still positively correlated to the MoCA score ($B = 0.10$, $p = 0.006$) and global Z score ($B = 0.02$, $p = 0.009$) (Table 4.3). The cGP/IGF-1 molar ratio was also still positively correlated to the global Z score ($B = 0.07$, $p = 0.02$) and the MoCA score ($B = 0.25$, $p = 0.02$) (Table 4.3). Other IGF-1-related changes were still not related to cognitive scores (Table 4.3).

Table 4.3 Association between biological measures and cognitive scores after being adjusted for age in the control group

Groups	Dependent variable (Independent variable)		IGF-1 (ng / ml)	cGP (ng / ml)	cGP /IGF-1 molar ratio	IGFBP-3 (ng / ml)	IGF-1 /IGFBP-3 molar ratio
Control	MoCA (Age) n=23	B	0.005	0.10	0.25	0.001	12.09
		P	0.42	**0.006****	**0.01***	0.35	0.07
	Global Z score (Age) n=23	B	0.000	0.02	0.07	0.000	1.86
		P	0.99	**0.009****	**0.02***	0.27	0.35

MoCA: Montreal Cognitive Assessment; Global Z score: global measure of cognitive function derived from comprehensive neuropsychological testing. B: unstandardized co-efficiency. *P < 0.05; **P < 0.01

4.4.4 The relationship between age and plasma cGP/IGF-1 molar ratio in PD-N, PD-MCI and PD-D

To explore if age-related changes in plasma cGP/IGF-1 ratio are related to cognitive status in PD, relationships between age and plasma cGP/IGF-1 ratio in PD-N, PD-MCI and PD-D were analysed. The moderation analysis showed that there was an interaction effect of age and PD-D on cGP/IGF-1 molar ratio, B = -0.18, 95%CI [-0.29, -0.06], t = -3.04, p = 0.003 (Table 4.4), suggesting that the relationship of age with cGP/IGF-1 molar ratio is significantly different between the PD-D and the PD-N group.

Table 4.4 the relationship between age and cGP/IGF-1 in each PD group

	B	95%CI	t	p
Age	0.09	[0.04, 0.14]	3.53	<0.001
PD-MCI	-0.13	[-0.65, 0.39]	1.16	0.24
PD-D	0.25	[-0.51, 1.01]	2.98	0.003
Age×PD-MCI	-0.05	[-0.12, 0.03]	-1.20	0.23
Age×PD-D	-0.18	[-0.29, -0.06]	-3.04	0.003

B: beta coefficients; 95%CI: 95% confidence intervals.

Figure 4.2 shows the relationship between age and cGP/IGF-1 molar ratio across the PD groups. There was a positive association between age and cGP/IGF-1 molar ratio in the PD-N group (B = 0.09, p<0.001). There was non-significant positive correlation between age and cGP/IGF-1 molar ratio in the PD-MCI group (B = 0.04, p = 0.13). In contrast to the PD-N group, the PD-D group showed a significant negative correlation between age and cGP/IGF-1 molar ratio (B = -0.08, p = 0.04).

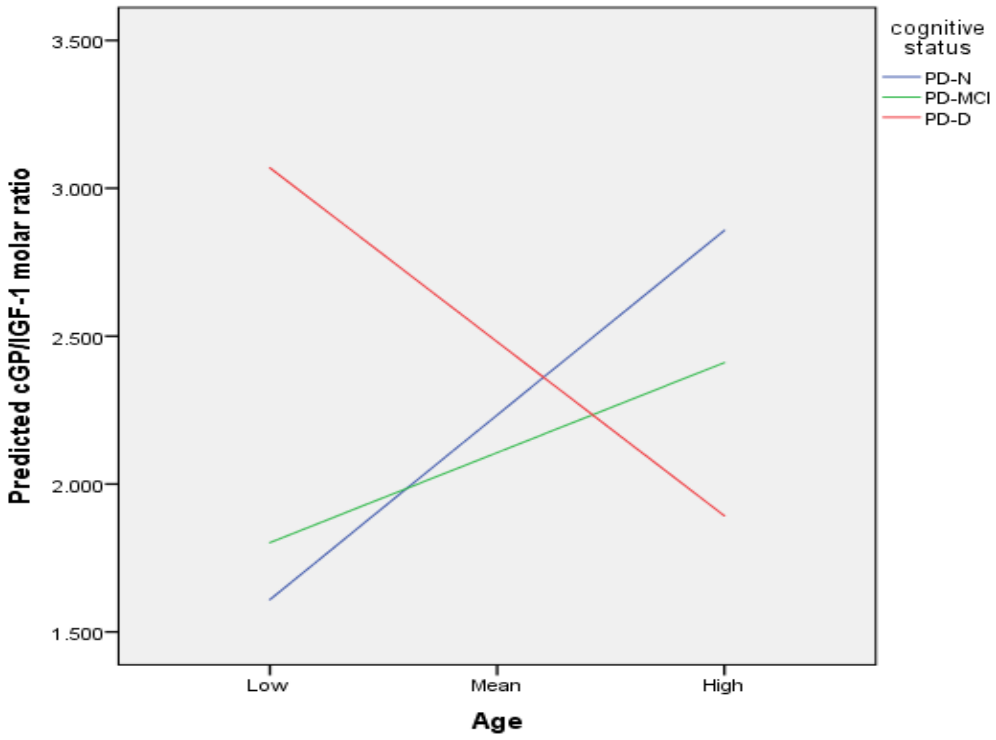


Figure 4.2 The relationship between age and cGP/IGF-1 molar ratio at three levels of cognitive status in PD

4.4.5 The relationship between age and plasma IGF-1 in PD-N, PD-MCI and PD-D

Plasma IGF-1 is involved in the autocrine regulation of IGF-1 function and thus its relationship with age was analysed. The moderation analysis showed that there were no significant interaction effects of age and cognitive status on cGP (Table 4.5), suggesting that the relationship of age with IGF-1 is not significantly different in each PD group. However, the PD-N group showed a negative association between age and cGP (B = - 2.85, p <0.001)

Table 4.5 the relationship between age and IGF-1 in each PD group

	B	95%CI	t	p
Age	-2.85	[-4.43, -1.27]	-3.56	<0.001
PD-MCI	6.04	[-10.21, 22.30]	0.73	0.46
PD-D	-21.36	[-44.54, 1.81]	-1.82	0.07
Age×PD-MCI	1.87	[-0.51, 4.24]	1.54	0.12
Age×PD-D	3.45	[-0.09, 6.99]	1.92	0.06

B: beta coefficients; 95%CI: 95% confidence intervals.

4.4.6 The relationship between age and plasma cGP in PD-N, PD-MCI and PD-D

The moderation analysis showed that there were no significant interaction effects of age and cognitive status on cGP (Table 4.6). However, the PD-N group showed a positive association between age and cGP concentrations (B = 0.16, p = 0.04)

Table 4.6 the relationship between age and cGP in each PD group

	B	95%CI	t	p
Age	0.16	[0.01, 0.31]	2.12	0.04
PD-MCI	-0.18	[-1.75, 1.40]	-0.22	0.82
PD-D	-0.65	[-2.94, 1.63]	-0.56	0.57
Age×PD-MCI	-0.02	[-0.25, 0.21]	-0.17	0.86
Age×PD-D	-0.33	[-0.68, 0.01]	-1.91	0.06

B: beta coefficients; 95%CI: 95% confidence intervals.

4.4.7 The relationship between age and plasma IGFBP-3 in PD-N, PD-MCI and PD-D

The moderation analysis showed that there were no significant interaction effects of age and cognitive status on cGP (Table 4.7), suggesting that IGFBP-3 is not significantly different for different cognitive status in our PD population. Moreover, no significant association between age and cGP concentrations was shown in all PD groups.

Table 4.7 the relationship between age and IGFBP-3 in each PD group

	B	95%CI	t	p
Age	-11.65	[-33.58, 10.26]	-1.05	0.30
PD-MCI	58.85	[-171.30, 289.00]	0.50	0.61
PD-D	-267.08	[-588.42, 54.26]	-1.64	0.10
Age×PD-MCI	11.36	[-21.87, 44.59]	0.67	0.50
Age×PD-D	18.64	[-30.30, 67.58]	0.75	0.45

B: beta coefficients; 95%CI: 95% confidence intervals.

4.4.8 The relationship between age and plasma IGF-1/IGFBP-3 in PD-N, PD-MCI and PD-D

The moderation analysis showed that there were no significant interaction effects of age and cognitive status on the IGF-1/IGFBP-3 molar ratio (Table 4.8), suggesting that the effect of age on plasma cGP is not significantly different for different cognitive status in our PD population. However, the PD-N group showed a positive association between age and IGF-1/IGFBP-3 ($B = -0.003$, $p = 0.005$)

Table 4.8 the relationship between age and IGF-1/IGFBP-3 in each PD group

	B	95%CI	t	p
Age	-0.003	[-0.004, 0.001]	-2.83	0.005
PD-MCI	0.002	[-0.02, 0.02]	0.25	0.80
PD-D	-0.02	[-0.04, 0.01]	-0.56	0.22
Age×PD-MCI	0.001	[-0.002, 0.004]	-0.17	0.37
Age×PD-D	0.003	[-0.001, 0.008]	-1.91	0.10

B: beta coefficients; 95%CI: 95% confidence intervals.

4.5 Discussion

To the best of my knowledge, this is the first clinical exploration on whether plasma cGP/IGF-1 molar ratio is a biomarker for IGF-1 function and thus could be related to changes in the cognitive status of PD patients. In general, our findings suggest that the higher molar ratio of cGP/IGF-1 has an association with better preserved cognitive function, possibly by improving the amount of bioavailable IGF-1 in plasma. In PD patients, the cGP/IGF-1 molar ratio increased with age in the PD-N group. Such an association was absent in PD-MCI and reversed to a decrease with age in PD-D groups. On the contrary, plasma IGF-1 or IGF-1/IGFBP-3 was lack of this sort of variation. Thus, these age-related variations in the cGP/IGF-1 molar ratio may assist in predicting cognitive status and the risk of advancing cognitive impairments in PD. This finding, if further confirmed through longitudinal studies, has the potential to be developed for clinical use as a prognostic biomarker for cognitive impairment in PD.

Inverse effects between aging and PD pathology on plasma IGF-1 have been reported (Godau et al., 2010; Muller et al., 2012). Because of difference in the severity of motor and cognitive

deficits, and age between PD groups, the collective pathophysiology of brain aging, cognitive impairments and PD motor deficits could lead to a moderate decrease of plasma IGF-1 in PD-D patients with cGP and IGFBP-3 remaining similar between the groups (Table 4.2).

A role of cGP in cognitive function has been previously demonstrated in rats (Guan, Zhang, Dale-Gandar, Hodgkinson, & Vickers, 2010; Singh-Mallah et al., 2016). The higher cGP/IGF-1 molar ratio, but not the IGF-1/IGFBP-3 molar ratio, was significantly associated with better global cognitive function in the non-PD participants and this association was independent of age. These results suggest that the old healthy people with a higher cGP/IGF-1 ratio may have a better global cognitive function.

Age-related decline of IGF-1 is a major contributor to cognitive impairment (Muller et al., 2012; O. Okereke et al., 2007; O. I. Okereke et al., 2006). Plasma IGF-1 decreased with age in PD-N patients (Table 4.5). As an autocrine response to IGF-1 deficiency to improve the bioavailability of IGF-1 in plasma (Guan et al., 2018; Guan et al., 2014; Singh-Mallah et al., 2016), plasma cGP and cGP/IGF-1 ratio correspondingly increased with age in this group (Table 4.6 and Table 4.4). These data suggest that an older person with a higher cGP/IGF-1 ratio may have better-preserved cognition. Compared to the non-PD controls, the PD-N patients were younger but showed lower global cognitive scores. This initial 'decline' in cognitive function may result from accelerated brain aging (Schneider et al., 2017). Although the mechanism of increasing enzymatic production of cGP is not known, the changes of cGP/IGF-1 along aging reflect the decline of IGF-1 function with age and the increase of cGP is to improve IGF-1 function (Guan et al., 2018). An increasing cGP/IGF-1 ratio with age in PD-N due to premature brain aging may serve as a potential biomarker for cognitive risk, but this notion needs to be examined in a future longitudinal study.

The association of a higher cGP/IGF-1 ratio with greater age in PD-N patients was absent in the PD-MCI patients and reversed in the PD-D patients, driven by the low plasma cGP. Low plasma cGP impairs autocrine regulation which has been suggested in hypertension (Guan et al., 2018) and stroke patients (Fan et al., 2019). Intervention with cGP protects against vascular and neuronal damages after ischemic brain injury (Guan et al., 2014) and normalizes systolic blood pressure in rats (manuscript under the review with Journal of Functional Foods). This impairment of autocrine regulation may similarly contribute to cognitive impairment in PD. The absence and reversed relationships of cGP/IGF-1 ratio with age in PD-MCI and PD-D patients may be the result of progressive impairment of autocrine regulation. The results from this study suggest that an initial or progressive loss of positive association between cGP/IGF-1 ratio and age may potentially be developed as a biomarker for the early diagnosis or the prognosis of cognitive function in PD patients.

IGF-1 is essential for vascular remodeling of the adult brain acting from the blood face of the blood-brain barrier (Lopez-Lopez et al., 2004). The mechanism of cGP in neuroprotection is mediated through improving vascular remodeling/function of IGF-1 in rat brain (Guan et al., 2014). Given the nature of vascular degeneration in cognitive impairments (Vemuri et al., 2017), the changes in plasma cGP/IGF-1 ratio that represent the amount of bioavailable IGF-1 could represent the IGF-1 function in cerebral vascular remodeling related to cognitive function.

MoCA is a brief test of cognitive function that uses short questions across domains to test global function. The global Z, derived from an extensive battery of tests, uses multiple tests within any one domain to test function. It is uncertain which one is better. Both have their place and reporting both has the advantage. Direct comparisons with other studies will be possible when using the MoCA given that this measure is now a general standard. The global Z probably

gives a better reflection of the cognitive function of an individual, given it is derived from more extensive testing. We use these scores for the relationship analysis in controls but not PD patients because the PD-N, PD-MCI and PD-D group is not only defined by these scores and thus the relationship between IGF-1 function and cognitive scores may be biased.

There are advantages in this study. Compared with other studies investigating IGF-1 function and global cognitive scores in PD, this study managed to include three groups of PD patients with more accurate cognitive status. Additionally, the recruitment of dementia patients in research can be challenging (Bartlett et al., 2018). Including a group with PD-D in the study provides a more complete picture of IGF-1 function in PD cognitive impairment.

There are limitations in this study. First, the sample size in the controls and PD-D group is small. However, a correlation between cGP and cognitive function suggests a promising application of cGP on monitoring cognitive function in normal people. Secondly, the cross-sectional design limits the ability of biomarkers for predicting longitudinal outcomes. A larger longitudinal study is needed to validate our results.

4.6 Summary

The increase of cGP/IGF-1 ratio with age preserved cognitive function in PD patients whereas the decrease of cGP/IGF-1 ratio with age is associated with dementia, with the MCI patients a transition phase between them. These observations raise the possibility that the associations of cGP/IGF-1 ratio with age may assist to predict cognitive status and the risk of advancing cognitive impairment in PD patients, if further confirmed in a larger and longitudinal trial.

Chapter 4 – Changes of plasma cGP/IGF-1 molar ratio with age is associated with the cognitive status of Parkinson's disease

5 Supplementation of blackcurrant anthocyanins increased cyclic glycine-proline in the cerebrospinal fluid of Parkinson patients: potential treatment to improve insulin-like growth factor-1 function

5.1 Summary of chapter contents

This chapter may provide a natural and achievable way to improve IGF-1 function and thus stroke recovery and cognition. The collection of samples and clinical data was accomplished by Yassar Alamri and the clinical data were used for his thesis. Therefore, this study only included clinical information as a background and the analysis of biological measures in my thesis. The analysis related to clinical data was not included in this thesis. Although no data show the relationship between cGP/IGF-1 molar ratio and clinical outcomes, this chapter suggests that plasma cGP have a better ability than IGF-1 to cross the blood-CSF barrier and could be a likely source for the increase of CSF cGP. This chapter firstly investigates the presence of cGP in the blackcurrant anthocyanins (BCA) and then the changes in concentrations of cGP, cGP/IGF-1 molar ratio, IGF-1 and IGFBP-3 in both plasma and CSF of PD patients before and after a 28-day BCA supplementation. Moreover, this chapter compared the central-uptaking efficiency of cGP with IGF-1 in plasma. This chapter was recently published as an original research article in Nutrients.

5.2 Introduction

PD is the second most common neurodegenerative condition. IGF-1, a neurotrophic factor, plays an essential role in neuronal survival and brain functions. IGF-1 resistance, characterized by the increase of circulating IGF-1 with impaired IGF-1 function, plays a role in the disease progression, cognitive impairment and pathology of idiopathic PD (Baldini et al., 2013; Cassilhas et al., 2010; Kao, 2009; Picillo et al., 2013). Thus, changes of plasma

IGF-1 have been under clinical development for monitoring the changes of IGF-1 function, in order to predict the prognosis and treatment responses in PD (Ma et al., 2015). However, the measurable IGF-1 in plasma is largely inactive. The majority of plasma IGF-1 is bound to IGFBPs, in which more than 75% is IGFBP-3 (Binoux, 1995). Binding to IGFBP-3 prevents IGF-1 from activating IGF-1 receptors, also from being metabolized (Binoux, 1995). Therefore, IGFBP-3 regulates IGF-1 function in both stimulatory and inhibitory manners, namely the autocrine regulation of IGF-1 (Binoux, 1995). Only a small amount of unbound IGF-1 in plasma is bioactive and the window of opportunity for directly detecting unbound IGF-1 is small because unbound IGF-1 is either rapidly metabolized or internalized after interacting with IGF receptors. Therefore, plasma IGF-1 concentration does not represent the function of IGF-1. Nonetheless, the IGF-1 concentration in plasma is still frequently evaluated for indicating IGF-1 function (Guan et al., 2015; Ma et al., 2015). As an alternative, the ratio of IGF-1/IGFBP-3 has been used for indicating 'free' bioactive IGF-1. Yet, the measurement includes a large amount of free IGFBP-3 (Guan et al., 2018); thus, is still not a reliable representation of bioavailable IGF-1.

A better representation of the bioavailability of IGF-1 is instead feasible by measuring the levels of cGP, a metabolite of 'free', bioactive IGF-1 (Guan et al., 2018; Singh-Mallah et al., 2016). The cGP is formed from the N-terminal tripeptide of IGF-1 after being cleaved by an acid enzyme (Bourguignon & Gérard, 1999; Guan et al., 2015; Nilsson-Håkansson et al., 1993; Sara et al., 1993; Yamamoto & Murphy, 1994, 1995). The N-terminal of IGF-1 is a primary binding site for IGFBP-3 (Sara et al., 1993) and cGP remains the same affinity of interacting with IGFBP-3 in a concentration-dependent manner (Guan et al., 2014). The function of cGP is mediated through competing with the binding of IGF-1 to IGFBP-3 (Guan et al., 2014), in which the higher ratio of cGP/IGF-1 associates with a greater bioavailable IGF-1 and better IGF-1 function (Guan et al., 2014). Administration of cGP protects the brain from ischemic injury in rats by improving IGF-1 function (Guan et al., 2007; Guan et al., 2014). A structure analog of

cGP also protects dopamine neurons from 6-hydroxydopamine induced injury and improves long-term functional recovery in a rat model of PD (Guan, Krishnamurthi, et al., 2000; Krishnamurthi et al., 2009).

High consumption of berry-fruits has been reported to be associated with a lower risk of PD (Gao, Cassidy, Schwarzschild, Rimm, & Ascherio, 2012). In an open-label study, PD patients show lower Hospital-associated Anxiety and Depression Scale (HADS) score after the supplementation of BCA (Alamri et al., 2016). However, the authors have disassociated the improved neuropsychological outcome with the supplementation, thus the mechanism underlying the beneficial effect of BCA remains unclear. It has been reported that the effects of anthocyanin on preventing apoptosis and cardiac dysfunction are mediated through activating IGF-1 receptors and signaling pathways in diabetic rats (P.-C. Huang et al., 2017). In addition, purple wheat is high in anthocyanins and the effects of purple wheat on prolonging the life span of Caenorhabditis elegans are associated with the IGF-1 signaling pathway (Chen et al., 2013). We, therefore, sought to examine the possibility of BCA intake on altering IGF-1 function by examining CSF and plasma levels of cGP, IGF-1 and IGFBPs.

5.3 Materials and Methods

5.3.1 Recruitment and clinical information of PD patients

The recruitment and clinical background of the patients in the clinical trial have been described in a previous publication (Alamri et al., 2016). Briefly, the patients were recruited form the Van der Veer Movement Disorders clinic and from the patient database of New Zealand Brain Research Institute. Patients were eligible to enroll in the study irrespective of the stage of disease and time since diagnosis. The patients were aged 40 years or older and met the UK Brain Bank criteria for idiopathic PD confirmed by a movement disorders neurologist. The study was approved by the Upper South A Regional Ethics Committee (reference: URA/10/03/022). A flowchart of the study population shows the patients recruiting (Figure 5.1).

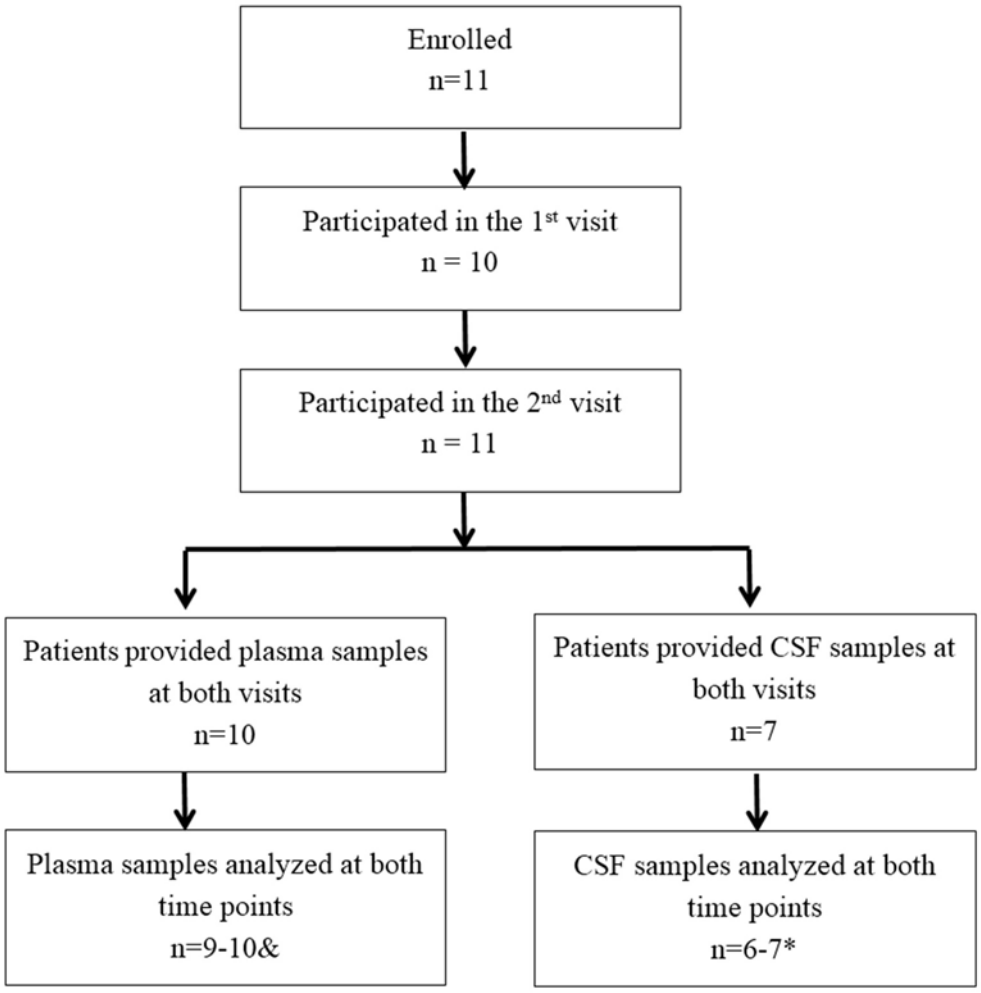


Figure 5.1 Flowchart of the study population.

*, & due to the amount of CSF samples available for analysis, seven pairs of CSF samples were analysed for cGP and six pairs for IGF-1 and IGFBP-3. The data from one participant were excluded from statistical analysis due to a 15-time increase in CSF cGP and 19 times increase in plasma cGP after supplementation.

Patients were assessed by experts using UPDRSIII and also administered a battery of psychocognitive tests before obtaining the samples at the first visit. These included the HADS, the Mini-Mental State Examination (MMSE), MoCA and the PD Questionnaire (PDQ-39). To avoid any learning effects different versions of the tests were used for the second visit if available. Table 5.1 shows the clinical information and assessments of the patients prior to the trial. The majority of patients were diagnosed as idiopathic PD without obvious cognitive impairment.

Table 5.1 Clinical information of 10 PD patients before BCA supplementation

Case	Age	Clinical Diagnosis	UPDRSIII	MMSE	MoCA	HADS	PDQ-39
BM02BC	61	Idiopathic PD	15	29	29	6	43.75
LE14BC	77	Idiopathic PD	48	27	22	3	31.25
SE07BC	73	Idiopathic PD	36	30	24	9	90.63
YY03BC	48	Idiopathic PD	33	29	29	8	59.83
EK05BC	80	Idiopathic PD	52	29	27	6	156.25
GD08BC	80	Idiopathic PD	34	28	24	11	134.88
ED12BC	60	Idiopathic PD	33	30	25	3	65.63
EY17BC	56	Idiopathic PD	27	30	28	21	159.38
TN15BC	70	Idiopathic PD	51	28	22	21	250
KT16BC	55	Idiopathic PD	31	28	26	4	71.88

HADS, Hospital Anxiety and Depression Scale; MMSE, Mini-mental State Examination; MoCA, Montreal Cognitive Assessment; PDQ-39, PD Questionnaire; UPDRS-III, Unified Parkinson's Disease Rating Scale-part three

5.3.2 Study design

The current study analysed the biological changes using the plasma and CSF collected from the clinical trial. The trial was run over two visits with 28 days apart. During each visit plasma and CSF samples were collected. Patients were instructed to consume a "low-anthocyanin diet" (i.e. white rice, white bread, tuna, chicken, coffee and non-herbal tea) 12 hours before each visit. Following the first visit, patients were supplemented with blackcurrant capsules over the next 28 days. The dose of BCA concentrated capsules (Super Currantex® 20, Vitality New Zealand, previous Just The Berries Ltd. New Zealand) was 300mg taken twice daily for a period of four weeks. The last dose was taken during or just before the second visit.

5.3.3 Sample collection and storage

The plasma and CSF samples were provided by the New Zealand Brain Research Institute, Christchurch, New Zealand. The BCA powder was provided by the Vitality New Zealand. The plasma samples were transported on dry ice to the University of Auckland and frozen under -80 °C immediately. When needed, the samples were thawed at 4 °C and thoroughly vortexed.

The CSF samples were obtained by lumbar punctures. The CSF sample, collected in a plain tube, was transported on wet ice to Endolab, Christchurch, New Zealand, within 15 min of collection. The samples were then centrifuged at 3000 rpm for 15 min at room temperature, and the supernatant was aliquoted equally between two plain tubes and frozen at −80 °C within 30 min of sample-receipt. After that, the samples were transported on dry ice to the University of Auckland and frozen under -80 °C immediately. When needed, the samples were thawed at 4 °C and thoroughly vortexed.

***In vitro* samples**

Powder from BCA concentrate capsules (Super Currantex® 20, Vitality New Zealand (previous Just The Berries Ltd. New Zealand) were store at 4 °C in a glass bottle sealed with parafilm. To analyze the potential presence of cGP in the BCA, the powder was dissolved in Milli-Q water with 3 different concentrations of 5, 50, and 100 mg/mL. Each concentration has been analyzed in five duplicates.

5.3.4 Measurement of concentrations of cGP in plasma using HPLC-MS

HPLC-MS was utilized to measure the concentration of total cGP in the samples. The methodology for plasma cGP extraction and HPLC-MS has been described in section 2.2.4 and 2.2.5.

5.3.5 Measurement of plasma concentration of IGF-1 and IGFBP-3 using ELISA

The concentrations of IGF-1 and IGFBP-3 were measured by ELISA. The methodology has been described in section 2.2.3.

5.3.6 Statistical Analysis

A paired t-test was used for analyzing the changes in cGP, IGF-1, IGFBP-3, and the ratio of cGP/IGF-1 before and after the BCA supplementation. Two-tailed one samples t-test was used for analyzing the percentage changes of concentrations. One-way ANOVA was used for analyzing the concentration of cGP of the BCA. Correlations between the biological changes were calculated using Pearson tests. A p-value of less than 0.05 is considered to be significant. Percentage change was calculated by the following formula: (value after supplementation − value before supplementation)/value before supplementation) × 100. The data were presented as mean ± SME and the percentage changes after supplementation.

5.4 Results

5.4.1 Analysis of cGP concentration in the BCA

One-way ANOVA suggested the concentration of cGP was significantly different between the samples with different dose of BCA ($p < 0.0001$, $n = 5$, Figure 5.2), with a dose-dependent increase of cGP concentration in the BCA. Compared to the samples with low dose of BCA (5 mg/mL), the concentration of cGP was significantly increased in the samples with 50 mg/mL BCA ($p < 0.0001$, $n = 5$) and further increased when the BCA dose increased to 100 mg/mL ($p < 0.0001$, $n = 5$).

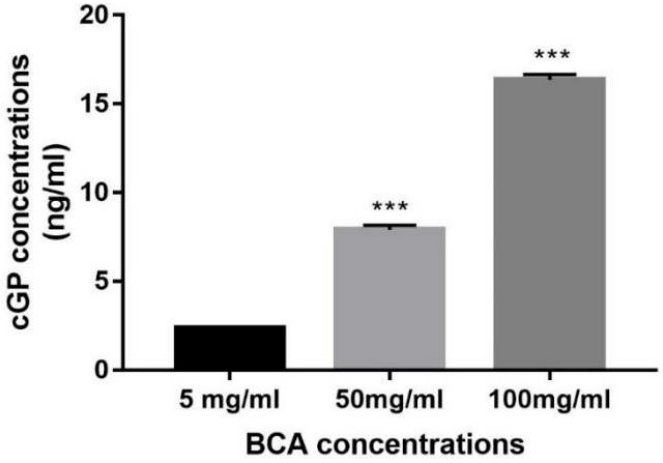


Figure 5.2 A dose-dependent increase of cGP concentration in the BCA.

Data presented as mean ± SEM, *** $p < 0.000$

5.4.2 Analysis in CSF and plasma

CSF

There was a significant increase in the concentration of CSF cGP after BCA supplementation (from ± 0.67 ng/mL to 12.12 ± 0.94 ng/mL, $p < 0.01$, $n = 6$, Figure 5.3a). The mean of percentage changes in cGP concentrations was increased by 74.36% after supplementation ($p < 0.05$, $t (5) = 3.989$). Amongst total seven pairs of samples, six of them showed an increase after supplementation (Figure 5.3b). One patient showed 16.9 times increase (11.30 ng/mL to 191.80 ng/mL) of cGP in the CSF, which has been eliminated from the statistical analysis as an outlier (15.7 times of mean, Figure 5.3b). One patient did not respond to the supplementation and the cGP concentration of this patient remained the same after the supplementation (from 8.8–8.7 ng/mL, Figure 5.3b). There was no significant change in the concentrations of IGF-1, cGP/IGF-1 ratio (Figure 5.3c, d), and IGFBP-3 (Table 5.2)

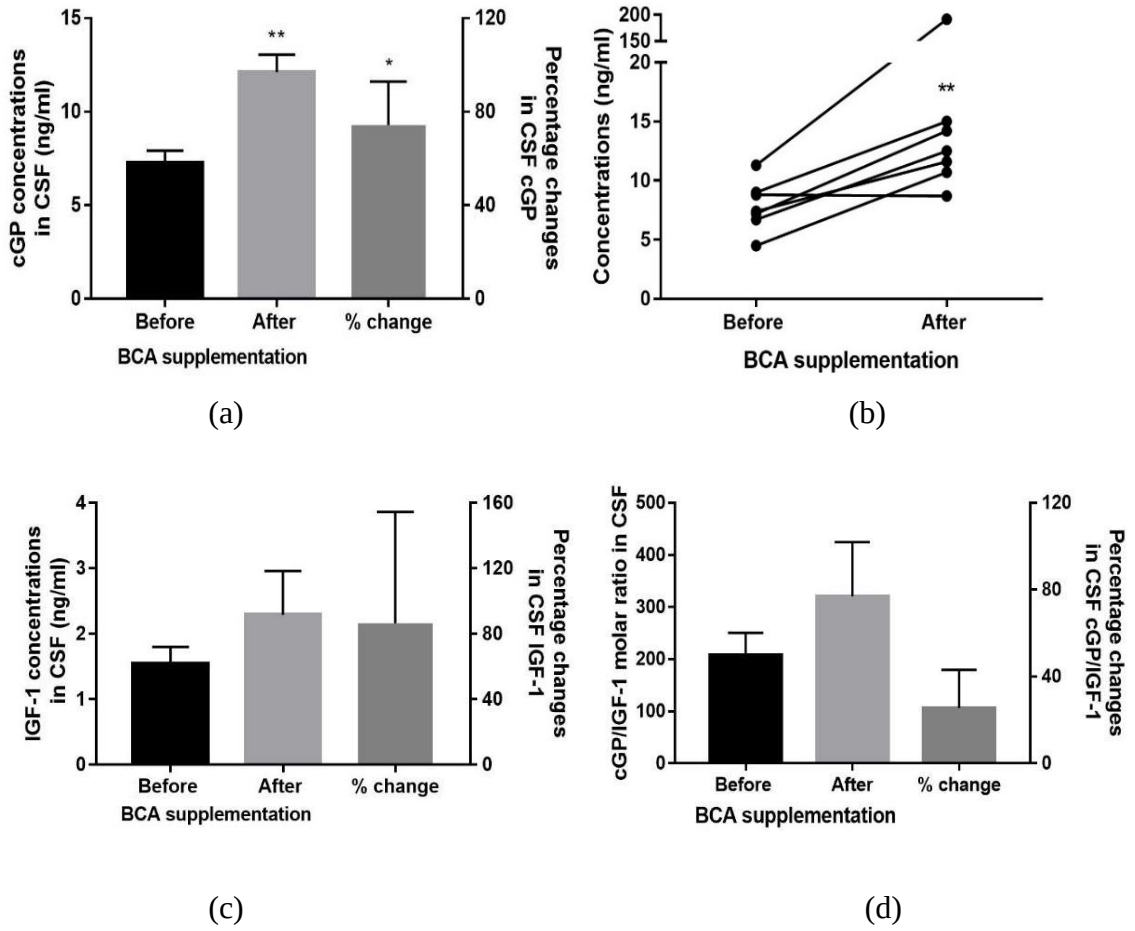


Figure 5.3 The changes in the CSF before and after the supplementation of the BCA.

(a) The concentration of cGP in the CSF was significantly increased after the supplementation compared to that prior to the supplementation. The percentage changes of cGP concentrations were significantly increased in the CSF. (b)There 6/7 paired samples showed an increase in cGP after supplementation, with one pair of samples increased by 16.9 times. cGP concentration of 1 pair of samples remained the same after the supplementation. (c)There was no statistical difference in IGF-1 concentration and percentage change of IGF-1 in the CSF. (d)There was no statistical difference in the cGP/IGF-1 ratio and percentage change of the ratio. Data presented as mean ± SEM and the percentage change from the baseline, * $p < 0.05$, ** $p < 0.01$.

Table 5.2 CSF and plasma concentrations of cGP, IGF-1 and IGFBP-3 before and after BCA supplementation

Before the supplementation

	CSF (ng/ml) Mean ± SEM (n=6)	Plasma (ng/ml) Mean ± SEM (n=9-10)	The ratio of CSF to plasma (%)
IGF-1	1.54 ± 0.26	179.04 ±14.89 (n=10)	0.86%
cGP	7.27 ±0.67	13.96 ± 1.33 (n=9)	52.01%
IGFBP-3	26.16 ±2.79	3038.92 ± 111.90 (n=10)	0.86%

After the supplementation

	CSF (ng/ml) Mean ± SEM (n=6)	Plasma (ng/ml) Mean ± SEM (n=9-10)	The ratio of CSF to plasma
IGF-1	2.29 ± 0.67	176.07 ± 14.13 (n=10)	1.30%
cGP	12.12 ± 0.94	16.92 ±2.79 (n=9)	71.63%
IGFBP-3	27.69 ± 3.53	3029.09 ± 59.35 (n=10)	0.91%

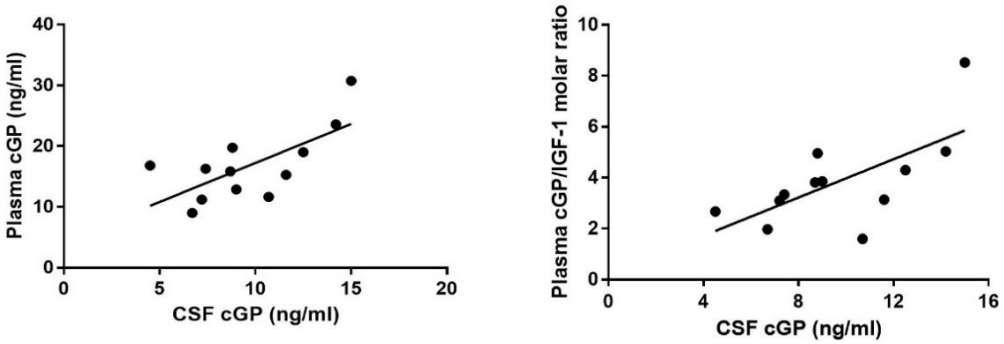
The CSF/plasma ratio was <1% in both IGF-1 and IGFBP-3 and 52% in cGP before supplementation and increased to 71% after the supplementation

Plasma

There were no changes in the concentrations of cGP, IGF-1, and IGFBP-3 following BCA supplementation (Table 5.2). Table 5.2 shows the relative values of cGP, IGF-1, and IGFBP-3 in CSF and plasma before and after supplementation. The CSF/plasma ratio was <1% in both IGF-1 and IGFBP-3 and 52% in cGP before supplementation and increased to 71% after the supplementation (Table 5.2).

5.4.3 Correlation Analysis

Pearson tests revealed strong correlation between the concentrations of cGP in CSF and plasma ($r = 0.68$, $p = 0.01$ Figure 5.4a, $n = 12$), as well as between the molar ratio of cGP/IGF-1 in plasma and cGP concentrations in CSF ($r = 0.66$, $p = 0.01$, Figure 5.4b, $n = 12$).



(a) (b)

Figure 5.4 Correlation between the concentrations of cGP in CSF and plasma.

(a) There was a significant positive correlation between CSF and plasma cGP ($r = 0.68$, $p < 0.01$). (b) There was a significant positive correlation between CSF cGP and plasma cGP/IGF-1 ($r = 0.66$, $p < 0.01$).

There was no correlation in IGF-1 concentration between the CSF and plasma ($r = -0.10$, $p = 0.75$) and no correlation between cGP and IGF-1 concentration in both CSF ($r = -0.13$, $p = 0.69$) and plasma ($r = 0.05$, $p = 0.85$).

5.5 Discussion

As a neuropeptide, cGP is a natural nutrient of the BCA. It is unclear if cGP is derived from IGF-1 or other proteins in blackcurrant in the blackcurrant extracts because IGF-1 was the only protein known to form cGP and, to my knowledge, has not been measured in blackcurrant extracts. The supplementation of the BCA led to an increase of CSF cGP in PD patients, suggesting oral availability and effective brain uptake of cGP. The cGP concentrations were correlated between the CSF and plasma, suggesting the plasma cGP may be the potential source.

Given the well-characterized function and mechanisms of IGF-1 and cGP in brain protection, the supplementation of the BCA may be further developed for treating neurological conditions through a larger clinical trial.

Neuroprotective effects of cGP have been demonstrated in a rat model with ischemic brain injury (Guan et al., 2007). The treatment effects of cGP are mediated by improving IGF-1 function by increasing bioavailable IGF-1 (Guan et al., 2014). Administration of a structure cGP analogue, cyclic Glycine-2ally-Proline (NNZ 2591), after the onset of motor deficits, improves long-term motor function in a rat model of PD (Krishnamurthi et al., 2009) and normalizes neuroplasticity in a rat model with acute memory impairment (Guan et al., 2010). The effectiveness of cGP and cGP analogue in brain protection and functional recovery proves that cGP is a neurotrophic agent. Supplementation of the BCA, which has such neurotrophic nutrients, increased CSF cGP concentration in PD patients. The data provide the first clinical evidence for oral availability and effective central uptake of cGP following the intake of foods.

cGP is a small and lipophilic molecule (192 d) and is able to cross the BBB in vivo (Li et al., 2019). Approximately 52% of plasma (endogenous) cGP found in the CSF before the supplementation, which increased to 71% after the supplementation. Given the ability of cGP crossing the BBB, the significant correlation between CSF and plasma concentration of cGP may suggest that the plasma cGP could be a likely source for the increase of CSF cGP, even though the supplementation did not change plasma concentration of cGP. We did not see a correlation between cGP and IGF-1 in the CSF, which could not exclude the possibility that a small part of CSF cGP forms from IGF-1 in the central nerve system (CNS), as the enzyme that cleaves IGF-1 also occurs in the CNS (Yamamoto & Murphy, 1995).

In contrast to cGP, the CSF concentration of IGF-1 was about 1% of that in the plasma, suggesting that CSF IGF-1 was largely independent of circulating IGF-1. IGF-1 is a larger molecule (7600 d) than cGP, with limited ability to cross the BBB (Thorne et al., 2004). Using

post-mortem human brain tissue, we have recently reported that the BBB function is not compromised in most of the brain regions of idiopathic PD cases that show the absence of dementia-related pathology (Yang et al., 2015). It is possible that the demand for trophic supports of degenerating brains promotes cGP transfer from plasma to CSF. Indeed, the direct administration of cGP to CSF protects brains from ischemic injury (Guan et al., 2014). Therefore, further increase of CSF cGP after supplementation of anthocyanin may potentially lead to the improvement of IGF-1 function in PD brains. Activating IGF-1 receptors and signaling pathways has been suggested to be the mechanism underlying the treatment effects of anthocyanin that prevents apoptosis and cardiac dysfunction in diabetic rats (P.C. Huang et al., 2017). However, clinical benefits from BCA supplementation were not conclusive, as the changes of CSF cGP were not significantly correlated with the HADS scores ($p = 0.18$, $r = 0.39$, data not shown). Large clinical trials are essential to confirm the efficacy of BCA supplementation in the clinical outcome of PD.

The majority of plasma IGF-1 is inactive due to the binding to IGFBPs (Binoux, 1995). Nonetheless, the increase in plasma IGF-1 has been used for indicating IGF-1 resistances in PD patients (Picillo et al., 2013). However, such changes in plasma IGF-1 are not always observed (Tuncel et al., 2009). The increased plasma IGF-1 in PD may be an ineffective endocrine response to IGF-1 deficiency and fail to produce more circulating IGF-1. The limited central uptake of IGF-1 could be a contributing factor to IGF-1 resistance in PD patients (Picillo et al., 2013). Circulating IGF-1 may decline when the condition of PD deteriorates to the stage with the complication of cognitive impairment (Ma et al., 2015; Tong et al., 2009).

Apart from increasing IGF-1 production, namely, endocrine regulation, the IGF-1 function is also regulated by improving autocrine/paracrine regulations, particularly under the situation that IGF-1 production is insufficient (Gluckman et al., 1992; Guan et al., 2018). The deficiency of IGF-1 could be the result of impaired autocrine regulation through interacting with IGFBPs

(Guan et al., 2018). We have reported the role of cGP in regulating IGF-1 bioavailability by competing with IGF-1 binding to IGFBP-3 (Guan et al., 2014) under both physiological (Singh-Mallah et al., 2016; Singh-Mallah et al., 2017) and pathological conditions (Guan et al., 2018). The competitive binding between IGF-1 and cGP is concentration-dependent, resulting in more cGP and more active IGF-1. Our observations from other clinical and experimental studies show that the increase of cGP and/or cGP/IGF-1 ratio is associated with weight changes in obese women (Guan et al., 2018), post-natal development (Singh-Mallah et al., 2016), and spontaneous recovery in stroke patients (Fan et al., 2019). Therefore, the changes in plasma cGP and/or cGP/IGF-1 ratio may provide an additional indication of IGF-1 function. Autocrine regulation of IGF-1 may present in the CNS, which could be different from that in plasma (Binoux, 1995). Given the limited access to CSF samples, a larger clinical trial is essential for determining whether plasma cGP and cGP/IGF-1 ratio would provide a reliable indication for IGF-1 function in PD and other neurological conditions with intact BBB.

Even though the results were significant, this pilot study has a clear limitation due to the small sample size. The interpretation of the results should be cautious until a confirmation is received from large clinical trials. A total of 6 out of 7 patients had positively responded to the supplementation by showing increases of cGP in the CSF, suggesting the change is sensitive. One patient did not respond to the treatment and had the highest score of PDQ-39. With 10 patients in each group, we also detected a significant decrease in plasma cGP in hypertensive women and an increase of cGP/IGF-1 ratio in obese women (Guan et al., 2018). The longitudinal design used in the current trial may eliminate some clinical variations and improve the sensitivity of the changes in plasma cGP and cGP/IGF-1 ratio (Guan et al., 2018). The clinical research for evaluating cGP and cGP/IGF-1 ratio for the IGF-1 function is still in its infancy. If it confirmed through large trials, the changes of plasma cGP would help to individualize BCA intervention.

My study only focused on the oral bioavailability of the cGP and did not study the long-term tolerability and side effects of the of BCA treatment. To my knowledge, the long-term tolerability of BCA treatment has not been reported, while a cGP analogue is generally recognized as safe (Bachurin et al., 2018). However, clinical data is currently unavailable. In the future, a long-term tolerability of BCA treatment could be researched in larger clinical studies.

5.6 Summary

This study for the first time provided clinical evidence of oral availability and better brain uptake of cGP than that of plasma IGF-1 after supplementation of the BCA. The increased cGP in the CSF of PD patients may be the result of the central uptake of plasma cGP. This is a potential treatment for improving circulating IGF-1 function if confirmed by larger clinical trials.

6 General discussion

This thesis addressed the hypothesis that plasma cGP/IGF-1 molar ratio could be a novel biomarker for circulating IGF-1 function and apply to predict stroke recovery and monitor cognitive status in PD. The first study examined the changes in plasma cGP/IGF-1 molar ratio over 90 days and its relationship with the functional recovery in stroke patients. The second study investigated the relationship between age-related changes of plasma cGP/IGF-1 molar ratio and cognitive status in PD. A final study examined the oral bioavailability of cGP after the BCA supplement. The thesis demonstrated the relationship between cGP/IGF-1 and circulating IGF-1 function and its potential application in the stroke and age-related cognitive impairment.

The low cGP/IGF-1 molar ratio in stroke patients suggested an impaired autocrine regulation. The increase of cGP/IGF-1 molar ratio in the initial 90 days of stroke recovery paralleled the functional recovery, suggesting an effective cGP-mediated autocrine regulation and an IGF-1 function-mediated recovery (Chapter 3). Moreover, a longitudinal association between 90 days of recovery and baseline plasma cGP/IGF-1 molar ratio suggests that the cGP/IGF-1 ratio may predict stroke recovery (Chapter 3).

In PD, the age-related changes of cGP/IGF-1 molar ratio are differently associated with cognitive status. We found an increase of cGP/IGF-1 molar ratio with age in PD with normal function, suggesting that a higher plasma cGP/IGF-1 molar ratio may maintain the cognitive function in PD. In contrast, we detected a decrease of cGP/IGF-1 molar ratio with age in PD patients with dementia, suggesting an impaired autocrine regulation of IGF-1 in dementia patients. The relationship of plasma cGP/IGF-1 ratio with clinical outcomes indicates the key role for cGP in regulating the function of circulating IGF-1 in cerebral vessel

dysfunction/degeneration, for example, stroke and age-related cognitive impairment. If confirmed in future, such findings may provide evidence of plasma cGP/IGF-1 as a reliable biomarker for IGF-1 function in circulation and could be applied to the prediction of stroke recovery and cognitive status. Given both stroke recovery and cognitive impairment are related to vascular function and microcirculation in the brain, this biomarker could have clinical applications in other neurological conditions with cerebrovascular dysfunctions.

The oral bioavailability of cGP is demonstrated in Chapter 5 by showing that oral administration of a cGP-rich BCA supplement increased the CSF cGP concentration. Finally, this chapter discusses the strengths and limitations and recommends future directions.

6.1 Changes of plasma cGP/IGF-1 molar ratio influence IGF-1 function in stroke and cognitive impairment

The changes of cGP/IGF-1 molar ratio is driven by the concentration of cGP and/or IGF-1 in plasma. The data in this thesis showed increased plasma cGP but stable IGF-1 concentrations during stroke recovery (Chapter 3). Plasma cGP also rose but IGF-1 declined with age in the PD-N patients (Chapter 4). The increase in plasma cGP levels could be a response to IGF-1 deficiency to increase IGF-1 in circulation in the situations of low plasma IGF-1 concentration during aging (absolute deficiency) and increased demand for IGF-1 activity during stroke recovery (relative deficiency). In support, the administration of cGP further improves stroke recovery (Guan et al., 2014) and the ability of learning and memory in developing rats (Singh-Mallah et al., 2016). A recent clinical study also shows increased plasma concentrations of cGP responding to decreased IGF-1 in obese women (Guan et al., 2018).

This endogenous compensatory response of cGP in normalizing IGF-1 function may be not always fully effective. The increased plasma cGP leads to a partial recovery in function (Chapter 3). In PD, although cognitive function in PD-N patients was ‘normal’, their cognitive

scores were lower than the normal controls, suggesting an initial decline in cognition (Chapter 4).

On the other hand, the reduced cGP concentrations in the stroke patients at hospital admission (Chapter 3) and an age-related reducing cGP concentration in the PD patients with dementia (Chapter 4) were observed in this thesis. These results suggest that the low cGP concentration in plasma may impair the autocrine regulation leading to IGF-1 dysfunction. In support, a clinical observation study showed a low plasma cGP concentration in hypertension patients (Guan et al., 2018). On the contrary, the cGP administration normalized the blood pressure in high-fat diet-induced hypertensive rats (unpublished data).

A decrease of IGFBP-3 concentration is also a response to the deficiency of IGF-1 to improve the amount of bioavailable IGF-1 (Guan et al., 2018; Singh-Mallah et al., 2016). The plasma IGFBP-3 is lower in stroke patients than the controls (Chapter 3) indicating the role of IGFBP-3-related autocrine regulation of IGF-1 in stroke. However, the concentration of IGFBP-3 remained the same while stroke patients made a partial recovery and there was no association with age and cognitive status in PD groups (Chapter 4). These data suggest IGFBP-3 may be less involved in IGF-1 associated vascular function than that of cGP, which needs further investigation.

Taken together, plasma cGP/IGF-1 is a biomarker for circulating IGF-1 function and could be applied to monitor and predict stroke recovery and cognitive status in PD.

6.2 Plasma cGP/IGF-1 molar ratio may be related to other neurological conditions with vascular origin

Physiologically, the majority of plasma IGF-1 is not bioactive and IGF-1 function depends on bioactive or bioavailable IGF-1 freed from IGFBP binding. Apart from the enzymes that break down IGFBPs (which not studied in the thesis), the amount of plasma cGP plays a critical role

in determining the amount of bioavailable IGF-1 (Guan et al., 2014). This autocrine regulation has a direct association with IGF-1 function in circulation but may be associated with the function of IGF-1 in brain tissues differently. A recent publication from our group described the changes of IGF-1, IGFBP-2 and cGP concentration in rat brain tissues and their association with synaptic function (Li et al., 2019). The authors suggested that the cGP-related autocrine regulation plays a minor role in the brain tissue as the brain IGF-1 is largely bioactive with a close correlation to synaptic function. It has been suggested that the truncated IGF-1, des-IGF-1, is a tissue form of IGF-1(Li et al., 2019). Even though the enzyme related to IGF-1 metabolism is present in brain tissues (Yamamoto & Murphy, 1995), the enzymatic activity is relatively less compared to that in plasma (Baker et al., 2005). These findings suggest that plasma cGP/IGF-1 may be not closely related to neurological conditions with neuronal origin.

However, plasma cGP/IGF-1 may be related to neurological conditions with vascular origin. As a systemic small vessel disease, hypertension is a life-long risk factor and a common contributor to stroke (Owolabi & Agunloye, 2013). Similar changes in endogenous cGP and response to cGP treatment in stroke and hypertension have been found (Guan et al., 2014 and unpublished data), suggesting the association of cGP-mediated autoregulation with small vessel diseases. Poor vascular health is a common risk factor contributing to cognitive impairment (Williams et al., 2018). Age-related vascular dysfunction contributes to cognitive decline (Raz et al., 2007; Toth et al., 2016). Variation of age-related cGP/IGF-1 molar ratio across the cognitive status in PD (Chapter 4) suggests the role of cGP-related regulation in maintaining age-related cognition. Given the nature of cerebral vascular degeneration/dysfunction in age-related cognitive impairment (Zhang et al., 2012), the changes in the plasma cGP/IGF-1 molar ratio may represent IGF-1-mediated cerebral vascular function/remodeling and its association with cognitive function.

Given both stroke and age-related cognitive impairment are neurological conditions with vascular origin, we speculate that the plasma cGP/IGF-1 ratio may have a limited application in other neurological conditions with the vascular origin. This needs further investigation.

cGP itself could be a marker of IGF-1 function when cGP and IGF-1 have the same changes, that is, both cGP and IGF-1 increase or decrease (shown in Table 4.3, Chapter 4). However, when they have different changes, cGP is less sensitive compared with cGP/IGF-1 as a biomarker of IGF-1 function (see relationship between NIHSS score and cGP in Chapter 3).

6.3 The oral bioavailability of cGP through natural products

Oral supplementation of cGP-rich BCA for 28 days increased the concentrations of cGP in the CSF of PD patients (Chapter 5). Taken together, these data suggest the oral bioavailability of cGP through natural products. In support of oral bioavailability of cGP, oral administration of cGP to rat dams increases the levels of cGP in the plasma and this has an association with an improvement in the learning and memory of offspring (Singh-Mallah et al., 2016). Another support is that oral administration of cGP analog to adult rats prevented scopolamine-induced memory deficits (Guan et al., 2010). Oral administration of cGP improves the concentration of cGP in brain tissues and synaptic expressions of rats (Li et al., 2019).

6.4 Advantages and limitations

This thesis demonstrated the cGP-related autocrine regulation of circulating IGF-1 function in both stroke and maintenance of cognition in PD in clinical settings. However, the low number of participants in each chapter could compromise the generalization of our conclusion. The outcome of my stroke study needs to be further confirmed in a larger, longer-term longitudinal recovery study. Likewise, longitudinal follow-up, with continued cognitive decline and plasma sample collection, of the PD-N and PD-MCI groups is needed to establish if the change in age-related cGP/IGF-1 ratio is, at the individual level, associated with cognitive decline as indicated

by the cross-sectional data presented in Chapter 4. Besides, stroke and cognitive impairment are related to vascular dysfunction and thus it is still not clear whether cGP/IGF-1 ratio could be applied to predict deficits caused by a neurodegenerative process such as motor deficits in PD. Nutritional factors are critical regulators of circulating IGF-1 levels. In particular, undernutrition of either protein or energy intake substantially lowers IGF-1 levels and the deficiency of essential amino acids has a severe depressing effect on IGF-1 levels (Thissen et al., 1998). Unfortunately, clinical data related to nutritional status were not collected in my studies. In future, this information will be included to give a more accurate assessment of the associations between cGP or cGP/IGF-1 and IGF-1 function. The current evidence hasn't shown the direct interaction of cGP with nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). However, it is possible that cGP also has effects on NGF and BDNF because these neurotrophins share the downstream signaling pathway with IGF-1 (Koh & Lo, 2015).

The high-performance liquid-chromatography mass spectrometry assay is currently not an easily available method for clinical practice. As a high complexity system, this assay requires a high level of technical expertise to develop and validate methods as well as troubleshoot the instruments (Adaway et al., 2014). However, further advancements may enable this assay to be used as a convenient clinical analytical approach.

6.5 Future directions and applications

6.5.1 The molar ratio of cGP/IGF-1 as a potential biomarker in conditions with vascular origin

The increasing studies show the involvement of cerebrovascular dysfunction in the underlying mechanisms of various neurological conditions. Cerebrovascular alterations play a role in

various neurological conditions (Cai et al., 2017; Tarantini et al., n.d.). Given that stroke and cognition are closely related to cerebrovascular function and integrity, this plasma biomarker may apply to other neurological conditions with associated changes in cerebral vascular, like Alzheimer's disease. The most dominant hypothesis of Alzheimer's disease is amyloid β-protein aggregation (Selkoe & Hardy, 2016). Vascular dysfunction is related to amyloid β-protein accumulation in the parenchyma and blood vessels due to the inability of amyloid protein clearance from the brain (Canobbio et al., 2015; Janota et al., 2016). Therefore, plasma cGP/IGF-1 molar ratio may be applied to Alzheimer's disease.

6.5.2 Intervention with cGP-rich natural products for stroke and PD patients

The limited ability to cross the BBB and the potential adverse effects of exogenous IGF-1 prevents the pharmaceutical using IGF-1 (Kemp, Fowlkes, & Thrailkill, 2006). Given that cGP normalizes IGF-1 function (Guan et al., 2014), oral administration of cGP by nature products may provide an easily achievable approach for improving IGF-1 function without inducing side effects. cGP treatment restores IGF-1 receptor-associated angiogenic capillaries following ischemic injury in the rats (Guan et al., 2014). Peripheral administration of a cGP analogue improves long-term motor function in the 6-OHDA lesioned rat (Krishnamurthi, Mathai, Kim, Zhang, & Guan, 2009) and cognitive function in rats (Guan et al., 2010; Singh-Mallah et al., 2016). Therefore, intervention with a cGP-rich natural product might potentially improve stroke recovery and prevent cognitive impairment in PD patients.

7 Conclusion

This thesis reports the representation of plasma cGP/IGF-1 molar ratio for the function of circulating IGF-1. This suggestive biomarker was validated by its association with clinical outcomes during stroke recovery, cognitive retention of old healthy people and changing

cognitive status in PD. The oral bioavailability of cGP by natural products is also explored. Increased cGP/IGF-1 suggests cGP-mediated autoregulation to improve circulating IGF-1 function while the declined plasma cGP/IGF-1 suggests the impaired autoregulation to decrease circulating IGF-1 function. Plasma cGP/IGF-1 molar ratio could be applied to predict stroke recovery and monitor age-related cognitive impairment, if further confirmed in larger and longitudinal trials. Given that stroke and cognitive impairments are associated with vascular health and function, this plasma biomarker might be also relevant to other neurological conditions with poor vascular functions. The oral bioavailability of cGP suggests a potentially effective intervention for improving IGF-1 function.

References

Aarsland, D., & Kurz, M. W. (2010). The epidemiology of dementia associated with Parkinson disease. *Journal of the Neurological Sciences*, *289*(1), 18–22. https://doi.org/10.1016/j.jns.2009.08.034

Åberg, D., Jood, K., Blomstrand, C., Jern, C., Nilsson, M., Isgaard, J., & Aberg, N. D. (2011). Serum IGF-I levels correlate to improvement of functional outcome after ischemic stroke. *The Journal of Clinical Endocrinology and Metabolism*, *96*(7), E1055-1064. https://doi.org/10.1210/jc.2010-2802

Adaway, J. E., Keevil, B. G., & Owen, L. J. (2014). Liquid chromatography tandem mass spectrometry in the clinical laboratory: *Annals of Clinical Biochemistry*. https://doi.org/10.1177/0004563214557678

Ajo, R., Cacicedo, L., Navarro, C., & Sánchez-Franco, F. (2003). Growth Hormone Action on Proliferation and Differentiation of Cerebral Cortical Cells from Fetal Rat. *Endocrinology*, *144*(3), 1086–1097. https://doi.org/10.1210/en.2002-220667

Akbari, E., Asemi, Z., Daneshvar Kakhaki, R., Bahmani, F., Kouchaki, E., Tamtaji, O. R., Hamidi, G. A., & Salami, M. (2016). Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial. *Frontiers in Aging Neuroscience*, *8*. https://doi.org/10.3389/fnagi.2016.00256

Alamri, Y., MacAskill, M., & Anderson, T. (2016). Repeated Lumbar Punctures for Non-Clinical Indications: How Do Patients Feel? *European Neurology*, *76*(3–4), 123–124. https://doi.org/10.1159/000449056

References

Aleman, A., & Torres-Alemán, I. (2009). Circulating insulin-like growth factor I and cognitive function: Neuromodulation throughout the lifespan. *Progress in Neurobiology*, *89*(3), 256–265. https://doi.org/10.1016/j.pneurobio.2009.07.008

Allegra, C. J., Jessup, J. M., Somerfield, M. R., Hamilton, S. R., Hammond, E. H., Hayes, D. F., McAllister, P. K., Morton, R. F., & Schilsky, R. L. (2009). American Society of Clinical Oncology Provisional Clinical Opinion: Testing for KRAS Gene Mutations in Patients With Metastatic Colorectal Carcinoma to Predict Response to Anti–Epidermal Growth Factor Receptor Monoclonal Antibody Therapy. *Journal of Clinical Oncology*, *27*(12), 2091–2096. https://doi.org/10.1200/JCO.2009.21.9170

Babyak, M. A. (2004). What You See May Not Be What You Get: A Brief, Nontechnical Introduction to Overfitting in Regression-type Models. *Psychosomatic Medicine*, *66*(3), 411–421.

Bach, L. A. (2015). Endothelial cells and the IGF system. *Journal of Molecular Endocrinology*, *54*(1), R1–R13. https://doi.org/10.1530/JME-14-0215

Bachurin, S. O., Gavrilova, S. I., Samsonova, A., Barreto, G. E., & Aliev, G. (2018). Mild cognitive impairment due to Alzheimer disease: Contemporary approaches to diagnostics and pharmacological intervention. *Pharmacological Research*, *129*, 216–226. https://doi.org/10.1016/j.phrs.2017.11.021

Bake, S., Okoreeh, A. K., Alaniz, R. C., & Sohrabji, F. (2016). Insulin-Like Growth Factor (IGF)-I Modulates Endothelial Blood-Brain Barrier Function in Ischemic Middle-Aged Female Rats. *Endocrinology*, *157*(1), 61–69. https://doi.org/10.1210/en.2015-1840

Baker, A. M., Batchelor, D. C., Thomas, G. B., Wen, J. Y., Rafiee, M., Lin, H., & Guan, J. (2005). Central penetration and stability of N-terminal tripeptide of insulin-like growth factor-I, glycine-proline-glutamate in adult rat. *Neuropeptides*, *39*(2), 81–87. https://doi.org/10.1016/j.npep.2004.11.001

References

Baldini, S., Restani, L., Baroncelli, L., Coltelli, M., Franco, R., Cenni, M. C., Maffei, L., & Berardi, N. (2013). Enriched Early Life Experiences Reduce Adult Anxiety-Like Behavior in Rats: A Role for Insulin-Like Growth Factor 1. *Journal of Neuroscience*, *33*(28), 11715–11723. https://doi.org/10.1523/JNEUROSCI.3541-12.2013

Bartlett, R., Milne, R., & Croucher, R. (2018). Strategies to improve recruitment of people with dementia to research studies. *Dementia*, 1471301217748503. https://doi.org/10.1177/1471301217748503

Batchelor, D. C., Lin, H., Wen, J.-Y., Keven, C., Zijl, P. L. V., Breier, B. H., Gluckman, P. D., & Thomas, G. B. (2003). Pharmacokinetics of glycine–proline–glutamate, the N-terminal tripeptide of insulin-like growth factor-1, in rats. *Analytical Biochemistry*, *323*(2), 156–163. https://doi.org/10.1016/j.ab.2003.08.032

Beach, T. G., Adler, C. H., Serrano, G., Sue, L. I., Walker, D. G., Dugger, B. N., Shill, H. A., Driver-Dunckley, E., Caviness, J. N., Intorcia, A., Filon, J., Scott, S., Garcia, A., Hoffman, B., Belden, C. M., Davis, K. J., & Sabbagh, M. N. (2016). Prevalence of Submandibular Gland Synucleinopathy in Parkinson's Disease, Dementia with Lewy Bodies and other Lewy Body Disorders. *Journal of Parkinson's Disease*, *6*(1), 153–163. https://doi.org/10.3233/JPD-150680

Beilharz, E. J., Russo, V. C., Butler, G., Baker, N. L., Connor, B., Sirimanne, E. S., Dragunow, M., Werther, G. A., Gluckman, P. D., Williams, C. E., & Scheepens, A. (1998). Co-ordinated and cellular specific induction of the components of the IGF/IGFBP axis in the rat brain following hypoxic–ischemic injury. *Molecular Brain Research*, *59*(2), 119–134. https://doi.org/10.1016/S0169-328X(98)00122-3

Bergman, D., Halje, M., Nordin, M., & Engström, W. (2013). Insulin-Like Growth Factor 2 in Development and Disease: A Mini-Review. *Gerontology*, *59*(3), 240–249. https://doi.org/10.1159/000343995

References

Binoux, M. (1995). The IGF system in metabolism regulation. *Diabete & Metabolisme*, *21*(5), 330–337.

Biomarkers Definitions Working Group. (2001). Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clinical Pharmacology & Therapeutics*, *69*(3), 89–95. https://doi.org/10.1067/mcp.2001.113989

Bondanelli, M., Ambrosio, M. R., Onofri, A., Bergonzoni, A., Lavezzi, S., Zatelli, M. C., Valle, D., Basaglia, N., & degli Uberti, E. C. (2006). Predictive Value of Circulating Insulin-Like Growth Factor I Levels in Ischemic Stroke Outcome. *The Journal of Clinical Endocrinology & Metabolism*, *91*(10), 3928–3934. https://doi.org/10.1210/jc.2006-1040

Bourguignon, J.-P., & Gérard, A. (1999). Role of insulin-like growth factor binding proteins in limitation of IGF-I degradation into the N-methyl-d-aspartate receptor antagonist GPE: Evidence from gonadotrophin-releasing hormone secretion in vitro at two developmental stages. *Brain Research*, *847*(2), 247–252. https://doi.org/10.1016/S0006-8993(99)02051-X

Cai, W., Zhang, K., Li, P., Zhu, L., Xu, J., Yang, B., Hu, X., Lu, Z., & Chen, J. (2017). Dysfunction of the neurovascular unit in ischemic stroke and neurodegenerative diseases: An aging effect. *Ageing Research Reviews*, *34*, 77–87. https://doi.org/10.1016/j.arr.2016.09.006

Canobbio, I., Abubaker, A. A., Visconte, C., Torti, M., & Pula, G. (2015). Role of amyloid peptides in vascular dysfunction and platelet dysregulation in Alzheimer's disease. *Frontiers in Cellular Neuroscience*, *9*. https://doi.org/10.3389/fncel.2015.00065

Carro, E., Trejo, J. L., Núñez, A., & Torres-Aleman, I. (2003). Brain repair and neuroprotection by serum insulin-like growth factor I. *Molecular Neurobiology*, *27*(2), 153–162. https://doi.org/10.1385/MN:27:2:153

References

Cassilhas, R. C., Antunes, H. K. M., Tufik, S., & de Mello, M. T. (2010). Mood, Anxiety, and Serum IGF-1 in Elderly Men Given 24 Weeks of High Resistance Exercise. *Perceptual and Motor Skills*, *110*(1), 265–276. https://doi.org/10.2466/pms.110.1.265-276

Chen, W., Müller, D., Richling, E., & Wink, M. (2013). Anthocyanin-rich Purple Wheat Prolongs the Life Span of Caenorhabditis elegans Probably by Activating the DAF-16/FOXO Transcription Factor. *Journal of Agricultural and Food Chemistry*, *61*(12), 3047–3053. https://doi.org/10.1021/jf3054643

Collett-Solberg, P. F., & Cohen, P. (1996). The role of the insulin-like growth factor binding proteins and the IGFBP proteases in modulating IGF action. *Endocrinology and Metabolism Clinics of North America*, *25*(3), 591–614. https://doi.org/10.1016/S0889-8529(05)70342-X

Corpas, E., Harman, S. M., & Blackman, M. R. (1993). Human growth hormone and human aging. *Endocrine Reviews*, *14*(1), 20–39. https://doi.org/10.1210/edrv-14-1-20

Counsell, C., Dennis, M., & McDowall, M. (2004). Predicting functional outcome in acute stroke: Comparison of a simple six variable model with other predictive systems and informal clinical prediction. *Journal of Neurology, Neurosurgery & Psychiatry*, *75*(3), 401–405. https://doi.org/10.1136/jnnp.2003.018085

Cramer, S. C. (2008). Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. *Annals of Neurology*, *63*(3), 272–287. https://doi.org/10.1002/ana.21393

Dai, Z., Wu, F., Yeung, E. W., & Li, Y. (2010). IGF-IEc expression, regulation and biological function in different tissues. *Growth Hormone & IGF Research*, *20*(4), 275–281. https://doi.org/10.1016/j.ghir.2010.03.005

Dalrymple-Alford, J. C., Livingston, L., MacAskill, M. R., Graham, C., Melzer, T. R., Porter, R. J., Watts, R., & Anderson, T. J. (2011). Characterizing mild cognitive impairment in

Parkinson's disease. *Movement Disorders*, *26*(4), 629–636. https://doi.org/10.1002/mds.23592

De Smedt, A., Brouns, R., Uyttenboogaart, M., De Raedt, S., Moens, M., Wilczak, N., Luijckx, G.-J., & De Keyser, J. (2011). Insulin-like growth factor I serum levels influence ischemic stroke outcome. *Stroke; a Journal of Cerebral Circulation*, *42*(8), 2180–2185. https://doi.org/10.1161/STROKEAHA.110.600783

Dong, X., Chang, G., Ji, X.-F., Tao, D.-B., & Wang, Y.-X. (2014). The Relationship between Serum Insulin-Like Growth Factor I Levels and Ischemic Stroke Risk. *PLoS ONE*, *9*(4), e94845. https://doi.org/10.1371/journal.pone.0094845

Douillard, J.-Y., Oliner, K. S., Siena, S., Tabernero, J., Burkes, R., Barugel, M., Humblet, Y., Bodoky, G., Cunningham, D., Jassem, J., Rivera, F., Kocákova, I., Ruff, P., Błasińska-Morawiec, M., Šmakal, M., Canon, J. L., Rother, M., Williams, R., Rong, A., … Patterson, S. D. (2013). Panitumumab–FOLFOX4 Treatment and RAS Mutations in Colorectal Cancer. *New England Journal of Medicine*, *369*(11), 1023–1034. https://doi.org/10.1056/NEJMoa1305275

Duan, C., & Xu, Q. (2005). Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. *General and Comparative Endocrinology*, *142*(1–2), 44–52. https://doi.org/10.1016/j.ygcen.2004.12.022

Dyer, A. H., Vahdatpour, C., Sanfeliu, A., & Tropea, D. (2016). The role of Insulin-Like Growth Factor 1 (IGF-1) in brain development, maturation and neuroplasticity. *Neuroscience*, *325*, 89–99. https://doi.org/10.1016/j.neuroscience.2016.03.056

Ebinger, M., Ipsen, N., Leonards, C., Empl, L., Hanne, L., Liman, T., Mai, K., Strasburger, C., Spranger, J., & Endres, M. (2015). Circulating Insulin-like Growth Factor Binding Protein-3 Predicts One-year Outcome after Ischemic Stroke. *Experimental and Clinical*

Endocrinology & Diabetes, *123*(08), 461–465. https://doi.org/10.1055/s-0035-1554632

Emre, M., Aarsland, D., Brown, R., Burn, D. J., Duyckaerts, C., Mizuno, Y., Broe, G. A., Cummings, J., Dickson, D. W., Gauthier, S., Goldman, J., Goetz, C., Korczyn, A., Lees, A., Levy, R., Litvan, I., McKeith, I., Olanow, W., Poewe, W., … Dubois, B. (2007). Clinical diagnostic criteria for dementia associated with Parkinson's disease. *Movement Disorders: Official Journal of the Movement Disorder Society*, *22*(12), 1689–1707; quiz 1837. https://doi.org/10.1002/mds.21507

Fan, D., Alamri, Y., Liu, K., MacAskill, M., Harris, P., Brimble, M., Dalrymple-Alford, J., Prickett, T., Menzies, O., Laurenson, A., Anderson, T., & Guan, J. (2018). Supplementation of Blackcurrant Anthocyanins Increased Cyclic Glycine-Proline in the Cerebrospinal Fluid of Parkinson Patients: Potential Treatment to Improve Insulin-Like Growth Factor-1 Function. *Nutrients*, *10*(6), 714. https://doi.org/10.3390/nu10060714

Fan, D., Krishnamurthi, R., Harris, P., Barber, P. A., & Guan, J. (2019). Plasma cyclic glycine proline/IGF-1 ratio predicts clinical outcome and recovery in stroke patients. *Annals of Clinical and Translational Neurology*, *0*(0). https://doi.org/10.1002/acn3.743

Feigin, V. L., Forouzanfar, M. H., Krishnamurthi, R., Mensah, G. A., Connor, M., Bennett, D. A., Moran, A. E., Sacco, R. L., Anderson, L., Truelsen, T., O'Donnell, M., Venketasubramanian, N., Barker-Collo, S., Lawes, C. M. M., Wang, W., Shinohara, Y., Witt, E., Ezzati, M., Naghavi, M., & Murray, C. (2014). Global and regional burden of stroke during 1990–2010: Findings from the Global Burden of Disease Study 2010. *The Lancet*, *383*(9913), 245–255. https://doi.org/10.1016/S0140-6736(13)61953-4

References

Fernandez, A. M., & Torres-Alemán, I. (2012). The many faces of insulin-like peptide signalling in the brain. *Nature Reviews Neuroscience*, *13*(4), 225–239. https://doi.org/10.1038/nrn3209

Filiou, M. D., & Turck, C. W. (2011). Chapter 1 - General overview: Biomarkers in neuroscience research. In P. C. G. and S. Bahn (Ed.), *International Review of Neurobiology* (Vol. 101, pp. 1–17). Academic Press. http://www.sciencedirect.com/science/article/pii/B9780123877185000018

Firth, S. M., & Baxter, R. C. (2002). Cellular actions of the insulin-like growth factor binding proteins. *Endocrine Reviews*, *23*(6), 824–854. https://doi.org/10.1210/er.2001-0033

Fowlkes, J. L., Serra, D. M., Bunn, R. C., Thrailkill, K. M., Enghild, J. J., & Nagase, H. (2004). Regulation of Insulin-Like Growth Factor (IGF)-I Action by Matrix Metalloproteinase-3 Involves Selective Disruption of IGF-I/IGF-Binding Protein-3 Complexes. *Endocrinology*, *145*(2), 620–626. https://doi.org/10.1210/en.2003-0636

Fowlkes, J. L., Thrailkill, K. M., Serra, D. M., Suzuki, K., & Nagase, H. (1995). Matrix metalloproteinases as insulin-like growth factor binding protein-degrading proteinases. *Progress in Growth Factor Research*, *6*(2), 255–263. https://doi.org/10.1016/0955-2235(95)00017-8

Frater, J., Lie, D., Bartlett, P., & McGrath, J. J. (2017). Insulin-like Growth Factor 1 (IGF-1) as a marker of cognitive decline in normal ageing: A review. *Ageing Research Reviews*, *42*, 14–27. https://doi.org/10.1016/j.arr.2017.12.002

Gandolfi, M., Smania, N., Vella, A., Picelli, A., & Chirumbolo, S. (2017). Assessed and Emerging Biomarkers in Stroke and Training-Mediated Stroke Recovery: State of the Art. *Neural Plasticity*, *2017*, 1389475. https://doi.org/10.1155/2017/1389475

References

Gao, X., Cassidy, A., Schwarzschild, M. A., Rimm, E. B., & Ascherio, A. (2012). Habitual intake of dietary flavonoids and risk of Parkinson disease. *Neurology*, *78*(15), 1138–1145. https://doi.org/10.1212/WNL.0b013e31824f7fc4

Gluckman, P., Klempt, N., Guan, J., Mallard, C., Sirimanne, E., Dragunow, M., Klempt, M., Singh, K., Williams, C., & Nikolics, K. (1992). A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochemical and Biophysical Research Communications*, *182*(2), 593–599.

Godau, J., Herfurth, M., Kattner, B., Gasser, T., & Berg, D. (2010). Increased serum insulin-like growth factor 1 in early idiopathic Parkinson's disease. *Journal of Neurology, Neurosurgery and Psychiatry*, *81*(5), 536–538. Scopus. https://doi.org/10.1136/jnnp.2009.175752

Gorelick, P. B., Scuteri, A., Black, S. E., DeCarli, C., Greenberg, S. M., Iadecola, C., Launer, L. J., Laurent, S., Lopez, O. L., Nyenhuis, D., Petersen, R. C., Schneider, J. A., Tzourio, C., Arnett, D. K., Bennett, D. A., Chui, H. C., Higashida, R. T., Lindquist, R., Nilsson, P. M., … Seshadri, S. (2011). Vascular Contributions to Cognitive Impairment and Dementia. *Stroke; a Journal of Cerebral Circulation*, *42*(9), 2672–2713. https://doi.org/10.1161/STR.0b013e3182299496

Guan, Gluckman, P., Yang, P., Krissansen, G., Sun, X., Zhou, Y., Wen, J., Phillips, G., Shorten, P. R., McMahon, C. D., Wake, G. C., Chan, W. H. K., Thomas, M. F., Ren, A., Moon, S., & Liu, D.-X. (2014). Cyclic glycine-proline regulates IGF-1 homeostasis by altering the binding of IGFBP-3 to IGF-1. *Scientific Reports*, *4*. https://doi.org/10.1038/srep04388

Guan, J, Bennet, L., Gluckman, P. D., & Gunn, A. J. (2003). Insulin-like growth factor-1 and post-ischemic brain injury. *Progress in Neurobiology*, *70*(6), 443–462. https://doi.org/10.1016/j.pneurobio.2003.08.002

References

Guan, J., Gunn, A. J., Sirimanne, E. S., Tuffin, J., Gunning, M. I., Clark, R., & Gluckman, P. D. (2000). The window of opportunity for neuronal rescue with insulin-like growth factor-1 after hypoxia-ischemia in rats is critically modulated by cerebral temperature during recovery. *Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *20*(3), 513–519. https://doi.org/10.1097/00004647-200003000-00010

Guan, J., Krishnamurthi, R., Waldvogel, H. J., Faull, R. L., Clark, R., & Gluckman, P. (2000). N-terminal tripeptide of IGF-1 (GPE) prevents the loss of TH positive neurons after 6-OHDA induced nigral lesion in rats. *Brain Research*, *859*(2), 286–292.

Guan, J., Miller, O. T., Waugh, K. M., McCarthy, D. C., & Gluckman, P. D. (2001). Insulin-like growth factor-1 improves somatosensory function and reduces the extent of cortical infarction and ongoing neuronal loss after hypoxia-ischemia in rats. *Neuroscience*, *105*(2), 299–306.

Guan, J, Waldvogel, H. J., Faull, R. L. M., Gluckman, P. D., & Williams, C. E. (1999). The effects of the N-terminal tripeptide of insulin-like growth factor-1, glycine-proline-glutamate in different regions following hypoxic-ischemic brain injury in adult rats. *Neuroscience*, *89*(3), 649–659. https://doi.org/10.1016/S0306-4522(98)00338-8

Guan, J., Williams, C., Gunning, M., Mallard, C., & Gluckman, P. (1993). The effects of IGF-1 treatment after hypoxic-ischemic brain injury in adult rats. *Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *13*(4), 609–616. https://doi.org/10.1038/jcbfm.1993.79

Guan, Jian, & Gluckman, P. D. (2009). IGF-1 derived small neuropeptides and analogues: A novel strategy for the development of pharmaceuticals for neurological conditions. *British Journal of Pharmacology*, *157*(6), 881–891. https://doi.org/10.1111/j.1476-5381.2009.00256.x

References

Guan, Jian, Harris, P., Brimble, M., Lei, Y., Lu, J., Yang, Y., & Gunn, A. J. (2015). The role for IGF-1-derived small neuropeptides as a therapeutic target for neurological disorders. *Expert Opinion on Therapeutic Targets*, *19*(6), 785–793. https://doi.org/10.1517/14728222.2015.1010514

Guan, Mathai, S., Harris, P., Wen, J.-Y., Zhang, R., Brimble, M., & Gluckman, P. (2007). Peripheral administration of a novel diketopiperazine, NNZ 2591, prevents brain injury and improves somatosensory-motor function following hypoxia–ischemia in adult rats. *Neuropharmacology*, *53*(6), 749–762. https://doi.org/10.1016/j.neuropharm.2007.08.010

Guan, Singh-Mallah, G., Liu, K., Thorstensen, E., Shorten, P., Mitchell, E. A., Taylor, R., Harris, P., Brimble, M., Thompson, J. M. D., & Murphy, R. (2018). The role for cyclic Glycine-Proline, a biological regulator of insulin-like growth factor-1 in pregnancy-related obesity and weight changes. *Journal of Biological Regulators and Homeostatic Agents*, *32*(3), 465–478.

Guan, Zhang, R., Dale-Gandar, L., Hodgkinson, S., & Vickers, M. H. (2010). NNZ-2591, a novel diketopiperazine, prevented scopolamine-induced acute memory impairment in the adult rat. *Behavioural Brain Research*, *210*(2), 221–228. https://doi.org/10.1016/j.bbr.2010.02.039

Gudasheva, T. A., Boyko, S. S., Akparov, V. K., Ostrovskaya, R. U., Skoldinov, S. P., Rozantsev, G. G., Voronina, T. A., Zherdev, V. P., & Seredenin, S. B. (1996). Identification of a novel endogenous memory facilitating cyclic dipeptide cyclo-prolylglycine in rat brain. *FEBS Letters*, *391*(1–2), 149–152.

Hankey, G. J. (2017). Stroke. *The Lancet*, *389*(10069), 641–654. https://doi.org/10.1016/S0140-6736(16)30962-X

References

Harada, C. N., Natelson Love, M. C., & Triebel, K. (2013). Normal Cognitive Aging. *Clinics in Geriatric Medicine*, *29*(4), 737–752. https://doi.org/10.1016/j.cger.2013.07.002

Hawkins, D. M. (2004). The Problem of Overfitting. *Journal of Chemical Information and Computer Sciences*, *44*(1), 1–12. https://doi.org/10.1021/ci0342472

Huang, P.-C., Wang, G.-J., Fan, M.-J., Asokan Shibu, M., Liu, Y.-T., Padma Viswanadha, V., Lin, Y.-L., Lai, C.-H., Chen, Y.-F., Liao, H.-E., & Huang, C.-Y. (2017). Cellular apoptosis and cardiac dysfunction in STZ-induced diabetic rats attenuated by anthocyanins via activation of IGFI-R/PI3K/Akt survival signaling. *Environmental Toxicology*, *32*(12), 2471–2480. https://doi.org/10.1002/tox.22460

Huang, R., Wang, P., Han, J., Xia, W., Cai, R., Sun, H., Sun, J., & Wang, S. (2015). Decreased Serum IGF-1/IGFBP-3 Molar Ratio is Associated with Executive Function Behaviors in Type 2 Diabetic Patients with Mild Cognitive Impairment. *Journal of Alzheimer's Disease: JAD*, *47*(1), 85–94. https://doi.org/10.3233/JAD-150071

Janota, C., Lemere, C. A., & Brito, M. A. (2016). Dissecting the Contribution of Vascular Alterations and Aging to Alzheimer's Disease. *Molecular Neurobiology*, *53*(6), 3793–3811. https://doi.org/10.1007/s12035-015-9319-7

Jones, A. J., Kuijer, R. G., Livingston, L., Myall, D., Horne, K., MacAskill, M., Pitcher, T., Barrett, P. T., Anderson, T. J., & Dalrymple-Alford, J. C. (2017). Caregiver burden is increased in Parkinson's disease with mild cognitive impairment (PD-MCI). *Translational Neurodegeneration*, *6*. https://doi.org/10.1186/s40035-017-0085-5

Juul, A. (2003). Serum levels of insulin-like growth factor I and its binding proteins in health and disease. *Growth Hormone & IGF Research*, *13*(4), 113–170. https://doi.org/10.1016/S1096-6374(03)00038-8

Kalia, L. V., & Lang, A. E. (2015). Parkinson's disease. *The Lancet*, *386*(9996), 896–912. https://doi.org/10.1016/S0140-6736(14)61393-3

References

Kao, S.-Y. (2009). Rescue of alpha-synuclein cytotoxicity by insulin-like growth factors. *Biochemical and Biophysical Research Communications*, *385*(3), 434–438. https://doi.org/10.1016/j.bbrc.2009.05.089

Kapasi, A., & Schneider, J. A. (2016). Vascular contributions to cognitive impairment, clinical Alzheimer's disease, and dementia in older persons. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1862*(5), 878–886. https://doi.org/10.1016/j.bbadis.2015.12.023

Kelley, K. M., Schmidt, K. E., Berg, L., Sak, K., Galima, M. M., Gillespie, C., Balogh, L., Hawayek, A., Reyes, J. A., & Jamison, M. (2002). Comparative endocrinology of the insulin-like growth factor-binding protein. *Journal of Endocrinology*, *175*(1), 3–18. https://doi.org/10.1677/joe.0.1750003

Koh, S.-H., & Lo, E. H. (2015). The Role of the PI3K Pathway in the Regeneration of the Damaged Brain by Neural Stem Cells after Cerebral Infarction. *Journal of Clinical Neurology (Seoul, Korea)*, *11*(4), 297–304. https://doi.org/10.3988/jcn.2015.11.4.297

Krishnamurthi, R. V. M., Mathai, S., Kim, A. H., Zhang, R., & Guan, J. (2009a). A novel diketopiperazine improves functional recovery given after the onset of 6-OHDA-induced motor deficit in rats. *British Journal of Pharmacology*, *156*(4), 662–672. https://doi.org/10.1111/j.1476-5381.2008.00064.x

Krishnamurthi, R. V. M., Mathai, S., Kim, A. H., Zhang, R., & Guan, J. (2009b). A novel diketopiperazine improves functional recovery given after the onset of 6-OHDA-induced motor deficit in rats. *British Journal of Pharmacology*, *156*(4), 662–672. https://doi.org/10.1111/j.1476-5381.2008.00064.x

Langhorne, P., Bernhardt, J., & Kwakkel, G. (2011). Stroke rehabilitation. *The Lancet*, *377*(9778), 1693–1702. https://doi.org/10.1016/S0140-6736(11)60325-5

References

Langhorne, P., Coupar, F., & Pollock, A. (2009). Motor recovery after stroke: A systematic review. *The Lancet Neurology*, *8*(8), 741–754. https://doi.org/10.1016/S1474-4422(09)70150-4

Larpthaveesarp, A., Ferriero, D. M., & Gonzalez, F. F. (2015). Growth Factors for the Treatment of Ischemic Brain Injury (Growth Factor Treatment). *Brain Sciences*, *5*(2), 165–177. https://doi.org/10.3390/brainsci5020165

Laviola, L., Natalicchio, A., & Giorgino, F. (2007). The IGF-I signaling pathway. *Current Pharmaceutical Design*, *13*(7), 663–669.

Lawson, R. A., Yarnall, A. J., Duncan, G. W., Khoo, T. K., Breen, D. P., Barker, R. A., Collerton, D., Taylor, J.-P., & Burn, D. J. (2014). Severity of mild cognitive impairment in early Parkinson's disease contributes to poorer quality of life. *Parkinsonism & Related Disorders*, *20*(10), 1071–1075. https://doi.org/10.1016/j.parkreldis.2014.07.004

Lee, K. B., Lim, S. H., Kim, K. H., Kim, K. J., Kim, Y. R., Chang, W. N., Yeom, J. W., Kim, Y. D., & Hwang, B. Y. (2015). Six-month functional recovery of stroke patients: A multi-time-point study. *International Journal of Rehabilitation Research. Internationale Zeitschrift Fur Rehabilitationsforschung. Revue Internationale De Recherches De Readaptation*, *38*(2), 173–180. https://doi.org/10.1097/MRR.0000000000000108

Levy, G. (2007). The Relationship of Parkinson Disease With Aging. *Archives of Neurology*, *64*(9), 1242–1246. https://doi.org/10.1001/archneur.64.9.1242

Li, F., Liu, K., Wang, A., Harris, P. W. R., Vickers, M. H., & Guan, J. (2019). Cyclic glycine-proline administration normalizes high-fat diet-induced synaptophysin expression in obese rats. *Neuropeptides*. https://doi.org/10.1016/j.npep.2019.05.006

References

Litvan, I., Aarsland, D., Adler, C. H., Goldman, J. G., Kulisevsky, J., Mollenhauer, B., Rodriguez-Oroz, M. C., Tröster, A. I., & Weintraub, D. (2011). MDS Task Force on mild cognitive impairment in Parkinson's disease: Critical review of PD-MCI. *Movement Disorders: Official Journal of the Movement Disorder Society*, *26*(10), 1814–1824. https://doi.org/10.1002/mds.23823

Litvan, I., Goldman, J. G., Tröster, A. I., Schmand, B. A., Weintraub, D., Petersen, R. C., Mollenhauer, B., Adler, C. H., Marder, K., Williams-Gray, C. H., Aarsland, D., Kulisevsky, J., Rodriguez-Oroz, M. C., Burn, D. J., Barker, R. A., & Emre, M. (2012). Diagnostic criteria for mild cognitive impairment in Parkinson's disease: Movement Disorder Society Task Force guidelines. *Movement Disorders: Official Journal of the Movement Disorder Society*, *27*(3), 349–356. https://doi.org/10.1002/mds.24893

Liu, X. J., Xie, Q., Zhu, Y. F., Chen, C., & Ling, N. (2001). Identification of a nonpeptide ligand that releases bioactive insulin-like growth factor-I from its binding protein complex. *The Journal of Biological Chemistry*, *276*(35), 32419–32422. https://doi.org/10.1074/jbc.C100299200

Lopez-Lopez, C., LeRoith, D., & Torres-Aleman, I. (2004). Insulin-like growth factor I is required for vessel remodeling in the adult brain. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(26), 9833–9838. https://doi.org/10.1073/pnas.0400337101

Ma, J., Jiang, Q., Xu, J., Sun, Q., Qiao, Y., Chen, W., Wu, Y., Wang, Y., Xiao, Q., Liu, J., Tang, H., & Chen, S. (2015). Plasma insulin-like growth factor 1 is associated with cognitive impairment in Parkinson's disease. *Dementia and Geriatric Cognitive Disorders*, *39*(5–6), 251–256. https://doi.org/10.1159/000371510

Malek, N., Lawton, M. A., Swallow, D. M. A., Grosset, K. A., Marrinan, S. L., Bajaj, N., Barker, R. A., Burn, D. J., Hardy, J., Morris, H. R., Williams, N. M., Wood, N., Ben-

Shlomo, Y., Grosset, D. G., & on behalf of the PRoBaND Clinical Consortium. (2016). Vascular disease and vascular risk factors in relation to motor features and cognition in early Parkinson's disease. *Movement Disorders*, *31*(10), 1518–1526. https://doi.org/10.1002/mds.26698

Markowska, A. L., Mooney, M., & Sonntag, W. E. (1998). Insulin-like growth factor-1 ameliorates age-related behavioral deficits. *Neuroscience*, *87*(3), 559–569. https://doi.org/10.1016/S0306-4522(98)00143-2

Mattlage, A. E., Rippee, M. A., Sandt, J., & Billinger, S. A. (2016). Decrease in Insulin-Like Growth Factor-1 and Insulin-Like Growth Factor-1 Ratio in the First Week of Stroke Is Related to Positive Outcomes. *Journal of Stroke and Cerebrovascular Diseases*, *25*(7), 1800–1806. https://doi.org/10.1016/j.jstrokecerebrovasdis.2016.03.054

Michell, A. W., Lewis, S. J. G., Foltynie, T., & Barker, R. A. (2004). Biomarkers and Parkinson's disease. *Brain*, *127*(8), 1693–1705. https://doi.org/10.1093/brain/awh198

Mohr, J. P., Grotta, J. C., Wolf, P. A., Moskowitz, M. A., Mayberg, M. R., & Kummer, R. V. (2011). *Stroke: Pathophysiology, Diagnosis, and Management*. Elsevier Health Sciences.

Morel, G. R., León, M. L., Uriarte, M., Reggiani, P. C., & Goya, R. G. (2017). Therapeutic potential of IGF-I on hippocampal neurogenesis and function during aging. *Neurogenesis*, *4*(1), e1259709. https://doi.org/10.1080/23262133.2016.1259709

Morris, J. C. (1993). The Clinical Dementia Rating (CDR). *Neurology*, *43*(11), 2412. https://doi.org/10.1212/WNL.43.11.2412-a

Muller, A. P., Fernandez, A. M., Haas, C., Zimmer, E., Portela, L. V., & Torres-Aleman, I. (2012). Reduced brain insulin-like growth factor I function during aging. *Molecular and Cellular Neurosciences*, *49*(1), 9–12. https://doi.org/10.1016/j.mcn.2011.07.008

References

Nijland, R. H. M., van Wegen, E. E. H., Harmeling-van der Wel, B. C., Kwakkel, G., & EPOS Investigators. (2010). Presence of finger extension and shoulder abduction within 72 hours after stroke predicts functional recovery: Early prediction of functional outcome after stroke: the EPOS cohort study. *Stroke*, *41*(4), 745–750. https://doi.org/10.1161/STROKEAHA.109.572065

Nilsson-Håkansson, L., Civalero, I., Zhang, X., Carlsson-Skwirut, C., Sara, V. R., & Nordberg, A. (1993). Effects of IGF-1, truncated IGF-1 and the tripeptide Gly-Pro-Glu on acetylcholine release from parietal cortex of rat brain. *Neuroreport*, *4*(9), 1111–1114.

Nishijima, T., Piriz, J., Duflot, S., Fernandez, A. M., Gaitan, G., Gomez-Pinedo, U., Verdugo, J. M. G., Leroy, F., Soya, H., Nuñez, A., & Torres-Aleman, I. (2010). Neuronal Activity Drives Localized Blood-Brain-Barrier Transport of Serum Insulin-like Growth Factor-I into the CNS. *Neuron*, *67*(5), 834–846. https://doi.org/10.1016/j.neuron.2010.08.007

Okereke, O. I., Kang, J. H., Ma, J., Gaziano, J. M., & Grodstein, F. (2006). Midlife Plasma Insulin-Like Growth Factor I and Cognitive Function in Older Men. *The Journal of Clinical Endocrinology & Metabolism*, *91*(11), 4306–4312. https://doi.org/10.1210/jc.2006-1325

Okereke, O., Kang, J. H., Ma, J., Hankinson, S. E., Pollak, M. N., & Grodstein, F. (2007). Plasma IGF-I levels and cognitive performance in older women. *Neurobiology of Aging*, *28*(1), 135–142. https://doi.org/10.1016/j.neurobiolaging.2005.10.012

Owolabi, M. O., & Agunloye, A. M. (2013). Risk factors for stroke among patients with hypertension: A case-control study. *Journal of the Neurological Sciences*, *325*(1–2), 51–56. https://doi.org/10.1016/j.jns.2012.11.016

Pardo, J., Uriarte, M., Cónsole, G. M., Reggiani, P. C., Outeiro, T. F., Morel, G. R., & Goya, R. G. (2016). Insulin-like growth factor-I gene therapy increases hippocampal neurogenesis, astrocyte branching and improves spatial memory in female aging rats.

European Journal of Neuroscience, *44*(4), 2120–2128. https://doi.org/10.1111/ejn.13278

Pathipati, P., Gorba, T., Scheepens, A., Goffin, V., Sun, Y., & Fraser, M. (2011). Growth hormone and prolactin regulate human neural stem cell regenerative activity. *Neuroscience*, *190*, 409–427. https://doi.org/10.1016/j.neuroscience.2011.05.029

Philippou, A., Maridaki, M., Pneumaticos, S., & Koutsilieris, M. (2014). The Complexity of the IGF1 Gene Splicing, Posttranslational Modification and Bioactivity. *Molecular Medicine*, *20*(1), 202–214. https://doi.org/10.2119/molmed.2014.00011

Picillo, M., Erro, R., Santangelo, G., Pivonello, R., Longo, K., Pivonello, C., Vitale, C., Amboni, M., Moccia, M., Colao, A., Barone, P., & Pellecchia, M. T. (2013). Insulin-like growth factor-1 and progression of motor symptoms in early, drug-naïve Parkinson's disease. *Journal of Neurology*, *260*(7), 1724–1730. https://doi.org/10.1007/s00415-013-6851-0

Picillo, M., Pivonello, R., Santangelo, G., Pivonello, C., Savastano, R., Auriemma, R., Amboni, M., Scannapieco, S., Pierro, A., Colao, A., Barone, P., & Pellecchia, M. T. (2017). Serum IGF-1 is associated with cognitive functions in early, drug-naïve Parkinson's disease. *PLOS ONE*, *12*(10), e0186508. https://doi.org/10.1371/journal.pone.0186508

Pilotto, A., Turrone, R., Liepelt-Scarfone, I., Bianchi, M., Poli, L., Borroni, B., Alberici, A., Premi, E., Formenti, A., Bigni, B., Cosseddu, M., Cottini, E., Berg, D., & Padovani, A. (2016). Vascular Risk Factors and Cognition in Parkinson's Disease. *Journal of Alzheimer's Disease*, *51*(2), 563–570. https://doi.org/10.3233/JAD-150610

Pisa, D., Alonso, R., & Carrasco, L. (2020). Parkinson's Disease: A Comprehensive Analysis of Fungi and Bacteria in Brain Tissue. *International Journal of Biological Sciences*, *16*(7), 1135–1152. https://doi.org/10.7150/ijbs.42257

References

Pisa, D., Alonso, R., Rábano, A., & Carrasco, L. (2016). Corpora Amylacea of Brain Tissue from Neurodegenerative Diseases Are Stained with Specific Antifungal Antibodies. *Frontiers in Neuroscience*, *10*. https://doi.org/10.3389/fnins.2016.00086

Powers, W. J., Derdeyn, C. P., Biller, J., Coffey, C. S., Hoh, B. L., Jauch, E. C., Johnston, K. C., Johnston, S. C., Khalessi, A. A., Kidwell, C. S., Meschia, J. F., Ovbiagele, B., & Yavagal, D. R. (2015). 2015 AHA/ASA Focused Update of the 2013 Guidelines for the Early Management of Patients With Acute Ischemic Stroke Regarding Endovascular Treatment A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke*, STR.0000000000000074. https://doi.org/10.1161/STR.0000000000000074

Ranke, M. B. (2015). Insulin-like growth factor binding-protein-3 (IGFBP–3). *Best Practice & Research Clinical Endocrinology & Metabolism*, *29*(5), 701–711. https://doi.org/10.1016/j.beem.2015.06.003

Raz, N., Rodrigue, K. M., Kennedy, K. M., & Acker, J. D. (2007). Vascular health and longitudinal changes in brain and cognition in middle-aged and older adults. *Neuropsychology*, *21*(2), 149–157. https://doi.org/10.1037/0894-4105.21.2.149

Reisberg, B., Ferris, S. H., de Leon, M. J., & Crook, T. (1982). The Global Deterioration Scale for assessment of primary degenerative dementia. *The American Journal of Psychiatry*, *139*(9), 1136–1139. https://doi.org/10.1176/ajp.139.9.1136

Reisberg, Barry, Finkel, S., Overall, J., Schmidt-Gollas, N., Kanowski, S., Lehfeld, H., Hulla, F., Sclan, S. G., Wilms, H.-U., Heininger, K., Hindmarch, I., Stemmler, M., Poon, L., Kluger, A., Cooler, C., Bergener, M., Hugonot-Diener, L., Robert, P. H., & Erzigkeit, H. (2001). The Alzheimer's Disease Activities of Daily Living International Scale (ADL-IS). *International Psychogeriatrics*, *13*(2), 163–181. https://doi.org/10.1017/S1041610201007566

References

Richards, C. L., Malouin, F., & Nadeau, S. (2015). Chapter 13 - Stroke rehabilitation: Clinical picture, assessment, and therapeutic challenge. In S. N. and S. R. Numa Dancause (Ed.), *Progress in Brain Research* (Vol. 218, pp. 253–280). Elsevier. http://www.sciencedirect.com/science/article/pii/S0079612315000047

Rost, N. S., Bottle, A., Lee, J.-M., Randall, M., Middleton, S., Shaw, L., Thijs, V., Rinkel, G. J. E., Hemmen, T. M., & Collaborators, the G. C. S. G. (2016). Stroke Severity Is a Crucial Predictor of Outcome: An International Prospective Validation Study. *Journal of the American Heart Association*, *5*(1), e002433. https://doi.org/10.1161/JAHA.115.002433

Sacco, R. L., Kasner, S. E., Broderick, J. P., Caplan, L. R., Connors, J. J. (Buddy), Culebras, A., Elkind, M. S. V., George, M. G., Hamdan, A. D., Higashida, R. T., Hoh, B. L., Janis, L. S., Kase, C. S., Kleindorfer, D. O., Lee, J.-M., Moseley, M. E., Peterson, E. D., Turan, T. N., Valderrama, A. L., & Vinters, H. V. (2013). An Updated Definition of Stroke for the 21st Century A Statement for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke*, *44*(7), 2064–2089. https://doi.org/10.1161/STR.0b013e318296aeca

Sadowski, T., Dietrich, S., Koschinsky, F., & Sedlacek, R. (2003). Matrix Metalloproteinase 19 Regulates Insulin-like Growth Factor-mediated Proliferation, Migration, and Adhesion in Human Keratinocytes through Proteolysis of Insulin-like Growth Factor Binding Protein-3. *Molecular Biology of the Cell*, *14*(11), 4569–4580. https://doi.org/10.1091/mbc.E03-01-0009

Salehi, Z., Mashayekhi, F., & Naji, M. (2008). Insulin like growth factor-1 and insulin like growth factor binding proteins in the cerebrospinal fluid and serum from patients with Alzheimer's disease. *BioFactors (Oxford, England)*, *33*(2), 99–106.

Santangelo, G., Vitale, C., Picillo, M., Moccia, M., Cuoco, S., Longo, K., Pezzella, D., di Grazia, A., Erro, R., Pellecchia, M. T., Amboni, M., Trojano, L., & Barone, P. (2015). Mild Cognitive Impairment in newly diagnosed Parkinson's disease: A longitudinal prospective study. *Parkinsonism & Related Disorders*, *21*(10), 1219–1226. https://doi.org/10.1016/j.parkreldis.2015.08.024

Sara, V. R., Carlsson-Skwirut, C., Bergman, T., Jörnvall, H., Roberts, P. J., Crawford, M., Håkansson, L. N., Civalero, I., & Nordberg, A. (1989). Identification of Gly-Pro-Glu (GPE), the aminoterminal tripeptide of insulin-like growth factor 1 which is truncated in brain, as a novel neuroactive peptide. *Biochemical and Biophysical Research Communications*, *165*(2), 766–771.

Sara, V. R., & Hall, K. (1990). Insulin-like growth factors and their binding proteins. *Physiological Reviews*, *70*(3), 591–614.

Sara, Vicki R., Carlsson-Skwirut, C., Drakenberg, K., Giacobini, M. B., Håkansson, L., Mirmiran, M., Nordberg, A., Olson, L., Reinecke, M., Ståhlbom, P. A., & Nordqvist, A. C. S. (1993). The Biological Role of Truncated Insulin-like Growth Factor-1 and the Tripeptide GPE in the Central Nervous Systema. *Annals of the New York Academy of Sciences*, *692*(1), 183–191. https://doi.org/10.1111/j.1749-6632.1993.tb26216.x

Schneider, C. B., Donix, M., Linse, K., Werner, A., Fauser, M., Klingelhoefer, L., Löhle, M., von Kummer, R., Reichmann, H., & Storch, A. (2017). Accelerated Age-Dependent Hippocampal Volume Loss in Parkinson Disease With Mild Cognitive Impairment. *American Journal of Alzheimer's Disease & Other Dementias®*, *32*(6), 313–319. https://doi.org/10.1177/1533317517698794

Schwab, S., Spranger, M., Krempien, S., Hacke, W., & Bettendorf, M. (1997). Plasma insulin-like growth factor I and IGF binding protein 3 levels in patients with acute cerebral ischemic injury. *Stroke; a Journal of Cerebral Circulation*, *28*(9), 1744–1748.

References

Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Molecular Medicine*, *8*(6), 595–608. https://doi.org/10.15252/emmm.201606210

Sherbet, G. V. (2011). 8—Insulin-Like Growth Factors. In G. V. Sherbet (Ed.), *Growth Factors and Their Receptors in Cell Differentiation, Cancer and Cancer Therapy* (pp. 87–104). Elsevier. http://www.sciencedirect.com/science/article/pii/B9780123878199000086

Shimizu, S., Hirose, D., Hatanaka, H., Takenoshita, N., Kaneko, Y., Ogawa, Y., Sakurai, H., & Hanyu, H. (2018). Role of Neuroimaging as a Biomarker for Neurodegenerative Diseases. *Frontiers in Neurology*, *9*. https://doi.org/10.3389/fneur.2018.00265

Singh-Mallah, G., McMahon, C. D., Guan, J., & Singh, K. (2017). Cyclic-glycine-proline accelerates mammary involution by promoting apoptosis and inhibiting IGF-1 function. *Journal of Cellular Physiology*, *232*(12), 3369–3383. https://doi.org/10.1002/jcp.25782

Singh-Mallah, G., Singh, K., McMahon, C. D., Harris, P., Brimble, M. A., Thorstensen, E., & Guan, J. (2016). Maternally Administered Cyclic Glycine-Proline Increases Insulin-Like Growth Factor-1 Bioavailability and Novelty Recognition in Developing Offspring. *Endocrinology*, *157*(8), 3130–3139. https://doi.org/10.1210/en.2016-1189

Sonntag, W. E., Ramsey, M., & Carter, C. S. (2005). Growth hormone and insulin-like growth factor-1 (IGF-1) and their influence on cognitive aging. *Ageing Research Reviews*, *4*(2), 195–212. https://doi.org/10.1016/j.arr.2005.02.001

Stinear, C. M. (2017). Prediction of motor recovery after stroke: Advances in biomarkers. *The Lancet. Neurology*, *16*(10), 826–836. https://doi.org/10.1016/S1474-4422(17)30283-1

Svensson, J., Diez, M., Engel, J., Wass, C., Tivesten, Å., Jansson, J.-O., Isaksson, O., Archer, T., Hökfelt, T., & Ohlsson, C. (2006). Endocrine, liver-derived IGF-I is of importance

for spatial learning and memory in old mice. *Journal of Endocrinology*, *189*(3), 617–627. https://doi.org/10.1677/joe.1.06631

Tang, J.-H., Ma, L.-L., Yu, T.-X., Zheng, J., Zhang, H.-J., Liang, H., & Shao, P. (2014). Insulin-Like Growth Factor-1 as a Prognostic Marker in Patients with Acute Ischemic Stroke. *PLoS ONE*, *9*(6). https://doi.org/10.1371/journal.pone.0099186

Tarantini, S., Tran, C. H. T., Gordon, G. R., Ungvari, Z., & Csiszar, A. (n.d.). Impaired neurovascular coupling in aging and Alzheimer's disease: Contribution of astrocyte dysfunction and endothelial impairment to cognitive decline. *Experimental Gerontology*. https://doi.org/10.1016/j.exger.2016.11.004

Thissen, J.-P., Underwood, L., & Ketelslegers, J.-M. (1998). Nutritional Regulation of Hepatic Production of IGF-1. *SpringerLink*, 119–130. https://doi.org/10.1007/978-1-4612-2228-6_12

Thomas, B., & Beal, M. F. (2007). Parkinson's disease. *Human Molecular Genetics*, *16*(R2), R183–R194. https://doi.org/10.1093/hmg/ddm159

Thorne, R. G., Pronk, G. J., Padmanabhan, V., & Frey, W. H. (2004). Delivery of insulin-like growth factor-I to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. *Neuroscience*, *127*(2), 481–496. https://doi.org/10.1016/j.neuroscience.2004.05.029

Tong, M., Dong, M., & de la Monte, S. M. (2009). Brain Insulin-Like Growth Factor and Neurotrophin Resistance in Parkinson's Disease and Dementia with Lewy Bodies: Potential Role of Manganese Neurotoxicity. *Journal of Alzheimer's Disease : JAD*, *16*(3), 585–599. https://doi.org/10.3233/JAD-2009-0995

Toth, P., Tarantini, S., Csiszar, A., & Ungvari, Z. (2016). Functional vascular contributions to cognitive impairment and dementia: Mechanisms and consequences of cerebral autoregulatory dysfunction, endothelial impairment, and neurovascular uncoupling in

aging. *American Journal of Physiology-Heart and Circulatory Physiology*, *312*(1), H1–H20. https://doi.org/10.1152/ajpheart.00581.2016

Trejo, J., Piriz, J., Llorens-Martin, M. V., Fernandez, A. M., Bolós, M., LeRoith, D., Nuñez, A., & Torres-Aleman, I. (2007). Central actions of liver-derived insulin-like growth factor I underlying its pro-cognitive effects. *Molecular Psychiatry*, *12*(12), 1118–1128. https://doi.org/10.1038/sj.mp.4002076

Tuncel, D., Inanc Tolun, F., & Toru, I. (2009). Serum Insulin-Like Growth Factor-1 and Nitric Oxide Levels in Parkinson's Disease. *Mediators of Inflammation*, *2009*. https://doi.org/10.1155/2009/132464

Vardy, E. R. L. C., Rice, P. J., Bowie, P. C. W., Holmes, J. D., Grant, P. J., & Hooper, N. M. (2007). Increased circulating insulin-like growth factor-1 in late-onset Alzheimer's disease. *Journal of Alzheimer's Disease: JAD*, *12*(4), 285–290.

Vemuri, P., Lesnick, T. G., Przybelski, S. A., Knopman, D. S., Lowe, V. J., Graff-Radford, J., Roberts, R. O., Mielke, M. M., Machulda, M. M., Petersen, R. C., & Jack, C. R. (2017). Age, Vascular Health, and Alzheimer's disease Biomarkers in an Elderly Sample. *Annals of Neurology*, *82*(5), 706–718. https://doi.org/10.1002/ana.25071

Vitiello, M. V., Moe, K. E., Merriam, G. R., Mazzoni, G., Buchner, D. H., & Schwartz, R. S. (2006). Growth hormone releasing hormone improves the cognition of healthy older adults. *Neurobiology of Aging*, *27*(2), 318–323. https://doi.org/10.1016/j.neurobiolaging.2005.01.010

Walton, E. L. (2018). Saliva biomarkers in neurological disorders: A "spitting image" of brain health? *Biomedical Journal*, *41*(2), 59–62. https://doi.org/10.1016/j.bj.2018.04.005

Williams, I. C., Park, M. H., Tsang, S., Sperling, S. A., & Manning, C. (2018). Cognitive Function and Vascular Risk Factors Among Older African American Adults. *Journal*

of Immigrant and Minority Health, *20*(3), 612–618. https://doi.org/10.1007/s10903-017-0583-7

Wood, K.-L., Myall, D. J., Livingston, L., Melzer, T. R., Pitcher, T. L., MacAskill, M. R., Geurtsen, G. J., Anderson, T. J., & Dalrymple-Alford, J. C. (2016). Different PD-MCI criteria and risk of dementia in Parkinson's disease: 4-year longitudinal study. *Npj Parkinson's Disease*, *2*, 15027. https://doi.org/10.1038/npjparkd.2015.27

Yamamoto, H., & Murphy, L. J. (1994). Generation of des-(1-3) insulin-like growth factor-I in serum by an acid protease. *Endocrinology*, *135*(6), 2432–2439. https://doi.org/10.1210/endo.135.6.7988428

Yamamoto, H., & Murphy, L. J. (1995). Enzymatic conversion of IGF-I to des(1–3)IGF-I in rat serum and tissues: A further potential site of growth hormone regulation of IGF-I action. *Journal of Endocrinology*, *146*(1), 141–148. https://doi.org/10.1677/joe.0.1460141

Yang, P., Pavlovic, D., Waldvogel, H., Dragunow, M., Synek, B., Turner, C., Faull, R., & Guan, J. (2015). String Vessel Formation is Increased in the Brain of Parkinson Disease. *Journal of Parkinson's Disease*, *5*(4), 821–836. https://doi.org/10.3233/JPD-140454

Zhang, R., Kadar, T., Sirimanne, E., MacGibbon, A., & Guan, J. (2012). Age-related memory decline is associated with vascular and microglial degeneration in aged rats. *Behavioural Brain Research*, *235*(2), 210–217. https://doi.org/10.1016/j.bbr.2012.08.002

Zhu, W., Fan, Y., Frenzel, T., Gasmi, M., Bartus, R. T., Young, W. L., Yang, G.-Y., & Chen, Y. (2008). Insulin Growth Factor-1 Gene Transfer Enhances Neurovascular Remodeling and Improves Long-Term Stroke Outcome in Mice. *Stroke; a Journal of Cerebral Circulation*, *39*(4), 1254–1261. https://doi.org/10.1161/STROKEAHA.107.500801