Exploring Novel Biomarkers of Brain Health in Midlife Type 2 Diabetes Mellitus (ENBIND)

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A thesis submitted to the University of Dublin, Trinity College, for the Degree of Masters by Research (Medical Gerontology)

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Declaration of Authorship

I declare that this thesis, and all of the work within is entirely my own and has not been submitted as an exercise for a degree at Trinity College Dublin or any other University.

The material of this study forms the cross-sectional analysis of baseline data from the ENBIND (Exploring Novel Biomarkers of Brain Health in Type 2 Diabetes Mellitus) Study. I declare that I performed all of the work contained within this thesis. My supervisors Prof Sean Kennelly and Dr Nollaig Bourke provided input on the hypothesis and design of the study.

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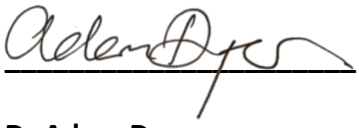

Date: 16.08.20

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Summary

Type 2 Diabetes Mellitus (T2DM) in midlife (between the ages of 35-65) is associated with a two-fold increased risk of dementia in later life. Despite this, knowledge around the pathophysiological mechanisms linking T2DM and dementia remain poorly explored. Further, knowledge around which individuals with T2DM in particular are at greatest risk of later cognitive decline is lacking. The aim of the current study was to address some of these open questions in a cohort of otherwise healthy individuals with T2DM and matched controls. We aimed to explore the determinants of cognitive function in midlife T2DM and assess the role of markers from two previously unexamined areas to predict deficits in T2DM - namely (i) midlife gait speed and (ii) innate immune system activity.

Otherwise healthy individuals with midlife T2DM (aged 35-65) were recruited and had a detailed history obtained and exam performed. Cognitive function was assessed using the Montreal Cognitive Assessment (MoCA) as a measure of general cognition in addition to a detailed computerised neuropsychological assessment (CANTAB) aimed at examining executive function, attention, working memory and reaction time.

Gait speed was assessed over three tasks: (i) 'usual' or 'self-selected' gait speed, (ii) 'fast' or 'maximal' gait speed as well as (iii) cognitive dual-task gait. Blood samples were taken for assessment of peripheral circulating cytokines (a pre-selected panel of six proteins was assessed) and isolation of Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were stimulated with several innate immune agonists directly chosen to target the innate immune NLRP3 inflammasome. NLRP3 activation was measured as the production of mature IL-1β (an inflammasome dependent cytokine).

Overall, participants with midlife T2DM had significantly poorer scores on the MoCA, and were more than twice as likely to make an error as their non-T2DM counterparts. This finding persisted after adjustment for age, sex, years or formal education and a family

history of cognitive impairment. We also observed strong trends for poorer performance on neuropsychological tests of executive function, attention, reaction time in T2DM.

Those with T2DM had significantly slower gait speed as measured across all three tasks, which persisted after controlling for covariates known to impact on gait speed in midlife. In the overall cohort, slower gait speeds on all three tasks were associated with a greater likelihood of error on the MoCA. Following robust covariate adjustment, only the association for greater dual task cost (a greater slowing effect on addition of the cognitive dual task paradigm) remained significantly predictive of MoCA score. We observed several significant associations between performance on both the self-selected and maximal gait speed tasks and measures of executive function and attention. However, none of the observed effects were specific to those with T2DM as assessed by an interaction term between T2DM status and the independent variable of interest incorporated into the regression models

There were no differences between individuals with T2DM and healthy controls in the serum levels of six pro-inflammatory markers. We observed several associations between elevated MCP-1 and greater likelihood of error on the MoCA in addition to associations between MCP-1 and measures of working memory. We demonstrated significant production of IL-1β on stimulation of the NLRP3 inflammasome with several agonists, including Aβ-42, the pathogenic protein in Alzheimer's Disease (AD). This did not differ between those with T2DM and healthy controls. We observed significant associations between greater inflammasome activation in response to Aβ-42 and greater likelihood of error on the MoCA in addition to poorer performance on the dual-task gait paradigm. There was a strong trend for a T2DM-specific effect.

In conclusion, we report significantly poorer cognitive and gait performance in midlife T2DM. Addition of a cognitive-dual task gait greatly enhances the ability of gait assessment to act as a predictor of cognitive performance. Whilst analysis of serum pro-inflammatory cytokines yielded no significant results, we observed significant associations between NLRP3 inflammasome responsiveness to Aβ-42 (and other NLRP3 agonists) and

performance on both general cognition (MoCA) and the cognitive dual-task gait paradigm. Future work will follow this cohort to analyse these markers longitudinally, adding further to our insight around the putative pathophysiological and potential underlying mechanisms linking T2DM in midlife and later dementia risk, with the ultimate aim of selecting out those most at risk for multi-domain preventative interventions.

Acknowledgements

This project was a massive undertaking and would not have been possible without a supportive team of talented and passionate individuals. My sincerest gratitude is extended to the more than one-hundred research participants who completed the study in order to better understand the links between Type 2 Diabetes and Brain Health. This thesis is dedicated to them.

Fulsome thanks are extended to Professor Sean Kennelly for conceiving and supporting the current study. Since my first encounter with Professor Kennelly as an undergraduate, I have always been struck by his patient-centred approach to both the practice of medicine and conduct of research. He has made an excellent mentor to myself and many other aspiring physicians and I am continually in awe of his ability to cross the boundaries between clinical and academic worlds with ease. I am excited to witness his growing contribution to brain health and dementia research over the coming years, and am sure that his contribution will better the care of those affected with cognitive impairment and dementia worldwide.

To Dr Nollaig Bourke I extend warmest thanks. Since first meeting with Dr Bourke I have been inspired by her infectious enthusiasm for answering the basic scientific questions behind the clinical realities we witness in our patients. Dr Bourke has been a constant source of support and encouragement throughout the year and I look forward to our future collaborations, bringing a translational gerontology to our practice as physicians – which whilst a nascent field is a very exciting one !

Further, to Isabella Batten, I will be forever grateful for the constant support and teaching in the lab. Her patience, technical wizardry and deep scientific understanding were invaluable resources throughout the year and I am very excited to see her future career as a scientist blossom ! I also wish to thank Dr Liam Townsend for the constant

supply of samples, mutual frustration and of course the terrible banter - another fantastic physician and scientist !

Finally, to all the staff at Trinity Translational Medicine Institute (TTMI) my warmest thanks (current and former members of the Little, Doherty, Brady and Genetics labs – you know who you are…). To all the members of the Bourke lab who passed through during the year (Avril & Conor Reddy, Darragh Lowndes, Nicole Mather & Eoin McManamon) my sincerest gratitude for the good times, help and observations which made traversing a once large gap between the bedside and the bench that much easier.

To all my colleagues and friends from the Department of Age-Related Healthcare in Tallaght University Hospital, I extend great appreciation and admiration. To my office-sharers Cathie Burke and Cathy McHale and all the staff of the Charlie O'Toole Day Centre – my thanks. A special thanks to Marian Hughes and Mary Kelly for all the support they have shown me throughout the year – it made the pursuit a lot more successful – and definitely more enjoyable !

Finally, to my parents (Mary and David), my sister (Emma) and my better half (Christine) who have had to put up with my excessive worrying, working and late nights in the lab. I will be forever indebted to them for their unwavering support in my career to date and simply would not have been able to complete this work without them - one could not ask for a better or more supportive family !

Publications and Funding

Peer-Reviewed Journal Publications:

Dyer AH, Briggs R, Mockler D, Gibney J, Kennelly SP (2019). Non-Pharmacological Interventions for Cognition in Type 2 Diabetes Mellitus: A Systematic Review. **QJM: Journal of the Association of Physicians** doi.org/10.1093/qjmed/hcz053. **PMID**: 30825309

Book Chapter:

Dyer AH, Kennelly SP (2019). Midlife Diabetes and Dementia: Understanding the Link. In Neuroscience of Dementia (Elsevier). Ed Prof Victor Preedy

Conference Proceedings:

Dyer AH, Batten I, Townsend L, Gibney J, Woods C, Bourke N, Kennelly SP (2019). Common Pathological Pathways Between Type 2 Diabetes and Dementia: Investigating the Role of the Innate Immune System. British Neuroscience Association Festival of Neuroscience. April 2019, Dublin, Ireland. **Rapid-Fire Oral Presentation (International)**

Dyer AH, Killane I, Campbell B, Tobin K, Batten I, Bourke N, Widdowson M, Woods CP, Gibney J, Reilly R, Kennelly SP (2019). Gait as a Potential Marker of Cognitive Decrements in Type 2 Diabetes (T2DM): Early Results from the ENBIND Study. International Society for Gait and Posture Research Congress. Edinburgh, July 2019. **Poster Presentation (International)**

Dyer A, Killane I, Bourke N, Woods CP, Gibeny J, O'Neill D, Reilly R, Kennelly S (2019). Does Dual-Task Gait Speed Predict Cognitive Performance in Midlife Type 2 Diabetes ? Baseline Results from the ENBIND Study. Accepted for Oral Presentation. Irish

Gerontological Society Annual Scientific Meeting, Cork, September 2019. **Oral Presentation (National). Accepted**

Dyer A, Batten I, Killane I, Woods CP, Gibney J, O'Neill D, Reilly R, Bourke N, Kennelly S (2019). Linking Diabetes and Dementia Risk: A Role for AB-42 Induced Activation of the NLRP3 Inflammasome: Results from the ENBIND Study. Accepted for Oral Presentation. Irish Endocrine Society Annual Scientific Meeting, Galway, October 2019. **Oral Presentation (National). Accepted**

Dyer A, Batten I, Killane I, Woods CP, Gibeny J, O'Neill D, Reilly R, Bourke N, Kennelly S (2019). Linking Diabetes and Dementia Risk: A Role for AB-42 Induced Activation of the NLRP3 Inflammasome: Results from the ENBIND Study. Accepted for Poster Presentation. Irish Gerontological Society Annual Scientific Meeting, Cork, September 2019. **Poster Presentation (National). Presented***

Dyer A, Killane I, Bourke N, Woods CP, Gibney J, O'Neill D, Reilly R, Kennelly S (2019). Does Dual-Task Gait Speed Predict Cognitive Performance in Midlife Type 2 Diabetes ? Baseline Results from the ENBIND Study. Accepted for Oral Presentation. Irish Endocrine Society Annual Scientific Meeting, Galway, October 2019. **Poster Presentation (National). Presented**

***Awarded the Irish Gerontological Society's Presidents Prize for Best Presentation**

Funding Received:

Building Engagements in Health Research (BEHR) Grant, Trinity College Dublin
Meath Foundation Grant, Tallaght University Hospital, Dublin
AMT Force and Motion Travel Grant – Enabling Presentation at ISPGR Conference
Department of Age-Related Healthcare, Tallaght University Hospital, Dublin

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List of Abbreviations

Aβ-42	Amyloid Beta – 42
ACTH	Adrenocorticotrophic Hormone
AD	Alzheimer's Disease
AD8	Ascertain Dementia 8 Questionnaire
AGE	Advanced Glycation End Product
ARHC	Age-Related Healthcare
ARIC	Atherosclerosis Risk in Communities
BBB	Blood Brain Barrier
BMI	Body Mass Index
CANTAB	Cambridge Automated Neuropsychological Assessment Battery
CCI	Charlson Comorbidity Index
CNS	Central Nervous System
CRP	C Reactive Protein
CSF	Cerebrospinal Fluid
CXCL10	C-X-C Motif Chemokine 10
DAMPs	Damage Associated Molecular Pathogens
DNSS	Diabetic Neuropathy Symptom Score
DSST	Digit Symbol Substitution Test
DTI	Diffusion Tensor Imaging
DTC	Dual Task Cost
EDTA	Ethylene-Diamene Tetra-Acetic Acid
ELSA	English Longitudinal Study on Ageing
ELISA	Enzyme Linked Immunosorbent Assay
ENBIND	Exploring Novel Biomarkers of Brain Health in Type 2 Diabetes
FA	Fractional Anisotrophy
FBS	Fetal Bovine Serum
FFA	Free Fatty Acids
GLP-1	Glucacon-Like Peptide 1

HbA1c	Glycated Haemoglobin
HOMA-IR	Homeostatic Model Assessment – Insulin Resistance
HPA	Hypothalamic-Pituitary-Adrenal Axis
HR	Hazard Ratio
HVLT	Harvard Verbal Learning Test
IAPP	Islet Associated Amyloid Peptide
IDE	Insulin Degrading Enzyme
IGF-1	Insulin Like Growth Factor 1
IL-1β	Interleukin 1 β
IL-6	Interleukin 6
IL-12p70	Interleukin 12 p 70
IMU	Inertial Motion Unit
IR	Insulin Resistance
IRS1	Insulin Receptor Substrate 1
LPS	Lipopolysaccharide
MCI	Mild Cognitive Impairment
MCP-1	Monocyte Chemoattractant Protein 1
MMSE	Mini-Mental State Examination
MoCA	Montreal Cognitive Assessment
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
NLRP3	NOD-, LRR- and pyrin- domain containing protein 3
NLRs	Nucleotide Binding Oligimerisation Domain Receptors
NT-proBNP	N Terminal pro-Brain Natriuretic Peptide
OTS	One Touch Stockings of Cambridge (CANTAB)
OTS-PFS	Percentage Correct on First Choice (CANTAB)
OTS-MDLFC	Mean Latency to First Choice (CANTAB)
pAD	prodomal Alzheimer's Disease
PAL	Paired Associates Learning (CANTAB)

PAL-FAMS (CANTAB)	Paired Associates Learning – First Attempt Memory Score
PAL-TEA	Paired Associates Learning – Total Errors Adjusted (CANTAB)
PAMPs	Pathogen Associated Molecular Pathogens
PAT	Poly dA:dT
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PET	Positron Emission Tomography
PRM	Pattern Recognition Memory (CANTAB)
PRM-PCD (CANTAB)	Pattern Recognition Memory – Percent Correct Delayed
PRM-PCI (CANTAB)	Pattern Recognition Memory – Percent Correct Immediate
qPCR	quantitative Polymerase Chain Reaction
RAGE	Receptor for Advanced Glycation End Products
RAVLT	Rey Auditory Visual Learning Test
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RTI	Reaction Time Task (CANTAB)
RTI-MDRT	Reaction Time Task – Median Duration Reaction Time (CANTAB)
RTI-MDMT (CANTAB)	Reaction Time Task – Median Duration Movement Time
RVP	Rapid Visual Processing (CANTAB)
RVPA	Rapid Visual Processing - A Prime (CANTAB)
RVP-PFA	Rapid Visual Processing – Probability of False Alarm (CANTAB)
SWM	Spatial Working Memory (CANTAB)
SWM-BE	Spatial Working Memory – Between Errors
SWM-S	Spatial Working Memory – Strategy
T2DM	Type 2 Diabetes Mellitus
TCD	Trinity College Dublin

TILDA	The Irish Longitudinal Study on Ageing
TLR	Toll Like Receptor
TMT	Trail Making Test
TTMI	Trinity Translational Medicine Institute
TUH	Tallaght University Hospital
WMS	Wechsler Memory Scale

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Chapter 1: The Need for New Understanding of the Diabetes-Dementia Link and New Biomarkers of Brain Health in Type 2 Diabetes Mellitus (T2DM)

" The diabetic patient, on his own part, complains of loss of memory and of poor ability to concentrate the attention…"

(Miles & Root, JAMA, 1922)

Almost one-hundred years since the first reference in the academic literate to cognitive decrements experienced those with diabetes (and in particular Type 2 Diabetes), it has been firmly established that diabetes in midlife is one of the greatest risk factors for later cognitive decline and dementia. Through initial epidemiological evidence, longitudinal studies of ageing and now to complex modelling of midlife risk factors for later cognitive decline, the link between Type 2 Diabetes (T2DM) and dementia has been widely reported. But despite the growing evidence for the link, there are still many unknowns. In the first instance, the basic mechanisms by which midlife T2DM can increase dementia risk have yet to be uncovered. Additionally, knowledge of which patients are particularly at risk remains poorly explored. Few interventions have been trialled to mitigate the risk of later cognitive decline in midlife T2DM, and in particular, multi-domain interventions have been virtually non-existent. It is the central aim of this thesis, and the greater **ENBIND (Exploring Novel Biomarkers of Brain Health in Type 2 Diabetes Mellitus)** Study to uncover these mechanisms, to identify those in midlife at greatest risk and design novel multi-domain interventions to combat dementia risk in T2DM.

T2DM is a common and complex metabolic disorder of altered carbohydrate metabolism characterised by insulin resistance and the later development on numerous microvascular and macrovascular complications (deFronzo et al. 2016). The prevalence of T2DM has greatly increased in recent years and has quadrupled from 1980 (affecting around 108 million people globally) to the present date (415 million affected) (deFronzo

et al. 2016). Future projections expand this number further, with T2DM estimated to affect around 650 million people by 2035 (American Diabetes Association, 2016). T2DM is most often diagnosed in the 4^{th}-6^{th} decades of life and may be characterised by a pre-diabetes stage of impaired fasting glucose or carbohydrate tolerance. Many commentators have noted T2DM as a disease of ageing and in fact, T2DM would affect every single individual living to 108 years if the population were to experience such longevity ! T2DM shares many of the hallmarks of an age-related disease such as altered cellular senescence as well as low-grade chronic inflammation (or "inflammaging") (Franchesci et al. 2018). Further, the number of adults growing older with T2DM is rapidly increasing, on foot of global demographic trends.

Dementia is a massive concern for public health at present due to our ageing demography as well as the lack of new pharmacological treatments. One of the most exciting developments in recent years in dementia research is the efficacy of multi-domain preventative interventions targeted at individuals at high risk of developing dementia (Ngandu et al. 2015). Addressing risk factors for dementia is believed to be therapeutically more successful than the further development of agents aimed at targeting the consequences of the disease (such as anti-amyloid therapy). For instance, if the symptoms of dementia were to be delayed by as small as one year in every person affected, it could potentially lower the prevalence of dementia by over 9 million people in the next 30 to 40 years (Gustafson & McFarlane, 2018). A Lancet Commission has estimated that 35% of the overall burden of dementia is due to modifiable risk factors, such as T2DM (Livingston et al. 2017)

Given the evidence for the increased dementia risk in T2DM as well as the fact that most of those affected with T2DM are frequently exposed other midlife risk factors for dementia (such as hypertension, obesity and smoking), those affected by T2DM represent an attractive cohort to study with the aim of characterising cognitive trajectories and novel biomarkers in order to select-out individuals at risk of later cognitive decline. Whilst there has been a wealth of studies examining T2DM and

cognition in adults in the 6th decade of life onwards, there has been a dearth of research on midlife T2DM, the period of life where T2DM is acting as a risk factor in the first instance. Both T2DM and dementia are characterised by long prodromal periods and such opportunities represent an ideal target for intervention. In the coming chapter, the evidence linking T2DM and dementia is discussed, which is followed by a discussion of the potential mechanisms and biomarkers as well as potential for future treatments to mitigate the increased dementia risk seen in Type 2 Diabetes.

1.1 Evidence for increased dementia risk in Type 2 Diabetes Mellitus (T2DM)

The first epidemiological evidence for the increased risk of dementia in T2DM was published exactly thirty years ago (Katzman et al. 1989). In this study examining the development of dementia in volunteers aged 75-85 who were followed up for five years, T2DM was first reported as a risk factor for the later development of dementia (Katzman et al. 1989). Reports that followed in the early exploration of this link were mainly cross-sectional in nature and yielded conflicting results. It wasn't until the first longitudinal ageing studies were published in the late 1990s and early in the turn of the Century that the picture became a little clearer. Longitudinal ageing studies, characterised by a high fidelity of follow up and deep phenotyping of individuals across waves, provided a unique opportunity to study links between risk factors such as T2DM and the later development of dementia. Two prime examples of these studies are the Rotterdam (Ott et al. 1999) and Rochester (Liebson et al. 1997) studies. In the Rotterdam study, over 6,000 non-demented community-dwelling older adults were followed and T2DM was associated with a two-fold increased risk of dementia over follow-up. In the Rochester study, a total of 1,500 participants with T2DM were followed up for nearly 10,000 person years (participants were aged 45 years and older), T2DM was associated with a significantly increased risk of All-Type Dementia (ATD). Following the publication of these two studies and other longitudinal studies exploring the link, a meta-analyses of 28 studies has concluded that T2DM is associated with a risk ratio of 1.73 for the later development of ATD (Gudala et al. 2013).

More recent examples of well-characterised cohorts yielding novel insights into the T2DM-dementia link include the Atherosclerosis Risk in Communities Cohort (ARIC). In this study, participants were assessed between 1987-89 with regular follow up until 2011-2013, a total follow up period of 25 years. In the total cohort of around 15,000, 1,500 participants developed dementia. Comparative analysis of risk factors included midlife smoking, diabetes, prehypertension and hypertension. Apart from older age (HR: 8.06, 6.69-9.72) and APOE allele (HR: 1.98: 1.78-2.21), T2DM was associated with the greatest hazard ratio (HR 1.77, 1.53-2.04) which was greater than effect observed from race, educational attainment, hypertension, prehypertension and smoking (Gottesman et al. 2018). The authors noted the finding the risk posed by T2DM was almost as great as that posed by APOE genotype, one of the most studied risk factors for dementia to date. This finding has led to renewed interest in the link between T2DM and dementia.

One of the most interesting findings from the epidemiological literature are the insights into the life-course aetiology of the T2DM-dementia link. It appears that T2DM acts as a risk factor for dementia in midlife, largely between the ages of 35-65 years (Gottesman et al. 2018, Pelimanni et al. 2018) . Interestingly in those that develop T2DM aged 65 years and older, the risk does not appear to be as great (van den Berg et al. 2006). This suggests that midlife may be a particularly important period in which to intervene with potential preventative interventions, advancing the case for preventative biomarkers and risk assessment in midlife T2DM.

Not only does T2DM increase the risk of dementia, but it also acts as a potent risk factor for Mild Cognitive Impairment (MCI). Further, it appears that T2DM may additionally accelerate the transition from MCI to dementia in those affected (Luchsinger et al. 2007, Xu et al. 2010). It is presently unclear the exact cognitive trajectories seen in those with T2DM who go on to develop dementia. Some authors comment that T2DM may be associated with cognitive impairment which may be different from the MCI seen in prodromal Alzheimer's Disease (AD) (Jan Biessels et al. 2018). A recent expert review

has conceptualised the T2DM-dementia link as follows: (i) cognitive decrements seen across the lifespan in those with T2DM, that do not necessarily progress to dementia, (ii) cognitive impairment that is seen in those with T2DM who are at an increased risk of dementia and (iii) dementia in those with T2DM. Such a conceptualisation is more in-keeping with a life course aetiology of dementia, potentially providing more targets for cognitive interventions (Jan Biessels et al. 2018). For most authors, dementia seen in those with T2DM is likely the end-point of a gradually progressive multifactorial process which may occur over several decades. Much about the exact trajectories, timeline and the cognitive domains affected at the different stages of the association remain unexplored.

1.2 Cognitive Impairment in Type 2 Diabetes

There have been numerous studies examining cognitive performance in T2DM in comparison to age-matched healthy controls. These have mainly been conducted based on the initial epidemiological evidence from studies such as those discussed above. Consistently, in older adults (aged 60-80 years), T2DM has been associated with significantly poorer performance on all cognitive subdomains (namely, memory attention, processing speed, executive function and language/verbal fluency) in cross-sectional analyses, which has been confirmed by meta-analytic studies (Ganmore & Beeri, 2018)

One of the most interesting findings comes from the Whitehall II study. Analysis of the 302 participants with diabetes, in comparison to the remainder of the cohort, found that those with midlife T2DM (mean age: 54 years) demonstrated a 45% faster decline in memory, 29% faster decline in reasoning and a 24% faster decline in global cognitive over 10 years (Rawlings et al. 2014). This study is unique in demonstrating cognitive decline in those with T2DM at the very time in life that T2DM appears to be acting as a risk factor for later cognitive decline. This is in-keeping with prior studies demonstrating no significant differences in those with very early T2DM, demonstrating that the brain

may need exposure to the deleterious effects of T2DM for a significant amount of time for damage to occur. Overall, the evidence seems to suggest that differences may be subtle in the initial stages of T2DM, but over time, with long-enough follow up, cognitive deficits begin to emerge.

Further evidence from the ARIC cohort discussed above demonstrated a 19% greater cognitive decline in nearly two-thousand individuals with midlife T2DM over 20 years (Tuligenga et al. 2014). Combined with the Whitehall II Study above, the ARIC study demonstrates a highly significant impact of midlife T2DM on later cognitive function. These stand in stark contrast to studies with shorter follow up, such as five years, which demonstrate no difference in rate of decline (Nooyens et al. 2010). The findings can be summarised as follows: it appears that T2DM in midlife acts as a risk factor for later cognitive decline and dementia, however the effects are only clinically detectable after 10 years or greater of follow-up. This long prodromal period experienced by those with T2DM before cognitive impairment becomes evident provides an exciting opportunity for biomarker development and potential intervention.

1.3 Mechanisms Mediating the Link Between Type 2 Diabetes and Dementia

One of the most significant unanswered questions which remains in the link between T2DM and dementia is how this link is mediated. The underlying pathological processes remain to be elucidated, although several important hypotheses have been advanced and are gaining significant traction in the literature. To view such hypotheses as independent is probably incorrect, and the process of cognitive impairment in T2DM most likely develops due to a large number of interacting factors over a significant period of time. The most common putative pathophysiological hypotheses which have been developed and are discussed here include: (i) the role of hyper and hypoglycaemia, (ii) insulin resistance, common to both dementia and T2DM, (iii) neuro-inflammation and the role of innate immune activity, (iv) glucocorticoid excess and alterations HPA

reactivity and (v) the role of vascular pathology. These are discussed in turn with an integrative overview presented in **Figure 1.1**

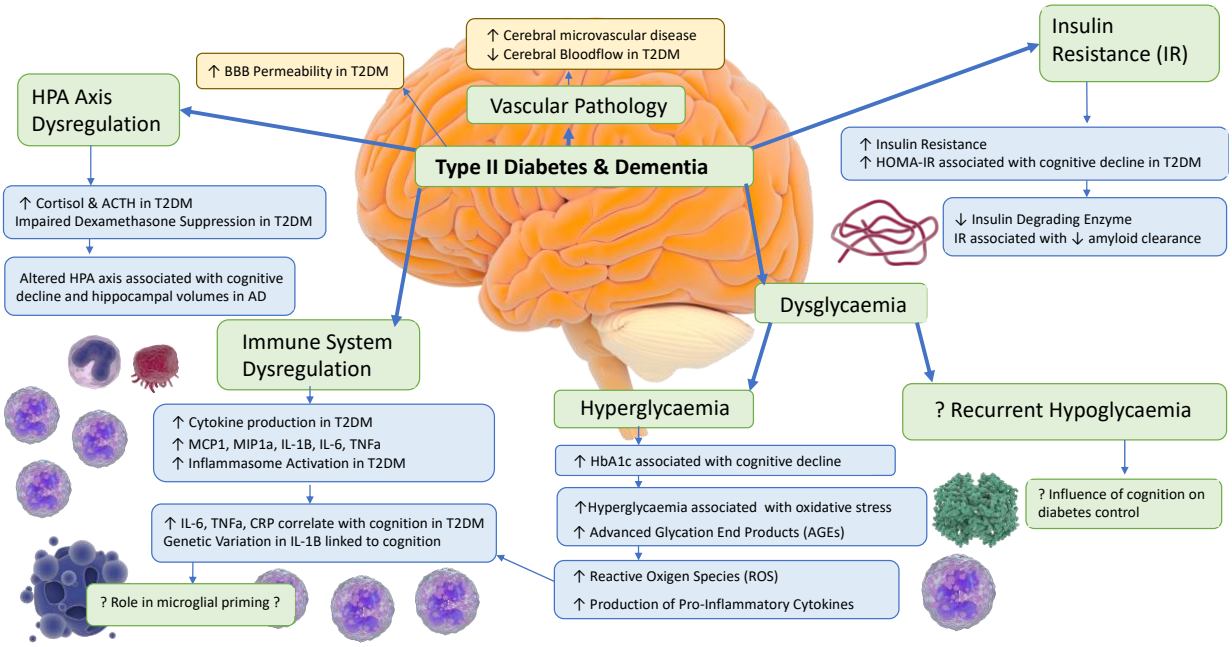

Figure 1.1 Putative Pathophysiological Hypotheses Explaining the T2DM-Dementia Link (Adapted from Dyer & Kennelly, 2019). *Principal hypotheses which have been advanced in order to explain the link between T2DM and dementia include: dysglycaemia (hyperglycaemia and hypoglycaemia), insulin resistance, dysregulation of the hypothalamic-pituitary-adrenal axis, dysregulation of the innate immune system and vascular pathology. It is likely that these aetiologies act in combination and not isolation, in line with the complex nature of the T2DM-dementia link*

1.3.1 Dysglycaemia (Hypo and Hyperglyceamia)

Type 2 Diabetes Mellitus is characterised by hyperglycaemia (high blood glucose), and the finding of hyperglycaemia leads many on to a diagnosis of T2DM in the first instance. There is a wealth of evidence in the literature that exposure to high levels of blood glucose impairs cognition both in the acute and chronic contexts. One of the first studies to support this demonstrated impairments in working memory and attention in those with T2DM in hyper-insulinaemic glucose clamps to maintain hyperglycaemia

(Sommerfield et al. 2004). Further evidence has emerged supporting the relationship between hyperglycaemia and poorer cognitive performance when measured simultaneously (Crane et al. 2013). Whilst the study of acute hyperglycaemia effects and cognition is important, the relationship between chronic hyperglycaemia and cognition is probably more worthy of interrogation in the T2DM-dementia link.

In the landmark ACCORD-MIND Study (The Memory in Diabetes Sub-study), a 1% higher glycated haemoglobin (HbA1c; representing overall blood sugars over the lifespan of a Red-Blood Cell – around 3 months) was associated with significantly poorer performance on both the Digit Symbol Substitution Test (DSST) and the Mini-Mental State Examination (MMSE) (Cukerman-Yaffe et al. 2009). Further, studies carried out in participants with T2DM have demonstrated a significant relationship between diet-based glycaemic load/hyperglycaemia and poorer performance on measures of perceptual speed and visuospatial tasks. Moreover, studies examining individuals with MCI have demonstrated an accelerated conversion to dementia in those with hyperglycaemia, with decreased frontal grey matter volumes noted (Seetherman et al. 2015, Morris et al. 2014)

Interestingly, the association between hyperglycaemia and cognition is also seen in individuals who do not have T2DM. In longitudinal analysis of non-diabetic adults, hyperglycaemia has been associated with a greater risk of dementia overall. Further evidence from the English Longitudinal Study of Ageing (ELSA) has demonstrated that even in individuals without T2DM, levels of glycated haemoglobin (HbA1c) are associated with rate of cognitive decline over 8 year follow up (Zheng et al. 2018). This is particularly interesting in shedding new light on the complex relationship between hyperglycaemia and later risk of cognitive decline.

Whilst the relationship for hyperglycaemia and later cognitive impairment/decline has been well demonstrated, the case for hypoglycaemia (low blood sugars) is less well demonstrated. Studies, mainly conducted on participants with Type 1 Diabetes (also

characterised by a later risk of cognitive decline and similar micro/macrovascular complications as T2DM) have demonstrated an increased risk of later cognitive decline in individuals with three or more episodes of severe hypoglycaemia, with risk increasing alongside each additional episode of severe hypoglycaemia (Whitmer et al. 2009). One of the problems in assessing the reliability of such findings is that hypoglycaemia may result from problems with treatment adherence, particularly where individuals are using their own insulin medication, which may be impaired in those with cognitive problems. Thus the direction of causality remains difficult to prove. More pragmatically, it may be that increased variability in blood sugars may have a pronounced effect in cognition and interestingly, a recent well-conducted pooled analysis of two longitudinal studies (including the aforementioned English Longitudinal Study of Ageing) demonstrated that variability in HbA1c measurements within individuals may actually be a predictor of longitudinal cognitive decline in T2DM (Rawlings et al. 2017)

One of the open questions in the literature currently is the exact mechanisms by which hyperglycaemia can cause neuronal damage in T2DM. Again, the effects of hyperglycaemia on the Central Nervous System (CNS) may be divided into acute and chronic effects. In the acute phase of hyperglycaemia, hyperglycaemia may affect changes on cerebral blood flow and cause osmotic changes in neurons. Such changes may result in oxidative stress and subsequent neuronal damage. In the longer term, chronic hyperglycaemia may result in the formation of Advanced Glycation End Products (AGEs). AGEs consist of both lipids and proteins which, as a result of exposure to high levels of blood sugars undergo a glycation reaction (Dhananjayan et al. 2018)
AGEs are implicated in diabetes as well as many other degenerative and age-related conditions such as AD, chronic kidney disease and atherosclerosis. One of the principal ways in which AGEs play a role in these diseases is through activation of the innate immune system. AGEs may act as Damage Associated Molecular Pathogens (DAMPs), acting on innate immune receptors, resulting in increased systemic inflammation. This is seen through the formation of Reactive Oxygen Species (ROS) and the production of pro-inflammatory cytokines. Such low grade “sterile” inflammation has a role to play in

both T2DM and Alzheimer Disease, both diseases of ageing and inflammation (or "inflammaging"), discussed in more detail in the neuro-inflammation section and additionally in Chapters 5 and 6. Notably, AGEs have been implicated in both T2DM and dementia individually, and levels have been associated with cognitive decline in T2DM, therefore acting to accelerate deficits seen across cognitive domains in T2DM (Yaffe et al. 2011)

1.3.2 Insulin Resistance

Insulin Resistance (IR) refers the reduced ability of the peptide insulin to activate insulin receptors and is a central part of both T2DM and is associated with many features of the Metabolic Syndrome. Insulin, produced by the beta-cells of the pancreas, is released in response to increased glucose and acts to store glucose in organs such as the liver and muscle. Insulin receptors are found all over the body and are abundantly expressed in the brain including in high density in areas implicated in memory, attention and executive function such as the hippocampus, entorhinal cortex and frontal lobes. Insulin produced from the pancreas and released into the bloodstream is rapidly transported over the blood brain barrier and acts on all major cell types in the Central Nervous System (CNS) (Dyer et al. 2016). In insulin resistance, post-translational modification of the insulin receptor at a serine-threonine residue, located on the cytosolic side of the receptor, induces resistance to the effects of insulin and may be one of the key pathophysiological events in the later development of T2DM (Ma et al. 2015).

Clinically, there are a number of ways to assess for insulin resistance and numerous criteria exist. Traditionally, the most widely used assessment has been the homeostatic model assessment of insulin resistance (HOMA-IR) (Matthews et al. 1985). This modelling takes into account the relevant concentrations of glucose and insulin and expresses their product, divided by a constant. Measurements of insulin resistance have been shown to predict later cognitive decline in those with and without T2DM (Ronnema et al. 2008). Interestingly, in one of the main studies examining this

relationship, a longitudinal study of over 2,000 participants, a low insulin response (consistent with insulin resistance) assessed on an intravenous glucose test and HOMA-IR assessment, predicted the development of AD over a mean follow up period of 32 years (Ronnema et al. 2011). Such findings are striking in implicating the phenomenon of insulin resistance in cognitive impairment, especially as a link between midlife T2DM and the later development of dementia.

Again, there is much work to be done in order to characterise the mechanisms by which insulin resistance may cause neuronal damage in T2DM. One of the most fascinating findings from this area relates to the impact on Insulin Degrading Enzyme (IDE) (Umegaki et al. 2014, El Khoury 2014). IDE is a zinc-binding protease of the metalloproteinase family which is responsible for the degradation of insulin and other peptides, including atrial and brain naturietic peptides in addition to amyloid-β (the putative pathogenic protein implicated in Alzheimer Disease) (Pivovarova et al. 2016). Insulin resistance is associated with decreased levels of IDE able to clear other peptides, and may result in a decreased degradation of amyloid-β, the constituent of plaques in Alzheimer Disease, one of the principal pathological findings noted by Alois Alzheimer at the beginning of the previous century. There has been interest in the development of substrate specific IDE therapies in the potential treatment of Alzheimer Disease. Thus, the impact of IDE may play an important part in insulin resistance mediating the T2DM-dementia link.

Independent of T2DM, IR is now being seen as an important part in the pathophysiology of Alzheimer Disease. In post-mortem studies carried out on patients with Alzheimer Disease, markers on insulin resistance have been noted. In particular, it appears that markers of insulin signalling (such as phosphorylation of the Insulin Receptor Substrate 1 or IRS1) are greatly reduced (Umegaki et al. 2014). Similarly, it appears that Insulin-Like Growth Factor 1 (IGF-1) signalling is also affected (in addition to its binding proteins such as IGFBP3) in Alzheimer Disease, consistent with the pleiotropic effects of IGF-1 as a growth factor in the CNS with involvement in processes such as memory formation

and neurogenesis (Arafa et al. 2017, Dyer et al. 2016, Duron et al. 2012). Thus, insulin resistance, and the effects on implicated pathways may have a central role to play in mediating the link between midlife T2DM and the later development of cognitive impairment/dementia.

One of the most interesting developments in this area is the conceptualisation of the concept of brain insulin resistance (Goldstein, 2002). In a unique experiment using Positron Emission Tomography (PET) scanning and a hyperinsulinemic clamp, it has been demonstrated that both global and regional changes in metabolic activity in response to insulin was much greater in insulin sensitive individuals in comparison to those with insulin resistance (Anthony et al. 2006). Such findings directly implicate IR in the brain in T2DM. However, the relationship between Brain Insulin Resistance and the later development of dementia in those with T2DM remains unclear, and will demand novel experimental, and by necessity, longitudinal designs in order to address this question.

1.3.3 Neuro-inflammation

Systemic inflammation appears to have an important role to play in mediating the T2DM-dementia link, although much of the evidence is circumstantial and limited to either disease individually. Fundamentally, T2DM can be conceptualised as a disease of peripheral insulin resistance and defective insulin secretion by pancreatic β cells. Both of these phenomena have a strong association with systemic inflammation (Donah & Sholeson, 2012, Donath 2014). One of the instigating events in the pathophysiology of T2DM is localised inflammation in the pancreas in response to dysfunctional beta cells (with glucotoxicity, liptoxicity, oxidative stress and endoplasmic reticulum stress). This damage results in activation of the innate immune system by Damage Associated Molecular Pathogens (DAMPs) and ultimately activation of pro-inflammatory macrophages resulting in the secretion of pro-inflammatory chemokines and cytokines such as Tumor Necrosis Factor (TNF), Interleukin 1β (IL-1β) and Monocyte Chemoattractant Protein 1 (MCP-1). Such

a cascade results in systemic inflammation which is detectable in the serum and plasma of those with T2DM (Donath & Sholeson, 2012). Further, obesity, common amongst individuals with T2DM, is associated with "meta-inflammation" and the production of pro-inflammatory cytokines, as is insulin resistance (Donath & Sholeson, 2012).

How this systemic inflammation may relate to the later risk of dementia and cognitive impairment is not clear at present, but theories have been proposed. A central part of this link may be the activation of innate immune receptors by Damage Associated Molecular Pathogens (DAMPs). There are a wide variety of DAMPs that could be acting on Pathogen Recognition Receptors (PRRs) to cause this systemic inflammation. Important DAMPs which have been studied in T2DM include Islet Associated Amyloid Peptide (IAPP), Free Fatty Acids (FFA) as well as AGEs discussed above. These stimuli have also been shown to activate the nucleotide-binding oligimerisation domain receptors (NLRs) and formation of the NLRP3 inflammasome with the subsequent production of IL-1β (Kono et al. 2008). Such a pathway is supported by evidence reported altered activation of the NLRP3 inflammasome in individuals with newly diagnosed diabetes (Lee et al. 2013).

Activation of the innate immune inflammasome complex and the release of cytokines such as IL-1β and TNF may have crucial roles to play in mediating the T2DM-dementia link. Increased cytokine gradients in peripheral blood have previously been associated with continuing pro-inflammatory reactions within the brain. Exposure to circulating IL-1β may induce pro-inflammatory changes in microglial cell populations, similar to the peripheral effect of systemic inflammation on macrophages, and cause either acute inflammation and neurodegeneration or prime microglia to produce a pro-inflammatory response when stimulated by other DAMPs within the brain (Heneka et al. 2018, Block et al. 2007).

The role for the NLPR3 inflammasome is elegant in this model, as it proposes that T2DM could potentially act as a priming factor to make microglia more pro-inflammatory in the brain, resulting in greater degrees of nuero-inflammation in response to neurodegenerative insults. For instance, the NLRP3 inflammasome is capable of being activated by amyloid- β with the formation of ASC-Specks following a priming step, and a subsequent cleavage of

pro-IL-1β to release mature IL-1β in response to amyloid protein itself (Heneka et al. 2013). Such an attractive hypothesis for the role of the innate immune inflammasome in the T2DM-dementia link is explored further in Chapter 6 where a description of innate immune signalling via the NLRP3 inflammasome and its potential role in the T2DM-discussed.

Intense interest in the fields of neuro-immunology and neurodegeneration have focussed in recent years on the role of microglial priming. Microglial phenotype and number usually undergo strict regulation in the CNS, but may expand, increase engulfment and become activated (to an "alternative phenotype"), a response which may be termed "priming" (Perry & Holmes, 2014). The systemic immune activation in both T2DM and obesity, seen in midlife when these states are associated with meta-inflammation and circulating pro-inflammatory cytokines, may induce this response in microglia, although this is yet to be explored. One would hypothesise that microglia, once primed by the systemic immune activation seen in these conditions, could respond in an exaggerated manner to either age-related changes or changes seen in early AD, resulting in more neuronal damage in T2DM and hence the earlier development of overt signs of cognitive impairment and dementia. This hypothesis is displayed graphically in Figure 2.

Another important consideration in conceptualising the role of neuro-inflammation in T2DM-dementia is the concept of both T2DM and dementia being diseases of ageing. Ageing represents the greatest individual risk factor for dementia and T2DM is also frequently described as a age-related pathology. The nascent field of "inflammageing", which refers to the long-term result of chronic, low-grade innate immune system activation which may become damaging with age is a useful lens through which to view the T2DM-dementia link. As a result of increasingly longevity and those with T2DM living longer with T2DM, the chronic low grade systemic inflammation experienced in T2DM may accelerate the effects of age-related degeneration in the CNS. Interestingly, one of the experts in the field, Claudio Francheschi, points out that inflammation and "meta-inflammation" largely share the same molecular mechanisms in low-grade systemic inflammation in response to damage associated signals. The key pathways involved in inflamageing converge on cellular

senescence, defective autophagy and mitophagy, innate immune inflammasome activation (as discussed above and in chapter 6), activation of the DNA damage system and ubiquitin-protease systems (Francheschi et al. 2018).

Interestingly, in the Edinburgh Type 2 Diabetes Study, associations between increased levels of pro-inflammatory cytokines in the plasma of individuals with T2DM and poorer cognitive function has been observed, giving direct support to the role of peripheral inflammation in mediating the T2DM-dementia link (Marioni et al. 2010). Similarly, findings in other studies have demonstrated associations between serum levels of IL-1β and cognitive performance in older individuals with T2DM (Tian et al. 2018). Such studies are few in the literature, but lend support to the role of the innate immune system in mediating T2DM-related cognitive impairment and hence later dementia risk.

Finally, one of the open questions about how peripheral inflammation seen in T2DM may result in microglial activation centres on the role of the Blood-Brain Barrier (BBB). Whilst the brain was once thought of as an immune-privileged organ, this may not be fully true (Perry & Holmes, 2014). There is a great degree of communication between the periphery and CNS when it comes to immune system activation. One of the most fascinating findings in this area is the demonstration of increased BBB permeability in those with T2DM, possibly as the result of systemic inflammation (Janelidze et al. 2017). Weakened functioning of the BBB may leave the CNS exposed to greater degrees of systemic inflammation, priming microglia and making the brain less resilient to neurodegenerative or age-related insults.

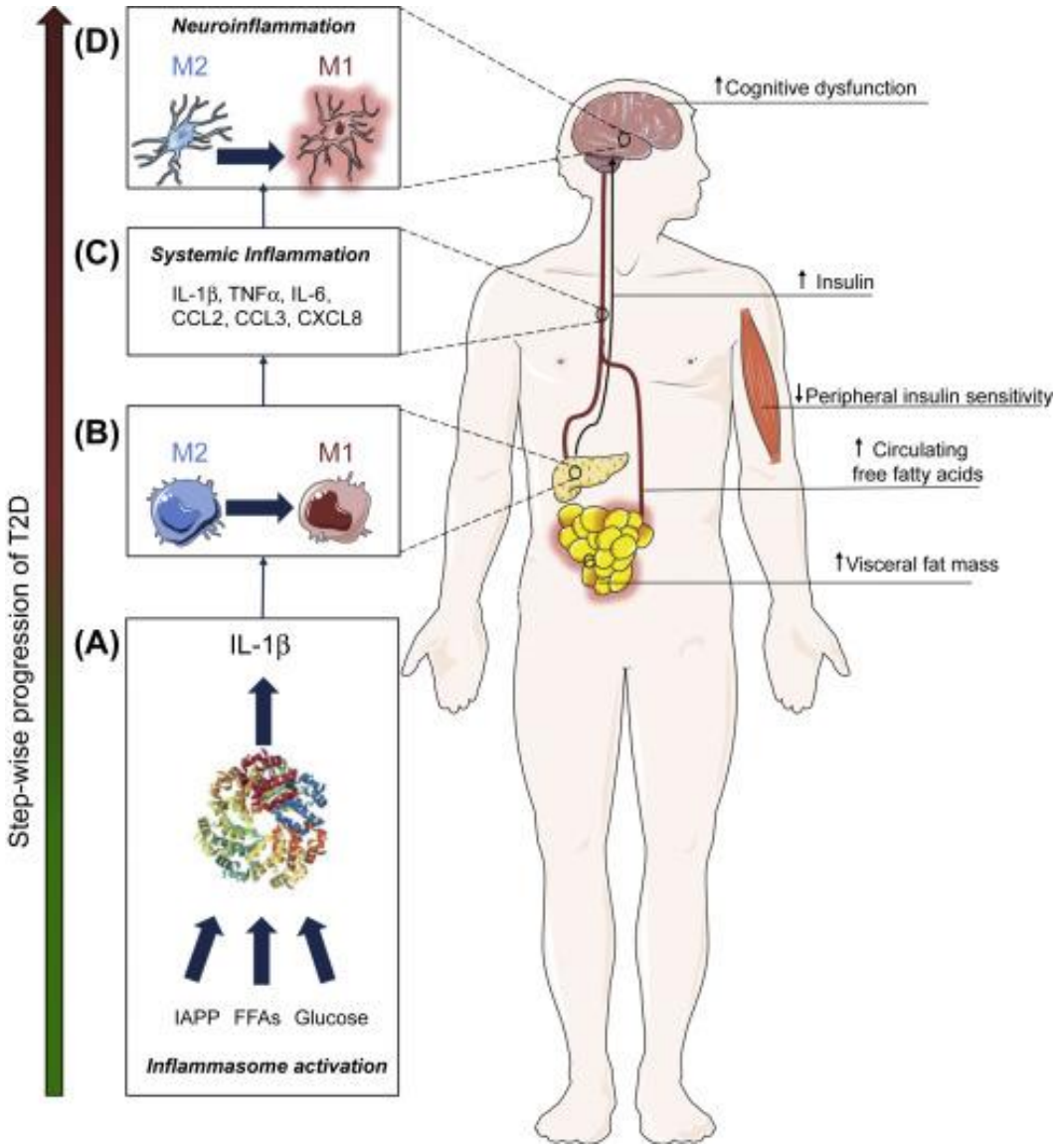

Figure 1.2. The Potential Neuro-Immune Pathway by which Diabetes may Increase Dementia Risk (Adopted from Wong et al. Type 2 Diabetes and Dementia. Elsevier Academic Press). *In this model, localised inflammation in the pancreas in response to insulin resistance and defective pancreatic cell function leads to an increased activation of the innate immune inflammasome and the production of inflammasome-dependent cytokines such as IL-1β. The result of this is activation of pro-inflammatory macrophages and the subsequent increase in circulating pro-inflammatory cytokines and chemokines seen. Systemic inflammation may affect the Central Nervous System, and prime microglia to*

react to Damage-Associated Molecular Pathogens which may be seen in neurodegenerative or age related states.

1.3.4 Glucocorticoid Excess and Perturbed Hypothalamic Pituitary Adrenal Axis Functioning

Another hypothesis gaining traction in the link between T2DM in midlife and the later development of cognitive decline/dementia is that of perturbed glucocorticoid levels and signalling. It has been well-reported that individuals with T2DM demonstrate raised plasma cortisol levels in addition to increased Adrenocorticotrophic Hormone (ACTH) levels. Further, studies have demonstrated that individuals with T2DM have impaired dexamethasone suppression. Importantly, it is widely known in the literature that glucocorticoid excess can have deleterious effects on hippocampal and entorhinal neuronal function, areas particularly important in the pathophysiology of cognitive decline and dementia (Strachan et al. 2008) .

In animal models of insulin resistance and Type 2 Diabetes Mellitus is has been shown that the presence of T2DM is associated with impaired hippocampal function mediated through glucocorticoid effects on both new and mature neuronal cell populations (Stranahan et al. 2009). This is on foot of previous evidence in both animal models and humans that increased levels of circulating glucocorticoids may have deleterious effects on hippocampal cell number and function (Strachan et al. 2008).

In individuals with AD, altered functioning of the Hypothalamic-Adrenal Pituitary (HPA) axis has been documented with raised levels of plasma cortisol which have been directly associated with both cognitive decline and altered hippocampal volumes (de Leon et al. 1988, O'Brien et al. 1996). One of the most interesting findings in this area again comes from the Edinburgh Type 2 Diabetes Study. In this study of 1,066 men and women aged 60-75 years, higher fasting cortisol was associated with greater cognitive decline, specifically affecting working memory and processing speed (Reynolds et al. 2010).

Taken with the known aberrations in HPA axis activity in both T2DM and dementia, this important finding adds significant weight to the hypothesis supporting the role of the HPA axis and glucocorticoid excess in mediating the link between T2DM and dementia (Reynolds et al. 2010).

1.3.5 The Role of Vascular Pathology

Despite a large number of cross-sectional and indeed longitudinal reports in the literature, the amount of studies supporting the role of vascular risk factors and vascular pathology in mediating the link between T2DM and cognitive decline remains rather small (Jan Biessels and Despa, 2018). One would hypothesise that given the strong vascular basis for many other microvascular and macrovascular complications of T2DM, that vascular risk factors would have a strong role to play in determining cognitive impairment in T2DM. The strongest evidence to date that midlife vascular risk factors such as hypertension, dyslipidaemia and smoking may be important in mediating cognitive decrements in T2DM are based on the adverse vascular profile typically seen in prediabetes and diabetes. Taken in the context of the strong role of these midlife vascular risk factors in dementia aetiology (See Fig 1.3), a hypothesis supporting a strong vascular basis to the cognitive impairment seen in T2DM is one of the most plausible routes by which T2DM may increase dementia risk.

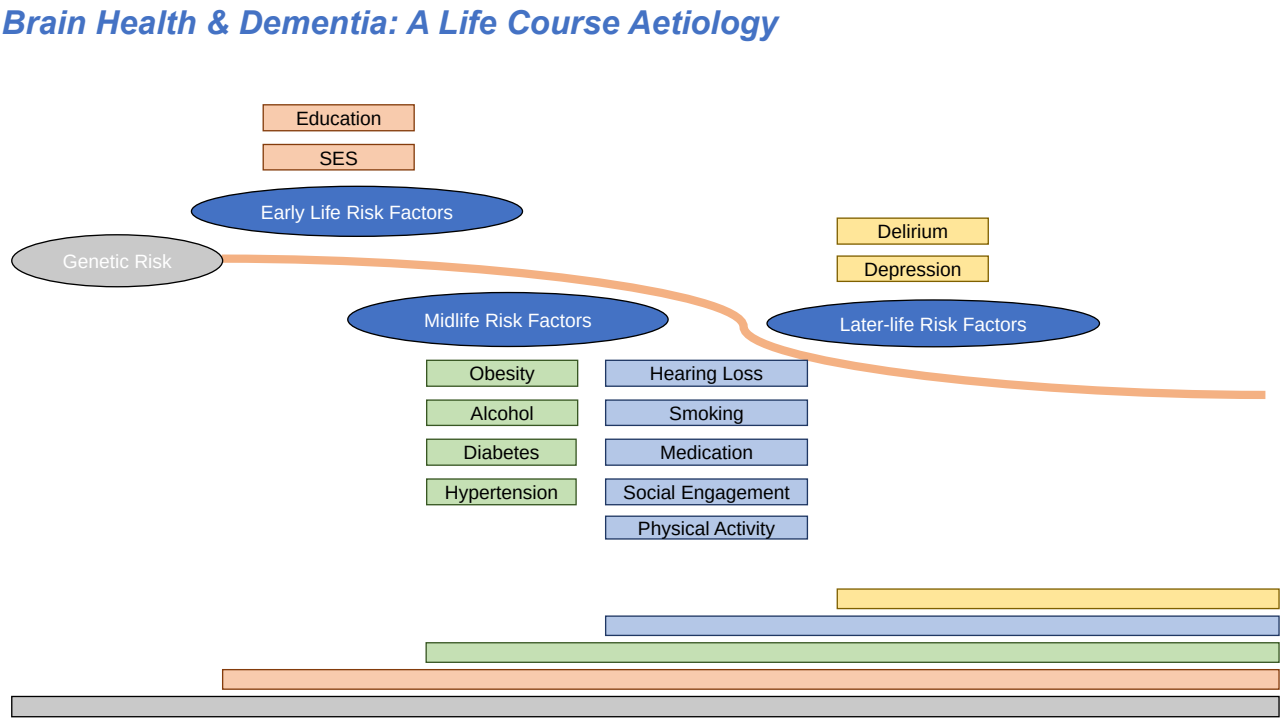

Figure 1.3. A Life Course Aetiology for Dementia. *Midlife vascular risk factors such as obesity, alcohol consumption, diabetes and hypertension are important in the pathophysiology of dementia, particularly when dementia is considered not just a disease of later life, but a disease with a life course aetiology*

Few studies have assessed how particular vascular risk factors impact on the later risk of cognitive decline and dementia in T2DM (Haroon et al. 2015, Feinkohl et al. 2015). Interesting data comes from brain imaging studies. Such studies carried out in individuals with T2DM demonstrate an increase in the number of lacunes, white matter hyperintensities, perivascular spaces and microinfarcts (Wardlaw et al. 2013). Whilst these signs are classically considered to be related to a vascular aetiology of brain disease, they are by no means specific. However, they do suggest a role for vascular processes in the aetiology of cognitive impairment in T2DM. Interestingly, studies have also demonstrated that individuals with T2DM also have decreased cerebral blood flow, which may support a vascular basis for cognitive decrements and impairments (Claffey et al. 2018).

One of the prevailing hypothesis about the role of vascular pathology in the development of T2DM related cognitive impairment considers the role of iscahemia and hyperglycaemia in individuals with long standing T2DM where vascular disease (and particularly small vessel vascular disease) may lower the threshold for the development of cognitive impairment and dementia (Umegaki, 2014). Such an approach is commonly cited in the development of Alzheimer Disease for instance, where it is now recognised that neurodegeneration and amyloid accumulation may in fact occur on a vascular platform. Such an approach is an integrative one and accommodates the other principal hypothesis of T2DM related cognitive impairment such as hyperglycaemia and the role of neuroinflammation.

Another mechanistic pathway linking the role of vascular disease and diabetes refers to the roles of lipid accumulation, increased levels of AGEs and aggregated protein in the vasculature. Such accumulation may cause a localised inflammatory reaction in the vessel wall, with the subsequent production of Reactive Oxygen Species (ROS), which serve to damage the vascular endothelium, impairing the release of vasodilatory mediators and impairing cerebral perfusion (Quaegebeur et al. 2011, Iadecola 2013). Such impairments in blood-flow have the potential to lead to neuro-vascular uncoupling (an impairment in the relationship between neural activity and cerebral blood flow). Such endothelial damage and chronic innate inflammatory insults may result in the microhaemorraghes and dysfunctional brain perfusion already noted to be present in individuals with T2DM. This vascular inflammation may also have the role of priming the innate immune system, linking the vascular hypothesis with that of neuro-inflammation above, and indeed vascular disease is a common driver of more general "inflammageing", the phenomenon introduced above (Franchesci et al. 2018)

In particular, cerebral hypoperfusion may have a central role to play in mediating many of the vascular changes seen in those with cognitive impairment. For instance, in an important study by Suter and colleagues (2002), cerebral hypoperfusion was found to have an important role to play in both white matter changes and cortical watershed

microinfarcts, further exacerbating neurodegenerative changes in those with pre-existing dementia. Such findings have been followed by other studies demonstrating an important role for borderzone microinfarcts in those with cognitive dysfunction (Capasi et al. 2019) and a role for leukoariosis and lacunar infarcts on cognitive dysfunction (McMurtray et al. 2007). Whilst similar studies have not been conducted in those with T2DM, it is plausible that such pathological processes may underlie the cognitive dysfunction apparent in later life in those with midlife T2DM.

1.3.6 Understanding the Basic Mechanisms: Drawing it All Together

Interestingly, it appears that whilst T2DM increases risk for all-type dementia, it is simply not the case that T2DM associated cognitive deficits and dementia can be classified as either vascular or Alzheimer type dementia. Such an approach, whilst seems pragmatic at first, creates a false dichotomy that simply isn't borne out by the most recent research. In fact, one of the biggest questions to date in studying the T2DM-dementia connection is whether T2DM accelerates the neurodegeneration by non-Alzheimer or non-vascular mechanisms. For instance, neuropathological studies demonstrate that the classic neuropathological features of Alzheimer Disease are not more common in those with T2DM (Arvanitakis et al. 2006). Neither is T2DM associated with Cerebrospinal Fluid (CSF) or Positron Emission Tomography (PET) biomarkers of increased amyloid deposition or tau pathology (Gottesman et al. 2017, Moran et al. 2015, Vemuri et al. 2017)

It may also be case that there are different phenotypes of cognitive impairment in T2DM. Not all dementia seen in individuals with T2DM may be the same and no studies to date have ever attempted to sub-classify by the different cognitive domains or upstream pathophysiological processes. One of the great challenges for this area of research will be dissociating the T2DM specific processes leading to cognitive dysfunction and impairment, and those that are more generally seen in dementia. For instance, whilst insulin resistance is seen as a potential prominent process in T2DM

related cognitive impairment, mounting evidence also implicates the role of cerebral insulin resistance in Alzheimer Disease in general. Understanding the difference in those with T2DM and those without will be of paramount importance in the coming years of research studying the pathophysiological processes linking T2DM and dementia.

1.4 Brain Imaging in Individuals with Type 2 Diabetes Mellitus

A surprisingly-large number of studies have been dedicated to examining differences in brain structure and function via neuroimaging between individuals with T2DM and healthy controls. Studies using Magnetic Resonance Imaging (MRI) have demonstrated significantly greater cortical and subcortical atrophy in addition to atrophy of the hippocampus and amygdala and decreased grey matter volumes in frontal and parietal cortices (Garcia-Vasares et al. 2014, Kumar et al. 2008, den Heijer et al. 2003, Moran et al. 2013). Such findings are noteworthy, considering the similarity in the nature and distribution of differences to those seen in AD. In a study examining individuals with both MCI and T2DM, Zhang et al. 2014 reported a greater decrease in medial temporal gyral grey matter in comparison to either condition alone. One of the most interesting findings in this expanding area of research is the correlation with many of the putative pathophysiological hypotheses explaining the T2DM-dementia link. For instance, studies have demonstrated that HbA1c, perturbation of the HPA axis and cortisol excess in addition to insulin resistance were all predictive of grey matter volumes in those with T2DM (Gold et al. 2007, Bruehl et al. 2010).

One of the most startling findings from the T2DM neuroimaging literature is a study published recently by researchers based in the University of Birmingham (Nouwen et al. 2017). They studied adolescents affected by T2DM, obese adolescents and healthy weight controls using MRI. Those with T2DM had reduced grey matter volume in the right hippocampus, left putamen and caudate, bilateral amygdala and left thalamus. Many of these are similar to areas affected in cognitive impairment and highlight the

urgent need for the better characterisation and treatment of brain health in T2DM and particularly for biomarkers at the earliest possible stage.

Other than studies comparing grey matter volumes and atrophy in those with T2DM and healthy controls, a small body of literature has also examined differences using Diffusion Tensor Imaging (DTI), used as a method of examining white matter changes and tractography. Interestingly, T2DM is associated with decreased Fractional Anisotrophy (FA) in frontero-temporal regions (Falvey et al. 2013). One of the most striking findings from this work is that of Hsu et al. 2012 who demonstrated that in individuals with T2DM free from cognitive impairment or cognitive decrements, there was a direct link demonstrated between disease duration and mean, axial and tansverse diffusivity on DTI (Hsu et al. 2012). This later finding deserves some pause and reflection – even in individuals with no clinical symptoms of cognitive impairment, T2DM is associated with white matter changes readily assessed by neuroimaging technology, solidifying the evidence for increased risk of cognitive impairment and dementia in T2DM and adding support to the need to explore new biomarkers which can detect subclinical disease. Further evidence has also demonstrated deficits in the attentional network and abnormalities in resting state activity in T2DM (Xia et al. 2015) and again, many of these changes recapitulate those seen in AD.

Functional neuroimaging studies have also been carried out to examine the differences between those with T2DM and healthy controls. Of note, decreased connectivity between fronto-temporal regions and the hippocampus in addition to reduced fronto-temporal blood flow and glucose utilisation have all been noted (possibly reflecting cerebral insulin resistance) (Jones et al. 2014, Niwa et al. 2006). Again, the involvement of fronto-temporal and hippocampal regions in these studies directly echo the earlier findings from both structural neuroimaging of brain volumes and diffusion tensor imaging and tractography.

In sum, it appears that T2DM, even without any overt evidence of cognitive impairment or cognitive decrements is associated with structural and functional deficits on neuroimaging. Such findings add direct support to the deleterious effects of T2DM on brain function and brain healthy and advocate for further development of neuroimaging markers of cognitive decline, to select out individuals at the earliest possible stages who may be at highest risk.

1.5 Biomarkers of Cognitive Impairment and Dementia in Type 2 Diabetes Mellitus

Perhaps the greatest unmet need at present in research examining the T2DM-dementia link is the lack of any substantial biomarker research with the ability to select out individuals who are particularly at risk. Phenotypically, T2DM is an extremely heterogenous disease, as has been recognised in a recent landmark study dividing T2DM into at least five discrete types (Ahlqvist et al. 2018).One of the principal findings of research to date is that T2DM appears to act as a risk factor for the later development of cognitive impairment and dementia in midlife, not later life, and on foot of this, it makes logical sense that the biomarkers with the greatest utility will be discovered in midlife, when risk is taking place and not in later life, when much of the pathophysiological damage caused by T2DM has already occurred.

One of the most substantial studies to date examining risk prediction in T2DM using a particularly elegant study design was carried out by Exalto and colleagues (Exalto et al. 2013). In this study, researchers examined two longitudinal cohorts of participants with T2DM (aged 60 years of older) who were followed up for 10 years. They evaluated 45 predictors and replicated in an independent sample. They demonstrated that microvascular disease, diabetic foot disease, cerebrovascular disease, cardiovascular disease, acute metabolic events, depression, age and education were most strongly predictive of dementia (Exalto et al. 2013). This is the first validated score and has the ability to predict 10 year dementia risk in those with T2DM.

The associations between cognitive impairment/dementia in T2DM and the presence of other T2DM related microvascular and macrovascular complications in the literature has been well demonstrated both cross-sectionally and longitudinally (Bruce et al. 2018), so it is no surprise that they constituted the main risk factors integrated in the above risk score. Suffice to say, it appears from the literature that if one's T2DM is severe enough to cause diabetic nephropathy, retinopathy or diabetic neuropathy, it is probably severe enough to cause damage to the brain. Such approaches confirm the presence of cognitive impairment/dementia as a complication of diabetes, but fall very short when it comes to risk prediction at the time when risk factors are exerting their effect. Consistent with recent evidence demonstrating the efficacy of multi-domain interventions in the mitigation of dementia risk in high risk groups (Ngandu et al. 2015), we need biomarkers in midlife, at least ten years before cognitive decrements and deficits are clinically evident, in order to select out those who are most at risk. This is an urgent priority for the T2DM-dementia research field.

In addition to the role of hyperglycaemia in mediating T2DM-related cognitive deficits/impairment discussed above, it appears that HbA1c may be one of the most important predictors in the later development of memory issues in those with T2DM. HbA1c shows strong correlation with the presence of cognitive impairment in older adults affected by T2DM (Cukerman-Yaffe et al. 2019). As mentioned above, HbA1c has even demonstrated a link with cognition in those without T2DM (Zheng et al. 2018). Again, this appears to fit with the hypothesis that the more severe T2DM affects an individual, the more likely it is to affect the brain. This is seen in studies demonstrating an impact of greater duration of diabetes (Roberts et al. 2008) and the presence of T2DM related complications (Bruce et al. 2008) being associated with a greater risk of cognitive impairment/dementia.

However, in considering the HbA1c-cognition link it must also be acknowledged that HbA1c is influenced by both treatment of T2DM and compliance with T2DM treatment. Thus, treatment compliance is an important consideration in assessing the HbA1c-

cognition link. No studies to have adequately assessed the association between T2DM treatment compliance and later cognitive dysfunction. Similarly, the development of cognitive impairment may impair individual's ability comply with treatment regimes for their T2DM which is an important confounding factor which must be acknowledged in exploring the HbA1c-cognition link. Poor compliance with treatment may be an early indicator of cognitive dysfunction in older adults with T2DM.

On foot of evidence above discussing the roles of vascular risk factors in mediating the T2DM-dementia link, lifestyle vascular risk factors may play an important role such as sedentary lifestyle/lack of exercise, smoking and alcohol consumption (Feinkohl et al. 2015). Further, simple anthropometric measurements of central adiposity, measures of total fat mass, hypertension (specifically elevated systolic blood pressure), dyslipidaemia and arterial stiffness may all have a role to play in predicting cognitive impairment and dementia in T2DM (Abbatecola et al. 2010a; Petrova, Prolopenko, Pronina & Mozheyko, 2010, Mehrabian et al. 2012). However, such studies are cross sectional in nature and largely limited to those affected by T2DM-related cognitive impairment aged 65 or older. Studies on such markers in midlife are scarce and badly needed.

As mentioned, the presence of diabetic microvascular/macrovascular disease affecting other organs seems to increase one's risk of developing T2DM-related cognitive impairment/dementia. One of these which is particularly interesting and may have a role in the future of alerting the clinician to cognitive impairment is diabetic retinopathy. A well-conducted meta-analytic study has confirmed the association between T2DM-related cognitive impairment and the presence of diabetic retinopathy (Crosby-Nwaobi et al. 2012). Such a complication may be a clinically-accessible window into assessing the degree of microvascular disease assessing the central nervous system. Studies are needed to look at the relationship between cognitive impairment in T2DM and the earliest signs of diabetic eye disease, again particularly in midlife, when the greatest risk is occurring. It is also important to note the well-documented link between retinal

disease and lacunar disease (Doubal et al. 2009). Pathology in retinal vessels, which share common ontogeny, size and physiological characteristics and cerebral small vessels, may be a reflection of cortical microvascular disease and lacunar pathology. This may reflect the importance of vascular pathology in mediating the T2DM-cognitive dysfunction link.

Finally, there is a small number of studies which have examined the link between cognitive function in T2DM (usually measured on the standardised MoCA or MMSE) and serological measurements. Studies have examined NT-proBNP (Feinkohl et al. 2012), cortisol (Reynolds et al. 2010), soluble RAGE (the receptor for AGEs discussed above) (Chen et al. 2011), BDNF (Brain Derived Neurotrophic Factor) (Zhen et al. 2013), leptin levels (Labad et al. 2012) and vitamin D levels (Chen et al. 2014). Interestingly, a handful of studies have also examined cross-sectional relationships between metabolites and proteins in urine (such as microalbuminaemia and urinary pentosidine – an indicator of RAGE levels) (Yaffe et al 2011, Bruce et al. 2008). Such studies are limited to cross-sectional designs, and usually assess links in older adults.

The literature reviewed above concerning biomarkers of cognitive impairment and T2DM is generally limited to cross sectional reports in older adults. It is therefore not surprising that there are significant findings linking many of the clinical and laboratory parameters with the presence of cognitive dysfunction in T2DM. These markers have not been assessed in adults added 40-60 yeas, when most of the risk T2DM is imposing on brain health is taking place. There is urgent need for new research to investigate the link between midlife markers of brain health/cognitive function, and later cognitive decline in those affected by T2DM.

Such biomarkers, studied in individuals usually in later life, offer a limited window to understand the underlying pathophysiological processes affecting the brain in T2DM and offer an even further limited window for potential intervention. Further, it is not even known if these markers act as a predictor of later brain health problems when

analysed in midlife. It is imperative going forward that longitudinal studies are developed in individuals with midlife T2DM to assess the predictive value of these, and more novel markers of brain health in selecting out those with T2DM who are at the greatest risk of later cognitive decline and dementia.

1.6 Potential Treatments for T2DM-Related Cognitive Impairment

There are a number of studies in the literature which have assessed potential treatments for T2DM associated cognitive impairment and dementia. Despite a growing number and variety of interventions that have been trialled, most of these trials have taken place once cognitive decline has already taken place to some degree, and hence most of the pathophysiological damage on brain health and brain function has occurred. Based on findings such as those from the FINGER Study reporting significant benefits of multi-domain interventions in individuals at risk of later cognitive decline (Ngandu et al. 2015), there is an urgent need for trials of multi-domain interventions in midlife in order to prevent later cognitive decline.

In summing the evidence base concerning treatments to mitigate the risk of cognitive impairment/dementia in midlife Type 2 Diabetes (T2DM), it be concluded that there is no good evidence at the current time for any particular treatment strategy. One of the key factors behind this is the lack of recognition of cognitive impairment as a complication of Diabetes (Dolan et al. 2018). Indeed, the American Diabetes Association recommends screening for cognitive impairment in those with T2DM, but only in individuals aged 65 years and older. Early identification is desperately needed in order to maximise the likelihood of successful intervention. However, as discussed above, a lack of fundamental knowledge surrounding the putative pathophysiology and a lack of biomarkers for cognitive impairment in midlife, when the risk is occurring seriously hampers attempts at early detection and treatment. Here we discuss the literature behind specific treatment strategies aimed at reducing cognitive impairment and

dementia risk in T2DM. The separate interventions are also summarised in **Tables 1.1, 1.2 and 1.3.**

1.6.1 Intensive Glycaemic Control

One of the important findings discussed in the sections above on hyperglycaemia in the underlying pathophysiology of the T2DM-dementia link in addition to the potential biomarkers section is the relationship between HbA1c and cognitive impairment in T2DM. It may be hypothesised from this evidence that lowering HbA1c may have a beneficial on cognition in T2DM. One of the most important studies to assess this was the ADVANCE Study (Aerosa-Sastre et al. 2017). The ADVANCE study randomised over 11,000 (mean age of 66 years) to either intensive glycaemic control regime or to a treatment as usual control. The main target in this study for the intervention group was to achieve a HbA1c of 6.5% or lower. After a median follow-up duration of 5 years, there was no difference between intervention or control groups. No benefit on cognition was observed, which may be partly due to the age of study participants as well as the specific cognitive assessments performed.

Similarly, in the ACCORD-MIND sub-study of the larger ACCORD study, just under 3,000 patients (mean age of included participants was 62.5 years) with T2DM at risk of cardiovascular events with an elevated HbA1c were assigned to either an intensive control (HbA1c target of 6% or lower) treatment or to standard strategy treatment (HbA1c of 7-9%). Again there was no effect on cognition, this time at 40 months (Launer et al. 2011). Finally, another trial (n = 1,200 participants), with a mean age of a decade older than the other trials (72 years) aimed at reducing HbA1c, intensive blood pressure lowering (<130/85), treatment of hyperlipidaemia with use of hypoglycaemics, anti-hypertensives and statins as required. There was no effect on MMSE scores (Araki et al. 2012).

At first, the results of such trials appears disappointing. However, most trials were conducted in older adults with diabetes with a long duration of T2DM and the presence of other T2DM related complications. We know from the evidence above that if participants have poor control of their T2DM in addition to the presence of T2DM related microvascular and macrovascular complications, they are likely to also have problems with cognition. Thus, these trials are being conducted in populations where much of the pathophysiological risk posed by T2DM may have already taken place and it is unlikely that such interventions will improve existing impairment. It is absolutely imperative that well-designed clinical trials in midlife populations are conducted in order to examine the effect of these interventions in younger individuals in order to act as preventative treatment. A summary of these studies is provided in **Table 1.1**

Study	Participants	Age	Intervention	Duration	Comparison	Outcome	Result
ADVANCE Study	11,140 with first diagnosis of T2DM aged >30	Mean age: 66.6 years	Intensive glucose control regime including glicazide and other treatments at physicians discretion and also randomised to perindopril-indapamide/other anti-hypertensives	Five Years	Standard Control Group	Number of patients declining by 3 points on MMSE from baseline	No Difference
ACCORD-MIND Study	2,794 with T2DM and HbA1c of >7.5%	Mean age: 62.3 years	40 months of either intensive glycaemic control with an HbA1c target of 7.0-7.9%, in addition to intensive blood pressure lowering	Forty Months	Diabetes education, glucose monitoring equipment and antidiabetic medications	MMSE, RAVLT, Stroop Test, DSST	No Difference
Araki et al. 2012	1,173 with T2DM and other cardiovascular risk factors	Mean age: 71.9 years	Intensive regime of treatment for diabetes, hypertension and dyslipidaemia or treatment as usual over 6 years	Six Years	Treatment as usual	MMSE	No Difference

Table 1.1 Studies aimed at Intensive Glucose Lowering in Type 2 Diabetes analysing effects on Cognition. *T2DM: Type 2 Diabetes Mellitus; MMSE: Mini-Mental State Examination; RAVLT: Rey Auditory Visual Learning Test, DSST: Digital Symbol Substitution Test*.

1.6.2 Metformin

Metformin remains the first line pharmacological treatment for T2DM. It has attracted a great deal of attention recently due to its potential as an anti-ageing drug (based on findings from the UKPDS study) and is now under investigation as an anti-ageing drug in non-diabetic cohorts (the TAME Study) (UKPDS, 2018; Barzilai et al. 2018). Much remains to be uncovered about the precise mechanism of action of metformin, but suffice to say it appears to have profound effects on reducing T2DM related complications and mortality (UKPDS, 2018). It remains the single most common hypoglycaemic in T2DM.

In assessing individuals with comorbid T2DM and depression, treatment with metformin was noted to improve cognitive function on the Wechsler Memory Scale (Guo et al. 2014). However this trial was small (n = 58). Other studies are conflicting. The most recent report on metformin use and cognitive impairment/dementia in T2DM comes from a study of American Veterans aged 65 years and older. Authors reported a decreased risk of dementia when metformin was compared to sulfonylurea use (Orkaby et al. 2017). One of the difficulties in knowing if metformin has any effect on cognition in those with diabetes is that it is typically prescribed to all individuals with a diagnosis of T2DM and there is a lack of an untreated T2DM cohort to compare the effects of no treatment.

1.6.3 Other Oral Hypoglycaemic Agents

Recent advances in the development of oral hypoglycamic agents in T2DM has not necessarily been accompanied by an increase in the understanding of the efficacy of such oral hypoglycaemic agents in mitigating cognitive decline/dementia risk in T2DM. In one study examining differences between repaglinide (n = 77) or glibenclamide (n = 79), the authors observed a decline in cognition in the group prescribed glibenclamide but not repaglinide (Abbatecola et al. 2006). This difference was ascribed to postprandial glucose excursion by the authors. In a trial of a similar size (n = 145 total), rosiglitazone or glyburide as add-on therapy to metformin was associated with improvement on certain cognitive domains

including performance on the Paired Associates Learning (PAL) test. These benefits also correlated with improved glycaemic control in this group (Ryan et al. 2006). Finally, in a small study of just under one-hundred patients, Abbetacola et al. 2010 report a protective cognitive effect of Rosiglitazone.

Overall, large studies are needed to examine the differences in cognitive trajectories in different individuals at risk of cognitive decline in T2DM. One of the first ways that this can be achieved is that cognitive function is measured at baseline in any trial examining the potential efficacy of a new agent for the treatment of T2DM. Another way is by specifically examining different combinations of medications and examine effects of different cognitive domains, not only scores on MMSE or other commonly used cognitive tests. A summary of the above studies is provided in Table 1.2

Study	Participants	Age	Intervention	Duration	Comparison	Outcome	Result
Abbetacola et al. 2006	156 Patients	Mean age 74.3 Years	Glibenclamide (glyburide)	One Year	Repaglinide	MMSE, global cognitive score (TMT, DSP, DSST, Verbal Fluency Test)	Decline in Cognition only seen in the Glibenclamide group
Ryan et al. 2006	145 Adults with Type 2 Diabetes receiving oral metformin combination therapy	Mean age 60.7 Years	Rosiglitazone Plus Metformin	Two Years	Glibenclamide (glyburide)	DSST, RAVLT, Paired Associates Learning, Pattern Recognition Memory, Spatial Working Memory Test, Reaction time, Rapid Visual Information Processing	Improvements in working memory correlated with glycaemic control
Guo et al. 2014	58 Patients with T2DM and Depression	Mean age 54.7 years	Treatment with metformin	Two Years	Placebo (Vitamin C)	Wechsler Memory Scale Revised (WMS)	Improved cognition as measured by the WMS
Abbetacola et al. 2010	97 older patients who had recently started treatment for T2DM	Mean age 76 years	Metformin +/- rosiglitazone	Nine Months	Diet	MMSE, RVALT, TMT	Results for metformin/rosiglitazone group remained stable for RAVLT

Table 1.2 Effects of Hypoglyceamic Agents on Cognition in Type 2 Diabetes. *T2DM: Type 2 Diabetes Mellitus; MMSE: Mini-Mental State Examination; RAVLT: Rey Auditory Visual Learning Test, DSST: Digital Symbol Substitution Test; TMT: Trail Making Test; WMS: Wechsler Memory Scale*

1.6.4 Diet and Exercise Based Interventions for Cognition in T2DM

Two important reports have explored the link between Diet and Exercise based interventions for cognition in T2DM: (i) The Look-AHEAD (Action for Health in Diabetes) study which was a large randomised controlled trial examining the impact of a ten year intensive lifestyle intervention (ILI) consisting of a reduction in daily calorie goals in addition to at least 175 minutes per week of physical activity (Espeland et al. 2017a, Espeland et al. 2017b, Rapp et al. 2017) and (ii) Secondary Analysis of the LIFE (Lifestyle Intervention and Independence for Elders) trial which analysed a 2 year period of physical activity intervention in older sedentary adults with functional limitations (Espeland et al. 2018). The Look-AHEAD study showed no effect of the intensive lifestyle intervention on cognition. Once again in similarly to trials above, participants were aged in the 6th and 7th decades of life in the Look-AHEAD Trial. For the LIFE trial, secondary analysis was conducted to see if outcomes differed by the presence of T2DM. The authors reported cognitive benefit only in the group with T2DM but not in the group without T2DM. Again, this trial was conducted in those aged greater than 70 years.

The above trials come with several caveats. For the Look-AHEAD study it is particularly noteworthy that cognitive assessment was not undertaken at baseline and so authors were not able to control for baseline cognition – this is a particularly important limitation. It made no assessment of cognitive trajectories. Further, we must remember that the positive results demonstrated in the LIFE trial are only an exploratory secondary analysis. Again, multi-domain interventions in midlife are needed. Study details for the Look-AHEAD and LIFE studies are provided in Table 1.3 below

Study	Participants	Age	Intervention	Duration	Comparison	Outcome	Result
Look-AHEAD Study	3,802 overweight and obese with T2DM	45-76 years	Intensive Lifestyle Intervention (calorie goals of 1,200-1,800 and >175 minutes/week activity)	Ten Years	Diabetes Support and Education	Diagnosis of MCI/dementia, MMSE, Functional Assessment Questionnaire, TMT-A, RAVLT, DSST, Stroop Test	No Difference
LIFE Study	1,476 older adults with functional limitations (post-hoc analysis of 415 with diabetes)	70-89 years	Physical Activity (walking, resistance training, flexibility)	Two Years	Health Education	MMSE, DSC, HVLT-D	Improvements in global cognitive function in patients with T2DM

Table 1.3 Effects of Diet and Lifestyle Based Interventions for Cognition in Type 2 Diabetes. *T2DM: Type 2 Diabetes Mellitus; MMSE: Mini-Mental State Examination; RAVLT: Rey Auditory Visual Learning Test, DSST: Digital Symbol Substitution Test; TMT: Trail Making Test; WMS: Wechsler Memory Scale; HVLT: Harvard Verbal Learning Test; MCI: Mild Cognitive Impairment*

The Need for New Understanding, Novel Biomarkers and Multi-Domain Treatment Interventions in T2DM

The current chapter has discussed the evidence linking midlife T2DM to the later development of cognitive decline and dementia, in addition to the pathophysiological hypotheses behind this link and the need for new biomarkers and treatments in midlife T2DM in order to prevent the later development of cognitive decline and dementia. It is clear that a significant shift in research examining the T2DM-dementia link is needed away from only examining clinical and research based correlations in older adults to a life-course approach, primarily targeted at those with midlife T2DM.

It is more than evident from the above literature that T2DM confers an increased risk of later cognitive decline and dementia. Whilst this has been well established from well-conducted cohort studies already mentioned (such as the ARIC or Whitehall II studies), knowledge of the exact cognitive trajectories in T2DM are lacking. Whilst it appears that T2DM is associated with cognitive decrements across the lifespan, the effect of T2DM is particularly pertinent in midlife and may only become clinically evident after about 10 years. We have seen from much of the literature that diabetes duration, diabetes control and the presence of complications predicts the development of cognitive impairment and dementia. Thus, it appears that there is a limited window of opportunity to prevent cognitive decline in T2DM, preferably as early as possible in the disease course, before pathophysiological damage has occurred.

Both T2DM and dementia/cognitive impairment may be characterised by long prodromal phases before clinical disease becomes clinically apparent. At present, this is driving the need for new disease biomarkers in dementia and cognitive impairment (for instance, the PREVENT study is currently examining biomarker and cognitive trajectories across midlife) (Ritchie et al. 2012). Such an approach is needed specifically in those with T2DM in order to characterise cognitive trajectories and novel biomarkers. Due to the high burden of other midlife vascular risk factors for later cognitive impairment and

dementia in T2DM such as hypertension and obesity, T2DM represents a particularly at-risk population for later development of cognitive decline and dementia. The breath of biomarkers needs further expansion beyond current research examining mainly clinical parameters (such as diabetes control, treatments, vascular risk factor control) to novel markers (such as spatiotemporal gait characteristics or novel immunological assays). Such an approach is aimed at essentially selecting out the high-risk amongst the high-risk.

It is also a key research priority to develop multi-domain interventions specifically targeted at individuals in midlife who may be at greatest risk. Building on the development of novel biomarkers, future work should aim to select-out individuals at most risk for targeted multi-domain interventions. Much like the putative hypotheses behind the T2DM-dementia link are yet to be fully elucidated and are multi-factorial in nature, multi-domain interventions will be needed to target the separate elements of this risk (for instance interventions consisting of vascular risk factor management, management of dyslipidaemia and other risk factors). Such interventions may eventually be invidualised targeting specific risk factors and affected cognitive domains. Such a vision carries the promise of a personalised medicine approach and may eventually be used to mitigate and ultimately prevent the later development of dementia in individuals with midlife T2DM

Chapter 2: Materials & Methods

2. Methods

I performed recruitment and assessment of all participants in the current study (the ENBIND Study), in addition to all of the laboratory work on participant blood samples. Experiments were designed in collaboration with my supervisors Prof Kennelly and Dr Bourke.

2. 1 Study Background & Setting

The study protocol was granted ethical approval by the joint TUH-St James's Hospital Ethics Committee in October 2018 (Reference: 2018/09/02/2018-10 List 34(4)). Participant recruitment occurred in the T2DM clinics based at the Robert Graves Institute of Endocrinology, Simms Building, Tallaght University Hospital (TUH) and participant assessment occurred in the Department of Age-Related Healthcare, TUH. All laboratory work was carried out in the Trinity Translational Medicine Institute (TTMI). All assessments were carried out over a 9 month period (October 2018 – July 2019).

2.1.1 Participant Recruitment

In accordance with the approved protocol for the ENBIND study, potential participants were approached at the time of their routine T2DM appointment. Briefly, participants were provided with an introduction to the purposes and nature of the study and asked if they would be interested in participating. If interested, participants were then given an information leaflet detailing the requirements and protocol of the study in addition to contact details for the study investigator, should they choose to participate. Participants medical notes were briefly reviewed to assess for exclusion criteria (below). A separate visit was arranged for those wishing to part-take in the study in order to complete the health, diabetes and memory assessments in addition to the blood draw. Participants were provided with a text reminder the day prior to this visit which also provided an opportunity to withdraw should they so wish. A control group of healthy

middle-aged adults without T2DM were also recruited by local advertisement in accordance with the approved protocol.

2.1.2 Inclusion and Exclusion Criteria

Participants were screened for inclusion based on the following criteria:

Inclusion Criteria:

1. Aged 35-65 years old
2. Both males and females
3. Diagnosed Type 2 Diabetes Mellitus (T2DM)
4. Montreal Cognitive Assessment (MoCA) score >19.

Exclusion Criteria:

1. Diagnosis of Non-T2DM Diabetes: Type 1 Diabetes, Gestational Diabetes, Other non-T2DM: e.g pancreatogenic diabetes
2. Macrovascular Complications of T2DM: Previous Stroke, Previous Myocardial Infarction/significant Ischaemic Heart Disease, Peripheral Vascular Disease,
3. Microvascular Complications of T2DM: Significant Diabetic Retinopathy , Peripheral Neuropathy or Diabetic Nephropathy.
4. Pregnancy
5. Established Diagnosis of cognitive impairment/dementia, or a diagnosis of a neurological cognition known to impact on cognitive function
6. Active diagnosis of depression within the past 6 months
7. Unstable angina, previous myocardial infarction or intermittent claudication
8. Significant cardiorespiratory illness
9. Use of psychotropic medications
10. Previous knee, hip replacement or lower limb/back surgery
11. Previous neurosurgery or traumatic brain injury

12. Significant musculoskeletal or rheumatological disorder known to impact on musculoskeletal function
13. Parkinson's Disease or Parkinsonism or other neurological disorder such as Multiple Sclerosis

2.2 Participant Assessment

Participants completed a study visit of approximately 90 minutes duration in the Department of Age-Related Healthcare (ARHC), Tallaght University Hospital (TUH). Participants were instructed to eat a regular meal before taking part in the study to avoid potential hypoglycaemia and not to take part if they felt unwell on the day of study visit.

The medical/diabetes assessment, screening questionnaire and cognitive assessment were carried out in the research office, ARHC, TUH. The gait assessment took place for all participants in a designated corridor in the department. Finally, participants returned to the research office for the blood draw and debriefing. Before leaving, participants were consented to future contact in three to five years-time to allow for the first follow up wave of the longitudinal ENBIND study.

2.2.1 Health and Diabetes History

Routine demographic information was obtained from participants including age, gender, height, weight and Body Mass Index (BMI). In addition, a history of vascular risk factors (hyperlipidaemia, hypertension, smoking) and the management of same was obtained. Variables with a known impact on cognition were also obtained such as years of education and family history of cognitive/impairment dementia (including relative affected and age). A comprehensive list of medical comorbidities was collected, and a Charlson Comorbidity Score calculated for each participant (Charlson et al. 1987). Further a comprehensive list of medication was obtained from each participant and the

number of daily medications calculated. The exclusion criteria were then re-reviewed before proceeding with cognitive/gait assessment and blood draw.

A diabetes history was then obtained from each participant (with T2DM) and included years since diagnosis and a diabetes medication history. Following this, the Diabetic Neuropathy Symptom Score (DNSS) was administered (Meijer et al. 2003, Meijer et al. 2002). Finally, participants were examined clinically for the presence of neuropathy and excluded from further participation if present.

2.3 Cognitive Assessments

Participants were assessed using the: (1) Montreal Cognitive Assessment (MoCA) for overall cognition and the (2) Ascertain Dementia 8-item (AD8) Questionnaire for dementia if MoCA score was <23. Patients then underwent detailed computerised neuropsychological assessment using a customised battery from the Cambridge Neuropsychological Test Automated Battery (CANTAB) in order to obtain a detailed assessment of cognition.

2.3.1 Montreal Cognitive Assessment (MoCA)

The Montreal Cognitive Assessment (MoCA) is a standardized screening tool for cognitive impairment/dementia and is believed to reflect general cognitive function. It was developed in Canada in 1995 and is used clinically in the detection of cognitive impairment/dementia (Nasreddine et al. 2005). In comparison with other cognitive tests, such as Folstein's Mini-Mental State Examination (MMSE), the MoCA is believed to be more sensitive in the detection of mild cognitive impairment. Further, in comparison to the commonly used MMSE, the MoCA additionally assesses executive function, abstraction and visuospatial ability (Nasreddine et al. 2005, Folstein et al. 1975).

The MoCA is scored out of 30 points and assesses the following domains: (a) Visuospatial/Executive Function (5 Points): Trail Making, Copying a Cube and Clock Drawing, (b) Naming (3 Points): Naming three animals, (c) Memory (5 Points): Recall of 5 words after 5 minutes, (d) Attention (5 Points): Repeating a list of digits in forward and backwards order, Tapping to the letter 'A' in a string of letters, serial 7 subtractions from 100 (e) Language (3 Points): repeating sentences and verbal fluency, (f) Abstraction (2 Points): describing similarities and (6) Orientation: to date, month, year, time, place and city.

Several cut-offs for the MoCA have been proposed. In data from TILDA (the Irish Longitudinal Study of Ageing), normative means for those aged 50, 55 years and 60 years with secondary level of education were between 25 and 26 with those for primary education only slightly lower (24-25) and those for tertiary education higher (27-28) (Kenny et al. 2013). Commonly, the cut-off of 26 is applied for detection of mild cognitive impairment although recent evidence suggest that a score of 23 or lower results in lower false positives without affecting detection rates (Carson et al. 2018, Nasreddine et al. 2005).

These scores vary widely in population based studies and are very sensitive to educational background, as recognised I the original report. In line with standard protocol, a correction of +1 point for <12 years of education was applied to MoCA scores in the current study. Participants with a score <19 consistent with the lowest 10% of those with primary education from normative TILDA data were excluded from further participation. Those with scores of <23 had the Ascertain Dementia 8 Questionnaire (AD8) administered directly to distinguish between MCI and dementia.

2.3.2 Ascertain Dementia 8 Questionnaire (AD8)

The AD8 is a functional questionnaire which assesses for functional impairment suggestive of potential dementia (Galvin et al. 2006, Galvin et al. 2005). The AD8 has

been validated both in primary care and memory clinic contexts (Hendry et al. 2019). It consists of 8 questions which focus on functional abilities and probes for a change in these abilities over recent months. It is usually administered to a family member or informant, but has shown equal sensitivity and specificity when administered directly (Galvin et al. 2007).

2.3.5 Cambridge Automated Neuropsychological Test Battery (CANTAB)

A unique study-specific CANTAB assessment battery was used in the current study and administered using an Apple iPad. CANTAB assessments are the most validated and widely-used computerised neuropsychological assessments developed to date. In order to screen for potential cognitive impairment/dementia in those with T2DM, a modified version of the prodromal Alzeimer's Disease (pAD) was used. The unique battery was devised to particularly assess visuospatial and executive function in addition to short term memory, domains known to be amongst the earliest affected in those with T2DM. This consisted of 6 individual tasks which lasted from 3-12 minutes each and constituted a total assessment time of 45-50 minutes.

The following tests were used:

1. **Reaction Time Task (RTI):** Assesses Reaction Time/Movement Time
2. **Paired Associates Learning (PAL):** Visuospatial Memory
3. **Spatial Working Memory (SWM):** Episodic Memory & Executive Function
4. **Pattern Recognition Memory (PRM):** Short Term & Delayed Memory
5. **One Touch Stockings of Cambridge (OTS):** Executive Function
6. **Rapid Visual Processing (RVP)**: Attention & Executive Function

Details of the nature of these tests are provided below. These assessments have been well validated in mild cognitive impairment and early Alzheimer Disease (Egerhazi et al. 2007; Saunders & Summers, 2011). They can be viewed largely as tasks with significant

memory demands or significant executive function demands. However, there is a large degree of overlap. Two principal outcome measures were derived from the available CANTAB output as detailed under each test below. A table of the main outcome measures is also provided below (Table 2.1).

2.3.5.1 Reaction Time Task (RTI)

The first task of the computerised battery designed to measure reaction and movement time is the Reaction Time (RTI) task. This brief task provides a screening assessment of motor and mental response speed in order to assess for movement time/reaction time and response accuracy which may confound further assessments. In this test, five circles appear displayed in an arc across the screen. The participant is instructed to press a main circle which appears at the bottom of the screen below the other five circles. On pressing this, one of the five circle changes from black to yellow. The participant is instructed to move his/her finger to the circle which has changed colour, and then back to the starting point. After a practice trial, the participant then performs the same task as fast as possible. This is repeated for 30 trials in total. If the participant is unable to complete the task, further computerised neuropsychological assessment is precluded and the following five tasks are not run.

The principal outcome measures extracted for this task are as follows:

1. **Median Duration Reaction Time (RTI-MDRT):** median duration to release response button after presentation of target stimulus in milliseconds. This is obtained across all correct assessed trials
2. **Mean Detection Movement Time (RTI-MDMT):** median time taken to move finger from response button to the target stimulus location following a yellow colour change in milliseconds

2.5.3.2 Paired Associates Learning (PAL)

The Paired Associates Learning (PAL) task assesses visual memory and the ability for new learning, which is a particularly sensitive tool for assessing episodic memory. In this task, a number of boxes are presented on the screen which open one-by-one to reveal a pattern in the box. After this a pattern is displayed in the middle of the screen and the participant is asked to detect the box in which the pattern first appeared. The initial practice trial consists of only two boxes and two patterns. For the main assessment, 4, 6 and 12 boxes are displayed, depending on participant performance. If a participant makes too many errors on the 4 box task, they will not progress to the 6 box task, and if too many errors are made on the 6 box task they will not progress to the 12 box task. Output measures take into account performance across all levels, as well as the number of errors that participants would have made should they advance to the next level (if they have not succeeded at the level reached)

The principal outcome measures extracted for this task are as follows:

1. **Total Errors Adjusted (PAL-TEA):** This parameter generates the total number of assessed trials where the correct answer was chosen by the participant on the first attempt. There is also a PAL-TEA28 version of this variable, which is identical but does not count the 12 box level, and makes an adjustment to account for errors not made due to early termination, this allows comparison to reference standard.
2. **First Attempt Memory Score (PAL-FAMS):** This score corresponds to the number of times the correct box was chosen by a participant on the first attempt in remembering the pattern locations. Again, it is calculated across all trials. There is also a PAL-FAMS28 version of this variable which is identical but omits the 12 box level to provide comparison the reference standard.

2.5.3.3 Spatial Working Memory (SWM)

The Spatial Working Memory (SWM) task is a particularly good assessment of executive function. There are several coloured boxes displayed on screen in the first instance. Participants are instructed that there are tokens hidden in the boxes on screen and that their task is to locate the hidden tokens. Further, they are told that once they have detected a token in a box, the token will not reappear in that box i.e a token will never be hidden in the same box more than once. On finding a token, participants have to click the outline in the shape of a token in a column on the side of the screen in order to collect the token. The task starts out with only two tokens to be found in two boxes, and then increases to 4, 6, 8 and 12 tokens. The patterns are changed at each stage in order to avoid learning effects and stereotyped search strategies. This task is designed as a test of spatial memory in addition to executive function and strategy (prior learning).

The principal outcome measures extracted for this task are as follows:

1. **Between Errors (SWM-BE):** This corresponds to the number of errors – the number of times a participant revisits a location in which a token has previously been found. There is a variable for this over all levels and one for each individual level.
2. **Strategy (SWM-S):** This refers to the number of times a participant begins a new search pattern from the same box started with previously (for this, it is implied that if a subject consistently starts from the same location where they found a token previously, they are employing a strategic search strategy). Again, there is a variable for this measure over all levels in addition to the overall score

2.3.5.4 Pattern Recognition Memory (PRM)

The Pattern Recognition Memory (PRM) is a test of visual memory recognition. In this task, participants watch a sequence of 12 patterns appear on screen. Patters are

specifically designed so that they cannot be given a verbal label. After viewing all 12 patterns, participants are asked to choose between two patterns and asked to detect the pattern that they have seen before. The task is then repeated with a new set of 12 patterns. Finally, a final set of patterns are introduced, again consisting of 12 new patterns which have not been seen before. Participants are instructed that they are to remember these patterns for a longer period of time. After 20 minutes (at the very end of the computerised assessment following the rapid visual information processing task), participants are asked to identify the pattern that they have seen before from two patterns presented on the screen. This is a test of delayed recall.

The principle measures extracted are as follows

1. **Percent Correct Immediate (RPM-PCI):** This corresponds to the number of correct patterns selected by the participant in the immediate condition, and is given as a percentage.
2. **Percent Correct Delayed (RPM-PCD):** Referring to the number of correct patterns selected after the 20 minute delay in the forced-choice condition.

2.3.5.5 Stockings of Cambridge (SOC)

In this task, a modified one-touch Stockings of Cambridge assessment, participants are presented with two displays on screen. Each display consists of three stockings which contain three balls arranged in different patterns. The rules are explained as follows: the participant is to move the balls in the bottom display in order to match the appearance of the top display. In the initial stage of the test, participants copy the moves made by the computer and are asked to count the number of moves made by the computer in order to familiarise participants with the test. Following this, they are exposed to the test proper, which asks them how many moves are made in order to get the patterns to match. They are given up to six options at the bottom of the screen. If they select an incorrect option, they are permitted to choose another option and repeat

this until the correct answer is reached. This task involves the use of problem solving strategies and has notable executive function demands.

The principal outcome measures for this task are as follows:

1. **Problems Solved on First Choice (OTS-PFS):** The total number of assessed trials where the correct answer is selected on the first attempt, calculated across all trials.
2. **Median Latency to First Choice (OTS-MDLFC):** This variable refers to the median latency, measured from the appearance of the stocking balls until the first choice is made. This is only calculated on those trials where the choice is correct.

2.3.6 Rapid Visual Information Processing (RVP)

The Rapid Visual Information Processing (RVP) is primarily a test of sustained attention. For this task, single digits appear at a rate of 100 digits per minute. Participants are instructed they are to detect a particular sequence of numbers (in the first instance, 3-5-7). In the practice trial, the digits in the sequence are highlighted in red, whilst all non-sequence digits are presented in grey. For the main task, all numbers are grey and participants are instructed to detect two further sequences (2-4-6 and 4-6-8). A total of 9 sequences appear every 100 seconds.

The main outcomes for this task are:

1. **A Prime (RVP-A):** This variable represents a signal detection measure of a subjects sensitivity to the target sequence, regardless of the response tendency. This is fundamentally a measure of performance corresponding to the participants ability to detect the target sequence.
2. **Probability of False Alarm (RVP-PFA):** This measure refers to the number of sequence presentations which were false alarms which is divided by the number of false alarms combined with correct rejections).

Assessment	Outcome Measure
Reaction Time Task (RTI)	Median Duration Reaction Time (RTI-MDRT)
	Median Detection Movement Time (RTI-MDMT)
Paired Associates Learning (PAL)	Total Errors Adjusted (PAL-TEA)
	First Attempt Memory Score (PAL-FAMS)
Spatial Working Memory (SWM)	Between Errors (SWM-BE)
	Strategy (SWM-S)
Pattern Recognition Memory (PRM)	Percent Correct Immediate (RPM-PCI)
	Percent Correct Delated (RPM-PCD)
Stockings of Cambridge (OTS)	Problems Solved on First Choice (OTS-PFS)
	Median Latency to First Choice (OTS-MDLFC)
Rapid Visual Processing (RVP)	A Prime (RVP-A)
	Probability of False Alarm (RVP-PFA)

Table 2.1. Details of the Cambridge Neuropsychological Assessment Battery (CANTAB) used in the current study. *The CANTAB battery was uniquely created for the ENBIND study and is specifically designed as a test with significant executive function and memory demands, as these are the domains most commonly affected in Type 2 Diabetes Mellitus and in early Mild Cognitive Impairment and Alzheimer Disease. Six tasks were selected with this in mind and two principal performance outcomes extracted in order to assess cognitive function in those with midlife Type 2 Diabetes Mellitus and those without (Healthy Controls).*

2.4 Gait Sped Assessment

Following cognitive assessment as above, gait speed was assessed across three conditions. A stopwatch with millisecond precision was used to measure walking speed. A designated corridor in ARHC, TUH was used for all gait assessments. The corridor was 20 metres long in total and had three markings: (i) **Mark 1**: a stop-start marking on the floor at the start of the corridor, located 1m from the second marking, (ii) **Mark 2**: a 15 metre mark which corresponded to the mark at which the stopwatch was started and stopped and (iii) **Mark 3:** a turning mark at the end of the corridor, marking the point at

which participants were to turn around to complete their walk. The 1 metre distance between marks (i) and (ii) allowed for acceleration and deceleration at the end of the walk. The walk totalled 30 metres, including a turn. Participants were asked to stand at mark 1 and to walk up to mark 3 and back (the end of the corridor). The stopwatch was commenced once his/her first foot crossed Mark 2 and was stopped once his/her following foot crossed Mark 2 at the end of the 30m walk. See Figure 2.1

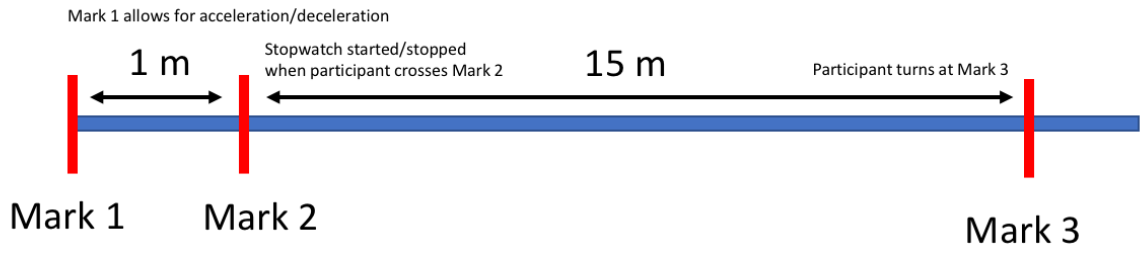

Figure 2.1. Markings for Assessment of Gait Speed. *A designated corridor in the Department of Age-Related Healthcare, Tallaght University Hospital (TUH) was used for all gait assessments. Participants were instructed to start at Mark 1 and walk to Mark 3 and back, having completed one full turn. The stopwatch was commenced once the first foot passed Mark 1 and stopped once the second foot crossed Mark 1 on returning. The total speed was calculated as per the formula speed = distance travelled/time taken in milliseconds. The inclusion of Mark 2, 1m ahead of Mark 1 allowed to acceleration and deceleration at the start/end of the walk respectively.*

Walking speed was measured across three tasks for each participant:

1. **Single Task Gait Speed – Self-Selected Pace:** Participants were instructed to walk from the start/stop mark at their usual pace, and gait speed was measured over 30 metres total using the stopwatch (Speed in ms^{-1} = 30 metres/time taken)
2. **Single Task Gait Speed – Maximum Pace:** Participants were instructed to walk from the stop/start mark at a brisk pace, without running from the start/stop

mark and back, with gait speed being measured over 30 metres total (Speed in ms^{-1} = 30 metres/time taken).

3. **Dual Task Gait Speed – Cognitive Task:** Participants were first asked, whilst standing stationary to recite every second letter of the alphabet, starting with A, C, E and so on until they reach the end. The amount of errors made and the time taken to complete this task was recorded. Then, participants were asked to repeat the cognitive task, but this time starting with B, D, F and so on whilst walking at their usual pace, from the start/stop mark and back. They were instructed that if they reached the end of the alphabet to continue on commencing again with the letter B. Again, speed was measured over 30 metres (Speed in ms^{-1} = 30 metres/time taken).

   There were three key outcomes calculated from the dual task condition:

   i. **Dual Task Difference:** The difference in walking speed (in ms^{-1}) between tasks 1 and 3 (calculated as task 3 speed – task 1 speed).

   ii. **Dual Task Cost:** The difference in walking speed (in ms^{-1}) expressed as a percentage of the single task (SUP) condition i.e the difference between single and dual task speeds expressed as a percentage change (either positive, indicating greater cost or negative, indicating less cost) of usual walking speed.

   iii. **Dual Task Error Rate:** This is the number of errors made by the participant in reciting the alternate letters of the alphabet on the dual task, divided by the number of letters recited.

2.6 Blood Draw

Following gait speed assessments, participants returned to the research office in ARHC, TUH. A blood draw was the performed using standard aseptic technique. Briefly, consent was obtained, a tourniquet applied and venepuncture performed using a

standard needle and collecting system. Blood samples were collected in the following order:

1. Three 3mL bottles coated with serum clot activator (serum tubes), for later analysis of serum proteins
2. One 3mL bottle coated with lithium heparin, for routine analysis of C-Reactive Protein in the hospital laboratory, TUH
3. In those participants where analysis of Peripheral Blood Mononuclear Cells (PBMCs) was being performed, three 9mL bottles coated with lithium heparin were collected. Lithium heparinised tubes prevented clotting of the blood sample and use of these tubes as opposed to other reagents, such as EDTA, has been shown to increase PBMC yield (Duffy et al. 2014).
4. Two 3mL bottles coated with Ethylene-Diamene-Tetra-Acetic (EDTA) acid were collected. One of these was sent for routine analysis of glycated haemoglobin (HbA1c) and the other stored at -80^{o}C for future genetic analysis.

Following venepuncture, samples for CRP and HbA1c were sent to the hospital laboratory for routine analysis in TUH. The other samples were transported to the Trinity Translational Medicine Institute (TTMI) immediately after participant for same day analysis by the study investigator.

2.7 Laboratory Materials and Methods

2.7.2 Blood Sample Processing

All blood processing occurred on the same day as samples were obtained. Every participant in the ENBIND study had a serum sample processed in addition to a sample banked for later genetic analysis, as per the ethics board approved protocol.

A subgroup of participants had Peripheral Blood Mononuclear Cells (PBMCs) isolated from whole blood samples and stimulated with a range of pro-inflammatory stimuli. Selection of samples for inclusion in this subgroup was based on time constraints and availability of laboratory equipment and assets on a given day. In as far as possible, samples were taken from participants with T2DM and Healthy Controls of similar age, sex and educational background and were obtained and analysed at the same time of day.

2.7.3 Serum Sample Preparation

Serum samples, collected in 3mL serum clot activator coated tubes, were centrifuged at 1,500rpm for ten minutes in total. This allowed separation of serum from clotting factors and red blood cells with the serum layer sitting on top. The serum layer when then removed and aliquoted into 1mL eppendorf tubes using a pipette which were labelled with anonymous participant numbers and stored at -80^{o}C for later analysis of pro-inflammatory cytokines using ELISA

2.7.3.1 Serum Protein Analysis

Enzyme-Linked Immunosorbent Assays (ELISAs) were used to quantify the levels of five pro-inflammatory proteins (IL-1β, IL-6, IL-12p70, CXCL10, MCP-1) in serum based on previous associations with T2DM, cognition/AD or both or known associations with pro-inflammatory pathways under study. Commercially available ELISAs kits were used and experiments carried out in accordance with manufacturers' instructions. Details on the ELISA kits used are provided in the table below (Table 2.2)

For IL-1β ELISAs (R&D Systems, Human IL-1 beta DuoSet ELISA), a 96 well micro-plate was coated overnight with the working concentration of capture antibody (diluted in PBS) and stored at 4^{o}C. Plates were sealed after each step using Parafilm. The following day, the capture antibody was aspirated and plates were washed thoroughly using

0.05% PBS-tween. Any excess was removed by blotting the plate on a paper towel. Plates were then blocked for 1 hour with 150uL assay diluent (1% FBS PBS). Plates were washed and dried as before. Following this, a serial dilution (2 fold dilutions) of IL-1β standard performed to give 8 solutions of known IL-1β concentration as per manufacturers protocol. 50uL of standards and samples were added in duplicate to a standard 96 well ELISA plate and samples incubated overnight at 4°C. Following this, samples were aspirated and plates washed as before. 50uL detection antibody, diluted in assay diluent (1% FBS PBS) was added and incubated for two hours at room temperature and plates washed/dried again. 50uL Streptavidin-Horseradish-Peroxidase (Strep-HRP) was then added for a 20 minute incubation at room temperature in the dark and plates aspirated and re-washed. Finally, 50uL substrate solution (3,3'5,5'-tetramethylbenzidine substrate solution) was added. After 20 minutes of incubation in the dark at room temperature, 25uL of 1M stop solution (H^2SO^4) was added. The plates were then covered in foil and analysed without delay using a plate-reader at 450nm. In order to analyse results, a standard curve was created using the known concentrations of protein from the standard curve, and values for serum samples calculated.

For the remaining kits (IL-6, CXCL10, IL-12p70, MCP-1; all BD Biosciences OptEIA™ kits), the same protocol was followed with the following notable differences:

1. Assay diluent for these kits was 10% FBS in PBS
2. Strep-HRP and detection antibody were added in the same step as per manufacturers' instructions and incubated for one-hour total.

For IL-1β, IL-6, CXCL10, serum samples were added undiluted. For IL12p70, a 10 fold dilution was carried out on serum samples and for MCP-1 a 2-fold dilution was carried out. These concentrations were determined from early optimisation experiments which demonstrated that the aforementioned dilutions ensured readings were taking place in the middle of the standard curve, in so far as possible, in order to avoid over-extrapolation of the curve and subsequent error.

Protein	Catalogue No.	Capture	Serum Dil.	Detection	Strep-Hrp	Standard
IL-1β	R&D; DY201	1:120	Undiluted	1:60	1:40	110ng/ml
IL-6	BD; 555220	1:250	Undiluted	1:250	1:250	145ng/ml
CXCL10	BD; 550926	1:250	Undiluted	1:500	1:250	120ng/ml
IL-12p70	BD; 555183	1:250	1:10	1:250	1:250	127ng/ml
MCP-1	BD; 555179	1:250	1:2	1:1000	1:250	140ng/ml

Table 2.2. Enzyme Linked Immunosorption Assay (ELISA) kits used in the analysis of pro-inflammatory cytokines. *All kits were commercially available and protocols carried out in accordance with the manufacturer's protocol. Of note, the kit for IL-1β was obtained from R&D Biosystems and required a 1% Fetal Calf Serum (FCS) in PBS assay diluent whereas all remaining ELISA kits were obtained from BD Oliptica and used 10% FCS in PBS assay diluent. Standards were aliquoted and stored at -80^{o}C as were capture and detection antibodies for the R&D kit, as per the manufacturer's instructions. Otherwise, kits were stored at 4-6^{o}C in accordance with kit instructions.*

2.7.4 Peripheral Blood Mononuclear Cell Isolation (PMBCs) from whole blood samples

2.7.4.1 Isolation of PBMCs using a Density Gradient

Isolation of PBMCs was carried out using a laminar flow hood and sterile conditions maintained throughout the isolation procedure. The 3x9mL lithium heparin samples were combined in a single sterile 50mL falcon tube. The same quantity of Histopaque-1077 was then added to a separate sterile 50mL falcon tube. Histopaque-1077 was used at it has a density which is greater than that of mononuclear cells, but lower than that of red blood cells. Thus, the result of separation using this density gradient means that red bloods cells pass to the bottom of the tube due to their lower density. PMBCs remain at the interface between the Histopaque medium and the plasma layer on top (see

Figure 2.2 below). The result of this process is a “buffy coat” consisting of the PBMC layer.

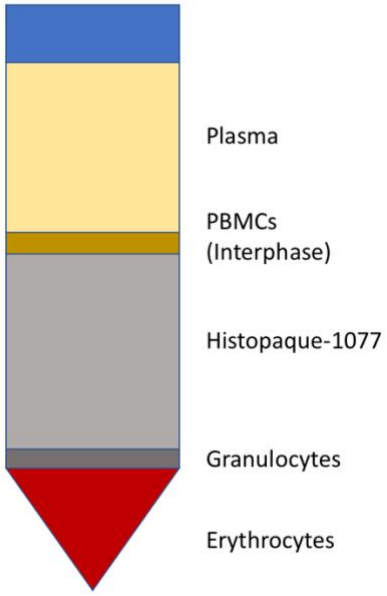

Figure 2.2. Layers obtained from passing whole blood over a Histopaque-1077 gradient. *Whole blood samples were obtained using standard aseptic venepuncture technique in Lithium Heparin Bottles and combined in a sterile falcon tube. Samples were then gently layered on a Histopaque-1077 gradient and centrifuged for 50 minutes at 700g. This results in the separation of the various blood cell layers as above, with the cells of interest, the Peripheral Blood Mononuclear Cells (PBMCs) suspended in the interphase between the Histopaque medium below and the plasma above. Red blood cells due to their greater density sink to the bottom of the falcon tube during density centrifugation.*

Gently, the whole blood was slowly layered down the side of the falcon tube on top of the Histopaque-1077, ensuring to maintain a 1:1 ratio, using a sterile Pasteur pipette. This prevented mixture of the whole blood with the Histopaque prior to centrifugation. These samples were then centrifuged at 700g for 50 minutes with the break off in order to ensure separation of the different layers. Following this, a sterile Pasteur pipette was

used to remove the buffy layer containing the PBMCs which were transferred to a sterile 50mL falcon tube. The PBMCs were then "washed" using 30mL of 0% FCS RPMI media for 5 minutes at 1,500 rpm. The supernatant was then discarded and the cell pellet "washed" for a second time in 30mL 0% FCS RPMI. Again, the supernatant was discarded and this time the cell pellet was re-suspended in 1mL of culture media (10% FCS 1% PS RPMI).

A cell count was then performed using trypan blue exclusion. For this, 2uL of the media containing PBMCs was added to 38uL of trypan blue. The sample was gently pipetted up and down and 10uL added to a haemocytometer. The number of viable cells in the outermost four sections (in the corners of the haemocytometer grid) was then counted using a cell counter and the following formula applied:

Number of cells/mL = *average count per section x 10^4 x 20 (dilution factor).*

Finally, using this count, the 1mL of cell-media solution was re-suspended in 10% FCS 1% PS RPMI in order to yield a solution of 1 x 10^6 cells per mL.

2.7.4.2 Cell Stimulations

PBMCs were stimulated for a total of 18 hours at 37.5^{o}C prior to harvesting. For each condition, between 1.5-2 mL of 1 x 10^6 cells per mL of cell-media suspension was gently added to six wells in total of a 6 well plate. Reagent were freshly prepared for each stimulation from stock solutions as follows:

1. **Lipopolysaccharide (LPS):** A solution of 100ng/mL was used. LPS was prepared from a stock solution of 1mg/mL by preparing a 1:10 dilution of LPS stock. Dilution was performed using 10% FCS RPMI. 1uL of the final solution was added to each 1mL of 1 x 10^6 cells per mL cell suspension.

LPS was used as it is an activator of the innate immune NF-κB pathway and is known to act as an initial signal (or 'signal 1') in innate immune NLRP3 pathway. Singaling via this mechanism is known to upregulate IL-1β mRNA transcription and the translation of pro-IL-1β which is later cleaved by a 'signal 2' to the active form of IL-1β.

2. **Nigericin:** 1uL of 10mM nigericin was added per 1mL of 1 x 10^6 PBMCs per mL. This yielded a final concentration of 1uM for nigericin stimulations.

   Nigericin is a potent activator of the innate immune inflammasome and is a strong 'signal 2' in NLRP3 signalling and the subsequent production of IL-1β

3. **Amyloid-β 42 (Aβ42):** A final solution of 10nM was prepared from a 0.5mM stock using a 1:50 dilution diluted in sterile PBS. 1uL of the final solution was then added to each 1mL of 1 x 10^6 cells per mL cell suspension.

   Amyloid Aβ42 is the putative pathogenic protein in Alzheimer Disease and is known to activate the innate immune NLRP3 inflammasome. This occurs by acting as a 'signal 2', cleaving mature IL-1β from pro IL-1β.

4. **Poly dA:dT (PAT):** A solution of 100ng/mL poly dA:dT/Lipofectamine was prepared. For Poly dA:dT (PAT), a 1:25 dilution of 1mg/mL stock solution was prepared using 0% FCS RPMI (0.4uL PAT per 9.6uL of media). A Lipofectamine solution was then prepared from stock solution in a 2:1 ratio to the PAT. Lipofectamine was used as a method to introduce the PAT into the PBMCs, ensuring adequate transfection. Five minutes after the PAT and lipofectamine were diluted, they were combined in a single Eppendorf tube. After 20 minutes, 10uL of the final solution was added to the PBMC solution per 1mL of 1 x 10^6

cells per mL. The final concentration of PAT used for transfection was 100ng/mL, as per manufacturers' instructions

Poly dA:dT (PAT) is an acid sodium salt which is a repetitive double stranded DNA sequence. It is a known activator of the innate immune cGAS-STING pathway which senses cytosolic DNA as a damage signal in response to cellular injury. As this pathway signals in the cytosol of the cell, the lipofectamine is used in order to ensure transfection of the Poly dA:dT across the cell membrane and into the cytosol where these receptors are located.

Following preparation of the reagents, the PBMC stimulations were arranged as follows:

Condition	Duration
Unstimulated (No reagent)	18 hours
LPS	18 hours
LPS & Nigericin	LPS for 18 hours, Nigericin added with 2 hours remaining
Aβ42	18 hours
LPS & Aβ42	Both added for 18 hours (combined)
PAT	18 hours

Table 2.3 Peripheral Blood Mononuclear Cell Stimulations (PBMCs). *1.5-2 mL of 1 x 10^6 per mL of cell-media suspension was added to each well and stimulated under the above conditions. LPS (a potent activator of the Nfκb pathway) was added alone in the first instance, but also as a primer of innate immune responses in the inflammasome medication stimulations. In these, nigericin (a potent activator of the innate immune NLRP3 inflammasome) in addition to Aβ42 (the putative pathogenic protein in Alzheimer Disease, which is also known to active the NLRP3 inflammasome) were added to LPS treated cells. In a final condition, Poly dA:dT, a DNA damage mimetic was added in order to stimulate activation of the cGAS-STING pathway*

2.7.4.5 Sample Harvesting

Following cell stimulation for a total of 18 hours, cells and supernatants were harvested for storage and further analysis. The plates containing cell stimulations were removed from the incubator and placed on ice. Cell-media suspensions from each well were removed individually and placed into pre-labelled sterile Eppendorf tubes. These tubes were then centrifuged at 1,500rpm for a total of five minutes in order to separate a cell pellet and cell supernatant. During the spin, a fresh solution of lysis buffer (PureLink RNA lysis buffer) containing 10uL/mL of β-mercaptoethanol was prepared in a fume hood. 300uL of this lysis buffer was then added to the empty wells. The bottom of the empty wells were scraped using the tip of the pipette and the solution pipetted up and down gently. Following the spin, the supernatant was removed from the Eppendorf tubes and aliquoted into separate sterile, pre-labelled Eppendorf tubes at -20^{o}C for later analysis. The 300uL of lysis buffer in each well was then combined with the corresponding cell pellet in the original Eppendorf tube and these were stored at -80^{o}C for later RNA extraction.

2.7.4.6 RNA Extraction

RNA was extracted from PBMCs using a PureLink mini RNA extraction kit (Thermo-Scientific) following the standardised protocol supplied with the kit. RNA was extracted from the Eppendorfs containing previously combined cell pellet and lysis-buffer from the appropriate wells. Briefly, Eppendorfs were removed from storage and allowed to thaw on ice. Following this, 300uL of 70% ethanol solution was added to each and samples were vortexed until well mixed. Prior to this, using a sterile syringe and fine needle, the samples were passed up and down the syringe to ensure adequate mixing. Sample-ethanol mixture was then vortex until well-mixed. Following this, 600uL of the final mixture was then added to pre-labelled spin cartridges which were then centrifuged at 12,000g for 20 seconds. After this, the flow-through was discarded. Then, 700uL of wash buffer 1 supplied with the kit was added to each cartridge and the

centrifuge step (12,000g for 20 seconds) repeated. Again, flow through was discarded and collection tubes replaced. 500uL of wash buffer 2 was then added and the spin cartridges spun at 12,000g for 1 minute. This step was then repeated and collection tubes discarded. Finally, samples were then centrifuged for 2 minutes and 12,000g to remove any excess ethanol. Collection tubes were then discarded and 30uL of RNAse free water was added to each cartridge, which was placed in a pre-labelled RNAse free Eppendorf tube. These were allowed to incubate at room temperature for one minute. They were then centrifuged for 1 minute at 8,000g in order to elute the purified RNA in the bottom of the Eppendorf tube. Following quantification of RNA yield using a NanoDrop spectrophotometer, samples were then stored for cDNA synthesis at -80^{o}C.

For quantification of RNA concentration, a blank reading was first obtained using RNAse free water. Then, 1.5uL of each sample was used to measure RNA concentration in ng/uL based on absorbance at 260nm. The ratio of absorbance at 260nm and 280nm was used to assess RNA purity, with samples having a ratio of around 2 indicating pure RNA. All samples that were used in the current study had a ratio of at least 1.6

2.7.4.7 DNAse treatment and cDNA Synthesis

Synthesis of cDNA from RNA was carried out using the High Capacity cDNA Reverse Transcription Kit (Thermo-Scientific). DNAse treatment was first carried out in order to degrade potential contaminating DNA from RNA samples using the DNase I, Amplification Grade kit (Thermo-Scientific).

For DNAse treatment, samples were first prepared using 250ng or greater purified RNA made up to 8.5uL with RNAse free water. The specific volume of purified RNA used depended on the RNA yield and was calculated for each batch of samples individually. The sample with the most abundant RNA yield was prepared in duplicate in order to act as the negative Reverse Transcriptase (-RT) sample in the cDNA synthesis stage. To the 8.5uL of RNA-water mixture, 1uL of 10X Assay Buffer and 0.5uL of DNAse I was added,

with the enzyme being added last and additions being performed quickly whilst working on ice. Tubes were incubated at room temperature for 15 minutes before the addition of 1uL EDTA solution. Samples were then heated in a heating block for 10 minutes at 65^{o}C. Samples were briefly spun to remove condensation.

Meanwhile, positive and negative reverse transcriptase master-mixes were prepared from the cDNA synthesis kit as below (Table 2.4). Following preparation of the appropriate master-mixes, 15uL of each was added to microcentrifuge/PCR tubes. Then, 10uL of DNAse treated sample was added to each. Finally, these samples were placed in a thermocycler to allow reverse transcription to occur. The settings on the thermocycler were set up for each run as follows:

1. **Step 1**: 25^{o}C for 10 minutes
2. **Step 2**: 37^{o}C for 120 minutes
3. **Step 3:** 85^{o}C for 5 minutes
4. **Step 4**: 20 ^{o}C till samples removed

Following this, samples were removed and stored at -20^{o}C for further analysis by qPCR.

	Pos. Reverse Transcriptase	**Neg. Reverse Transcriptase**
Assay Buffer (10 x)	2.5 ul	2.5 ul
dNTP (25 x)	0.8 ul	0.8 ul
Random Primers (10 x)	2 ul	2 ul
Reverse Transcriptase	0.5 ul	0 μl
RNAse Free Water	9.2 ul	9.7 ul

Table 2.4 Preparation of the Positive and Negative Reverse Transcriptase Mastermix. *The Positive Reverse Transcriptase mix was prepared for all samples in a given run using the above quantities of assay buffer, dNTPs, random primers and RNAse free water up*

to the required volumen. Reverse Transcriptase enzyme was added to the Positive Reverse Transcriptase mastermix, but not in the Negative Reverse Transcriptase mastermix. This acted as a negative control in the future qPCR experiments.

2.7.4.8 Polymerase Chain Reaction (qPCR) Quantification

For the current analysis, 8 primers were designed using the NCBI Primer BLAST database. Primers were designed to be intron spanning (so as not to amplify contaminant DNA) and were specifically designed to amplify a product of maximum 200 base pairs. Primers were then obtained from IDT technologies. There was a forward and reverse primer designed for each target gene. Details on specific sequences of primers designed and used are given below. Primer targets were selected based on involvement in key pro-inflammatory pathways under study and prior literature.

Target	Forward Sequence	Reverse Sequence
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
IL1β	TCGCCAGTGAAATGATGGCT	TGGAAGGAGCACTTCATCTGTT
IL-6	CCTTCTCCACAAACATGTAACAAGA	TCACCAGGCAAGTCTCCTCA

Table 2.5 Primer Sequences used for qPCR Analysis

Lypophylised primers were reconstituted in 250uL RNAse free water as per the manufacturers instructions. From this stock solution, 10uL of both the forward and reverse primers were combined with 80uL of RNAse free water to yield a working primer solution. Of this working concentration, 0.4uL was added to each qPCR well (final concentration 0.4 nM per well). Meanwhile, cDNA was diluted in a 1:10 ratio with RNAse free water, with 2uL of the final mix to be added per pPCR well. A master-mix for each gene was then prepared using the following quantities per well: 5uL Sybr Green (Thermo-Scientific), 0.4uL primer solution, 2.6uL RNAse free water.

A 384-well PCR plate was labelled and divided. Samples were analysed in technical triplicate with a space provided between each sample and each target gene. 2uL of diluted cDNA sample was first pipetted into the top left of each well. The plate was then gently tapped off the bench to allow samples fall to the bottom of the well. Following this, 8uL of the appropriate master-mix for each gene was added to the top left of each well, after turning the plate 180^{o}. The plate was then covered with a plate-sealer and centrifuged for 30 seconds in order to ensure both sample and master-mix travelled to the bottom of the well. The plate was then analysed using the QuantStudio 5 thermoscientific qPCR machine. Samples were subjected to a single run at the following settings:

In order to quantify the relative quantity of expression of each target gene, the $2^{-\Delta\Delta CT}$ method was used. For this method, the relative quantity of gene expression is calculated relative to a housekeeping gene (18S). CT values were extracted and entered into the $2^{\wedge-(CT)}$ formula. The ratio of the gene of interest was calculated relative to 18 was obtained as follows: $2^{\wedge-(CT)}$ (gene of interest)/$2^{\wedge-(CT)}$ (18s). This was used to calculate values for each sample. For PBMC stimulations, the sample under stimulation was divided by the expression in the unstimulated value for the same participant. This gave a ratio representing gene induction in response to cell stimulations.

2.7.3.1 Enzyme-Linked Immunoabsorbent Assays (ELISAs) of Cell Supernatants

IL-1β and IL-6 ELISAs were performed on previously aliquoted cell supernatants for all six stimulation conditions. ELISAs were carried out as detailed above under the serum sample section with the notable exception that supernatant samples were diluted to a concentration of 1:50-1:200 and values multiplied by the dilution factor after extrapolation from the standard curve.

2.8 Statistical Analysis

Between group differences comparing those with T2DM and healthy controls were carried out using t-tests and Wilcoxon rank-sum tests (depending on distribution) for continuous variables and chi-square tests for binary or categorical variables. Proportions were presented as % and continuous variables as means +/- standard deviations or medians with interquartile range depending on distribution. Normality of data was evaluated by Quantile-Quantile (Q-Q) plots, historgrams and the Shapiro-Wilk statistical tests as appropriate.

For analysis of cognitive assessments, between group differences were compared in the first instance. Data was then analysed using various regression models to examine predictors of cognitive performance in midlife T2DM. For analysis of MoCA scores, a MoCA error term was also created which accounted for the skew in scores and predictors of MoCA errors was analysed as count data using poisson regression. For analysis of detailed CANTAB performance, Z-scores were created by subtracting the overall mean from a participants score and dividing by the standard deviation of that particular measure. Linear regression was then used to examine predictors of cognitive performance. Where no between group differences were observed in the first instance, predictors of cognitive performance were examined using forward stepwise inclusion of variables in linear regression. All CANTAB output measures were evaluated by univariate association in addition to adjusted models including age, group (T2DM vs healthy controls), educational attainment (years of formal education) and a family history of cognitive impairment/dementia.

In order to analyse gait performance, data was examined for normality and between group differences examined in the first instance. As above, linear regressions were used to analyse the association between gait performance and cognitive variables in addition to important covariates known to impact on gait performance. For measures where there were no between group differences on cognitive variables, associations between

gait and cognition were examined using linear regressions with the introduction of group (T2DM vs healthy controls) as a covariate and additionally via an interaction term with the variable under study to examine if the association was specific to those with T2DM or to a midlife population in general. In order to explain the variation seen in gait speed, performance on all tasks was subsequently divided into quartiles and quartiles used as a predictor in regression models.

For analysing the laboratory data, between group differences were analysed for serum cytokines and PBMC stimulations. Serum cytokine values were all non-normally distributed and a log transformation was applied. Log transformations were applied to RNA values from unstimulated and stimulated PMCs that were non-normally distributed. Linear regression were used on log transformed data to analyse the relationship between serum cytokines, PBMC cytokine gene expression/protein and gait and cognitive function. Again, where no between group differences existed, associations were analysed in the whole study population and then group (T2DM vs healthy controls) introduced as a covariate and finally as an interaction term.

A threshold of <0.05 was applied for all tests of statistical significance in the current study. Linear models were examined for multicollinearity using variance inflation factors, with values >5 indicating significant multicollinearity. Residual-versus-fitted plots were also examined visually in order to ensure homogeneity of the residual distribution. Poisson models were evaluated for overdispersion by comparing results to negative binomial models and selecting the models with the overall best fit. All data analysis was carried out on STATA v15.0 and all graphs created using STATA v15.0 and GraphPad v7.0

2.9 Aims and Hypothesis

The aims of my thesis was to evaluate the following questions:

1. **Do otherwise healthy participants with Type 2 Diabetes Mellitus (T2DM) in midlife have poorer cognitive function in comparison to healthy controls ? (Chapter 3)**
    a. For what domains of cognitive function are deficits seen ?
    b. What are the clinical characteristics of those with T2DM which have demonstrable deficits in cognition ?
2. **To evaluate gait as a potential biomarker of cognitive deficits in midlife T2DM. Are cognitive deficits in midlife T2DM correlated with measures of gait speed and performance on gait tasks ? (Chapter 4)**
    a. Particularly for dual-task gait, which is known to strongly correlate with processing speed and executive function in later life
    b. For what domains of cognition is there an association seen between gait performance and cognitive impairment ?
3. **Is there a relationship between circulating pro-inflammatory mediators and cognitive dysfunction in T2DM ? (Chapter 5)**
    a. If so, which mediators are implicated and which domains of cognitive function
4. **Is there a relationship between circulating pro-inflammatory mediators and gait performance in T2DM (Chapter 5)**
    a. If so, for which makers and using which gait tasks is this seen ?
5. **Is Type 2 Diabetes Characterised by an aberrant immune response to the putative pathogenic protein in Alzheimer Disease, Amyloid-β 42 ? (Chapter 6)**
    a. Is there any relationship between peripheral blood cell immune responses, assayed using Peripheral Blood Mononuclear Cells (PBMCs) and cognitive/gait characteristics ?
    b. Is any such effect specific to T2DM ?

Chapter 3: Cognitive Function in Otherwise Healthy Adults with Midlife Type 2 Diabetes Mellitus

Abstract

Introduction: Midlife Type 2 Diabetes (T2DM) is associated with a two-fold increased risk of dementia in later life. Despite the fact that this has been known for nearly 30 years, how early cognitive deficits begin to emerge in T2DM is currently unclear. We aimed to assess the cognitive profile of otherwise-healthy adults diagnosed with T2DM in midlife.

Methods: Middle aged adults (aged 35-65) were recruited from T2DM clinics in addition to a cohort of age matched controls. A comprehensive medical history was taken and participants with T2DM only included if they had no micro/macrovascular complications of T2DM. Cognition was assessed using the standard Montreal Cognitive Assessment (MoCA) in addition to a customised neuropsychological assessment battery (CANTAB) aimed at assessing memory, executive function, attention and reaction time. Appropriate univariate statistics in addition to poisson and linear regression models were used to analyse results.

Results: One-hundred and two participants (N = 71 with T2DM & N = 31 healthy controls) were recruited [mean age: 52 (± 7.9); 53% female]. Those with T2DM had a median duration since diagnosis of 5 (Interquartile Range: 2-10) years and a mean HbA1c of 54.5 (46-70mmol/L). T2DM was associated with a greater likelihood of error on the MoCA (IRR = 2.36, 1.53-3.64, $p<0.001$) which persisted after adjustment for age, sex, a family history of dementia/cognitive impairment and years of formal education (IRR = 2.20, 1.41 – 3.64, $p<0.001$). Whilst T2DM was associated with poorer scores on tests of reaction/movement time, executive function, attention and visuospatial memory (both immediate and delayed), results were not statistically significant either alone or following adjustment for the above covariates. Greater T2DM duration was associated with a greater likelihood of error on the MoCA.

Conclusion: T2DM, in addition to duration of T2DM diagnosis, were associated with a greater likelihood of error on the MoCA, a measure of general cognitive function. However, there were no statistically significant between-group differences on the detailed neuropsychological assessment battery. Whilst our results demonstrated poorer general cognitive function in T2DM, deficits in specific cognitive domains may not emerge this early in T2DM and warrants longitudinal analysis.

3.0 Introduction

Type 2 Diabetes (T2DM) is midlife is associated with a two-fold increase in the risk for later development of dementia (Gustafson & McFarlane, 2018). Whilst the increased dementia risk seen in those with T2DM has been known for a long time, the specific cognitive deficits and cognitive trajectories seen in those with T2DM remain poorly explored. It appears that T2DM affecting those between the ages of 40-60 years poses the greatest risk, and may even increase dementia risk as much as APOE ε4 genotype, which apart from age, is the greatest risk factor for the later development of dementia (Gottesman et al. 2017).

According to a recent authoritative review on the T2DM-link, cognitive deficits seen in T2DM can be seen across the lifespan and characterised as follows: (i) cognitive decrements: subtle, slowly progressive cognitive decrements which occur across the lifespan in those with T2DM, (ii) cognitive impairments: demonstrable impairments in cognition occurring usually after 65 but may be seen earlier and (iii) dementia: after the age of 65, dementia seen in those affected by T2DM (Jan Biessels & Despa, 2018). However, the observation is made that not all those affected by T2DM will progress from cognitive decrements to cognitive impairment or dementia and that the cognitive decrements are not necessarily a "pre-dementia" state. However, the exact nature of these decrements in midlife as well as the transition from these to other forms of cognitive impairment across the lifespan, remain poorly explored. Moreover, it is not understood which patients with these decrements will eventually go on to develop cognitive impairment/dementia in later life.

There is a significant amount of variation in published studies in terms of cognitive domains studied, sample sizes, ages and populations and length of follow up (if any). Whilst many studies have examined cognitive domains affected in Type 2 Diabetes (T2DM), many are limited to those aged 60 and older. In fact, only nine studies have examined cognitive function specifically in midlife Type 2 Diabetes, which have all been

cross-sectional in nature (Biessels et al. 2001, Garcia-Casares et al. 2014, Kinga & Anett, 2016, Kumar et al. 2015, Mehrabian et al. 2012, Naseer et al. 2014, Nazaribadie et al. 2013, Ryan et al. 2000, Solanki et al. 2009, Yau et al. 2009). Some studies have drawn on longitudinal cohort studies with T2DM participants compared against a much greater number of healthy controls drawn from the larger population (Aberle et al. 2008, Rawlings et al. 2014). Many of these studies have suffered from small sample sizes and significant heterogeneity in terms of included participants.

In the largest specifically designed study to date on middle-aged participants with T2DM, T2DM was associated with poorer performance across all memory, executive function and attention assessments in comparison to age-matched healthy controls (Palacious-Mendoza et al. 2018). Previous studies have demonstrated poorer cognitive function in midlife T2M in terms of information processing speed (such as on the Groove Pegboard Test), psychomotor speed, attention and concentration (such as on the Digit Span Forward Test), working memory (such as the Serial Addition Test) and executive function (with consistent differences seen for performances on the Stroop task). Whilst there does seem to be demonstrable cognitive deficits in midlife T2DM, negative studies also exist (Ryan & Geckle, 2000). There is a paucity of evidence for longitudinal changes in cognition in midlife T2DM, and on longitudinal cognitive trajectories as people affected by T2DM in midlife age.

In the current study, we aim to recruit a sample of otherwise healthy participants with midlife T2DM. We have devised strict inclusion and exclusion criteria to select out only those participants with uncomplicated T2DM in midlife. Through detailed cognitive and neuropsychological assessment we aim to characterise the specific pattern of cognitive impairment seen in T2DM as well as examine the clinical predictors of these deficits. We aimed to include as healthy a population as possible, and targeted “young” T2DM clinics in our recruitment. The data presented here forms part of the baseline assessments for the ENBIND study, introduced above, which is the first purpose-designed longitudinal study of cognition in those affected by T2DM in midlife. This central aim of this chapter,

within the context of the ENBIND study, is to characterise the cognitive function of those affected by midlife T2DM.

3.1 Participant Characteristics

3.1.1 Overall Cohort Characteristics

Over the 9 month study period, one-hundred and two participants were recruited as part of the ENBIND study. Of these, 71 were otherwise healthy participants with midlife T2DM and 31 were healthy controls. The mean age of included patients was 52 (± 7.9) years and just over half (51.96%, N = 53) were female. Median Body Mass Index (BMI) was 25.65 (IQR: 25.85-34.9). Median years of full time education was 12 (IQR: 16-18). Just under one-fifth (17%) had a family history of dementia. Overall, 50 (49%) had a history of treated hypertension and 52 (51%) had treated hypercholesterolaemia. Median Charlson Comorbidity Index (CCI) was 1 (IQR: 1-2). Median number of daily medications was 3 (IQR: 1-5).

3.1.2 Between Group Differences (Type 2 Diabetes vs Controls)

On between group analysis, patients with T2DM had a significantly higher BMI (z = -4.35, p<0.001, wilcoxon rank-sum), greater number of daily medications (z = -6.73, p<0.001, wilcoxon rank-sum) and a greater burden of hypertension (χ^2 = 23.8, p<0.001, chi-square) and hyperlipidaemia (χ^2 = 31.23, p<0.001, chi-square). These are detailed below in Table 3.1

Characteristic	**T2DM** **N = 71**	**Healthy Controls** **N = 31**	**P-Value**
Age (mean, SD)	52.04 (± 8.01)	52.16 (± 7.82)	0.53
Gender (N Female, %)	20 (64.52%)	33 (46.48%)	0.10
Years of Education (median, IQR)	14 (13-15)	13 (12-14)	0.11
Body Mass Index (median, IQR)	31 (27.5-37.3)	26.8 (24.8-28.4)	<0.001

Family History of Dementia (N, %)	4 (13.79%)	13 (18.31)	0.59
Daily Medications (median, IQR)	4 (2-5)	1 (0-1)	<0.001
Hypertension (N, %)	46 (64.78%)	4 (12.9%)	<0.001
Hyperlipidaemia (N, %)	49 (69.01%)	3 (9.67%)	<0.001
Charlson Comorbidity Index (median, IQR)	2 (1-2)	1 (0-2)	<0.001
HbA1c (mmol) (median, IQR)	54.5 (46-79)	37.5 (34-39)	<0.001

Table 3.1. Between group differences in participants included in the ENBIND Study. *Normally distributed data were assessed using t-tests whilst non-parametric data were assessed using Wilcoxon rank-sum tests. Proportions were compared using a Chi-Square test (χ^2). Participants with diabetes had a greater body mass index, greater number of daily medications, greater burden of hypertension and hypercholesterolaemia than age, sex and education matched healthy controls.*

3.1.3 Type 2 Diabetes Characteristics

In those participants with midlife T2DM, the median years since diagnosis was 5 (Interquartile range: 2-10). One-quarter of those with T2DM were treated using metformin only (19/71, 26.76%). A further 43.66% (31/71) were prescribed metformin in combination with some other T2DM medication: 9 (12.67%) of these were co-prescribed a DPP-IV inhibitor, 5 (7.04%) were co-prescribed a SGLT2 inhibitor and 15 (21.13%) were co-prescribed glicazide. Just under one-quarter (23.94%, 17/71) were treated using GLP1 analogues ad just under one-tenth (8.45%, 6/71) were treated using insulin. In those with T2DM, the median number of daily medications was 4 (IQR: 2-5). Whilst all T2DM participants scored a single point for uncomplicated diabetes on the CCI, 5 participants with T2DM (7.04%, 5/71) scored an additional point for medical comorbidity. Median HbA1c was 54.5mmol (IQR: 46-79mmol)

3.2 Cognitive Performance in Type 2 Diabetes

3.2.1 T2DM and General Cognitive Function

All participants in the current study had a MoCA completed. Overall, the median MoCA score (corrected for educational attainment) in all included participants was 29 (IQR: 28-30). Only 10% of participants had a MoCA score of <26. Overall, MoCA scores showed a strong left skew (Kurtosis = 3.45) and the MoCA error term demonstrated a Poisson distribution. Distribution of total MoCA score in addition to MoCA errors are show below in Figure 3.1 & 3.2.

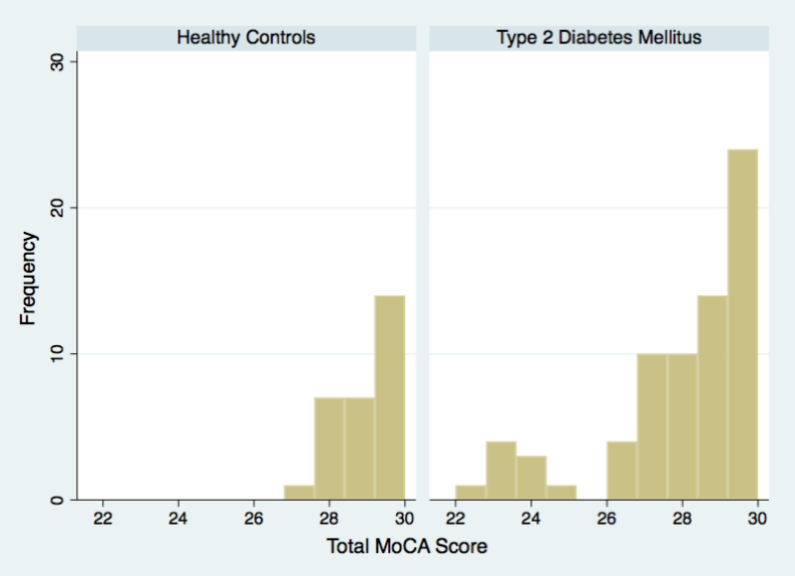

Figure 3.1 Overall MoCA Scores in Otherwise Healthy Participants with Type 2 Diabetes and Age, Sex and Education Matched Healthy Controls. *Median MoCA score in the study population as a whole was 29 (Interquartile range: 28-30). Only a single participant had a MoCA below the cut-off of 23/30 indicating potential cognitive impairment. MoCA scores demonstrated a strong left-skew.*

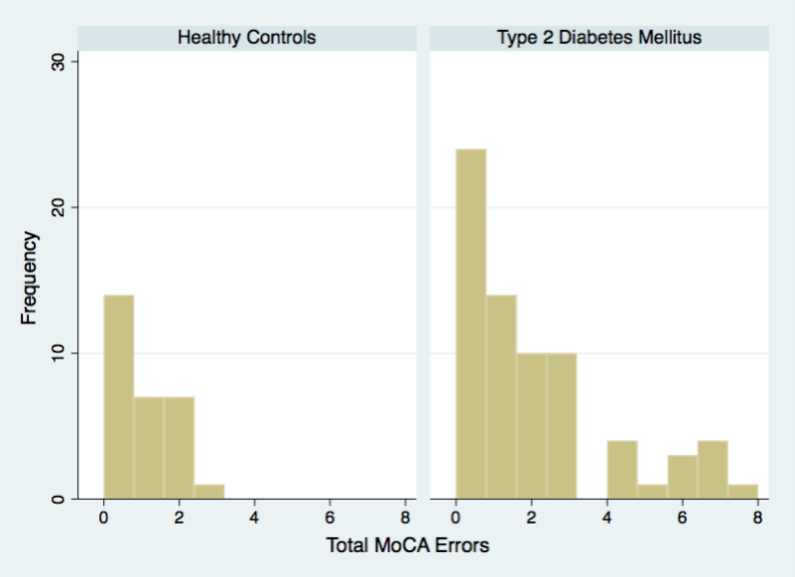

Figure 3.1 MoCA Errors in Otherwise Healthy Participants with Type 2 Diabetes and Age, Sex and Education Matched Healthy Controls. *As MoCA scores (Fig 3.1 above) demonstrated a strong left-skew, an error term was created by subtracting the total MoCA score from the total MoCA marks available (30 – score) and the result modelled as an error rate for regressions. MoCA error rate assumed a Poisson distribution.*

Overall, T2DM was associated with lower MoCA score on univariate analysis (z = -2.289, p = 0.02, Wilcoxon rank-sum). On unadjusted poisson regression, T2DM was associated with a greater likelihood of error on the MoCA with an Incident Rate Ratio (IRR) of 2.36 (95% CI: 1.53 – 3.64, p<0.001). This association persisted after adjustment for age, sex, years of formal education and family history of dementia (IRR: 2.20, 1.41 – 3.43, p = 0.001) (Table). Of note, no patients had a MoCA score of <19 warranting exclusion. Only a single participant had a MoCA score below the cut-off with a negative AD8 score indicating possible Mild Cognitive Impairment (MCI).

Independent Variable	Unadjusted IRR (95% CI)	P-Value	Adjusted IRR (95% CI)	P-Value
T2DM	2.36 (1.53-3.64)	<0.001	2.20 (1.41-3.43)	0.001
Age	-		1.04 (1.02-1.06)	0.001
Gender	-		0.85 (0.62–1.17)	0.312
Years of Formal Education	-		0.93 (0.87-0.99)	0.029
Family History of Dementia	-		0.79 (0.49-1.28)	0.333

Table 3.2 Poisson Regression of Total MoCA Errors. *Diabetes was associated with a significantly greater likelihood of error on the MoCA which persisted after adjustment for age, gender, years of formal education and family history of dementia.*

3.2.2 T2DM and Neuropsychological Test Performance

3.2.2.1 CANTAB Battery Assessment

Overall, 97 (60 with T2DM and 30 healthy controls) of the study participants completed the entire neuropsychological assessment battery. Three participants refused to take part in the full battery or left early. One participant was not assessed using the CANTAB battery as they had a score below the standard MoCA cut off for cognitive impairment (<23).

3.2.2.2 Reaction Time Task (RTI)

On the initial neuropsychiatric test for reaction and movement time in response to a circular stimulus changing colour (RTI: Reaction Time Task), mean reaction times were similar between groups (407 ± 50.95ms for T2DM vs 423 ± 59.30ms for healthy controls, t = -1.26, p = 0.89) as were mean movement times (299.5 ± 91.59ms for T2DM vs 282 ± 80.50ms for healthy controls, t = -0.90 p = 0.82). All 97 participants completed the 30 trials of this task.

3.2.2.3 T2DM and Performance on Memory-Dominant Tasks: Paired Associates Learning, Spatial Working Memory and Pattern Recognition Memory

On the Paired Associates Learning (PAL) task, there was a trend for better performance in healthy controls on univariate analysis of the first attempt memory score (11.17 ± 4.16 for healthy controls vs 10.31 ± 4.52 for T2DM, t = 1.49,p = 0.07). Participants with T2DM made more errors on the 4,6 and 8 stimuli tasks combined (22 [IQR: 8-40] for T2DM vs 14 [IQR: 12-18], z = -1.39 p = 0.16), although this result was also not statistically significant.

On the other task probing working and short term memory, the Spatial Working Memory (SWM) task, there were no differences between T2DM and healthy controls in terms of errors made (16.5 ± 10.55 for T2DM vs 16.05 ± 9.35, t = 0.21, p = 0.42) and Strategy scores (9.5 [IQR: 6-11] for healthy controls and 9 [IQR: 8-10], z = 0.23, p = 0.55 for T2DM).

On the final memory-dominant task, the Pattern Recognition Memory (PRM) test, there were no between-group differences in percentage detection of previously viewed patterns either immediately (88.89% [IQR: 77.78%-94.44%] for healthy controls vs 88.89% [IQR: 77.78-94.44%], z = -1.07, p = 0.29) or after a 20 minute delay (81.8 ± 13.83% for healthy controls vs 79.08 ± 12.74 % for T2DM, t = 0.93 ,p = 0.34). Results are demonstrated below in Figure 3.3

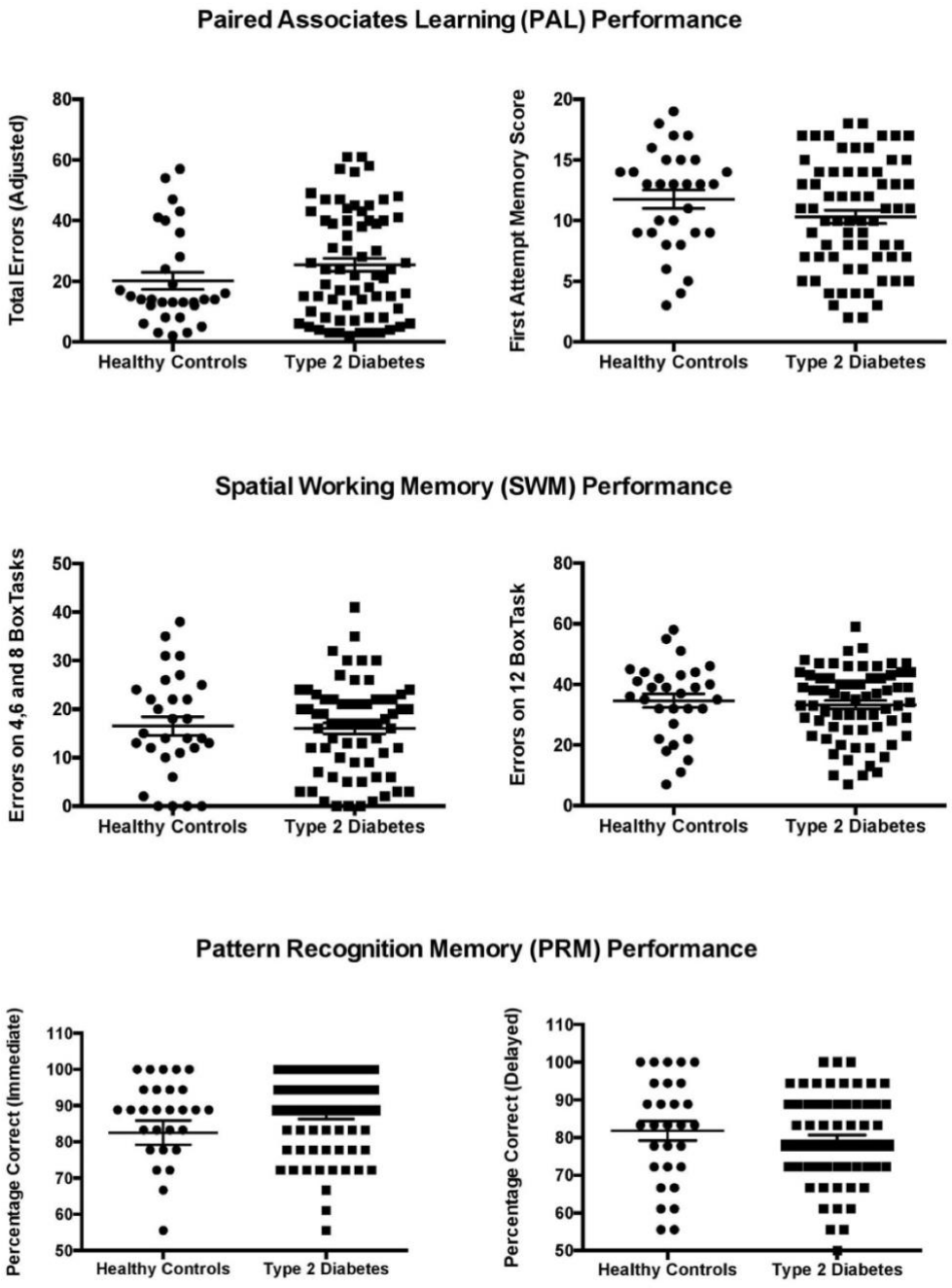

Figure 3.3 T2DM was not associated with poorer performance in any of the three memory-dominant neuropsychological tests. *No differences were seen between T2DM and control groups for performance on Paired Associates Learning (PAL) for either the total errors or first attempt memory score, for the errors on spatial working memory performance or for the percentage of correct trials either immediately or after a 20*

minute delay on the pattern recognition memory task. Results are graphed individuals and the mean (± Standard Error of the Mean) graphed.

3.2.2.4 T2DM and Performance on Executive Function Dominant Tasks: Rapid Visual Processing and One-Touch Stockings of Cambridge

For the remaining two tasks, which have high executive function demands, there were similarly no differences between T2DM and healthy control groups. For the Rapid Visual Processing Task (RVP), there was no difference in performance (A' Prime) between T2DM and Healthy Controls (0.87 ± 0.06 for T2DM vs 0.88 ± 0.06 for controls, t = -1.04, p = 0.85). Similarly, there was no difference in probability of false alarm between the two groups (0.007 [IQR:0.002-0.14] for T2DM vs 0.006 [IQR: 0.002—0.031, z = -0.85, p = 0.84] for healthy controls). For the One Touch Stockings of Cambridge task, there were no differences in the proportions of problems solved on first choice (9.1 ± 3.01 for healthy controls vs 8.46 ± 3.13 for T2DM, t = 0.94, p = 0.18) or in the latency to first choice (18.48s [IQR: 13.02-23.48] for HC vs 19.58s [IQR: 13.86 - 26.49 for T2DM z = -0.57, p = 0.57). The results for these tasks are given in Figure 3.4. Results of all twelve neuropsychological test performance measures are given below in Table 3.3.

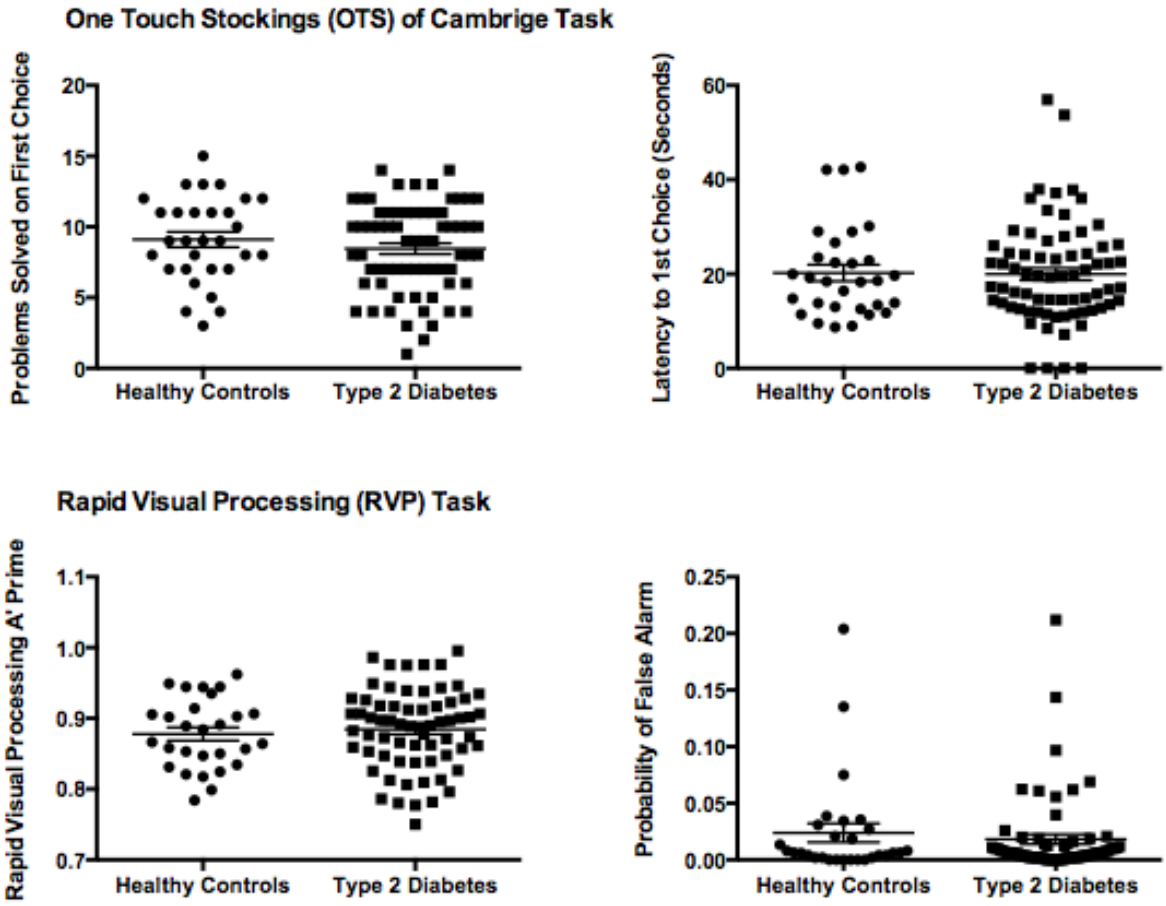

Figure 3.4 T2DM was not associated with poorer performance in any of the executive function-dominant neuropsychological tests. *No differences were seen between T2DM and control groups were observed for performance on the one touch stockings of Cambridge Task (where participants compute the number of moves to make two stimuli match) or in the Rapid Visual Processing Task (a test of attention and executive function). Individual Results are shown above and summary statistics graphed as mean (± Standard Error of the Mean)*

Assessment	Outcome Measure	Healthy Controls	T2DM	P-Value
Reaction Time Task (RTI)	Median Duration Reaction Time (RTI-MDRT)	407ms (50.95)	423ms (59.30)	0.89
	Median Detection Movement Time (RTI-MDMT)	282ms (80.50)	299.5ms (91.59)	0.82
Paired Associates Learning (PAL)	Total Errors Adjusted (PAL-TEA)	14 (12-18)	22 (8-40)	0.16
	First Attempt Memory Score (PAL-FAMS)	11.77 (4.16)	10.31 (4.52)	0.07
	Total Errors Adjusted (PAL-TEA): 12 boxes	29.5 (13-44)	44 (17-44)	0.13
Spatial Working Memory (SWM)	Between Errors (SWM-BE): 4,6 & 8 boxes	16.5 (10.55)	16.05 (9.35)	0.42
	Between Errors (SWM-BE): 12 boxes	34.63 (12.46)	33.30 (11.70)	0.31
	Strategy (SWM-S)	9.5 (6-11)	9 (8-10)	0.55
Pattern Recognition Memory (PRM)	Percent Correct Immediate (RPM-PCI)	88.89% (77.78-94.44%)	88.89% (77.78-94.44%)	0.29
	Percent Correct Delated (RPM-PCD)	81.80% (13.83%)	79.08% (12.74%)	0.34
Stockings of Cambridge (OTS)	Problems Solved on First Choice (OTS-PFS)	9.1 (3.01)	8.46 (3.13)	0.18
	Median Latency to First Choice (OTS-MDLFC)	18,479 (13,016-23.480)	19.584 (13,860-26,049)	0.57
Rapid Visual Processing (RVP)	A Prime (RVP-A)	0.87 (0.06)	0.88 (0.06)	0.85
	Probability of False Alarm (RVP-PFA)	0.00635 (0.002-0.0309)	0.00655 (0.002- 0.143)	0.84

Table 3.3. CANTAB results across all Neuropsychological Tasks. *Results are given in terms of means (± Standard Deviation of the Mean) were appropriate or as medians (Interquartile Range) where non-parametric. Univariate analysis was conducted using either two sample t-test or a wilxocon rank-sum test. Whilst T2DM was associated with poorer performance on average, none of these differences were stastically significant on univariate analysi*

3.2.2.5 T2DM and CANTAB Performance adjusting for Age, Gender, Educational Attainment and Family History

All twelve neuropsychological test outcome measures were analysed using linear regression modelling in order to adjust for important covariates with a known impact on cognitive function comprising age, gender, family history of cognitive impairment/dementia and years of formal education. The β -Coefficient and appropriate 95% Confidence Interval for those with T2DM vs healthy controls is given in the table below. T2DM was not associated with significantly poorer performance on any measure following adjustment for these important covariates. Across all twelve measures, age was the only consistent significant predictor of performance. However, when age was controlled for using these regressions, there was no T2DM effect observed. Whilst further adjustment for hypertension and hypercholesterolaemia were attempted, these covariates were omitted from the final model due to multicollinearity.

Assessment	Outcome Measure	β -Coefficient (95% CI)	P-Value
Reaction Time Task (RTI)	Median Duration Reaction Time (RTI-MDRT)	13.08 (-12.42 – 38.57)	0.31
	Median Detection Movement Time (RTI-MDMT)	16.35 (-23.23 – 56.02)	0.41
Paired Associates Learning (PAL)	Total Errors Adjusted (PAL-TEA)	4.83 (-2.46 – 12.12)	0.19
	First Attempt Memory Score (PAL-FAMS)	-1.25 (-3.12 – 0.63)	0.19
	Total Errors Adjusted (PAL-TEA): 12 boxes	4.74 (-2.06 – 11.53)	0.17
Spatial Working Memory (SWM)	Between Errors (SWM-BE): 4,6 & 8 boxes	0.38 (-3.77 – 4.53)	0.86
	Between Errors (SWM-BE): 12 boxes	0.09 (-5.14 – 5.31)	0.97
	Strategy (SWM-S)	0.18 (-0.85 – 1.22)	0.72
Pattern Recognition Memory (PRM)	Percent Correct Immediate (RPM-PCI)	3.55 (-2.48 – 9.59)	0.25
	Percent Correct Delated (RPM-PCD)	-3.66 (-9.46 – 2.14)	0.21
Stockings of Cambridge (OTS)	Problems Solved on First Choice (OTS-PFS)	-0.70 (-2.08 – 0.67)	0.31
	Median Latency to First Choice (OTS-MDLFC)	1222 (-3040 – 5485)	0.57
Rapid Visual Processing (RVP)	A Prime (RVP-A)	0.01 (-.017 – 0.35)	0.51
	Probability of False Alarm (RVP-PFA)	-0.02 (0.06 – 0.01)	0.23

Table 3.4 Cognitive Performance in Midlife Diabetes Mellitus on the CANTAB Assessment Battery (Adjusted). *Coefficients were generated from linear models controlling for the following covariates with a known impact on cognitive function: age, gender, years of formal education and*

family history of dementia/cognitive impairment. Coefficients, presented at the estimate with approximate 95% confidence interval represent the effect of cognitive function on the specific outcome measure of interest.

3.2.2.6 Standardized and Composite Scores on CANTAB Assessment

Scores on all neuropsychological test outcomes were also Z-transformed in order to standardise scores across all measures and enable the computation of composite scores for different test domains. The Z-scores by group are demonstrated in Table XX and ZZ below. Similarly there were no differences in cognitive performance associated with Type 2 Diabetes Mellitus on any task apart from the Pattern Recognition Task, where the percentage correct on the immediate task was poorer in those with T2DM ($t = 1.69$, $p = 0.04$, t-test). Several trends for worse performance in those with T2DM were seen on the Paired Associates Learning Task ($p = 0.08$ for Frist Attempt Memory Score, $p = 0.08$ for Total Adjusted Errors), but these were not statistically significant. See Table 3.5. Additionally, Table 3.6 gives the result of linear models examining the effect of T2DM on performance controlling for age, gender, family history and years of formal education as above. Neither the association/trends observed remained significant under this model (See Table 3.6).

An overall Z score for cognitive performance was created by summing the main two outcomes from each of the six tests to create a composite Z score. Z scores were given a plus or minus value depending on the sense of the score itself (for instance, errors were given a minus value whilst successful attempts were given a positive value). These scores were then re-standardised to provide an overall Z-score for cognitive function. Further, a composite Z score was then created for memory and executive function domains using the same method. Whilst those participants with midlife T2DM demonstrated poorer performance, there were no statistically significant differences between groups in overall cognitive function (Z-scores = 0.09 for healthy controls, -0.04 for T2DM, $t = 0.59$, $p = 0.28$), in memory (Z-scores = 0.17 for healthy controls, -0.08 for T2DM, $t = 1.13$, $p = 0.12$) or executive function (Z-scores = 0.12 for healthy controls, -0.06 for T2DM, $t = 0.80$, $p = 0.21$). These scores are graphed in Figure 3.5

Assessment	Outcome Measure	Healthy Controls (Z-Score)	T2DM (Z-Score)	P-Value
Reaction Time Task (RTI)	Median Duration Reaction Time (RTI-MDRT)	0.19 (0.89)	-0.09 (1.04)	0.10
	Median Detection Movement Time (RTI-MDMT)	0.14 (0.92)	-0.06 (1.04)	0.19
Paired Associates Learning (PAL)	Total Errors Adjusted (PAL-TEA)	0.21 (0.91)	-0.10 (1.03)	0.08
	First Attempt Memory Score (PAL-FAMS)	-2.11 (3.52)	-3.30 (3.06)	0.08
	Total Errors Adjusted (PAL-TEA): 12 boxes	2.11 (1.03)	-0.09 (0.98)	0.08
Spatial Working Memory (SWM)	Between Errors (SWM-BE): 4,6 & 8 boxes	-0.03 (1.09)	0.15 (0.97)	0.58
	Between Errors (SWM-BE): 12 boxes	-0.08 (1.05)	0.04 (0.98)	0.69
	Strategy (SWM-S)	-0.04 (1.15)	0.02 (0.94)	0.59
Pattern Recognition Memory (PRM)	Percent Correct Immediate (RPM-PCI)	0.25 (1.33)	-0.11 (0.78)	**0.04***
	Percent Correct Delated (RPM-PCD)	-0.14 (1.05)	0.06 (0.97)	0.82
Stockings of Cambridge (OTS)	Problems Solved on First Choice (OTS-PFS)	-0.14 (0.18)	0.06 (0.12)	0.82
	Median Latency to First Choice (OTS-MDLFC)	0.07 (0.98)	-0.03 (1.01)	0.33
Rapid Visual Processing (RVP)	A Prime (RVP-A)	0.16 (1.05)	-0.07 (0.98)	0.15
	Probability of False Alarm (RVP-PFA)	-0.19 (1.55)	0.09 (0.69)	0.89

Table 3.5 Standardised performance on the CANTAB assessment batter in those with Midlife Type 2 Diabetes Mellitus in comparison to Healthy Controls. *Raw scores were standardized using a Z-Score approach (individual score minus the mean divided by the standard deviation)*

to create a score representing performance relative to the rest of the cohort. T2DM was associated with slightly poorer performance on the pattern recognition memory task (immediate) (t = 1.69, p = 0.04, t-test). No other significant differences were observed

Assessment	**Outcome Measure**	**β -Coefficient (95% CI)**	**P-Value**
Reaction Time Task (RTI)	Median Duration Reaction Time (RTI-MDRT)	-.23 (-0.68 – 0.22)	0.31
	Median Detection Movement Time (RTI-MDMT)	-0.18 (-0.63 – 0.26)	0.41
Paired Associates Learning (PAL)	Total Errors Adjusted (PAL-TEA)	-0.28 (-0.06 – 0.01)	0.19
	First Attempt Memory Score (PAL-FAMS)	-1.08 (-2.78 – -0.56)	0.19
	Total Errors Adjusted (PAL-TEA): 12 boxes	-0.29 (-0.71 – 0.13)	0.17
Spatial Working Memory (SWM)	Between Errors (SWM-BE): 4,6 & 8 boxes	-0.04 (-0.06 – 0.13)	0.86
	Between Errors (SWM-BE): 12 boxes	-0.01 (-0.47 – 0.43)	0.97
	Strategy (SWM-S)	0.72 (-0.48 – 0.37)	0.72
Pattern Recognition Memory (PRM)	Percent Correct Immediate (RPM-PCI)	-0.26 (-0.70 – 0.18)	0.25
	Percent Correct Delated (RPM-PCD)	-0.28 (-0.16 – 0.72)	0.21
Stockings of Cambridge (OTS)	Problems Solved on First Choice (OTS-PFS)	0.22 (-0.22 – 0.67)	0.31
	Median Latency to First Choice (OTS-MDLFC)	-0.13 (-0.56 – 0.31)	0.57
Rapid Visual Processing (RVP)	A Prime (RVP-A)	-0.15 (-0.61 – 0.30)	0.51
	Probability of False Alarm (RVP-PFA)	0.03 (0.18 – 0.73)	0.23

Table 3.5 Adjusted performance on the CANTAB assessment batter in those with Midlife Type 2 Diabetes Mellitus vs. Healthy Controls. *Raw scores were standardized using a Z-Score approach as above. Linear regression models then examined the impact of T2DM status on cognitive performance controlling for important covariates such as age, gender, family history of cognitive impairment/dementia and years of formal education. Results are presented as Coefficients with the approximate 95% Confidence Intervals. There was no statistically significant association of T2DM status with performance on any metric under the adjusted model*

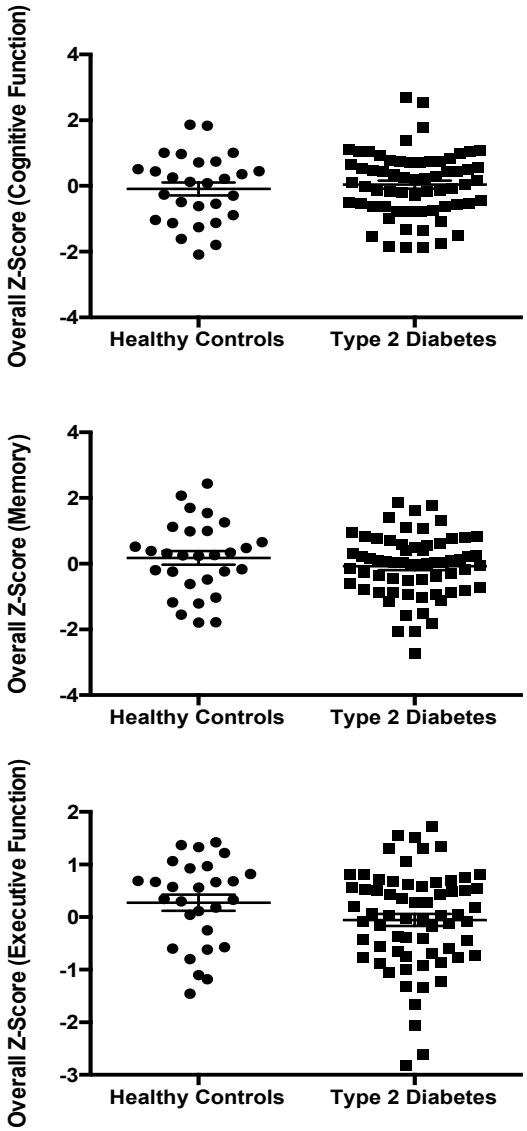

Figure 3.5 Composite Z-Scores for Overall Cognitive Function, Memory and Executive Function. *Scores were created by summing Z scores from individual tasks depending on the particular domain assessed and the sense (+/-) of the metric. Composite scores were re-standardised to obtain overall Z-Scores. There were no significant between group differences on any of the Z-Score*

3.2.2.7 Relationship Between T2DM Associated Clinical Variables and Cognition

In order to assess the relationship between T2DM associated clinical factors and cognitive function, regression models were fitted for both general cognition (MoCA scores) and Neuropsychological test outcomes. Poisson and Linear models were constructed which assessed the impact of diagnosis duration (years), glycaemic control (HbA1c), Body Mass Index (BMI) and treatment status (metformin alone/combination, GLP1 analogue use, SGLT2 inhibitor use, DPP-IV inhibitor use). Associations were first tested unadjusted and then in multivariate models controlling for the other clinical covariates in addition to age, gender, years of formal education and family history of diabetes.

On assessment of general cognitive function measured using the MoCA (which was significantly lower in those with T2DM), diagnosis duration (IRR 1.03, 1.00-1.06, p =0.05) was associated with a greater likelihood of poorer cognitive performance and being prescribed either an SGLT2 inhibitor or insulin were associated with a lesser likelihood of poor cognitive performance (IRR: 0.27, 0.08-0.89, p = 0.03; IRR: 0.42, 0.18-0.99, p = 0.04 respectively) in adjusted models. On analysing the predictive value of clinical variables with the twelve neuropsychological outcomes from the CANTAB battery, no linear regression models, either alone adjusted, demonstrated any association with cognitive outcomes. (Table 3.6).

Variable	IRR (95%) CI	P-Value
Diagnosis Duration (Years)	1.03 (1.00 – 1.06)	**0.05***
HbA1c (mmol)	0.99 (0.98 – 1.01)	0.46
Body Mass Index	0.98 (0.96 – 1.01)	0.30
Treatment Status		
Metformin	1.11 (0.73 – 1.68)	0.61
GLP-1	0.58 (0.32 – 1.05)	0.07
DPP-IV inhibitor	0.82 (0.45 – 1.49)	0.51
SGLT2 inhibitor	0.27 (0.08 – 0.89)	**0.03***
Insulin	0.42 (0.18 – 0.99)	**0.04***

Table 3.6 Association of Clinical Covariates with Likelihood of Error on the MoCA in a fully adjusted Poisson Model in those with midlife T2DM. *Diagnosis Duration was associated with a greater likelihood of error independent of age alone. Use of an SGLT2 inhibitor in addition to the use of insulin was associated with a lesser likelihood of error on the MoCA. * p = <0.05*

3.3 Discussion

The current chapter demonstrated poorer general cognitive function, but no demonstrable deficits in neuropsychological test outcomes, in midlife T2DM in comparison to healthy age-matched controls. Our study is the second largest study in the literature assessing cognition in T2DM in those aged <60 years, and is unique in its included population and detailed cognitive assessment, which represents the most extensive cognitive testing of any midlife T2DM to date.

Our findings reporting a greater likelihood of error on the MoCA in midlife T2DM is particularly striking. This finding persisted after robust adjustment for important covariates with a known impact on cognitive function inclusive of age and educational background. This observation was seen regardless of age and is particularly noteworthy in the context of the young age of included participants - the mean age of participants in each group was 52 years. We observed no MoCA scores consistent with cognitive impairment or dementia, as may be expected from an otherwise healthy cohort of participants in midlife.

The lack of significant between-group differences on neuropsychological testing is intriguing . Whilst prior literature has suggested that T2DM is associated with subtle cognitive decrements specifically affecting working memory, executive function and attention in midlife, we did not observe any on neuropsychological assessment (Pelimanni & Jehkonen, 2018). Results were standardised and we also controlled for the influence of age, gender, family history of dementia/cognitive impairment and years of formal education in our analysis, yielding no significant T2DM-effect.

One reason for this may be that our included participants were simply too young to demonstrate a cohort effect overall. Another reason may be that we excluded participants with any T2DM related complications including retinopathy, peripheral neuropathy and nephropathy. Previous evidence has demonstrated that these

complications are significant predictors of cognitive impairment in those with T2DM (Exalto et al. 2013). We also excluded participants with significant ischaemic heart disease, strokes and other neurological conditions also known to impact on cognitive function. Further, our population demonstrated good glycaemic control overall in addition to a high prevalence of treated hypertension and hypercholesterolaemia, known midlife risk factors for cognitive impairment (Gottesman et al. 2017). Such a cohort may be at risk of selection bias when comparing to other studies which have not included such strict exclusion criteria. These criteria do, however, provide a strong estimate of the T2DM-specific effect on cognition in midlife. Further, this cohort will represent an invaluable cohort for further follow up to assess potential T2DM-specific effects on cognition.

It cannot be ruled out that the inclusion of a larger sample size may have resulted in significant differences in cognition between those with T2DM in midlife and matched controls would have been observed. Despite the fact that our study included far less participants than large population based studies which have been used in analysing the T2DM-dementia link at an epidemiological level, it is the second largest study in the literature assessing cognition in midlife T2DM.

In comparison to the largest study in the literature in those aged <60 assessing cognitive decrements in midlife T2DM (Palacois-Mendoza et al. 2018), our participants were nearly 7 years younger on average, with an average of 4 years more formal education. Further, in our T2DM participants, mean HbA1c was nearly 1.5% lower and mean duration of diabetes nearly 5 years less than the aforementioned study (Palacois-Mendoza et al. 2018). Thus, our population may simply have been too young, educated and had too good diabetes control to capture any adverse cognitive effects. Whilst this may preclude stronger conclusions from our baseline analysis, it provides a fascinating cohort to profile longitudinally as part of the ongoing ENBIND study.

A further limitation may arise around the use of the CANTAB neuropsychological assessment battery. The CANTAB neuropsychological battery has been well validated in detecting mild cognitive impairment and prodromal AD, but has never before been used to capture subtle cognitive decrements or impairments that may affect those with T2DM. It may be that such decrements, if present in the domains tested, are too subtle to demonstrate on such computerised neuropsychological testing. It is noteworthy that we observed differing results from use of the traditional MoCA and CANTAB assessment battery. The true value of these assessments will again be seen in the next wave of the ENBIND study when we assess the same participants longitudinally using the same neuropsychological test battery.

An important limitation of the current study is that we did not control for hypertension and hypercholesterolaemia in our analysis. Due to the low prevalence of these in the control group and the high prevalence of them in our T2DM group, the inclusion of these variables introduced collinearity into our models. Future studies should examine both the impact of the presence of hypertension and hypercholesteraemia as well as the longitudinal variation in these as predictors of cognitive dysfunction in T2DM.

The results presented here also suggested a protective effect of insulin and SGLT-2 inhibitors on MoCA error rate in our cohort. Whilst there are plausible links between the use of these agents and improved cognition based on both biological mechanisms and by improvement of T2DM control, the fact that these results were seen with such small numbers and failed to replicate in the more comprehensive neuropsychological assessment battery suggests statistical anomaly and a strong likelihood of Type I Error. Further studies, and in particular longitudinal studies, will be needed to tease out the exact relationships between diabetes treatments and the development of cognitive impairment.

Conclusion

In conclusion, otherwise healthy participants with T2DM in midlife had poorer performance on general cognitive testing (MoCA), but not on specific domains or subdomains of neuropsychological assessment when compared to age, sex, gender and education matched healthy controls. We observed no significant differences for domains of memory, executive function or attention which have been previously demonstrated in older cohorts with a longer duration and poorer control of T2DM. We present the largest study to date examining cognition in those with diabetes and a mean age <55 years and the second largest in those <60 years. Repeat assessments, carried out as part of the longitudinal ENBIND study will enable characteristaion of cognitive deficits over time in T2DM and help understand the cognitive trajectories in this high risk group.

Chapter 4: Gait Speed and Dual-Task Gait Performance in Midlife Type 2 Diabetes Mellitus

Abstract

Introduction: Gait speed, and in particular dual-task gait speed, has been associated with cognitive deficits and dementia risk in several large longitudinal studies in community dwelling middle aged and older adults. Whether gait speed may predict cognitive deficits in T2DM has never been assessed.

Methods: ENBIND participants had gait speed assessed over three conditions: (i) self-selected gait speed (usual gait speed), (ii) maximal gait speed and (iii) dual task gait speed (with the addition of a cognitive task). Cognition was measured as above on the MoCA and a detailed computerised neuropsychological assessment battery (CANTAB). We assessed the ability of gait speed to predict cognitive performance.

Results: T2DM (N = 71) was associated with slower self-selected, maximal and dual-task gait speed (all $p<0.001$) in comparison to age-matched healthy controls (N = 31) which persisted after robust control for covariates. In the cohort overall, self-selected, maximal and dual-task speed were associated with greater likelihood of error on the MoCA (all $p<0.001$). However, after robust adjustment for covariates, only the association for Dual Task Cost (DTC) persisted ($p = 0.021$), but a DTC*T2DM interaction was not significant. Significant associations were seen between maximal and dual task speed and cognitive performance across the two neuropsychological tasks assessing executive function (One Touch Stockings of Cambridge and the Rapid Visual Processing Task), however these associations were once again not T2DM specific.

Conclusions: The current analysis showed a slowing effect of T2DM regardless of age, body mass index, comorbidity and medication burden on self-selected, maximal and dual-task gait speed. We observed significant associations between gait performance on the addition of a dual task and general cognitive function. Some associations were also seen for maximal gait speed and executive function, however, neither of these findings were specific for those with T2DM. Our findings support the ongoing

exploration and use of gait as a marker of overall cognitive function and warrants longitudinal investigation in this high risk group.

4.0 Introduction

A significant body of literature has emerged to suggest that gait speed, and in particular dual-task gait speed is associated with overall cognitive function in addition to the development of mild cognitive impairment and dementia. Given the risk of later cognitive impairment and dementia in those with T2DM, it follows that gait speed, especially assessed in a longitudinal fashion, may provide a potential marker of cognitive impairment/dementia in those at high risk of cognitive decline.

In a recent paper from the English Longitudinal Study on Ageing (ELSA), walking speed and incident dementia was assessed in community dwelling adults aged 60 years and older. Those with slower walking speeds at baseline and a greater decline in speed over time were at greater risk of dementia (Hackett et al. 2018). Similarly, in the Gait and Brain Study, a longitudinal analysis of older adults aged 65 and older, those who experienced a decline in gait speed were almost 7 times as likely to develop dementia (Montero-Odasso et al. 2018).

Studies like these follow numerous longitudinal studies which have demonstrated the link between slow gait speed and incident cognitive impairment and dementia, supporting the wealth of evidence demonstrating the utility of gait speed and gait speed decline as a potential marker of cognitive impairments and cognitive decline (Rosano & Snitz, 2018, Dumurgier et al. 2017, Welmer et al. 2014, Taniguchi et al. 2017, Doi et al. 2019). This association has been supported in a recent meta-analysis of 36 studies and just under 30,000 participants (Peel et al. 2019)

Whilst most of these studies have occurred in adults aged 60 and older, the longitudinal link between gait and cognition in younger participants remains poorly explored. Some studies have demonstrated an association between gait speed and different domains of cognition such as executive function and visuospatial memory (Jabourian et al. 2014). However, less well-explored is the relationship between midlife gait velocity and

longitudinal cognitive trajectories, and studies on middle-aged adults are limited to cross-sectional analysis.

One of the pertinent questions to incorporating the use of gait as a biomarker of later cognitive impairment/dementia risk is around the gait task which is used. Many studies use standardised assessments such as the timed-up and go test, which appears to have good reliability and validity in older populations. However, in midlife, such tasks may not correlate as well with cognitive function and hence dementia risk. Some investigators have incorporated the use of a dual-task gait paradigm where a participant is asked to walk whilst carrying out another task (usually reciting alternate letters of the alphabet aloud or subtracting serial 7s from 100 aloud). Such a multitasking paradigm has significant executive function and attentional demands and can be seen as more ecologically valid than standard assessments of walking speed.

Previous studies have demonstrated an association between slower dual task speed and greater impairment in executive function, short term memory and composite assessments of processing speed, attention and executive function (Holtzer et al. 2014, Holtzer et al. 2016). Impairment in dual-task paradigms is seen in both mild cognitive impairment as well as dementia (De Sanctis et al. 2014, Theill et al. 2011). In one of the largest studies to date examining dual-task gait and cognitive performance, and also one of the few unique studies to focus on non-older or non-impaired populations, significant associations have been found between processing speed, short term memory and sustained attention in adults aged 50 and older and performance on a dual-cognitive paradigm (Killane et al. 2014). Such studies support the potential use of dual-task gait as a potential marker of later cognitive impairment/dementia risk. Again, like standard measures of gait speed, trajectories of dual task gait and their relationship to cognitive remains virtually unexplored in midlife.

Whilst gait speed and characteristics have been well-researched in T2DM patients with peripheral neuropathy, it is not clear the effect of midlife T2DM on gait speed. Further,

the association between gait speed and cognitive function, specifically in T2DM, has never been assessed. Those studies on patients with T2DM have demonstrated a greater gait variability in addition to slower walking speeds overall (Allet et al. 2008). These effects have also been demonstrated in small numbers of participants without T2DM associated neuropathy (Allet et al. 2009). In the Rotterdam Study, T2DM was associated with worse tandem gait and slower pace in adults aged 45 yeas and older (Maksimovic et al. 2016). Despite these studies demonstrating slower walking speed in T2DM, the longitudinal changes in gait speed in T2DM have rarely been explored. Further, the relationship between gait, cognition and risk of cognitive impairment in T2DM has never been assessed.

In the current analysis, we aim to explore whether gait parameters (namely self-selected, fast and dual-task gait speed in addition to dual task cost) predict cognition in midlife T2DM. Given the increased risk of dementia and cognitive impairment seen in T2DM as well as the compelling evidence linking gait parameters and changes in gait speed to cognitive trajectories in community dwelling older adults, we aim to assess this relationship in an otherwise healthy cohort with T2DM in midlife. Further, beyond the scope of the current chapter, we aim for follow participants longitudinally to assess the changes in gait parameters over time and the impact of these on cognitive trajectories. It is our central hypothesis that gait parameters may have potential utiliy as biomarkers of later cognitive decline in T2DM.

4.1 Gait Speed in ENBIND Cohort

Gait speed was measured for all participants across three gait tasks: (i) self-selected gait speed (or usual gait speed), (ii) maximal gait speed and (iii) dual-task gait speed. Gait speeds were normally distributed and mean gait speeds across all study participants were as follows: (i) self-selected speed: 1.21 ± 0. 16ms^{-1}, (ii) maximal gait speed: 1.66 ± 0.25ms^{-1} (iii) dual-task speed: 1.21 ± 0. 19ms^{-1}. For the dual task condition, the Dual Task Cost (DTC) was calculated as the difference between self-selected and dual-task gait speeds divided by the initial self-selected speed. It was normally distributed with a mean percentage cost of +1.8 ± 0.9%. DTC was normally distributed with a range of observations from -20.5% to + 32%, with positive cost indicating a greater slowing effect on the addition of the dual task and negative cost indicating a quickening effect on addition of the cognitive task. DTC scores were Z-transformed as above in order to further facilitate linear analysis. The distribution of these four metrics is detailed below in Figure 4.1

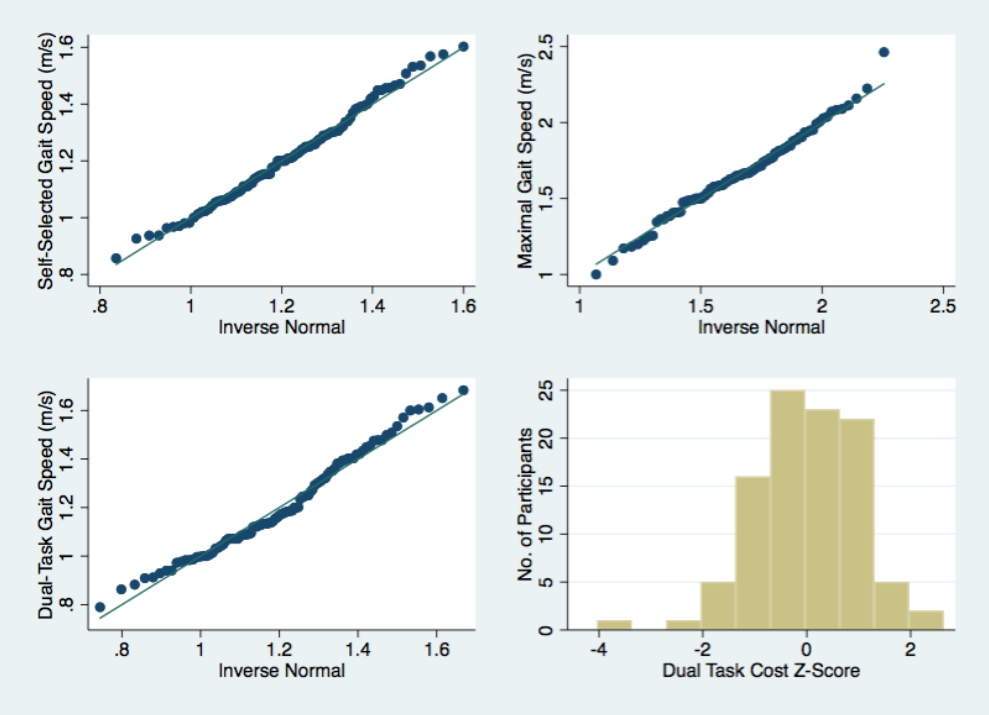

Figure 4.1. Distribution of (i) self-selected, (ii) maximal, (ii) dual task gait speeds in addition to the distribution of dual-task cost. *All metrics were normally distributed.*

Dual Task Cost (DTC) represents the difference between performance on the dual task vs the self-selected (or usual) gait speed, expressed as a percentage of self-selected gait speed.

4.2 Gait Speed in Type 2 Diabetes Mellitus (T2DM)

Type 2 Diabetes was associated a significantly slower gait speed on the self-selected task (1.36 ± 0.16ms^{-1} for T2DM vs 1.16 ± 0.13ms^{-1} for healthy controls, t = 6.55, p<0.001). Similarly, for the maximal gait speed task, T2DM was associated with a significant slowing effect (1.82 ± 0.20ms^{-1} for T2DM vs 1.59 ± 0.24ms^{-1} for healthy controls, t = 6.55, p<0.001). Finally, in the dual-task condition, T2DM was again associated with significantly poorer performance in terms of dual-task speed (1.37 ± 0.19ms^{-1} for T2DM vs 1.14 ± 0.16ms^{-1}, t = 6.22, p<0.001) and a trend towards poorer performance in terms of dual task cost (Z-Scores: 0.22 ± 0.9 for healthy controls, -0.09 ± 1.05 for T2DM, t = 1.34, p = 0.09). There was no significant between group difference on letter reached in the alphabet or number of errors made in the dual task (Z = -0.33, p = 0.74 15, 14-18 for T2DM, 14, 14-18 for healthy controls for letter reached; Z = -1.08, p = 0.28, 0 0-2 for healthy controls; 1, 0-2 for T2DM)

Multivariate analysis was conducted using fixed effects linear regression and included covariates known to impact on gait speed in midlife. After robust covariate adjustment controlling for age, gender, body mass index (BMI), total number of medications, use of antidepressant medication and Charlson Comorbidity index (CCI), T2DM was associated with significantly slower gait speed on both self-selected (β: -0.19, 95% CI -0.31 - -0.06, p = 0.004) and fast gait speed tasks (β: -0.10, 95% CI -0.30 - -0.09, p = 0.039). On the dual task, T2DM was associated with slower gait speed (β: -0.18, 95% CI -0.33 - -0.03, p = 0.023), but not associated with greater dual-task cost (β: -0.4, 95% CI -0.7 - 0.02, p = 0.639). Further, T2DM was not associated with greater likelihood of error (on poisson regression adjusting for the above covariates) in the cognitive component of the dual

task (IRR: 1.30, 0.70 – 2.41, p =0.40) or with the letter reached during the task (IRR: 1.08, 0.93- 1.26, p = 0.295).

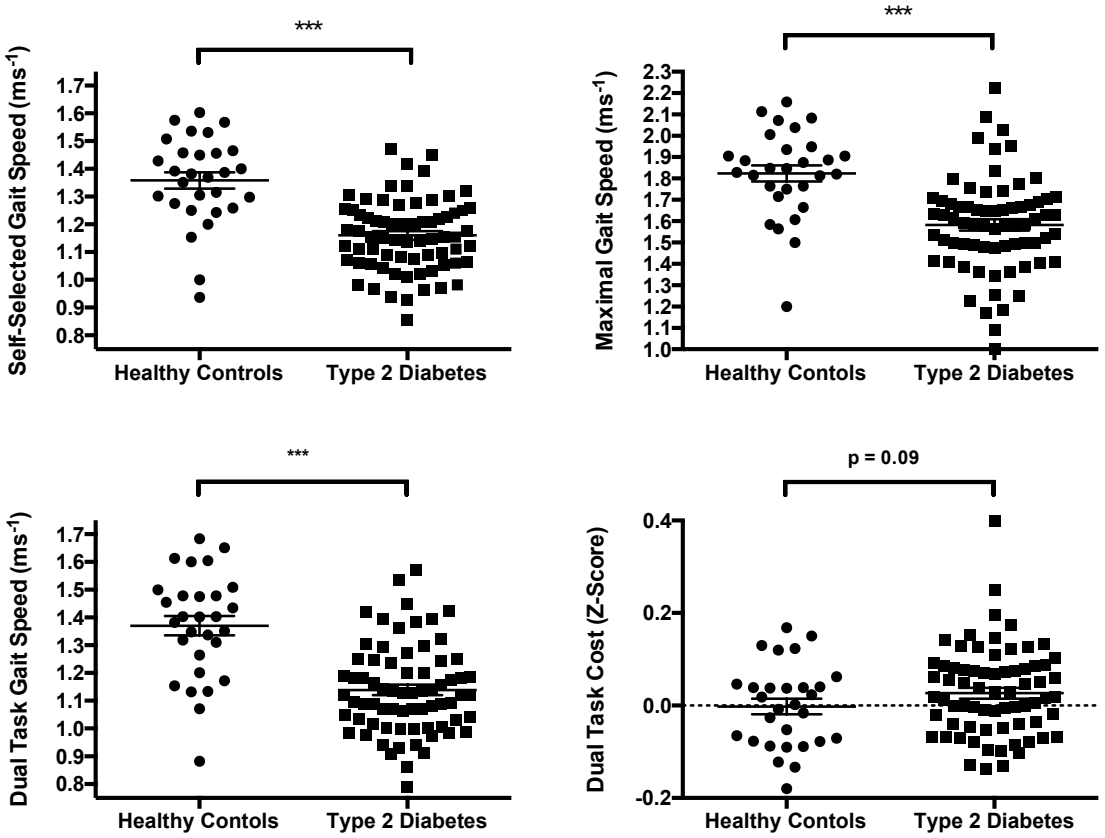

Figure 4.2 Type 2 Diabetes Mellitus (T2DM) is associated with slower self-selected and maximal gait speed in addition to slower speed on a dual task paradigm. *Whilst a significant slowing effect was seen for slow, fast and dual-task gait speed, there was no significant difference in terms of Dual Task Cost between those with midlife Type 2 Diabetes and healthy controls*. ** = p<0.05.*

	Self-Selected Gait Speed (m/s)		Maximal Gait Speed (m/s)		Dual Task Speed (m/s)		Dual Task Cost (%)	
Predictor	**β Coef. (95% CI)**	**P-Value**	**β -Coef. (95% CI)**	**P-Value**	**β -Coef. (95% CI)**	**P-Value**	**β -Coef. (95% CI)**	**P-Value**
T2DM	-0.19 (-0.31 - -0.06)	**0.004***	-0.10 (-0.30 – 0.09)	**0.039***	-0.18 (-0.33 – 0.03)	**0.023***	-0.02 (-0.07 – 0.04)	0.639
Age	-0.02 (-0.01 – 0.00)	0.254	-0.01 (-0.01 – 0.00)	**0.026***	-0.01 (-0.01 – 0.01)	0.712	-0.01 (-0.00 – 0.00)	0.510
Gender	-0.04 (-0.10 – 0.01)	0.129	-0.01 (-0.14 – 0.04)	0.270	-0.02 (-0.09 – 0.05)	0.653	-0.01 (-0.06 – 0.02)	0.220
Body Mass Index (BMI)	-0.00 (-0.01 – 0.01)	0.121	-0.00 (-0.01 – 0.00)	0.837	-0.01 (-0.01 – 0.00)	0.084	-0.00 (0.00 – 0.01)	0.333
Antidepressant Use	0.02 (-0.07 – 0.11)	0.697	0.01 (-0.13 – 0.16)	0.877	0.05 (-0.07 – 0.16)	0.417	-0.02 (-0.09 – 0.4)	0.505
Charlson Comorbidity (CCI)	0.02 (-0.08 – 0.13)	0.629	0.02 (-0.08 – 0.13)	0.549	0.03 (-0.10 – 0.16)	0.625	0.01 (-0.09 – 0.14)	0.831

Table 4.1. Type 2 Diabetes Mellitus (T2DM) was associated with a significant slowing effect on self-selected, maximal and dual-task gait speed.

*Whilst slower gait speeds were seen for those with T2DM on the dual-cognitive task, there was no significant effect of Type 2 Diabetes on Dual Task Cost (DTC). Other covariates associated with fast gait speed include age and number of medications * = p<0.05.*

4.3 General Cognitive Function and Gait Speed in T2DM

In order to analyse effects of gait speed on general cognitive function, we examined the association between gait speed and cognitive performance using the MoCA. As in the previous chapter, likelihood of error on the MoCA was analysed using a poisson regression model due to the left skew of the data. Further, in order to provide covariates with easier interpretation, quartiles of gait speed and Dual Task Cost were created. This resulted in the creation of a rate ratio (likelihood of error on MoCA) representing the change per change in quartile from fastest to slowest and from best performance to worst performance on the dual-task.

In all participants combined, self-selected gait speed quartile was associated with likelihood of error on the MoCA (IRR 1.32, 1.15 – 1.52, p <0.001). However, there was no evidence for a T2DM specific effect as assessed by an interaction between group status (T2DM vs healthy controls) and gait speed quartile (IRR for T2DM*self-selected speed quartile 1.31, 0.79 – 2.16, p = 0.23). Similar results were seen for the maximal gait speed task with a significant increase in likelihood of MoCA error associated with decreasing quartile (IRR 1.49, 1.29 – 1.73, p<0.001). Again, no T2DM-specific effect was observed (IRR for interaction 1.31, 0.79 – 2.16, p = 0.88). Finally, the results were similar for the dual task gait speed where gait quartile predicted likelihood of MoCA error (IRR: 1.63, 1.40-1.90, p<0.001). Again no specific interaction between group status and gait quartile was seen (IRR: 1.10, 0.70-1.71, p = 0.69)

On analysing likelihood of MoCA error by Dual Task Cost (DTC) quartile, there was a significant quartile effect on MoCA error (IRR: 1.64, 1.41 – 1.91, p<0.001). Again, no evidence was seen for a group-by-quartile interaction (IRR: 1.01, 0.67-1.52, p = 0.67) indicating that the association between dual task cost quartile and likelihood of MoCA error are independent of T2DM status. Analysis were then repeated controlling for age, gender, family history of years of formal education. Associations persisted only for the

effect of DTC quartile on likelihood of MoCA error, independent of group (IRR: 1.56, 1.07 – 2.28, p = 0.021). Results are given in detail below in Table 4.2

	Self-Selected Gait Speed		Maximal Gait Speed		Dual Task Speed		Dual Task Cost	
Unadjusted Model	**IRR (95% CI)**	**P-Value**	**IRR (95% CI)**	**P-Value**	**IRR (95% CI)**	**P-Value**	**IRR (95% CI)**	**P-Value**
Gait/Cost Quartile	1.32 (1.15 – 1.52)	**<0.001****	1.49 (1.29 – 1.73)	**<0.001****	1.63 (1.40 – 1.89)	**<0.001****	1.64 (1.41 – 1.91)	**<0.001***
Adjusted Model								
Gait/Cost Quartile	0.82 (0.49 - 1.35)	0.431	0.96 (0.60 – 1.53)	0.879	1.29 (0.85 - 1.95)	0.239	1.56 (1.07 – 2.29)	**0.021***
Type 2 Diabetes (T2DM)	0.89 (0.31 – 2.53)	0.824	0.92 (0.32 – 2.67)	0.876	1.01 (0.33 – 3.08)	0.986	1.77 (0.50 – 6.39)	0.378
Quartile*T2DM Interaction	1.48 (0.87 – 2.51)	0.145	1.35 (0.83 – 2.21)	0.227	1.18 (0.75 – 1.85)	0.486	1.03 (0.68 – 1.57)	0.879
Age	1.03 (1.01 – 1.06)	**0.002***	1.03 (1.01 – 1.05)	**0.023***	1.03 (1.01 – 1.06)	**0.002***	1.04 (1.02 – 1.06)	**<0.001***
Gender	0.81 (0.59 – 1.11)	0.200	0.82 (0.59 – 1.13)	0.216	0.81 (0.59 – 1.11)	0.186	0.88 (0.63 – 1.21)	0.423
Years of Formal Education	0.93 (0.87 – 0.99)	**0.028***	0.94 (0.88 – 1.00)	**0.046***	0.95 (0.89 – 1.01)	0.132	0.94 (0.88 – 1.01)	0.074
Family History of Dementia	0.77 (0.48 – 1.25)	0.282	0.75 (0.47 – 1.21)	0.244	0.77 (0.48 – 1.25)	0.295	0.78 (0.48 – 1.25)	0.303

Table 4.2. Fully adjusted models exploring the effect of fast, slow and dual task gait speeds on likelihood of error on the Montreal Cognitive Assessment (MoCA). *Overall, gait speed quartiles in addition to quartiles of Dual Task Cost (DTC) were analysed using adjusted Poisson Regression models adjusting for important covariates with a known impact on cognitive function. Unadjusted, there were significant associations between decreasing self-selected speed quartile, maximal gait speed quartile, dual task speed and DTC and greater likelihood of error on the MoCA. Under the fully adjusted model, only the association for Dual Task Cost (DTC) persisted, with greater cost on dual task speed associated with greater likelihood of error on the MoCA.*

4.3 Neuropsychological Test Scores and Gait Speed in T2DM

4.3.1 Reaction Time

Linear regression was performed on standardised scores (Z-Scores) on the initial reaction time task and analysis performed both in terms of Median Duration Reaction Time (MDRT) and Median Duration Movement Time (MDMT). Unadjusted, there was a trend for an association with self-selected gait speed and reaction time (β Coef: 1.16, -0.06 – 2.40 p 0.06) and a significant association with maximal gait speed (β Coef: 0.95, 0.17 – 1.73, p = 0.01). No association was seen for dual task speed (β Coef: 0.70, -0.32 – 1.72, p = 0.18) or dual-task cost (β Coef: 0.05, -2.04 – 2.14, p = 0.96). For the mean duration movement time, there was no association with self-selected gait speed (β Coef: 0.69, -0.55-1.94, p = 0.27), a trend for an association with maximal gait speed (β Coef:0.75, -0.04-1.53, p = 0.06) and no association for dual-task gait speed(β Coef: 0.25, -0.78 – 1.28, p = 0.63) or dual-task cost (β Coef: 0.62, -1.47 – 2.70, p = 0.56). Associations and trends did not persist following covariate adjustment and there were no significant results for group-speed or group-cost interactions in the regression models. Results are given in tabular format in Tables 4.3 – 4.6 (Unadjusted and Adjusted Models using both Z-scores and Non-Standardised Score).

4.3.2 Memory

4.3.2.1 Paired Associates Learning

There was no association between total errors made on the Paired Associates Learning Task and self-selected (β Coef: 0.18, -1.07 – 1.41, p = 0.78), fast (β Coef: 0.57, -0.22 – 1.36, p = 0.15), dual-task (β Coef: 0.33, -0.69 – 1.35, p = 0.52) gait speed quartiles and no association between dual-task cost (β Coef: 0.58, -2.65 – 1.49, p = 0.581). No associations were seen following robust covariate adjustment and adding in an interaction term for gait speed/cost and T2DM status. For the first attempt memory

score on the same task, there was no association with self-selected gait speed (β Coef: 0.68, -4.10 – 5.46, p = 0.78), fast gait speed (β Coef: 2.20, -0.83 – 5.23, p = 0.15), dual task speed (β Coef: 1.28, -2.65 – 5.20, p = 0.519) or dual-task cost (β Coef: -2.23, -10.2 – 5.74, p = 0.58). Again, covariate adjustment and interaction analysis produced no significant results (Tables 4.3/4.4)

4.3.2.2 Spatial Working Memory

Again on the spatial working memory task, no association was seen between self-selected gait speed and between error performance on the 4,6 and 8 box levels (β Coef: 0.53, 0.72 – 1.78, p = 0.4). A trend was observed for the association between maximal gait speed and between error performance (β Coef: 0.67, -0.11-1.46, p = 0.09) but not for dual task speed (β Coef: 0.26, -0.76 – 1.29, p = 0.61) or dual task cost (β Coef: 0.24, -1.85 – 2.32, p = 0.82). A trend was also observed between maximal gait speed and performance on the 12 box level (β Coef = 0.69, -0.10 – 1.48, p = 0.09) but not for other gait parameters. For the Strategy parameter, there was no association between self-selected gait speed and strategy score (β Coef: 0.92, -0.32 – 2.15, p = 0.14). However, an association was observed between maximal gait speed and strategy score (β Coef: 0.99, 0.21-1.77, p = 0.01), but not for dual-task speed (β Coef: 0.24, -0.78 – 1.27, p = 0.64) or dual-task cost (β Coef: 1.26, -0.81 – 3.33, p = 0.23). Again, associations did not persist after covariate adjustment and no T2DM specific effects were observed assessed using the T2DM*Quartile interaction term.

4.3.2.3 Pattern Recognition Memory

Self-selected gait speed was not associated with the percentage of correct responses in the immediate task (β Coef: 0.20, -1.41 – 1.02, p = 0.75) and neither was fast gait speed (β Coef: -0.04, -0.82 – 0.75, p = 0.93), dual-task speed (β Coef: -0.10, -1.09 – 0.91, p = 0.85) or dual-task cost (β Coef: -0.27, -2.31 - 1.76, p = 0.79). For the delayed task where the stimuli are re-presented after 20 minutes, there was a trend for association with

self-selected gait speed (β Coef: -1.06, -2.31 – 0.19, p= 0.09) but not with maximal gait speed (β Coef: -0.62, -1.45, 0.20, p = 0.13) or dual-task speed (β Coef: -0.85, -1.89 – 0.18, p = 0.10) and no association was seen for dual task cost (β = 0.11, -2.09 – 2.10, p = 0.99). No results were significant after covariate adjustment and addition of interaction variables to assess T2DM-specific associations (Tables 4.3/4.4).

4.3.3 Executive Function

4.3.3.1 One Touch Stockings of Cambridge

Performance on the One Touch Stockings of Cambridge is believed to have significant executive function demands. There was a significant association between the percentage correct on first choice and self-selected gait speed (β Coef: -1.54, -2.75 - -0.33, p = 0.01) in addition to maximal gait speed (β Coef: -1.08, 1.86 - -0.31, p = 0.006), dual-task gait speed (B Coef: -1.532, -2.51 - -0.55, p = 0.003) but not dual task cost (β Coef: 1.60, -0.46 - 3.66, p = 0.128). Only the association for dual task speed persisted after robust covariate adjustment (β Coef: -1.48, -2.7 – 0.29, p = 0.016), but the effect was independent of T2DM status (β Coef for interaction: 0.77, -1.64 – 3.18, p = 0.53). There was no association between the latency to first choice and any of the gait parameters (β Coef: -0.16, -1.41 – 1.09, p = 0.80 for self-selected, β Coef: 0.18, -0.62 – 0.98, p = 0.65 for maximal, β Coef: -0.09, -1.11 – 0.94, = 0.87 for dual-task and β = 0.09, -1.99 – 2.18, p = 0.93 for dual-task cost).

4.3.3.2 Rapid Visual Processing

Both self-selected (β Coef: -1.29, -2.52 – 0.57, p = 0.04) and maximal gait speed (β Coef: -0.8, -1.60 – -0.01, p = 0.05) was significantly associated with the A' Prime outcome measure on the Rapid Visual Processing Task. There was a trend for an association between dual task speed and A' prime score (β Coef: -0.93, -1.95 – 0.09, p = 0.07), but no association for dual task cost (β Coef: 0.48, -1.6 – 2.57, p = 0.65). The association

between self-selected gait speed and A' prime score strengthened after covariate adjustment (β Coef: -1.97, -3.45 – 0.49, p = 0.01) but there was no T2DM specific effect. The association with maximal gait speed was reduced to a trend after adjustment (β Coef: 0.87, -1.78 – 0.04, p = 0.06) whilst the association with dual task gait speed became significant (β Coef: -1.45, -2.64 – -0.26, p = 0.02), but again no T2DM-gait interactions were observed.

All analysis were repeated examining only the fastest vs slowest quartile in a binary fashion (N = 51 participants). Results of these models for unadjusted (Table 4.5) and fully adjusted for age, family history, gender and years of education (Table 4.6) are presented below.

Unadjusted Coefficients [Z-scored values]	**Self-Selected Gait Speed**		**Maximal Gait Speed**		**Dual Task Speed**		**Dual Task Cost**	
Predictor	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Reaction Time Task								
RTIF-MDRT	1.16 (-0.06 – 2.40)	0.060	0.95 (0.17 – 1.73)	**0.010***	0.70 (-0.32 – 1.72)	0.182	0.05 (-2.04-2.14)	0.960
RTIF-MDMT	0.69 (-0.55 – 1.94)	0.271	0.75 (-0.04 – 1.53)	0.060	0.25 (-0.78 – 1.28)	0.640	0.62 (-1.47 – 2.70)	0.550
Paired Associates Learning								
PAL-TEA	0.18 (-1.07 – 1.41)	0.782	0.57 (-0.22 – 1.36)	0.150	0.33 (-0.69-1.35)	0.520	0.58 (-2.65 – 1.49)	0.581
PAL-FAMS	0.68 (-4.10 – 5.46)	0.780	2.20 (-0.83 - 5.23)	0.154	1.28 (-2.65 – 5.20)	0.519	-2.23 (-10.2 – 5.74)	0.579
Spatial Working Memory								
SWM-BE468	0.53 (0.72 – 1.78)	0.400	0.67 (-0.11 – 1.46)	0.090	0.26 (-0.76 – 1.29)	0.612	0.24 (-1.85 – 2.32)	0.821
SWM-Strategy	0.92 (-0.32 – 2.15)	0.140	0.99 (0.21 – 1.77)	**0.010***	0.24 (-0.78 – 1.27)	0.640	1.26 (-0.81 – 3.33)	0.232
Pattern Recognition Memory								
PRM-PCI	0.20 (-1.41 – 1.02)	0.751	-0.04 (0.82 – 0.75)	0.929	-0.10 (-1.09 – 0.91)	0.850	-0.27 (-2.31 – 1.76)	0.791
PRM-PCD	-1.06 (-2.31 – 0.19)	0.091	-0.62 (-1.45-0.30)	0.130	-0.85 (-1.89 – 0.18)	0.099	0.11 (-2.09 – 2.10)	0.990
Stockings of Cambridge (OTS)								
OTS-PCFS	-1.54 (-2.75 - -0.33)	**0.010***	-1.08 (1.86 - -0.31)	**0.006***	-1.532 (-2.51 - --0.55)	**0.003***	1.60 (-0.46 – 3.66)	0.128
OTS-MLFC	-0.16 (-1.41 – 1.09)	0.801	0.18 (-0.62 – 0.98)	0.651	-0.09 (-1.11- 0.94)	0.869	0.09 (-1.99 – 2.18)	0.93
Rapid Visual Processing								
RVP-AP	-1.29 (-2.52 – 0.57)	**0.040***	0.80 (-1.60 - -0.01)	**0.049***	-0.93 (-1.95 – 0.09)	0.070	0.48 (-1.6 – 2.57)	0.648
RVP-PFA	0.03 (-1.22 – 1.29)	0.958	-0.45 (-1.26 – 0.36)	0.272	-0.10 (-1.14 – 0.93)	0.842	0.45 (-1.64 – 2.53)	0.672

Table 4.3 Unadjusted models exploring the effect of fast, slow and dual task gait speeds on cognitive performance using the CANTAB Neurospychological Assessment Battery (Z-Scores). *Overall, decreasing gait speed quartile on slow, maximal and dual task speed was associated with poorer performance on measures on measures of executive function such as the One Touch Stockings of Cambridge Task (OTS) or the Rapid Visual Processing Task. Poorer performance on gait speed and dual task cost was determined moving from fastest to slowest quartiles. RTIF-MDRT: Reaction Time Task Mean Duration Movement Time, MDMT: Reaction Speed Task Mean Duration Movement Time, PALTEA: Paired Associates Learning: Total Errors Adjusted, PALFAMS: Paired Associates Learning: First Attempt Memory Score, SWMBE468: Spatial Working Memory Between Errors 4,6,8 Boxes, SMWM: Spatial Working Memory Strategy, PRMPCI: Pattern Recognition Memory Percentage Correct Immediate, PRMPCD: Pattern Recognition Memory Percentage Correct Delayed. OTS PCFS: One Touch Stockings of Cambridge Percentage Correct in First Attempt, OTSMLFC: One Touch Stockings of Cambridge Median Latency to First Choice.*

Unadjusted Coefficients [Absolute Values]	**Self-Selected Gait Speed**		**Maximal Gait Speed**		**Dual Task Speed**		**Dual Task Cost**	
Predictor	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Reaction Time Task								
RTIF-MDRT	-0.01 (-0.01 – 0.01)	0.039	-0.00 (-0.01 - -0.00)	**0.005***	-0.00 (-0.00 -0.00)	0.159	-2.75 (-122 – 116)	0.964
RTIF-MDMT	-0.01 (-0.00 - 0.01)	0.422	-0.00 (-0.01 – 0.00)	0.053	-0.00 (-0.00 - 0.00)	0.599	-54.5 (-239 - 130)	0.558
Paired Associates Learning								
PAL-TEA	-0.01 (-0.01 - 0.00)	0.243	-0.00 (-0.01 - -0.00)	**0.035***	-0.00 (-0.00, 0.00)	0.519	9.88 (-25.5 – 45.2)	0.581
PAL-FAMS	0.03 (-0.03 - 0.09)	0.350	0.01 (-0.00 - 0.02)	0.092	0.00 (-0.01 – 0.01)	0.743	-0.48 (-9.59 - 8.64)	0.918
Spatial Working Memory								
SWM-BE468	-0.00 (-0.00, 0.00)	0.226	-0.00 (-0.01 - -0.00)	**0.043***	-0.00 (-0.01, 0.00)	0.623	-2.28 (-22.5 – 17.9)	0.823
SWM-Strategy	-0.01 (-0.03 - -0.00)	**0.027***	-0.03 (-0.05 - -0.01)	**0.013***	-0.00 (-0.02, 0.01)	0.639	-3.19 (-8.42 – 2.04)	0.229
Pattern Recognition Memory								
PRM-PCI	0.00 (-0.01, 0.01)	0.194	-0.01 (-0.06 - 0.06)	0.998	0.00 (-0.00, 0.00)	0.833	3.75 (-24.3 – 31.8)	0.791
PRM-PCD	-0.63 (-1.42 – 0.16)	0.114	-0.55 (-1.45 - 0.35)	0.226	0.00 (-0.00, 0.00)	0.102	-0.15 (-27.6 – 27.3)	0.992
Stockings of Cambridge (OTS)								
OTS-PCFS	-0.22 (-0.40 - -0.04)	**0.017***	0.03 (0.01 - 0.04)	**0.007***	0.02 (0.00, 0.03)	**0.003***	-4.95 (-11.3 - 1.44)	0.128
OTS-MLFC	0.00 (0.00 - 0.00)	**0.006***	-0.00 (-0.00 - 0.00)	0.614	0.00 (0.00, 0.00)	0.833	-943 (-2142 – 1954)	0.927
Rapid Visual Processing								
RVP-AP	0.62 (0.16 – 1.09)	**0.009***	0.98 (-0.01 - 1.79)	0.052	0.65 (-0.06 – 1.36)	**0.074***	-0.03 (-0.15 – 0.09)	0.648
RVP-PFA	-0.16 (-0.51 - 0.19)	0.360	0.35 (-0.29 – 0.99)	0.218	0.05 (-0.46 – 0.56)	0.847	-0.04 (-0.20, 0.13)	0.672

Table 4.4 Unadjusted models exploring the effect of fast, slow and dual task gait speeds on cognitive performance using the CANTAB Neurospychological Assessment Battery (Absolute Values). *Overall, decreasing gait speed quartile on slow, maximal and dual task speed was associated with poorer performance on measures on measures of executive function such as the One Touch Stockings of Cambridge Task (OTS) or the Rapid Visual Processing Task. Poorer performance on gait speed and dual task cost was determined moving from fastest to slowest quartiles. RTIF-MDRT: Reaction Time Task Mean Duration Movement Time, MDMT: Reaction Speed Task Mean Duration Movement Time, PALTEA: Paired Associates Learning: Total Errors Adjusted, PALFAMS: Paired Associates Learning: First Attempt Memory Score, SWMBE468: Spatial Working Memory Between Errors 4,6,8 Boxes, SMWM: Spatial Working Memory Strategy, PRMPCI: Pattern Recognition Memory Percentage Correct Immediate, PRMPCD: Pattern Recognition Memory Percentage Correct Delayed. OTS PCFS: One Touch Stockings of Cambridge Percentage Correct in First Attempt, OTSMLFC: One Touch Stockings of Cambridge Median Latency to First Choice.*

Adjusted Coefficients [Z-scores]	**Self-Selected Gait Speed**		**Maximal Gait Speed**		**Dual Task Speed**		**Dual Task Cost**	
Predictor	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Reaction Time Task								
RTIF-MDRT	1.06 (-0.19 – 2.31)	0.097	0.79 (-0.03 – 1.62)	0.060	0.60 (-0.44 – 1.64)	0.257	0.17 (-1.93 – 2.26)	0.876
RTIF-MDMT	0.44 (-0.83 – 1.72)	0.492	0.50 (-0.34 – 1.34)	0.237	0.06 (-0.99 - 1.11)	0.913	0.68 (-1.42 – 2.78)	0.522
Paired Associates Learning								
PAL-TEA	-0.28 (-1.50 – 0.93)	0.647	0.13 (-0.68 – 0.94)	0.749	0.03 (-0.98 – 1.03)	0.961	-0.58 (-2.58 – 1.43)	0.571
PAL-FAMS	-0.28 (-1.50 – 0.93)	0.647	0.13 (-0.68 – 0.94)	0.749	0.09 (-3.78 – 3.96)	0.961	-2.22 (-9.95 – 5.52)	0.571
Spatial Working Memory								
SWM-BE468	0.19 (-1.02 – 1.41)	0.753	0.28 (-0.52 – 1.09)	0.483	0.17 (-0.83 – 1.17)	0.738	-0.19 (-2.20 – 1.80)	0.848
SWM-Strategy	0.60 (-0.55 – 1.75)	0.305	0.58 (-0.18 – 1.34)	0.130	0.20 (-0.76 – 1.15)	0.681	0.69 (-1.21 – 2.60)	0.473
Pattern Recognition Memory								
PRM-PCI	-0.01 (-1.26 – 1.25)	0.992	0.18 (-0.65 – 1.01)	0.672	0.01 (-1.02 – 1.04)	0.989	-0.18 (-2.24 – 1.89)	0.867
PRM-PCD	-0.72 (-1.98 – 0.54)	0.259	-0.42 (-1.27 – 0.43)	0.326	0.55 (-1.59 – 0.49)	0.296	-0.19 (-2.25 – 1.87)	0.856
Stockings of Cambridge (OTS)								
OTS-PCFS	-1.23 (-2.47 – 0.00)	**0.050***	-0.84 (-1.65 - -0.19)	**0.045***	-1.34 (-2.34 – -0.34)	**0.009***	1.62 (-0.45 – 3.67)	0.121
OTS-MLFC	-0.31 (-1.54 – 0.93)	0.623	-0.07 (-0.89 – 0.75)	0.861	-0.18 (-1.19 – 0.84)	0.731	0.10 (-1.94 – 2.13)	0.926
Rapid Visual Processing								
RVP-AP	-1.10 (-2.34 – 0.14)	0.082	-0.60 (-1.43 – 0.23)	0.156	-0.09 (-1.89 – 0.16)	0.098	0.67 (-1.41 – 2.74)	0.524
RVP-PFA	0.13 (-1.17 – 1.43)	0.842	-0.47 (-1.33 – 0.39)	0.284	-0.03 (-1.10 – 1.04)	0.953	0.43 (-1.71 - 2.56)	0.693

Table 4.5 Association between Neuropsychological Performance and Gait Speed/Performance Quartiles adjusted for Age, Family History, Education and Gender (Z-Scores).

Unadjusted Coefficients [Absolute Values]	**Self-Selected Gait Speed**		**Maximal Gait Speed**		**Dual Task Speed**		**Dual Task Cost**	
Predictor	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Reaction Time Task								
RTIF-MDRT	-0.00 (-0.00 - 0.00)	0.074	-0.00 (-0.00 – -0.00)	**0.036***	-0.00 (-0.00 – 0.00)	0.750	-9.42 (-129 – 110)	0.876
RTIF-MDMT	-0.00 (-0.00 - 0.00)	0.362	-0.00 (-0.00 – 0.00)	0.149	-0.00 (-0.00 – 0.00)	0.232	-60 (-245 - 126)	0.522
Paired Associates Learning								
PAL-TEA	0.00 (-0.00 - 0.00)	0.689	-0.00 (-0.00 – 0.00)	0.681	-0.00 (-0.00 – 0.00)	0.902	9.8 (-24.5 – 44.2)	0.581
PAL-FAMS	-0.02 (-0.01 - 0.00)	0.694	0.00 (-0.02 - 0.01)	0.776	-0.00 (-0.00 – 0.00)	0.822	-0.64 (-9.48 – 8.21)	0.886
Spatial Working Memory								
SWM-BE468	-0.00 (-0.00 - 0.00)	0.935	-0.00 (-0.01 - 0.00)	0.630	-0.00 (-0.00 – 0.00)	0.919	1.88 (-17.52 – 21.3)	0.823
SWM-Strategy	-0.01 (-0.02 - 0.01)	0.395	-0.02 (-0.04 - 0.01)	0.178	-0.00 (-0.00 – 0.00)	0.832	-1.75 (-6.55 – 3.07)	0.473
Pattern Recognition Memory								
PRM-PCI	0.00 (-0.00 - 0.00)	0.996	-0.00 (-0.00 – 0.00)	0.705	-0.00 (-0.00 – 0.00)	0.950	2.41 (-26.0 – 30.9)	0.867
PRM-PCD	0.00 (-0.00 – 0.00)	0.166	0.00 (-0.00 – 0.00)	0.195	0.00 (-0.00 – 0.00)	0.177	2.49 (-24.5 – 29.4)	0.992
Stockings of Cambridge (OTS)								
OTS-PCFS	0.01 (-0.00 - 0.03)	**0.054***	0.02 (0.00 – 0.03)	**0.043***	0.02 (0.00 – 0.03)	**0.010***	-5.01 (-11.4, 1.35)	0.121
OTS-MLFC	0.00 (-0.00 - 0.00)	0.703	0.00 (-0.00 – 0.00)	0.941	0.00 (-0.00 – 0.00)	0.798	-935 (-2088 – 1904)	0.927
Rapid Visual Processing								
RVP-AP	0.51 (-0.11 - 1.13)	0.105	0.63 (-0.30 – 1.56)	0.182	0.58 (-0.18 – 1.34)	0.131	-0.04 (-0.16 – 0.08)	0.524
RVP-PFA	-0.03 (-0.44 – 0.40)	0.923	0.37 (-0.26 – 0.99)	0.252	0.05 (-0.46 – 0.57)	0.839	-0.04 (-0.21, 0.14)	0.693

Table 4.6 Association between Neuropsychological Performance and Gait Speed/Performance Quartiles adjusted for Age, Family History, Education and Gender (Absolute Values)

Unadjusted Coefficients	**Self-Selected Gait Speed**		**Maximal Gait Speed**		**Dual Task Speed**		**Dual Task Cost**	
Predictor	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Reaction Time Task								
RTIF-MDRT	-0.53 (-1.12 – 0.60)	0.077	-0.51 (-1.04 – 0.32)	0.064	-0.39 (-0.99 – 0.20)	0.191	-0.01 (-0.65 – 0.64)	0.987
RTIF-MDMT	-0.18 (-0.76 – 0.40)	0.542	-0.38 (-0.92 – 0.17)	0.173	-0.17 (-0.80 – 0.45)	0.579	0.01 (-0.55 – 0.56)	0.981
Paired Associates Learning								
PAL-TEA	0.03 (-0.56 – 0.62)	0.917	-0.05 (-1.09 – 0.03)	0.062	0.414 (-1.00 – 0.18)	0.163	-0.20 (-0.77 – 0.38)	0.501
PAL-FAMS	0.12 (-2.16 – 2.40)	0.917	-2.04 (-4.20 – 0.11)	0.062	-1.59 (-3.86 – 0.67)	0.163	-0.75 (-2.98 – 1.48)	0.501
Spatial Working Memory								
SWM-BE468	-0.19 (-0.85 – 0.48)	0.572	-0.05 (-1.18 – 0.18)	0.146	0.00 (-0.62 – 0.635)	0.990	0.06 (-0.55 – 0.66)	0.845
SWM-Strategy	-0.38 (-0.99 – 0.23)	0.213	-0.82 (-1.46 - -0.18)	0.013	-0.06 (-0.67 – 0.55)	0.843	0.37 (-0.19 – 0.92)	0.196
Pattern Recognition Memory								
PRM-PCI	0.03 (-0.61 – 0.68)	0.915	-0.37 (-1.05 – 0.32)	0.286	-0.01 (-0.45 – 0.43)	0.973	-0.06 (-0.72 – 0.60)	0.846
PRM-PCD	0.57 (0.02 – 1 12.)	0.044	0.42 (-0.18 – 1.02)	0.163	0.049 (-0.03 – 1.03)	0.066	0.33 (-0.24 – 0.91)	0.244
Stockings of Cambridge (OTS)								
OTS-PCFS	0.86 (0.3 – 0.28)	**0.004***	0.77 (0.16 – 1.40)	**0.014***	0.84 (0.24 – 1.44)	**0.007***	0.52 (-0.09 – 1.13)	0.092
OTS-MLFC	-0.02 (-0.54 – 0.50)	0.973	-0.22 (-0.81 – 0.38)	0.466	-0.09 (-0.64 – 0.45)	0.722	-0.14 (-0.71 – 0.42)	0.610
Rapid Visual Processing								
RVP-AP	0.68 (0.16 – 1.20)	**0.011***	0.47 (-0.09 – 1.04)	0.097	0.50 (-0.02 – 1.03)	0.059	0.12 (-0.40 – 0.65)	0.462
RVP-PFA	-0.077 (-0.33 – 0.18)	0.546	0.16 (-0.66 – 0.97)	0.704	0.10 (-0.67 – 0.85)	0.801	0.24 (-0.46 – 0.94)	0.501

Table 4.7. Unadjusted analysis comparing only those with in the fastest vs lowest quartile. *Those in the fastest vs lowest quartile demonstrated significant associations between executive function and gait speed quartile for measures of executive function.*

Unadjusted Coefficients	**Self-Selected Gait Speed**		**Maximal Gait Speed**		**Dual Task Speed**		**Dual Task Cost**	
Predictor	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Reaction Time Task								
RTIF-MDRT	0.43 (-1.00 – 0.14)	0.138	-0.29 (-0.90 – 0.34)	0.365	-0.20 (-0.83 – 0.42)	0.509	0.06 (-0.62 – 0.74)	0.863
RTIF-MDMT	-0.10 (-0.71 – 0.50)	0.734	-0.11 (-0.71 – 0.50)	0.725	0.06 (-0.58 – 0.70)	0.850	-0.01 (-0.55 – 0.53)	0.967
Paired Associates Learning								
PAL-TEA	0.16 (-0.43 – 0.74)	0.585	0.40 (-0.99 – 0.20)	0.184	-0.22 (-0.84 - 0.40)	0.474	-0.16 (-0.76 – 0.45)	0.609
PAL-FAMS	0.16 (-0.43 – 0.74)	0.585	0.40 (-0.99 – 0.20)	0.184	-0.22 (-0.84 - 0.40)	0.474	-0.16 (-0.76 – 0.45)	0.609
Spatial Working Memory								
SWM-BE468	0.00 (-0.65 – 0.65)	0.997	-0.08 (-0.83 – 0.66)	0.825	0.09 (-0.55 – 0.73)	0.778	-0.11 (-0.71 – 0.50)	0.722
SWM-Strategy	-0.17 (-0.71 – 0.38)	0.546	-0.41 (-1.11 – 0.29)	0.240	0.05 (-0.55 – 0.64)	0.881	0.12 (-0.41 – 0.65)	0.648
Pattern Recognition Memory								
PRM-PCI	0.00 (-0.68 – 0.68)	0.999	-0.57 (-1.35 – 0.21)	0.147	-0.05 (-0.47 – 0.37)	0.802	-0.07 (-0.77 – 0.64)	0.854
PRM-PCD	0.49 (-0.78 – 1.06)	0.088	0.27 (-0.42 - 0.96)	0.431	0.31 (-0.26 – 0.89)	0.275	0.16 (-0.42 – 0.74)	0.586
Stockings of Cambridge (OTS)								
OTS-PCFS	0.83 (0.23 – 1.47)	**0.008***	0.63 (-0.06 – 1.32)	0.074	0.78 (0.13 – 1.43)	**0.020***	0.50 (-0.15 – 1.15)	0.127
OTS-MLFC	0.05 (-0.48 – 0.58)	0.854	-0.13 (-0.79 – 0.53)	0.693	-0.03 (-0.60 – 0.54)	0.927	-0.09 (0.69 – 0.51)	0.773
Rapid Visual Processing								
RVP-AP	0.06 (0.07 – 1.13)	**0.027***	0.41 (-0.25 – 1.07)	0.212	0.51 (-0.04 – 1.06)	0.068	0.17 (-0.38 – 0.71)	0.548
RVP-PFA	-0.06 (-0.32 – 0.21)	0.665	-0.04 (-0.94 – 0.90)	0.958	0.07 (-0.78 – 0.91)	0.876	0.21 (-0.53 – 0.96)	0.568

Table 4.8. Analysis comparing only those with in the fastest vs lowest quartile adjusted for age, family history, gender and years of education.

Associations persisted for self-selected speed and measures of executive function

4.4 Discussion

In the current analysis, we demonstrated a significant effect of midlife T2DM on gait speed across all three gait tasks. This association persisted controlling for a number of important clinical covariates. We also demonstrated a significant association between slower gait and likelihood of error on the MoCA for all four parameters assessed, however, this only remained significant for Dual Task Cost after adjustment for covariates. Similarly, some associations were observed between maximal and dual-task gait speed and scores on neuropsychological tests of executive function and attention, consistent with previous literature. None of these associations were specific to T2DM and were observed in the cohort overall.

Our results are consistent with previous studies examining gait speed in diabetes. However, out study is notable for several reasons: (i) the exclusion of participants with neuropathy by both symptom screening and clinical assessment, (ii) the relatively shorter duration of diabetes, better diabetes control and younger age of our cohort overall and (iii) the addition of maximal speed and dual task conditions, which have not been explored in the literature on gait speed in T2DM to date.

One of the current questions in the literature addressing gait and cognitive performance is around the nature of the gait task used. In older cohorts than ours, there has been numerous associations between gait parameters and cognitive impairment/dementia in later life. However, in our study, we found that (after adjustment), only the dual task performance (and specifically dual task cost) predicted general cognitive performance. This is likely due to the additional executive function and attentional demands of completing a dual-task paradigm. Such a paradigm may be useful to 'bring out' cognitive deficits (in our study, likelihood of error on the MoCA), particularly in such a young cohort who are otherwise healthy. Our results advocate for the inclusion of the dual-task paradigm in future studies examining the links between cognition and gait.

Whilst the findings linking greater dual task cost to performance on the MoCA in midlife are interesting, it is worthy of note that the effects seen were not specific to those with T2DM. However, given that T2DM was associated with a greater likelihood of error on the MoCA in the first instance, it could inferred from our results that dual-task gait speed may be worthy of further investigation in the T2DM cohort, particularly in its relationship to cognition, given the increased risk of cognitive decline and dementia seen in T2DM. This represents the first investigation of the links between gait speed and cognition in T2DM and the subsequent waves of the ENBIND study will assess longitudinally in this high risk group.

The current study demonstrated a significant association between gait speed, measured across three different conditions (usual gait speed, maximal gait speed and dual-task gait speed) and executive function on the neuropsychological assessment battery (both Rapid Visual Processing and One-Touch Stockings of Cambridge tasks) and the MoCA, which has significant executive function demands. Our findings build on previous reports demonstrating the link between gait speed (particularly dual-task gait speed) and executive function (Killane et al. 2015).

A potential limitation of our study is the use of raw gait speed measures using a stopwatch and physical markers. Whilst such a method provides millisecond precision and whilst all assessments were performed by a single assessor, studies have demonstrated discrepancies between accelerometer and sensor based gait assessment and manual measurement (Maggio et al. 2016). Future work may incorporate wearable sensors and accelerometer/gyroscope Intertial Motion Units (IMU) to provide a more accurate measurement of gait. Further, IMU based measurement of gait may also allow the extraction of other gait parameters, such as gait variability, step width and stride time.

Conclusion

In conclusion, in this chapter we demonstrated a significant slowing effect on T2DM on gait speed across all three tasks. We observed a significant association between performance on a dual-task gait paradigm and liklelihood of error on the MoCA. We didn't observe any T2DM specific associations however, and any associations between gait and cognitive parameters were present in the cohort overall. We present the first analysis of dual-task gait in T2DM and further evidence for the incorporation of dual-tasking into gait analysis in midlife cohorts. Our findings supports the use of gait speed and dual-task gait in particular as markers of cognitive function in midlife and worthy of longitudinal study. It is planned to incorporate assessment of gait and in particular dual task gait into the longitudinal component of the ENBIND study in order to examine changes in gait parameters over time in addition to the ability of gait to predict cognitive decline longitudinally in those with T2DM.

Chapter 5: Examining the Relationship Between Serum Pro-Inflammatory Markers, Type 2 Diabetes, Cognitive Function and Gait Speed

Abstract

Introduction: Previous literature suggests that the immune system as a crucial role to play in the development, pathogenesis and clinical course of Type 2 Diabetes (T2DM). T2DM has been associated with higher levels of pro-inflammatory cytokines such as IL-1β, IL-6 and MCP-1 in plasma and serum. Despite this, the relationship between serum markers of immune system activation and cognitive decrements remains poorly explored.

Methods: Selected serum inflammatory proteins from the ENBIND cohort were analysed. Cognition was assessed using the MoCA & a neuropsychological battery (CANTAB). Levels of C-Reactive Protein (CRP) were analysed in the routine clinical laboratory. Using ELISA, we quantified serum levels of five inflammatory cytokines implicated in either T2DM, cognitive impairment and/or innate immunity: IL-6, IL-1β, IL-12p70, CXCL10 and MCP-1. Linear regression of log transformed values was used to evaluate the relationship between these markers and cognition.

Results: Overall, there were no differences in the levels of any of the six inflammatory proteins analysed between those with T2DM (n=70) and healthy controls (n=31). Examination of MoCA scores revealed that higher levels of both MCP-1 (IRR 0.21, 0.01-0.41, p = 0.04) and CXCL10 (IRR 0.21, 0.03-0.40,p = 0.02) were associated with greater likelihood of error on the MoCA. On assessment of neuropsychological outcome measures, we observed associations with greater levels of pro-inflammatory cytokines with poorer scores on reaction time (for IL-6 levels) and greater probability of error (with increasing MCP-1 levels) on the visual processing task. Finally, we observed a significant association between CRP levels and self-selected (usual) walking speed. None of the above associations persisted after adjusting for age, gender and other covariates with a known impact on cognitive function. No T2DM-specific associations were observed.

Conclusion: We observed no significant differences in levels of inflammatory cytokines analysed between those with T2DM and healthy controls within our ENBIND cohort. Observed relationships between MCP-1 and CXCL10 with general cognitive performance, in addition to associations with neuropsychological test outcomes for MCP-1 and IL-6, did not persist after covariate adjustment.

5.0 Introduction

Despite a large body of evidence demonstrating an increased risk of cognitive impairment or dementia in those with midlife T2DM (Gottesman et al. 2017), knowledge of which particular patients with T2DM who are most at risk is lacking, nor are there biomarkers to identify those individuals. As reviewed in Chapter 1, the few studies which have examined predictors of later cognitive risk in dementia are limited to studies carried out on older participants, with one of the most robust studies to date (Exalto et al. 2013) taking place in those aged 65 and older. Many of these have focused on conventional clinical and laboratory parameters, but few have explored the role of the immune system in mediating the T2DM-dementia link. Peripheral pro-inflammatory markers and cytokines offer a potential marker of assessing cognitive status and later dementia risk in those with T2DM.

The development, pathogenesis and clinical course of T2DM has been associated with greater levels of systemic inflammation, with elevation of pro-inflammatory cytokines such as IL-6, TNF-α and MCP-1 amongst others (Romeo et al. 2012). Macrophages, infiltrating into adipose tissue, liver and pancreatic beta cells have a key role to play in insulin resistance and the development of T2DM. Production of greater levels of cytokines such as IL-6 may result in adipose inflammation and hepatic insulin resistance, and can be detected by higher levels of circulating pro-inflammatory cytokines in sera in T2DM in comparison to age-matched heathy controls (de Fronzo et al. 2016). In fact, elevated levels of TNF-α in those without T2DM but display features of insulin resistance predicts the onset of T2DM (deFronzo et al. 2016). Similarly, in a large population-based study, elevated serum IL-6 has been associated with the later development of T2DM in otherwise healthy individuals (Spranger et al. 2003). Evidently, systemic inflammation has a central role to play in the pathogenesis of T2DM, and is detectable in serum of those with T2DM or at risk of developing T2DM.

Further, in those with T2DM, elevated levels of pro-inflammatory cytokines have been associated with the development of micro and macrovascular complications, including

diabetic retinopathy and neuropathy in addition to accelerated atherosclerosis in those affected by T2DM (King, 2008). Whilst this has been well-documented in the literature, the evidence is limited to cross sectional analysis, and so directionality of causation remains difficult to prove on the basis of such studies. Nevertheless, systemic inflammation has an important role to play in both the initial development of T2DM and the later development of T2DM related complications.

In the unrivalled Edinburgh Type 2 Diabetes Study, the largest detailed study to examine the relationship between type 2 diabetes and cognitive impairment to date, authors assessed the relationship of several pro-inflammatory markers and cognition. After adjusting for several clinical covariates in 1,066 participants, higher levels of IL-6 and TNF-α were associated with poorer cognitive scores and in particular, higher levels of IL-6 correlated with general cognitive function and tests of processing speed, executive function and mental flexibility (Marioni et al. 2010). Similarly, reports in those with T2DM and dementia demonstrate higher levels of CRP and TNF-α in those affected in comparison to either condition alone (Ragy & Kamal, 2017). In a study of two-hundred participants with T2DM with or without Mild Cognitive Impairment (MCI), MCI was associated with higher levels of circulating IL-1β (Forlenza et al. 2011). Whilst such studies have delivered a novel insight into the role of circulating inflammatory markers in the link between diabetes and dementia risk, no study to date has examined this association in those with midlife T2DM or in non-demented/impaired populations.

In the current analysis, we aim to explore whether circulating inflammatory markers have any association with general cognition or cognitive performance on specific tests of neuropsychological function in a non-impaired midlife population with T2DM. This is the first study to explore this association and aims to evaluate the potential utility of peripheral serum inflammatory mediators to predict risk of cognitive impairment/later risk of cognitive decline in those with midlife T2DM.

5.1 Measuring Circulating Pro-Inflammatory Proteins in the ENBIND Cohort

As outlined above, a serum sample was obtained and processed from each participant for the analysis of pro-inflammatory proteins. Candidate proteins for analysis were based on prior findings from the literature relating to both T2DM and cognitive impairment. The final panel of pro-inflammatory proteins chosen consisted of the following: IL-6, IL-1β, IL-12p70, CXCL10 (also known as IP-10), MCP-1 and CRP.

Overall, 101 participants had serum samples obtained (a single venepuncture was unsuccessful). Median (Interquartile Range) concentrations of the panel of pro-inflammatory cytokines in the combined cohort are as follows; IL-6: 4.55 (1.59-11.82) pg/mL, IL-1β: 2.07 (0-8.01) pg/mL, IL-12p70: 1900 (276-3929) pg/mL, CXCL10: 143 (96.16 – 234.89) pg/mL, MCP-1: 349.23 (231-519 pg/mL) and CRP: 1 (1-4) ng/mL. These concentrations were obtained from optimised ELISA experiments, ensuring in as far as possible that readings were taken from the middle of the curve, and not at the start or the end to avoid erroneous extrapolation. Due to the strong right-skew of the serum pro-inflammatory proteins (all demonstrated kurtosis >3), data were also log transformed in order to enable analysis in regression equations.

5.2 Between Group Differences in Pro-Inflammatory Proteins

In the initial analysis, the difference in levels of serum pro-inflammatory proteins were analysed using non-parametric univariate analysis. Of note, there was no significant difference in any pro-inflammatory protein between those with midlife Type 2 Diabetes Mellitus and Healthy Controls. This information is given below in tabular format with the appropriate univariate statistic in Table 5.1 and is illustrated in Figure 5.1

Protein	Healthy Controls	Type 2 Diabetes	z	P
IL-6	4.72 (0 – 11.3) pg/mL	4.53 (1.6 – 12.6) pg/mL	-0.24	0.80
IL-1β	2.07 (0 – 8.12) pg/mL	2.01 (0 – 9.36) pg/mL	0.14	0.89
IL-12p70	1908 (695 – 4931) pg/mL	1883 (147 – 3400) pg/mL	1.16	0.25
CXCL10	149 (107 – 239) pg/mL	142 (89 – 234) pg/mL	0.81	0.42
MCP-1	320 (224 – 458) pg/mL	383 (231 – 634) pg/mL	-1.05	0.29
CRP	1 (0-2) ng/mL	1.5 (1-4) ng/mL	-1.71	0.08

Table 5.1 Median (Interquartile Range) serum concentrations of pro-inflammatory proteins in those with and without Type 2 Diabetes Mellitus (T2DM) recruited into the ENBIND Study. *There were no significant between group differences in the levels of pro-inflammatory proteins on comparing those with midlife T2DM to healthy controls. Statistics given refer to the results of a two-tailed Wilcoxon Rank Sum test*

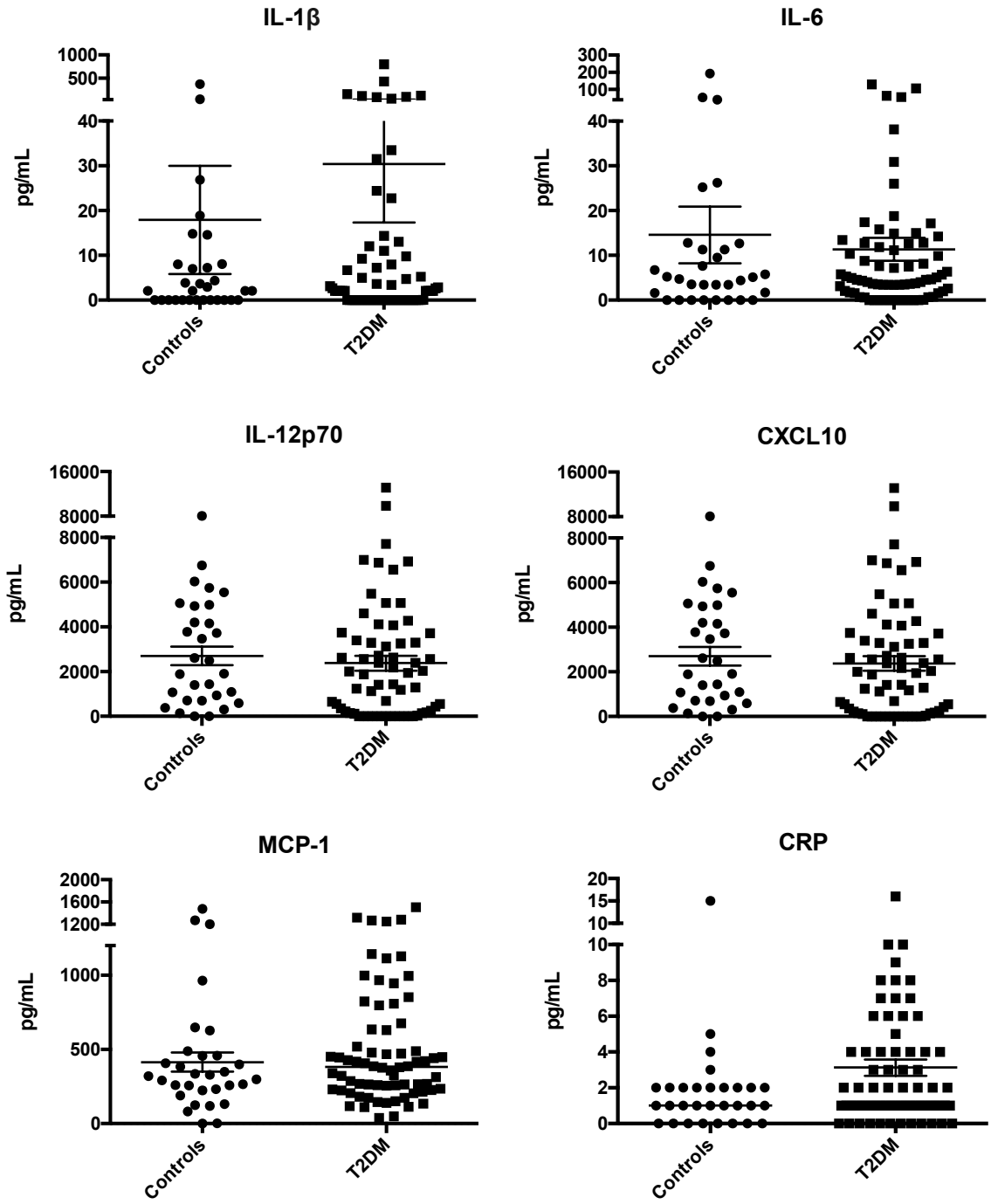

Figure 5.1. Serum Pro-Inflammatory Proteins in Midlife Type 2 Diabetes Mellitus vs. Healthy Controls. *Serum pro-inflammatory proteins were measured using standard ELISA kits at the appropriate dilution as per manufacturer's instructions. C-Reactive Protein (CRP) was measured in the routine hospital laboratory in Tallaght University*

Hospital (TUH), Dublin. There were no significant between group differences for any of the six pro-inflammatory proteins under study

5.2 Association between Serum Markers of Inflammation and General Cognition in Type 2 Diabetes

In order to analyse the relationship between serum pro-inflammatory cytokines and general cognition, we modelled the log transformed values for these proteins as a predictor of likelihood of error on the MoCA (as above in Chapters 4 & 5). This enabled us to examine the association of serum pro-inflammatory proteins on cognitive performance in the overall cohort (both T2DM and healthy controls). In adjusted models, we again used a T2DM*log(concentration) interaction term in order to capture any T2DM-specific associations with cognition.

Overall, a significant association was seen between both increasing MCP-1 levels and decreased likelihood of error on the MoCA (IRR: 0.82, 0.66 – 0.99, p = 0.046) as well as increasing CXCL10 concentration and increased likelihood of error on the MoCA (IRR: 1.24, 1.03 – 1.48, p = 0.022). However these associations did not persist after adjustment for covariates (age, sex, family history of dementia and education) and no T2DM specific interaction was seen. No T2DM specific associations of serum pro-inflammatory proteins and likelihood of error on the MoCA was seen. However, a trend was seen for increasing IL-12p70 concentrations and greater likelihood of error on the MoCA (IRR: 1.00, 1.00 – 1.01) in a fully adjusted model. These results are all provided in full in table 5.2 below with the relevant coefficients for both models.

	IL-1β		IL-6		IL-12p70		MCP-1		CXCL10		CRP	
Unadjusted	**Coef (95% CI)**	**P**	**Coef. (95% CI)**	**P**	**Coef. (95% CI)**	**P**	**Coef. (95% CI)**	**P**	**Coef (95% CI)**	**P**	**Coef (95% CI)**	**P**
Log (conc.)	-0.04 (-0.13-0.06)	0.43	0.00 (-0.0 – 0.01)	0.13	0.07 (-0.07 – 0.22)	0.32	0.21 (0.01 – 0.41)	**0.04**	0.21 (0.03 – 0.40)	**0.02**	0.03 (-0.2 – 0.06)	0.24
Adjusted												
Log (conc.)	0.02 (-0.03 – 0.03)	0.92	0.00 (-0.00 – 0.12)	0.35	-0.01 (-0.00 – 0.00)	0.31	0.50 (-0.12 – 1.10)	0.12	0.01 (-0.46 – 0.46)	0.99	0.05 (-0.05 – 0.15)	0.39
T2DM	1.04 (0.16 – 1.92)	0.02	0.82 (0.32 – 1.30)	0.01	0.30 (-0.34 – 0.95)	0.03	5.18 (1.26 – 9.11)	0.21	-0.20 (-2.84 – 2.43)	0.88	0.95 (0.40 – 1.51)	0.01
Conc.*T2DM	-0.1 (-0.43 - 0.23)	0.55	-0.00 (-0.01 – 0.01)	0.87	0.00 (-0.00 – 0.00)	0.07	0.75 (-0.43 – 0.98)	0.16	0.21 (-0.30 – 0.72)	0.43	-0.05 (-0.16 – 0.06)	0.41
Age	0.03 (0.01 – 0.06)	0.05	0.04 (0.01 – 0.06)	0.01	0.04 (0.02 – 0.05)	0.02	0.04 (0.02 – 0.06)	0.01	0.03 (0.01 – 0.06)	0.01	0.03 (-0.01 – 0.05)	0.01
Sex	-0.5 (-1.11 – 0.15)	0.06	-0.15 (-0.47 – 0.17)	0.36	0.19 (0.52 – 0.14)	0.05	-0.12 (-0.44 – 0.20)	0.48	-0.13 (-0.46 – 0.19)	0.42	-0.17 (0.50 – 0.15)	0.30
Education	-0.05 (-0.12 – 0.17)	0.14	-0.07 (-0.13 – 0.01)	0.03	-0.08 (-0.14 – -0.01)	0.02	-0.07 (-0.13 – 0.02)	0.04	-0.06 (-0.12 – 0.01)	0.08	-0.07 (-0.13 - -0.04)	0.04
Family History	-0.48 (-1.11 – 0.15)	0.13	-0.22 (-0.70 – 0.26)	0.37	-0.19 (-0.68 – 0.31)	0.46	-0.23 (-0.71 – 0.25)	0.35	-0.23 (0.72 – 0.25)	0.34	0.24 (-0.72 – 0.25)	0.44

Table 5.2. Serum Pro-Inflammatory Proteins and Likelihood of Error on the Montreal Cognitive Assessment (MoCA). *We modelled the relationship between the log concentration of serum pro-inflammatory proteins and likelihood of error on the MoCA using a Poisson Regression model. Models were run both unadjusted as well as adjusted for age, gender, years of formal education and family history of dementia. Additionally, we created an interaction term between the serum concentration of a given protein and diabetes status (T2DM vs healthy controls). This enabled us to evaluate for the presence of any T2DM specific effects.*

5.3 Association between Serum Markers of Inflammation and Neuropsychological Test Performance in Type 2 Diabetes Mellitus

In order to assess the relationship between circulating pro-inflammatory proteins and specific domains of cognition in T2DM, we modelled the previously transformed Z-scores across the twelve key neuropsychological outcome metrics using generalised linear regression. As before, the log concentration of the given serum protein was used as a predictor variable in order to assess this relationship. Significant results were obtained for inflammatory proteins and some metrics (below Table 5.3), namely for IL-6 and Mean Duration Movement Time on the Reaction Time Task, IL-12p70 and Percentage Correct on First Attempt on the Paired Associates Learning Task, MCP-1 and the Probability of False alarm on the Rapid Visual Processing Task and IL12p70 and the Percentage Correct in the Delayed Condition of the Pattern Recognition Memory Task. However none of these associations persisted under adjusted models. T2DM-specific effects were examined for by using an interaction between log of serum concentration of the pro-inflammatory proteins and T2DM status (T2DM vs no T2DM). No T2DM specific effects were observed for the association between T2DM, pro-inflammatory protein concentration and neuropsychological test performance. Overall results for all twelve measures are displayed in tabular format below (Table 5.3) and for the four significant associations on unadjusted analysis, a scatterplot with regression line of best fit is given in Figure 5.2.

Unadjusted Coefficients	**IL-1B**		**IL-6**		**IL12p70**		**MCP-1**	
Predictor	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Reaction Time Task								
RTIF-MDRT	-0.04 (-0.15 – 0.67)	0.439	-0.03 (-0.19 – 0.14)	0.759	0.16 (-0.18 – 0.21)	0.873	-0.19 (-0.08 – 0.46)	0.169
RTIF-MDMT	-0.03 (-0.14 – 0.84)	0.625	-0.17 (-0.34 - -0.15)	**0.032***	-0.02 (-0.22 – 0.18)	0.832	0.00 (-0.27 – 0.28)	0.984
Paired Associates Learning								
PAL-TEA	0.00 (-0.14 – 0.13)	0.970	-0.07 (-0.26 – 0.11)	0.430	-0.11 (-0.30 – 0.07)	0.227	-0.13 (-0.14 – 0.40)	0.324
PAL-FAMS	0.00 (-0.14 – 0.13)	0.970	-0.07 (-0.26 – 0.11)	0.430	-0.11 (-0.30 – 0.07)	0.227	-0.13 (-0.14 – 0.40)	0.324
Spatial Working Memory								
SWM-BE468	-0.03 (-0.17 – 0.11)	0.667	-0.07 (-0.25 – 0.11)	0.450	-0.08 (-0.27 – 0.11)	0.429	0.12 (-0.15 – 0.39)	0.362
SWM-Strategy	0.04 (0.17 – 0.10)	0.575	-0.03 (-0.20 – 0.14)	0.721	-0.09 (-0.28 – 0.10)	0.360	0.14 (-0.12 – 0.40)	0.287
Pattern Recognition Memory								
PRM-PCI	0.06 (-0.08 – 0.19)	0.403	-0.03 (-0.19 – 0.14)	0.754	0.08 (-0.11 – 0.28)	0.400	-0.08 (-0.35 – 0.20)	0.584
PRM-PCD	0.09 (-0.04 – 0.23)	0.152	0.10 (-0.08 – 0.28)	0.277	0.02 (-0.17 – 0.20)	0.856	0.02 (-0.09 – 0.46)	0.174
Stockings of Cambridge (OTS)								
OTS-PCFS	-0.01 (-0.13 – 0.11)	0.858	0.00 (-0.17 – 0.18)	0.935	0.21 (0.03 – 0.40)	**0.026***	-0.05 (-0.32 – 0.22)	0.700
OTS-MLFC	-0.05 (-0.16 – 0.07)	0.444	0.05 (-0.22 – 0.12)	0.580	-0.01 (-0.20 – 0.19)	0.941	0.15 (-0.29 – 0.26)	0.909
Rapid Visual Processing								
RVP-AP	0.01 (0.11 – 0.11)	0.933	0.13 (-0.03 – 0.30)	0.121	0.10 (-0.19 – 0.29)	0.282	-0.13 (-0.40 – 0.14)	0.344
RVP-PFA	0.03 (-0.13 – 0.19)	0.710	0.04 (-0.07 – 0.16)	0.436	0.12 (0.32 – 0.08)	0.242	0.40 (0.13 – 0.66)	**0.003***

Unadjusted Coefficients	**CXCL10**		**CRP**	
Predictor	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Reaction Time Task				
RTIF-MDRT	0.04 (-0.18 - 0.26)	0.752	0.02 (-0.04 – 0.08)	0.450
RTIF-MDMT	-0.04 (-0.27 – 0.18)	0.701	0.00 (-0.06 – 0.06)	0.972
Paired Associates Learning				
PAL-TEA	-0.13 (-0.21 – 0.24)	0.911	-0.04 (-0.10 – 0.13)	0.134
PAL-FAMS	-0.13 (-0.21 – 0.24)	0.911	-0.04 (-0.10 – 0.13)	0.134
Spatial Working Memory				
SWM-BE468	-0.00 (-0.22 – 0.22)	0.991	0.03 (-0.02 – 0.09)	0.216
4SWM-Strategy	-0.16 (-0.06 – 0.38)	0.159	0.04 (-0.02 – 0.09)	0.184
Pattern Recognition Memory				
PRM-PCI	0.09 (-0.14 – 0.31)	0.441	-0.02 (-0.08 – 0.04)	0.524
PRM-PCD	0.28 (0.05 – 0.509)	**0.018***	0.03 (-0.03 – 0.09)	0.292
Stockings of Cambridge (OTS)				
OTS-PCFS	0.11 (-0.11 – 0.34)	0.312	0.01 (-0.05 – 0.07)	0.775
OTS-MLFC	-0.02 (-0.25 – 0.21)	0.855	0.03 (-0.03 – 0.08)	0.394
Rapid Visual Processing				
RVP-AP	0.06 (-0.18 – 0.29)	0.634	0.00 (-0.05 – 0.06)	0.889

RVP-PFA	-0.10 (-0.33 – 0.13)	0.389	-0.03 (-0.09 – 0.03)	0.328

Table 5.3. Serum Pro-Inflammatory Proteins and Performance on the Neuropsychological Test Battery (CANTAB). *We modelled the relationship between the log concentration of serum pro-inflammatory proteins and performance on the 12 neuropsychological test outcomes (Z-Scores). Models were tested in the first instance unadjusted and revealed four significant associations at the threshold of p<0.05. However, none of these were significant in fully adjusted models and revealed no T2DM specific effects. Fully adjusted models, in addition to models with the addition of an interaction term for serum concentration and diabetes status (T2DM vs. Healthy Controls) revealed no T2DM specific associations.*

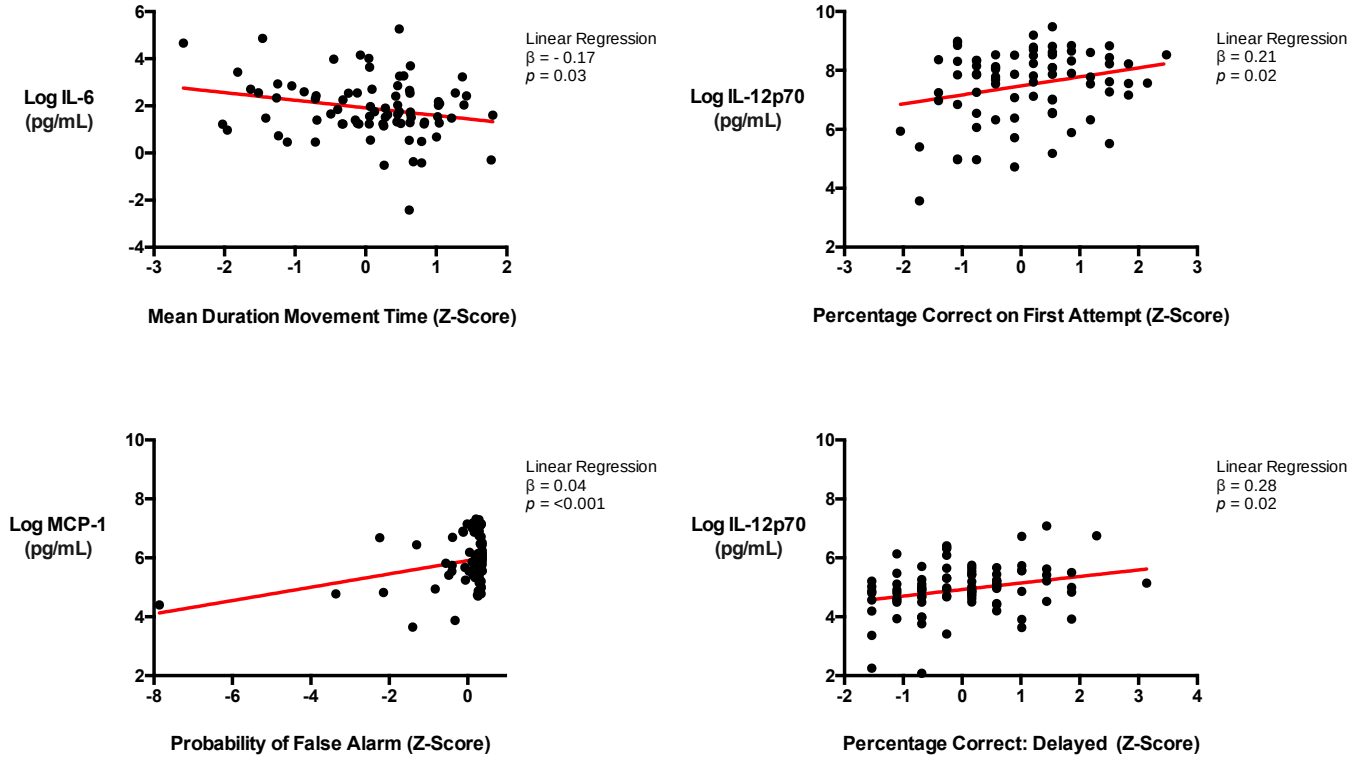

Figure 5.2. Serum Pro-Inflammatory Proteins and performance on the neuropsychological assessment battery (CANTAB). *Linear Regression modelling of the 12 neuropsychological outcomes using the 6 serum pro-inflammatory markers as predictor variables yielded four significant results on univariate analysis. Greater IL-6 concentration was associated with poorer performance on the Mean Duration Movement Time on the Reaction Time Task, with reduced IL-12p70 associated with better performance on the Percentage Correct on First Attempt on the Paired Associates Learning Task and Percentage Correct in the Delayed Condition of the Pattern Recognition Memory Task. Finally, Greater concentrations of MCP-1 was associated with a greater Probability of False alarm on the Rapid Visual Processing Task. None of these associations persisted following adjustment for age, gender, family history and years of formal education. No associations demonstrated a T2DM-specific effect.*

5.4 Association between Serum Markers of Inflammation and Gait Speed/Performance in Type 2 Diabetes Mellitus

In order to assess the relationship between peripheral inflammation assayed using the six selected markers (above) and gait speed/performance in T2DM, we used Spearman correlation analyses to assess the degree of correlation between log-transformed serum concentrations (which were non-parametric in distribution) and gait parameters obtained above.

For the self-selected gait speed task (or usual gait speed), we observed a positive correlation between higher serum CRP and slower gait speed (Spearman's rho = 0.26, p = 0.01). However, this was not specific to T2DM or controls individually. No association was seen between any other serum marker and self-selected gait speed. For maximal (or fast) gait speed, we observed no specific correlations with any serum markers. However a trend was seen for greater IL-1β concentrations and slower maximal gait speed (Spearman's rho = -0.22, p = 0.09). There were no notable trends or significant associations for the remaining gait parameters (dual task speed and dual task cost) with any serum marker. Correlations including results and lines of best fit are demonstrated in Figures 5.3-5.6 below.

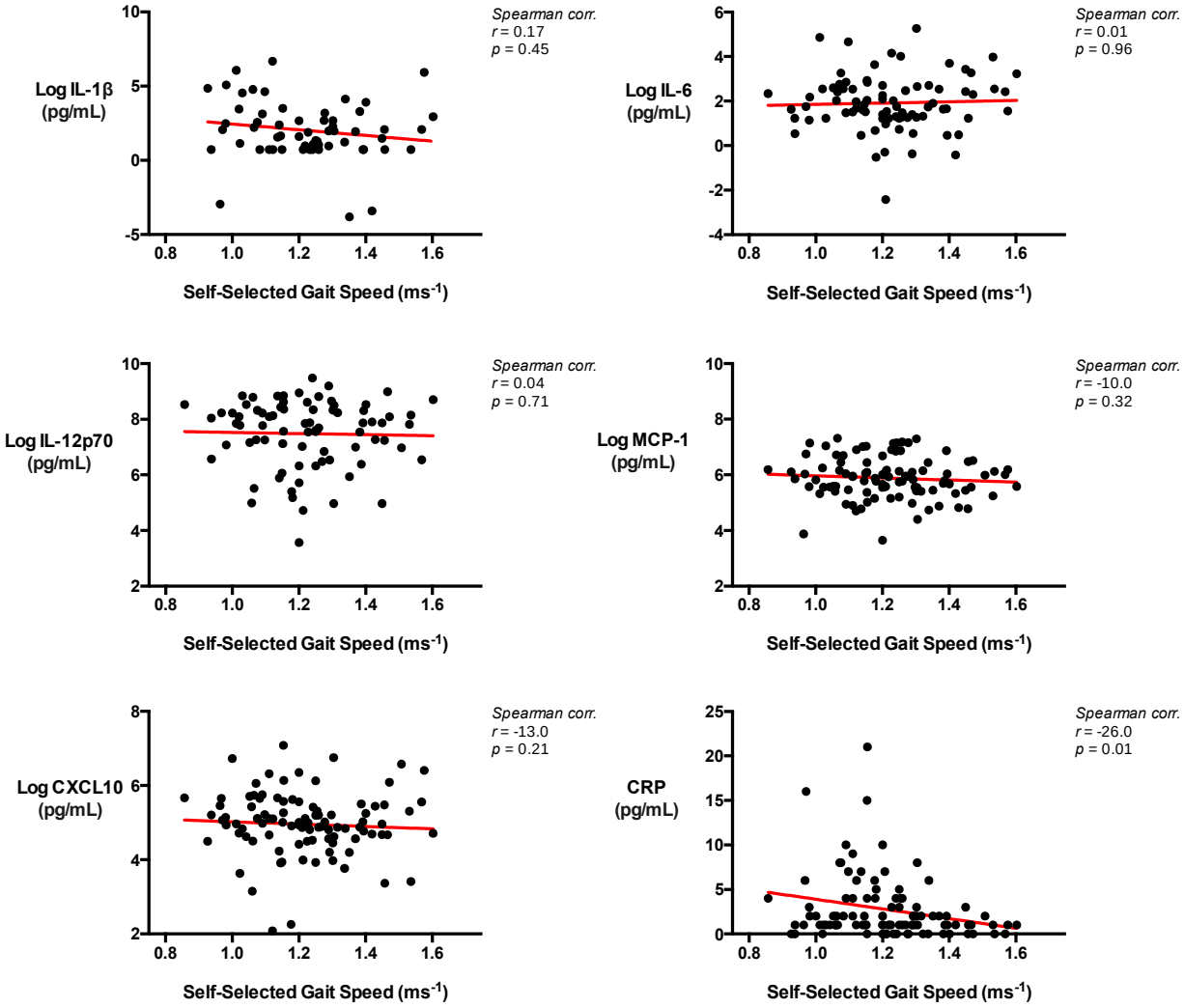

Figure 5.3. Correlation analysis between Serum Pro-Inflammatory Proteins and Self-Selected (or 'usual') Gait Speed. *Correlations between self-selected gait speed (or 'usual gait speed') were analysed using Spearman correlation analysis. There was a statistically significant association between slower self-selected gait speed and increasing CRP. However, there were no significant correlations between other markers of peripheral immune activity and walking speed.*

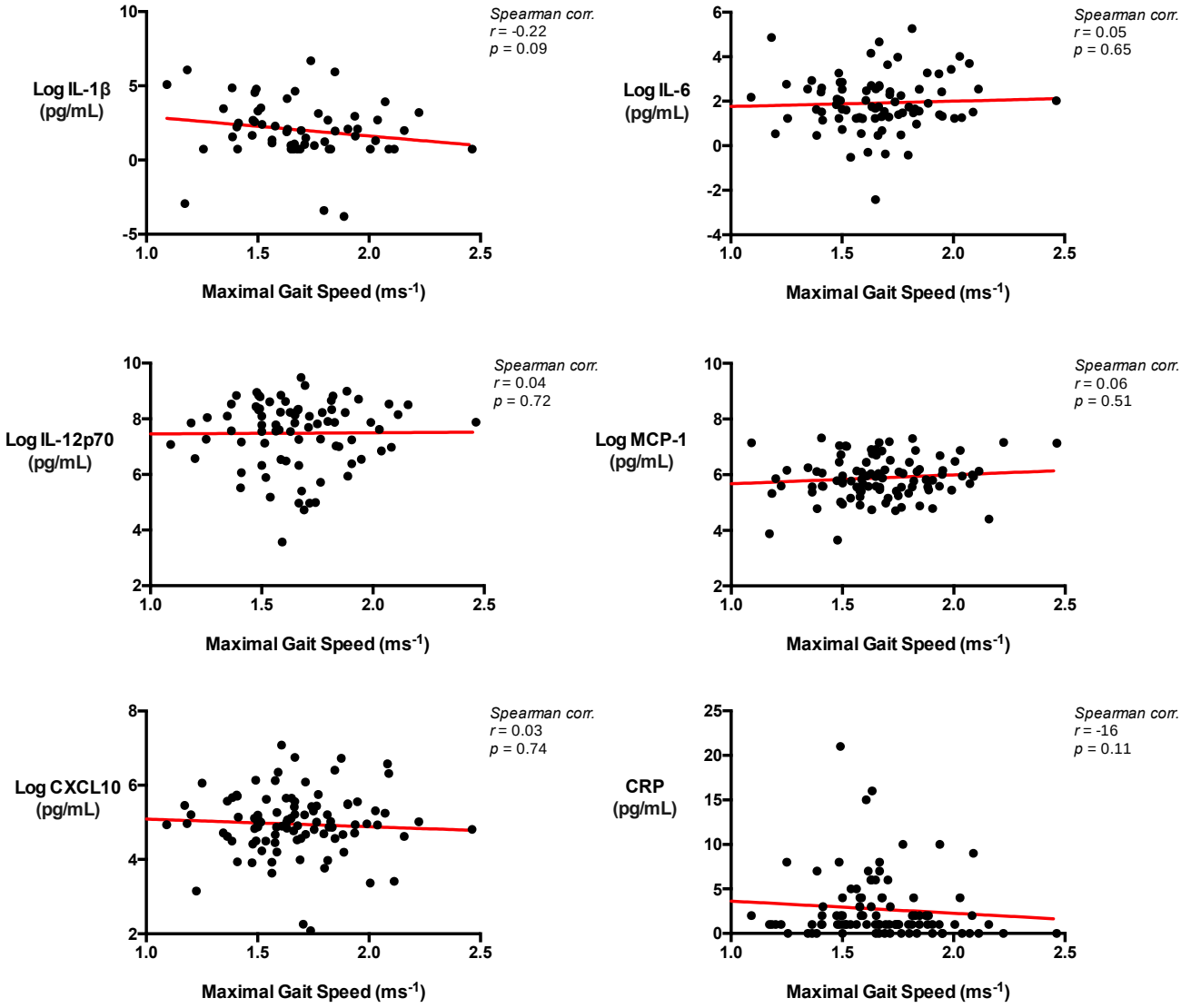

Figure 5.4. Correlation analysis between Serum Pro-Inflammatory Proteins and Dual Task Gait Speed . *Correlations between dual-task gait speed were analysed using Spearman correlation analysis. There were no significant correlations between markers of peripheral immune activity and dual task gait speed.*

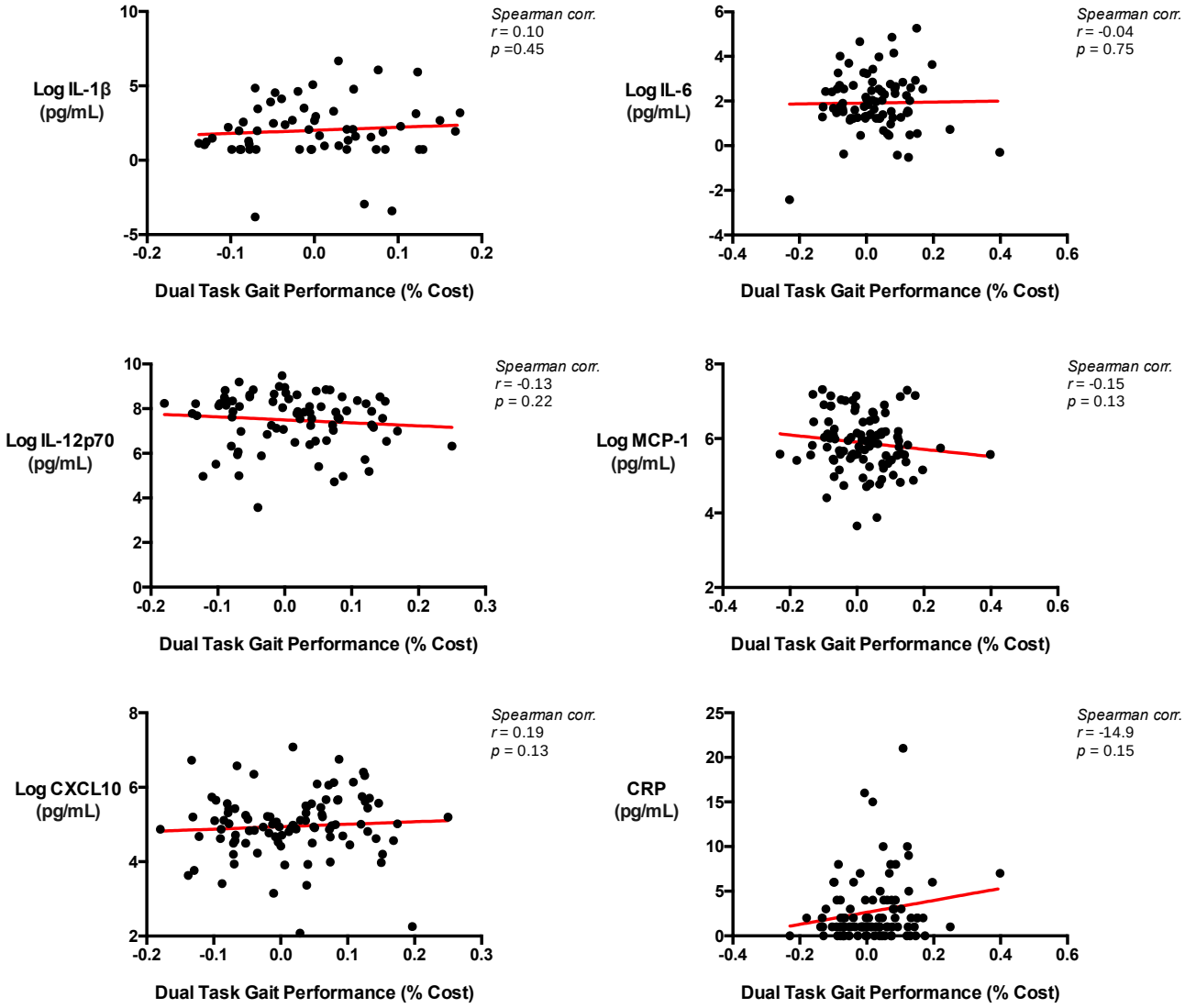

Figure 5.5. Correlation analysis between Serum Pro-Inflammatory Proteins and Dual Task Cost (DTC) . *Correlations between each serum marker of peripheral immune activity and performance on the dual task paradigm. Dual Task Cost (DTC), given as a percentage, refers to the difference in walking speed between the walking as usual task (self-selected gait speed) and the dual-cognitive task (reciting alternate letters of the alphabet), expressed as a percentage change of self-selected gait speed.*

Discussion

In this chapter, we analysed the relationship between circulating inflammatory markers and cognition/gait in midlife T2DM. We found no between group differences in the levels of these markers in sera between those with midlife T2DM and controls. We demonstrated a relationship between greater MCP-1 and CXCL10 levels and poorer general cognitive function (on the MoCA), and between IL-6 and MCP-1 and scores of neuropsychological function. However, none of these associations appeared unique to those with T2DM, and none of them persisted following adjustment for key clinical covariates. Our results are intriguing and worthy of further pause and discussion.

One of the striking results from the current analysis is the lack of between group differences in pro-inflammatory cytokines. Whilst the literature reports that those with T2DM typically demonstrate higher circulating levels of IL-6 and other pro-inflammatory cytokines, studies that have not found an association also exist. For instance, in a recent report, there was no difference in the levels of eight pro-inflammatory cytokines (including IL-6, IL-8 and IL-18) between T2DM and control groups, although differences in mRNA expression of pro-inflammatory genes in whole blood samples were observed (Gupta et al. 2017). In fact, it appears that whilst pro-inflammatory cytokines may be elevated early in a diagnosis of T2DM, as the disease progresses, elevated levels of serum pro-inflammatory cytokines may not persist (Gupta et al. 2017).

This is consistent with a high burden of inflammation at the onset of T2DM before treatment. In our cohort, the mean duration of T2DM was 5 years and nearly all participants were on some form of treatment. Furthermore, our participants demonstrated good glycaemic control. It has been shown that T2DM medication, particularly metformin or GLP-1 analogues, may have an anti-inflammatory effect and can restore some of the aberrant immune functioning in T2DM (Hogan et al. 2013). It may be the case that once treated, pro-inflammatory cytokine levels revert to normal.

Whether or not these increase with the presence of T2DM complications has never been assessed longitudinally.

Nevertheless, we observed some unadjusted associations between pro-inflammatory cytokines, namely MCP-1 (which has a wealth of evidence supporting increased levels in T2DM) and CXCL10 with likelihood of error on the MoCA. Whilst such associations didn't persist after adjusting for covariates and weren't specific to the T2DM group, they warrant further investigation. In the subsequent waves of the ENBIND study, it will be particularly interesting to see if baseline levels of these inflammatory mediators predict incident cognitive decline.

We failed to replicate previous associations of higher levels of IL-6 with poorer cognitive function in T2DM. The potential reasons for this are numerous. In the Edinburgh Type 2 Diabetes Study (Marioni et al. 2010) which reported that increased IL-6 levels were associated with poorer cognitive function, participants were, on average, 15 years older than participants in our study, and also had other complications of T2DM. Increased levels of IL-6 are associated with increasing age, and may reflect chronic inflammation in age-related diseases such as T2DM (Franchesci et al. 2018). Our cohort may simply be too young and well-treated to demonstrate these differences.

However, it must also be acknowledged that our study was relatively small in comparison to previous cohort studies. Given the wide variation seen in serum cytokine levels in the current cohort, it may be possible that our study is under-powered to detect such subtle differences as have been reported previously (Marioni et al. 2010). Future studies should aim to include a higher number of participants where possible and examine cytokine levels at multiple timepoints.

Conclusion

In conclusion, we found no evidence to support the role of circulating pro-inflammatory mediators as predictors of cognitive function in midlife T2DM. We found some associations for MCP-1 and CXCL10, however these were not significant after covariate adjustment and were not specific to T2DM participants. Our findings provide a fascinating insight into the inflammatory profile of those with midlife T2DM and good glycaemic control who are being treated for T2DM. We have demonstrated this in one of the youngest cohorts to date Further waves of the ENBIND study will allow evaluation of longitudinal associations between circulating pro-inflammatory mediators and the risk of cognitive impairment/decrements in T2DM. This will be particularly valuable on foot of the current characterisation of this midlife T2DM cohort, with detailed neuropsychological assessment captured in addition to the range of markers evaluated in the serum of included participants.

Chapter 6: The Relationship between activation of the innate immune system, Type 2 Diabetes, Cognition and Gait: A Role for the NLRP3 Inflammasome?

Abstract

Introduction: Whilst the link between T2DM and cognitive impairment has been widely replicated in the literature, the putative pathophysiology underlying this link has not been investigated. We examined if activation of the innate immune with inflammatory stimuli (particularly with stimulation of the NLRP3 inflammasome) can predict cognitive impairment and gait abnormalities in midlife, and in particular in those with T2DM.

Methods: Peripheral Blood Mononuclear Cells (PBMCs) were isolated from participants with T2DM (N = 39) and Healthy Controls (N = 21). Cells were incubated for 18 hours with a panel of inflammatory stimuli including: with Lipopolysaccharide (LPS; activator of TLR4), Amyloid β-42 (Aβ-42) – the putative pathogenic protein in Alzheimer's Disease, Nigericin (potent inflamamsome stimulator) and poly dA:dT (activator of cGAS-STING pathway). Cytokine production was measured via ELISA and mRNA expression quantified using qPCR. We assessed between group differences (T2DM vs controls) in addition to assessing the association between unstimulated and stimulated protein and mRNA and cognitive and gait parameters obtained in previous chapters.

Results: Treatment of PBMCs under all stimulated conditions resulted in a significant production of both of IL-1β (an inflammasome dependent cytokine) and IL-6. There were no differences between those with T2DM and healthy controls. Greater production of IL-1β following inflammasome stimulated conditions (LPS & Nigericin and LPS & Aβ-42) was significantly associated with greater likelihood of error on the MoCA, which persisted after covariate adjustment. A trend towards a T2DM-specific effect was observed. Furthermore, IL-1β production in response to Nigericin & LPS and Aβ-42 & LPS, in addition to poly dA:dT, was significantly associated with performance on the dual-task gait paradigm (a measure of multi-tasking, executive function and attention).

Conclusions: Our findings demonstrated no between group differences in PBMC responsiveness to innate immune inflammatory stimulants in T2DM. However,

inflammasome activation significantly predicted likelihood of error on the MoCA and dual-task gait performance.

6.0 Introduction

Evidence has accumulated that aberrant activation of the innate immune system, leading to increased production of inflammatory cytokines, has a central part to play in many age-related diseases, including Type 2 Diabetes Mellitus (T2DM) and Alzheimer's Dementia (AD). The innate immune system responds to danger signals which alert our bodies that cellular damage or pathogentic threat may be taking place. On foot of revolutionary discoveries about innate immune signalling, and in particular around Pathogen Recognition Receptors (PRRs) such as Toll Like Receptors (TLRs) and NOD-like receptors (NLRs) and their ligands, Damage-Associated Molecular Pathogens (DAMPs) and Pathogen Associated Molecular Pathogens (PAMPs), intense interest has focused on the more recently characterised inflammasome family of innate immune sensors, and in particularly the NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome.

TLRs are a conserved family of receptors which are involved in the recognition and conveying of pathogens to the immune system (Vidya etl al. 2018). Simulation of TLRs by specific ligands leads to signal transduction through nuclear factor kappa beta (NFκB) and other pathways, the end result of which is the production of pro-inflammatory cytokines (Vidya etl al. 2018). For instance, Toll Like Receptor 4 (TLR4) is specifically capable of responding to Lipopolysaccharide (or bacterial endotoxin). TLRs are capable of recognising both signals which indicate infection (PAMPs) as well as self-peptides which may indicate damage has occurred (DAMPs). Such activation has a key role to play in the development of many inflammatory diseases, including both T2DM and AD (O'Neill et al. 2013). Similarly, NLRs are transmembrane receptors which respond to both PAMPs and DAMPs in a similar fashion to TLRs and have broad effector functions such as: inflammasome assembly, signal transfuction, transcription activation and autophagy (Kim et al. 2016). NODs have also been implicated in a range of inflammatory diseases (Kim et al. 2016).

The NLRP3 inflammasome is a multiprotein complex formed of sensor molecules with the effector ASC and the effector Caspase 1 which mediate the production of mature forms of the highly inflammatory IL-1β family of cytokines, often concurrently triggering a form of cell death called pyroptosis (Heneka et al. 2018). Due to the strong inflammatory responses activated following inflammasome activation, inflammasomes require two signals for full activation (Heneka et al. 2018). In the first signal, innate immune receptors such as TLRs/NODs are stimulated by conserved peptides such as bacterial endotoxin (or Lipopolysaccharide: LPS) or other danger signals which leads to an increased production of pro-IL-1β (immature form of IL-1β) which cannot be released from the cell. Signal 2 leads to the formation of ASC specks, resulting in the recruitment of caspase-1 and subsequently to the mature form of IL-1β. Recent discoveries in both basic and translational laboratories have implicated activation of the NLRP3 inflammasome in both T2DM (Lee et al. 2013) and Alzheimer Disease (Sarsella et al. 2016). In both diseases, production of pro-inflammatory cytokines by the NLRP3 inflammasome may result in increasing immune-mediated cellular damage to neurons and other cell populations in the Central Nervous System.

One of the most interesting observations is that Aβ-42, the putative pathogenic protein in AD, may be a potent inflammasome activator (Heneka et al. 2013). Once primed, Aβ-42 is capable of acting as signal 2 and thus activates NLRP3, resulting in the production of the inflammasome-dependent cytokines IL-1β and IL-18. Whilst most of this work has been demonstrated in cultured micoglia and animal models of AD (Dempsey et al. 2017), aberrant NLRP3 inflammasome activation has also been documented in Peripheral Blood Mononuclear Cells (PBMCs) in individuals with both MCI and AD, with a significant upregulation in NLRP3 inflammasome related mRNA (NLRP3, PYCARD, Caspase 1) and downstream effectors (IL-18, IL-1β) (Saresella et al. 2016). Such findings offer fascinating insight into the potential pathogenesis of neuroinflammation in cognitive impairment and dementia, particularly in the era of inflammasome inhibitors which are under development.

In an elegant study of PBMCs from those individuals with IQ-discrepant memory vs IQ-concordant memory, co-treatment of PBMCs with Aβ-42 and LPS resulted in a greater

production of TNF-α in those with poorer memory scores, and was also accompanied by a shift towards glycolysis (Wolfe et al. 2018). In a further report, the same group found that incubation of THP-1 cells with plasma of individuals with IQ-discrepant knowledge resulted in a greater production of pro-inflammatory cytokines (Wolfe et al. 2019). Thus, even in unimpaired individuals, peripheral immune cells can respond to the pathogenic protein implicated in AD which produced an exaggerated pro-inflammatory changes in immune cells. Whether or not this is the case in T2DM-related cognitive decrements and impairments has never been investigated.

In a large study of 47 individuals with T2DM and 52 healthy controls, Lee et al. (2013) demonstrated increased expression of inflammasome related mRNA (NLRP3, ASC, pro-IL-1β) in untreated individuals with T2DM. Patients with T2DM also experienced greater maturation and production of IL-1β in addition to IL-18 in response to treatment with LPS and various additional innate immune stimuli in comparison to healthy controls. This landmark study presents firm evidence for NLRP3 upregulation in T2DM.

Another pathway which acts as an important sensor of cellular damage in the innate immune system is the cGAS-STING (cyclic GMP-AMP synthase-Stimulator of Interferon Genes) pathway, which has important roles in sensing damaged DNA, an indicator of cellular damage. Activation of this pathway may result in the production of type 1 interferons and other innate immune genes. Activation of the STING pathway and the type 1 interferon response has been implicated in neurodegeneration and AD (Mathur et al. 2018). Chronic neurodenegeration has also been noted to induce the type 1 interferon response via STING, accelerating disease progression in animal models (Nazmi et al. 2019). Similarly, damaged DNA is also capable of activating the absent in melanoma 2 (AIM2) inflammasome, and may form a caspase-1 activating inflammasome with ASC, as seen for the innate immune NLRP3 inflammasome above (Hornung et al. 2009). However, the role of this response in T2DM and its related complications has never been assessed.

Given the link between T2DM and the later development of dementia, we aimed to investigate the role of the NLRP3 Inflammasome and other innate immune pathways (namely the cGAS-STING pathway) in mediating the link between T2DM and cognition. We hypothesised that the PBMCs of individuals with T2DM may demonstrate an upregulated NLRP3 immune response on stimulation with Aβ-42 and that this upregulation may be correlated with cognitive function in those with T2DM. We also hypothesised that such upregulation might correlate with other potential markers of cognitive function in T2DM, such as gait speed and in particular, dual-task gait speed.

6.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation and Stimulation

6.1 Optimisation and Development of Study Protocol

The first step was to optimise and develop a protocol for analysis of PBMC responses to our panel of pro-inflammatory stimuli *in vitro.* We tested a range of stimuli concentrations over a range of stimulation times to determine optimal dose and timing for inflammasome activation in our cohort.

Initially, PBMCs were stimulated for a total duration of 3 hours with LPS (an activator of the Nf-kB pathway and primer of the innate immune inflammasome response) at three different concentrations, resulting in a significant upregulation in gene expression of IL-6 and IL1β (See Fig 6.1) in comparison to unstimulated controls. Whilst a dose-response relationship was initially observed with increasing concentrations of LPS added, the optimal upregulation of pro-inflammatory gene expression occurred at the concentration of 100 ng/mL. This concentration was used in subsequent experiments

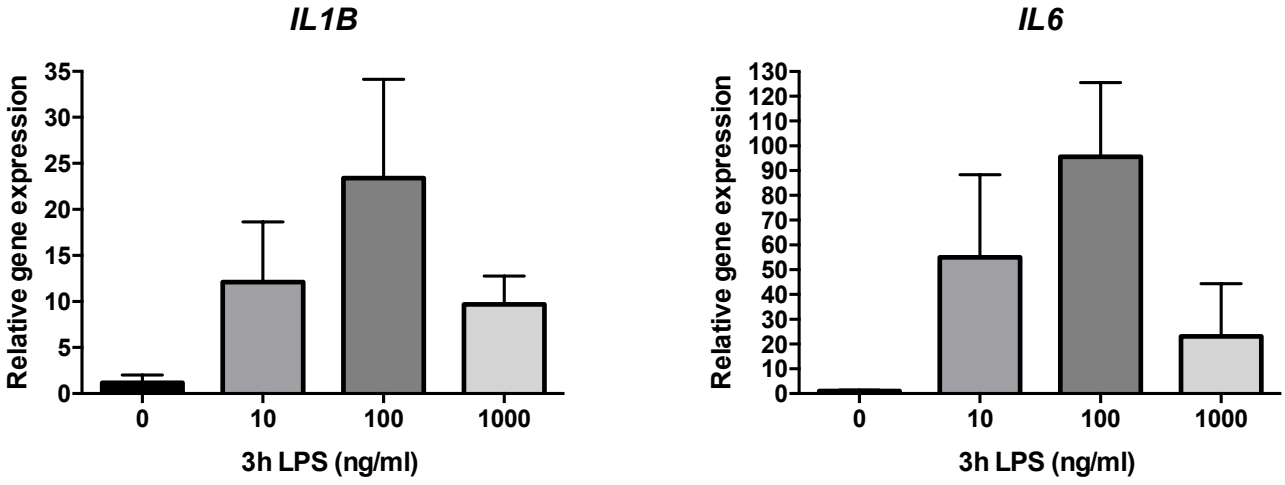

Figure 6.1 Inflammatory Gene Expression in Response to Stimulation with 3 hours. *PBMCs were isolated from two donors and stimulated in vitro with indicated concentrations of LPS for 3 hours. IL1β and IL6 gene expression was measured by qPCR. All gene expression was normalised to the expression of the endogenous control gene*

18S and stimulation data is expression relative to unstimulated levels. Data was analysed using Wilcoxon signed-rank test.

Following this, the duration of stimulation was tested in order to capture both up-regulation in pro-inflammatory gene expression and cytokine production following stimulation with innate immune agonists. In the first instance, agonists were applied for various durations for a maximum of eight hours total. LPS was applied at 2, 3, 4 and 6 hour intervals at the concentration optimised above (100ng/mL). Nigericin was applied to the LPS treated cells either 1 or 2 hours prior to harvesting. The greatest release of IL-1β (measured using ELISA) was observed in this initial experiment when at least 6 hours total of LPS was applied including 2 hours of nigericin stimulation at a standard concentration (as detailed above in the methods section). A further protocol tested stimulation of cells for 8 hours in total in comparison to eighteen hours. The most robust release of IL-1β was observed at 18 hours and so cell stimulation experiments were conducted for a total of 18 hours for the optimised study protocol (Figure 6.2).

Amyloid β-42 (the putative pathogenic protein implicated in Alzheimer Disease, which has been shown to active the innate immune NLRP3 inflammasome) was also added to LPS primed cells at two different concentrations in order to optimise pro-inflammatory cytokine production. In both experiments, co-treatment of LPS-stimulated cells with the lower concentration of Amyloid β-42 (10nM) resulted in equivocal IL-1β production as measured using ELISA as the higher concentration of Amyloid β-42 and so the lower concentration was taken for future experiments. (Figure 6.2).

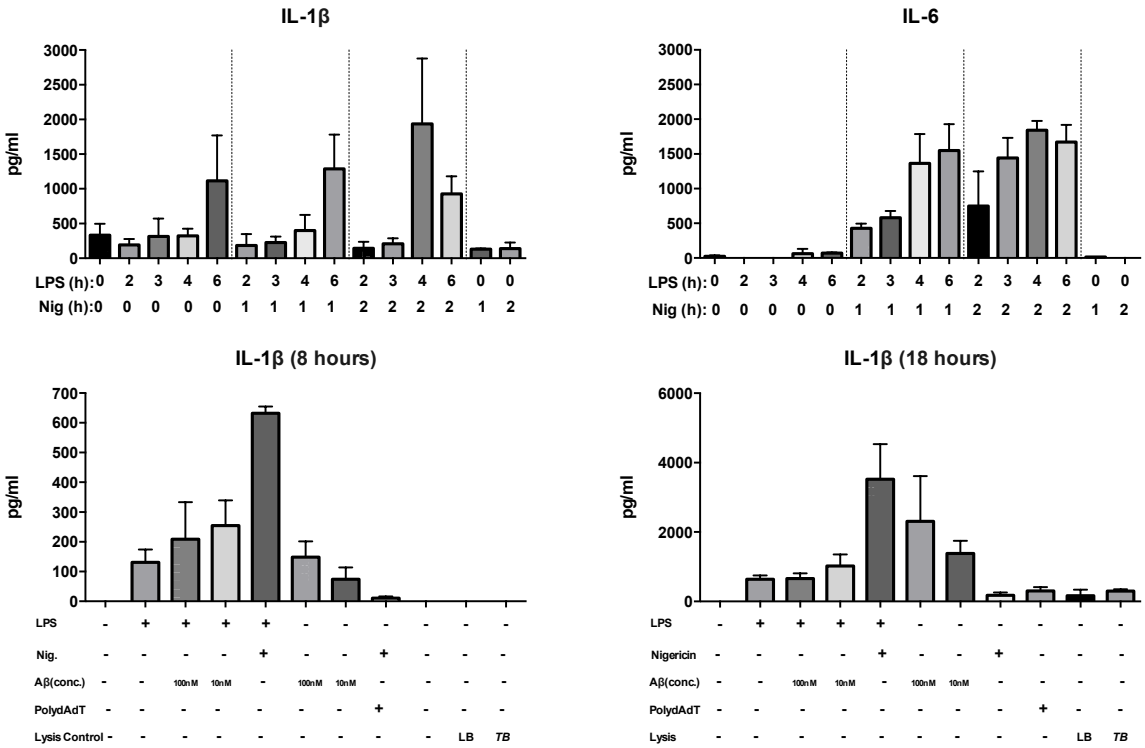

Figure 6.2 Stimulation of PBMCs with Innate Immune Stimuli. *PBMCs of healthy donors were isolated and stimulated with several pro-inflammatory agonists for various durations ranging from 2 hours to 18 hours. Nigericin was added for the remaining two hours in each experiment. Cells were also incubated with Amyloid β-42 for a total of eighteen hours, both alone and in combination with LPS at two different concentrations. Further, we assessed whether transfection with Poly dA:dT for 18 hours resulted in a proinflammatory response as determined by concentration of IL-1β protein measured using ELISA. These experiments were performed in order to optimise the stimulation conditions to be used for assaying innate immune responses in the ENBIND study. Data was analysed using Wilcoxon Rank Sum tests.*

6.2 PBMC Stimulation in the ENBIND Cohort

6.2.1 Included participants

In total, 60 participants (39 with midlife T2DM and 21 healthy controls) had PBMCs isolated and stimulated with various pro-inflammatory stimulants. In comparison to the overall study population, those with PBMC isolation performed did not differ in terms of age (52.32 ± 7.74 vs 51.66 ± 8.25 years, t = -0.44 p = 0.67), sex (χ^2 = 0.11, p = 0.74), years of formal education (17.64 ± 3.42 vs 17.62 ± 3.01, t = 0.03, p = 0.49), family history of dementia/cognitive impairment (χ^2 = 0.0057, p = 0.94) or BMI (32.21 8.62 vs 30.20 6.60, t = 1.38, p = 0.09). Specifically in those with midlife Type 2 Diabetes, there were no differences in terms of mean HBa1c (62.44 ± 19.54, t = 0.45, p = 0.32) (4 [IQR: 1-9] for those with PBMCs isolated vs 5 [IQR 2-9] for those without, z = 0.79, p = 0.43). There were no differences in overall T2DM medication usage in those with T2DM in comparison to the ENBIND cohort overall (χ^2 = 0.60, p 0.44). Therefore this sub-group were accepted to be a fair representation of the entire ENBIND cohort.

6.2.2 Baseline Expression of Pro-Inflammatory genes and Cytokines

Spontaneous production (i.e. unstimulated condition) of pro-inflammaory cytokiens by PBMCs was first examined. For IL-1β protein expression, 90% of participants (n = 54) had no detectable protein production on ELISA. For IL-6, baseline cytokine expression was slightly higher (596 ± 88.3 pg/mL). There were no significant between group differences for baseline production of pro-inflammatory cytokines (z = -0.50, p = 0.43 for IL-1β; z = -0.42, p = 0.67 for IL-6). Baseline expression of mRNA was also quantified using qPCR as detailed above. There were no significant between group differences in baseline pro-inflammatory gene expression (z = -0.58, p = 0.55 for IL-1β; z = -0.53, p = 0.60 for IL-6).

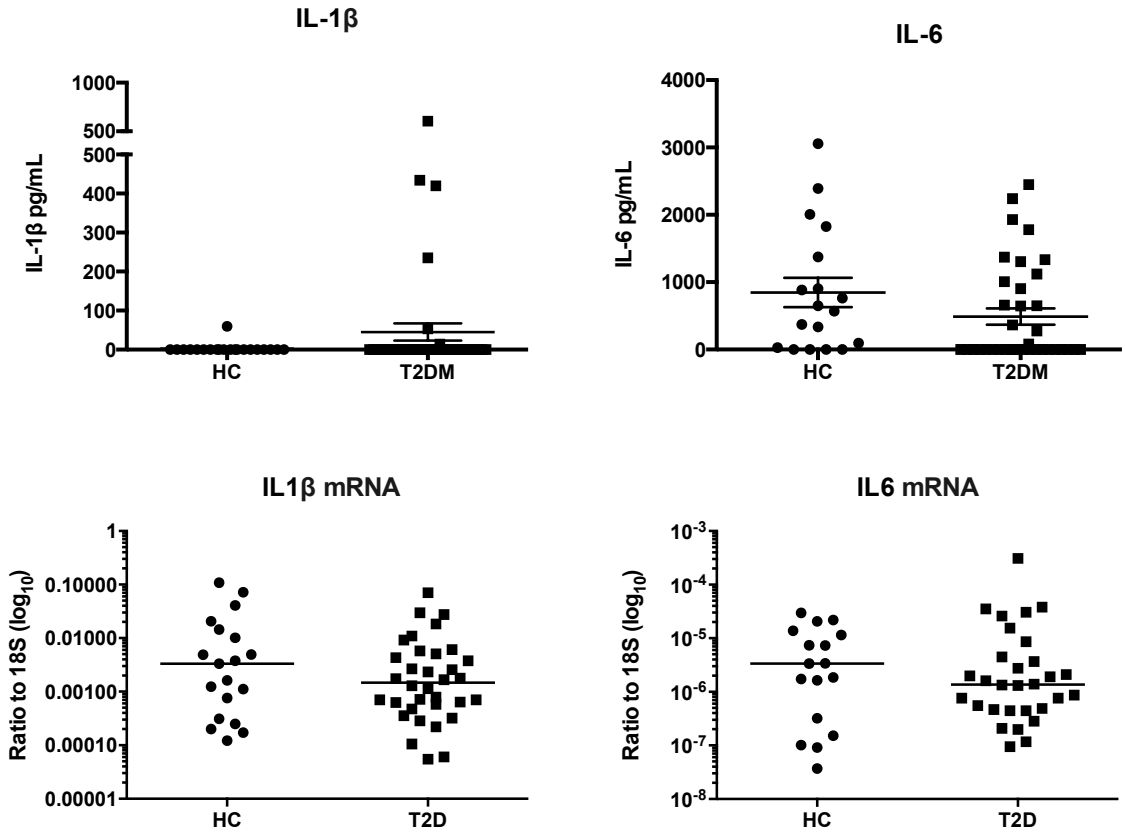

Figure 6.3 Unstimulated production of pro-inflammatory cytokines and expression of mRNA transcripts for pro-inflammatory genes. *One unstimulated condition per participant was incubated at 18 hours alongside each agonist-treated condition in order to provide a measure of baseline pro-inflammatory cytokine production and baseline pro-inflammatory gene expression*. *There were no differences in pro-inflammatory cytokine production measured using ELISA and no differences in mRNA pro-inflammatory gene expression measured using qPCR between T2DM and healthy controls (Wilcoxon Rank Sum Test). Protein results are expressed as pg/mL extrapolated from standard curve and mRNA results as Ratio of mRNA expression relative to a housekeeping gene, 18s.*

6.2.3 No difference in response of PBMCs to LPS stimulation in those with Midlife Type 2 Diabetes Mellitus (T2DM) in comparison to Healthy Controls

In order to analyse whether the presence of T2DM in midlife was associated with aberrant reponses of PBMCs to inflammatory stimuli, we incubated PBMCs of participants for a total of 18 hours duration with LPS (endotoxin). In all participants, regardless of T2DM status, there was a statistically significant increase in IL-1β cytokine production in response to LPS ($z = -6.68$, $p<0.001$, Wilcoxon matched-pairs). Similarly, there was a significant upregulation of IL-1β gene expression in PBMCs exposed to LPS ($z = -6.62$, $p<0.001$). The median fold increase in IL-1β was 37.77 (Interquartile Range: 13.53-124.07). There were no differences between groups (n = 60, n=39 T2DM and n=21 Healthy Controls) in terms of IL-1β cytokine production ($z = 0.37$, $p = 0.71$) or in the fold change in IL-1β relative to each participants own unstimulated condition ($U = 210$, $p = 0.20$).

Similarly, addition of LPS was associated with an increased production of the pro-inflammatory cytokine IL-6 ($z = 6.64$, $p<0.001$) in addition to a significantly increased expression of *IL6* mRNA relative to each participants own unstimulated condition ($z = -5.05$, $p<0.001$). The median fold increase in IL-6 expression on stimulation for 18 hours with LPS was 25.56 (Interquartile Range: 25.56, 3.38 – 128.2). Similar to IL-1β expression, there was no significant between group differences in the fold change in IL-6 between groups (T2DM vs Controls; $U = 187$, $p = 0.37$, Mann-Whitney U test). Cytokine production and gene expression results for the LPS condition are given below in Figure 6.4. Data is stratified by group (Midlife T2DM vs Healthy Controls)

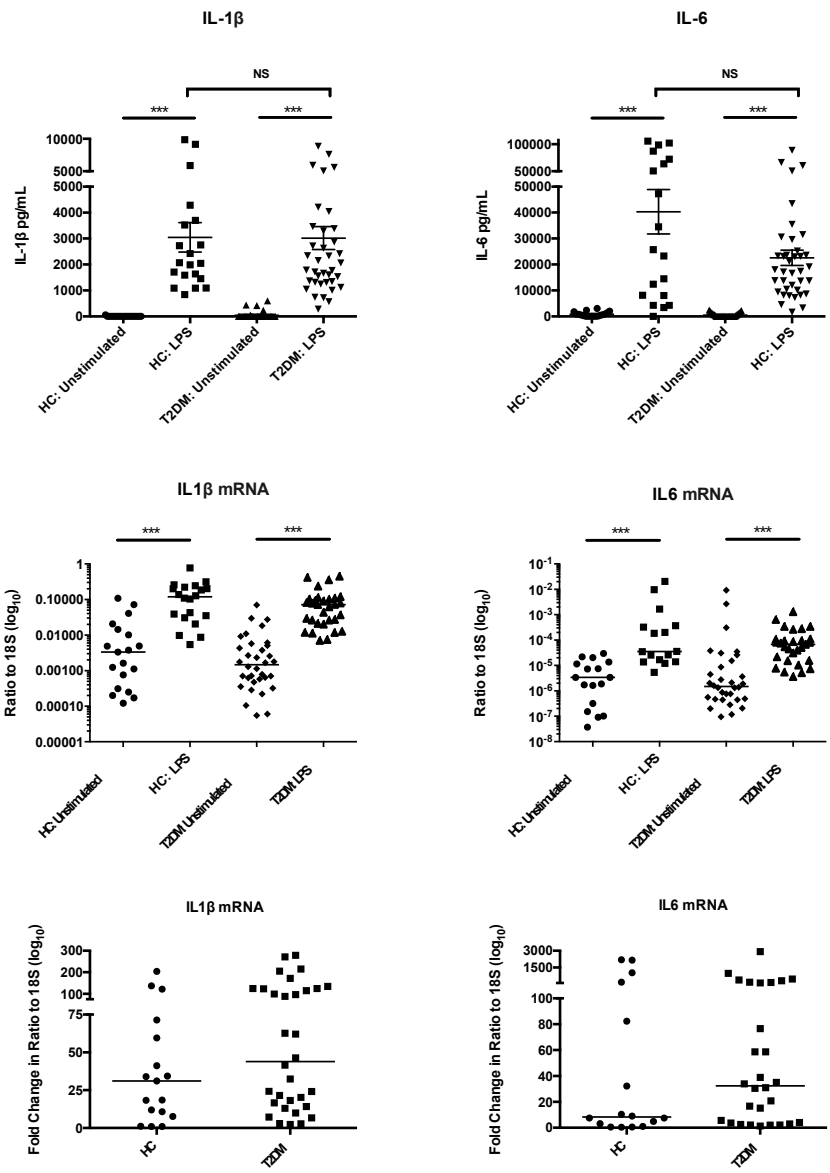

Figure 6.4. PBMC Responses to Stimulated with the TLR4 agonist LPS. *Peripheral Blood Mononuclear Cells (PBMCs) (n = 60) were isolated from participants in the ENBIND study and stimulated using LPS for 18 hours. Cytokine expression was measured using ELISA and mRNA measured using qPCR. Protein results are expressed as pg/mL and mRNA results as ratio relative to a housekeeping gene mRNA. Wilcoxon Matched Pairs tests were used for analysis in addition to Wilcoxon Rank Sum Tests/Mann-Whitney U Tests. *p<0.05, ***p<0.001*

6.2.4 There is no difference in NLRP3 inflammsome activation between people with midlife T2DM and healthy controls

In order to analyse innate immune NLRP3 inflammasome responses in those with T2DM and Healthy Controls, we stimulated PBMCs with LPS for a total of 18 hours in addition to adding nigericin (a potent inflammasome stimulator) for the final 2 hours of the incubation in order to induce a robust immune cell response. Co-incubation with LPS and nigericin resulted in a significantly increased release of IL-1β in comparison to the 18 hour incubation with LPS alone ($z = 6.25$, $p<0.001$). A similar increase in IL-6 was not observed ($z = 1.79$, $p = 0.86$). We observed no difference in mRNA expression between the co-incubation of LPS with nigericin than with LPS alone for both IL-1β mRNA ($z = 0.91$, $p = 0.81$) or IL-6 mRNA ($z = 1.10$, $p = 0.68$). In summary, co-incubation with nigericin for the final 2 hours of the 18 hour LPS stimulation had a highly significant effect on IL-1β cytokine production but not on IL-6 production or on gene expression encoding for these two pro-inflammatory cytokines, consistent with nigericin acting as a 'signal 2' resulting in activation of the NLRP3 innate immune inflammasome.

Of note there were no differences between groups (n = 60, 39 T2DM and 21 Healthy Controls) in terms of IL-1β production in response to LPS and nigericin co-incubation ($z = -0.65$, $p = 0.51$). IL-1β and IL-6 Cytokine production in addition to expression of IL-1β and IL-6 mRNA are detailed in Figures 6.5A and 6.5B below.

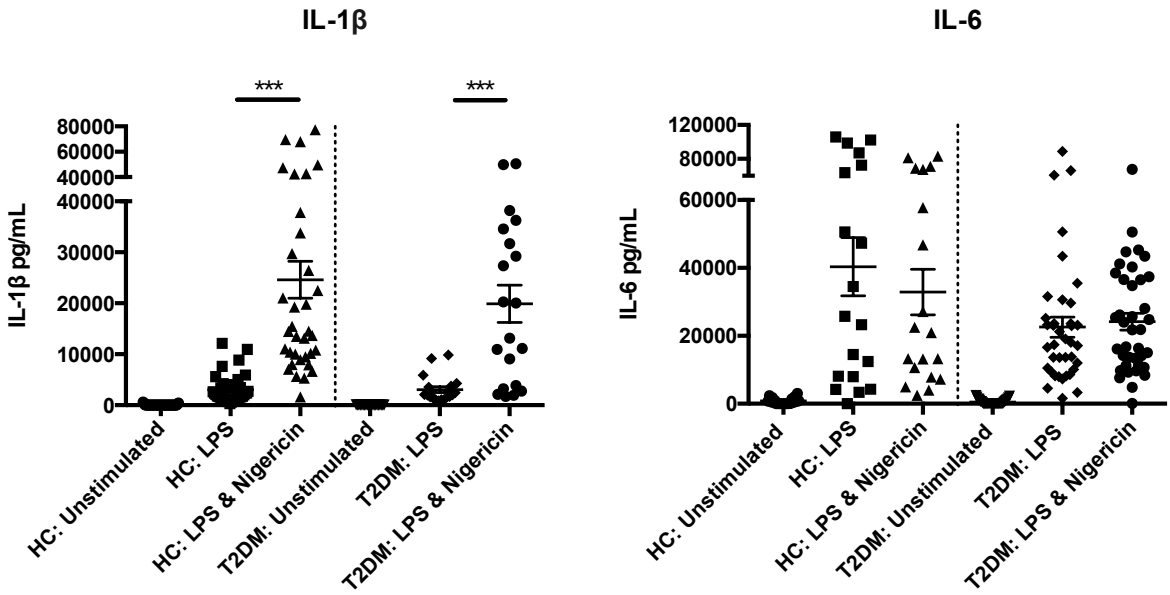

Figure 6.5A Stimulation of the NLRP3 inflammasome with LPS and Nigericin. *PBMCs (N = 60, 39 with T2DM, 21 Healthy Controls) were incubated for 18 hours with LPS as above with Nigericin added for the remaining 2 hours. Wilcoxon Match Pairs and Rank-Sum Tests were used for analysis. There were no significant between-group differences when comparing those with T2DM to healthy controls. ***p = <0.001*

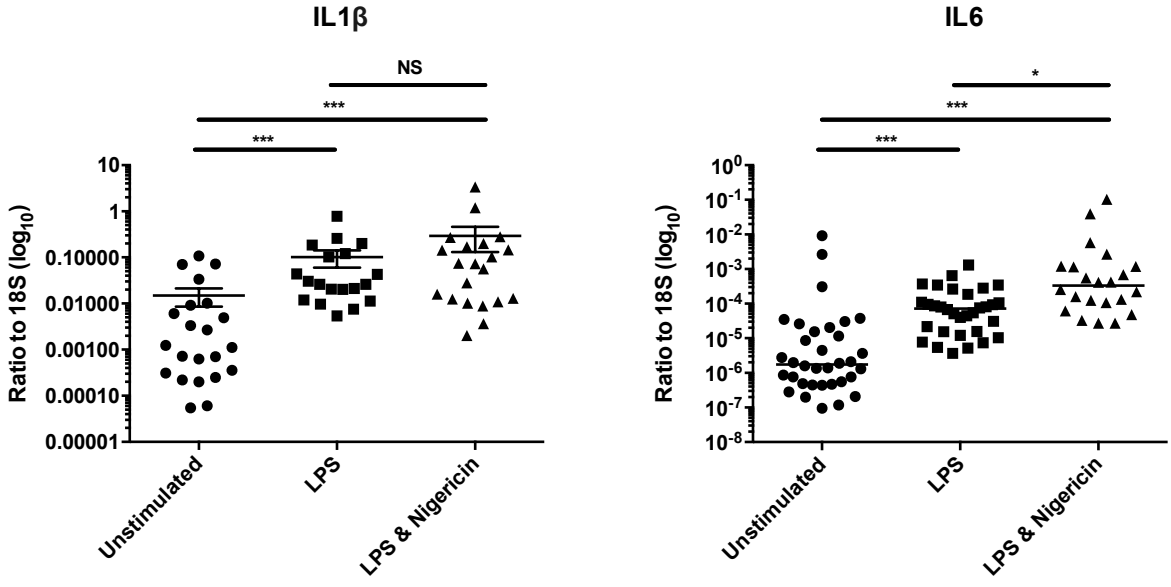

Figure 6.5B Stimulation of the NLRP3 inflammasome with LPS and Nigericin. *PBMCs (N = 60, 39 with T2DM, 21 Healthy Controls) were incubated for 18 hours with LPS as above with Nigericin added for the remaining 2 hours. Wilcoxon Match Pairs and Rank-Sum Tests*

*were used for analysis. Protein is expressed in pg/mL as per the standard curve and mRNA is expressed as ratio to a housekeeping gene, 18s. There were no significant between-group differences when comparing those with T2DM to healthy controls. ***p = <0.001*

6.2.5 Stimulation of the NLRP3 innate immune inflammasome with Amyloid β-42 results in significant IL-1β cytokine production

Previous studies demonstrated the ability of Amyloid β-42 to stimulate the innate immune NLRP3 inflammasome, we therefore hypothesised that T2DM may be associated with aberrant immune response to this protein. We stimulated PBMCs of patients with T2DM and Healthy Controls with Amyloid β-42 alone in addition to co-incubation with LPS as previous evidence suggests that in a similar manner to Nigericin, Amyloid β-42 may act as a 'signal 2' in activating the innate immune inflammasome resulting in the production of NLRP3 inflammasome-dependent pro-inflammatory cytokines such as IL-1β.

Incubation of PBMCs (n = 60, 39 T2DM vs 21 Healthy Controls) with Amyloid β-42 alone for 18 hours resulted in significantly increased production of both IL-1β and IL-6 cytokines (z = -5.61, p <0.001 for IL-1β; z = - 5.64, p<0.001). Similarly, co-incubation of PBMCs with both LPS and Amyloid β-42 resulted in a significant production of both IL-1β and IL-6 cytokines (z -6.64, p<0.001 for IL-1β; z -6.45, p<0.001 for IL-6). In comparison to LPS alone, the co-incubation with both LPS and Amyloid β-42 resulted in significantly greater release of IL-1β (z = 2.60, p = 0.047) but not in IL-6 (z = 0.99, p = 0.32). In a similar manner to stimulation with LPS and Nigericin, co-incubation of both LPS and Amyloid β-42 did not result in a significant up-regulation in gene expression for either IL-1β or IL-6 (z = -1.4, p = 0.67 for IL-1β; z = -1.8, p = 0.31).

On examining the difference between T2DM participants and matched Healthy Controls, we observed no difference in cytokine production on co-incubation with Amyloid β-42 alone for either IL-1β or IL-6 (1.69, p = 0.09, z = -1.45, p = 0.14 respectively) in addition to cytokine

responses to co-incubation with both LPS and Amyloid β-42 (z = 0.78, p = 0.44). Cytokine production and mRNA expression of IL-1β or IL-6 is demonstrated in Figures 6.6A and 6.6B.

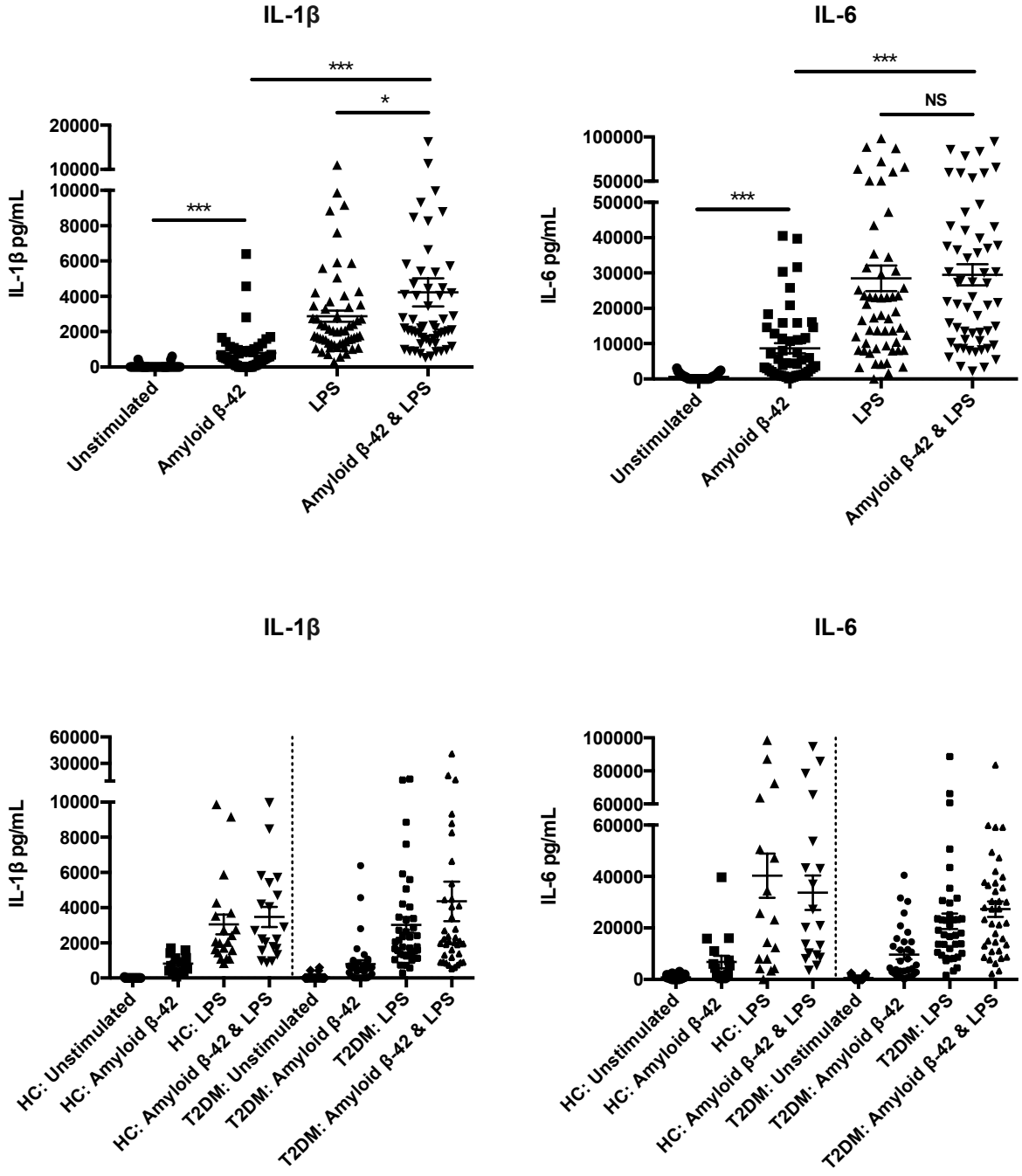

Figure 6.6A NLRP3 Dependent Cytokine Production by PBMCs incubated with Amyloid β-42 and LPS. *PBMCs (N = 60, 39 with T2DM and 21 Healthy Controls) were incubated with*

*Amyloid β-42 for a total of 18 hours, either alone or in addition to LPS. Protein is expressed as pg/mL as per extrapolation from the standard curve. Wilcoxon matched-pairs and Wilcoxon rank-sum tests were used to analyse results. *p<0.05, ***p<0.001*

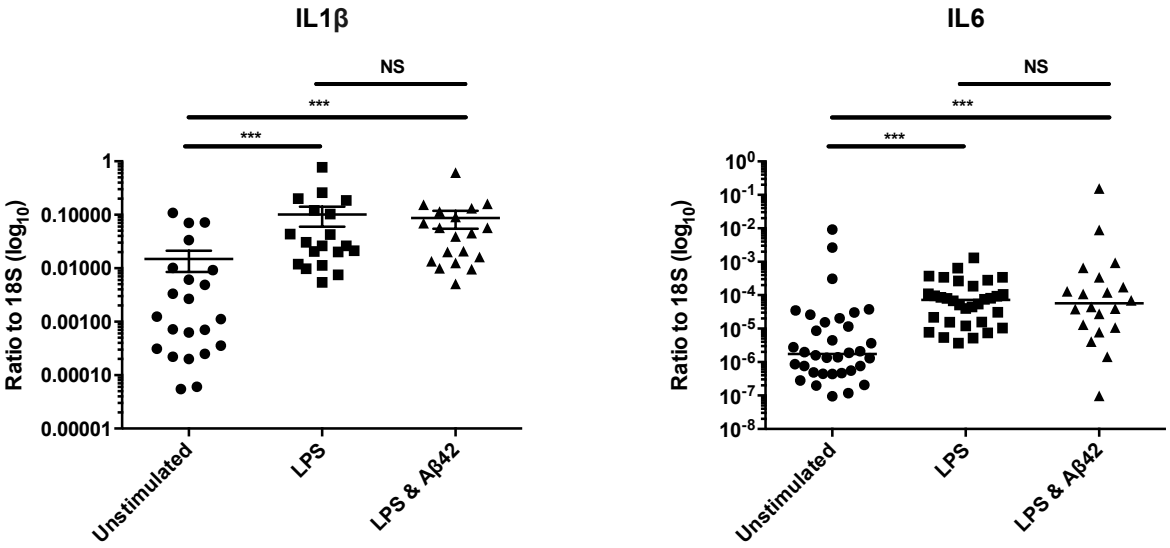

Figure 6.6B Pro-Inflammatory mRNA Gene Expression by PBMCs incubated with Amyloid β-42 and LPS . *PBMCs (N = 60, 39 with T2DM and 21 Healthy Controls) were incubated with Amyloid β-42 for a total of 18 hours in addition to LPS. mRNA is expressed relative to a housekeeping gene, 18s. Wilcoxon matched pairs and Wilcoxon rank-sum tests were used to carry out statistical analysis. *p<0.05, ***p<0.001, NS = Not Significant*

6.2.6 Stimulation of PBMCs with Poly dA:dT Reveals no Significant Differences in Cytokine Production or Pro-Inflammatory Gene Expression in Those with Midlife Type 2 Diabetes Mellitus vs Healthy Controls

In order to assess the activity of a different innate immune pathway, the cGAS-STING pathway in addition to the AIM2 pathway, we transfected cells with Poly dA:dT, a repetitive double strand of DNA introduced into the cytosol via lipofectamine transfection. Cells were incubated for a total duration of 18 hours before harvesting. Addition of Poly dA:dT resulted in a significant increase in the production of both IL-1β and IL-6 cytokines ($z = 6.12$, $p<0.001$ for IL-1β; $z = 4.49$, $p<0.001$) and in the expression of IL-6 ($z = -2.8$, $p = 0.03$) but not IL-1β mRNA ($z = -2.5$, $p = 0.06$). No between group differences were observed between T2DM and healthy controls for production of IL-1β ($z = 0.15$, $p = 0.88$) and IL-6 ($z = 1.43$, $p = 0.15$). Results are presented below in Figures 6.7A and 6.7B

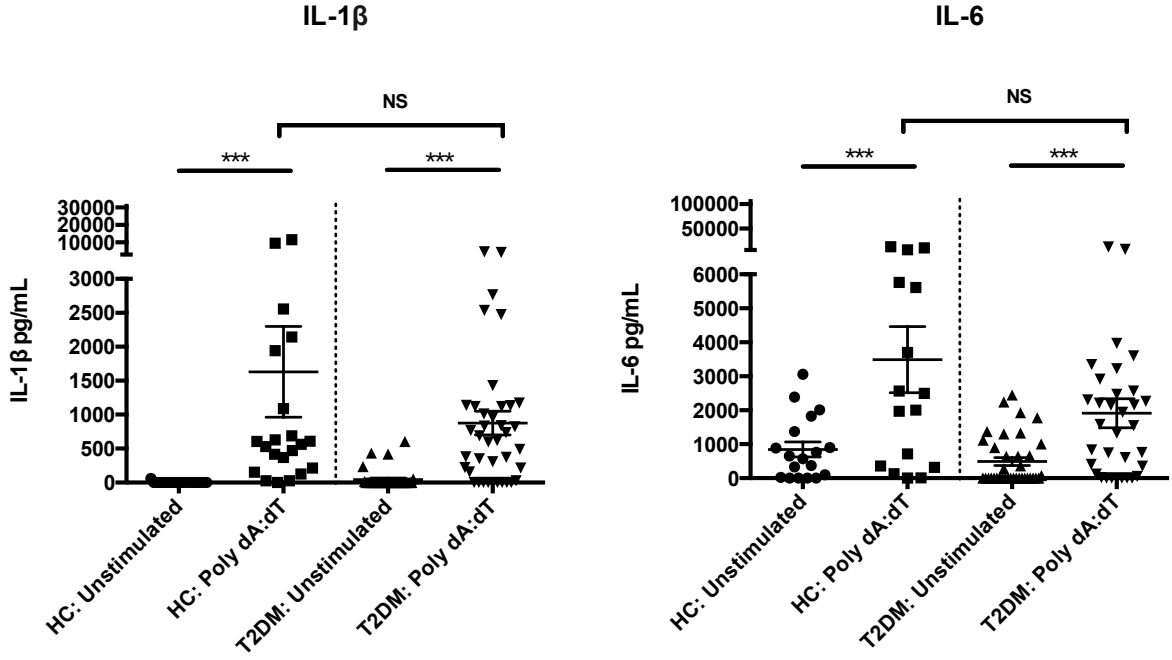

Figure 6.7A. Cytokine Production on Transfection of PBMCs with Poly dA:dT. PBMCs isolated from those with T2DM (N = 39) and Healthy Controls (N = 21) were treated for 18 hours with the innate immune agonist Poly dA:dT. Cytokine production was measured using standardised ELISA, with proteins expressed as pg/mL as per extrapolation from

the standard curve. Wilcoxon rank sum and matched pairs tests were used in analysis ***p<0.001, NS = Not Significant

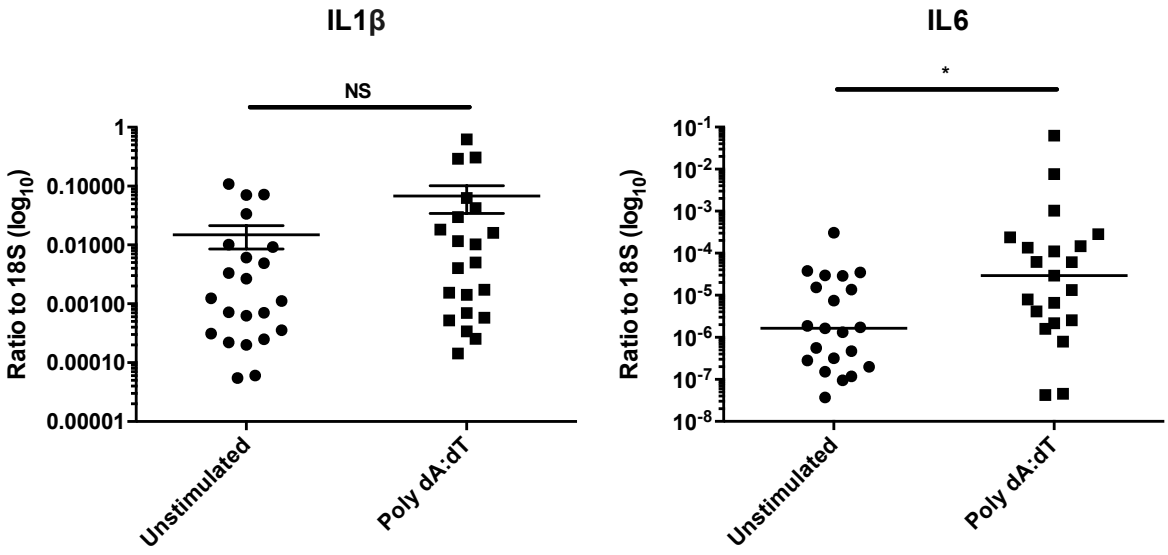

Figure 6.7B. IL-1β and IL-6 Gene Expression in PBMCs in response to 18 hour incubation with Poly dA:dT. PBMCs isolated from those with T2DM (N = 39) and Healthy Controls (N = 21) were treated for 18 hours with the innate immune agonist Poly dA:dT. Gene Expression was measured using standardised qPCR and expressed relative to a housekeeping gene, 18s. Wilcoxon rank sum and matched pairs tests were used in analysis ***p<0.001, NS = Not Significant

6.3 Examining the Link Between General Cognitive Function and Peripheral Blood Mononuclear Cell Responses to Stimulation in Type 2 Diabetes

6.3.1 Examining the link between PMBC immune responses and MoCA performance

In order to examine the link between PBMC immune responses and general cognitive function, we performed a series of correlational analysis between cytokine production and gene expression changes and performance on the MoCA. In the first instance this was tested individually using a Spearman correlation statistic. We analysed all PBMC responses irrespective of group, given that our initial analysis demonstrated no between-group differences.

Poisson regression models were then constructed, as above, to model the likelihood of error on the MoCA (as a count variable), to account for the strong left skew of the data. Production of IL-1β and IL-6 by PBMCs stimulated *in vitro*, expressed in terms of pg/mL, in each condition was log transformed and included as the dependent variable of interest in each model. Associations were first tested unadjusted, followed by a model adjusting for age, gender, family history of dementia/cognitive impairment and years of formal education. Finally, an interaction term between diabetes status (Type 2 Diabetes vs Healthy Controls) and the variable of interest (log of cytokine produced) was created in order to examine if any T2DM specific associations were seen. Analysis was also run for IL-1β and IL-6 mRNA which was log transformed and examined using the same models.

6.3.2 PBMC Immune Response to Stimulations and MoCA Performance

Analysis examining the correlation between the log of cytokine production and score on the MoCA revealed a significant correlation between immune cell responses to LPS stimulated IL-1β production and poorer MoCA score (Spearman's rho = -0.28, p = 0.04). Similarly, there was a non-significant trend for greater IL-1β production when cells were stimulated with poly dA:dT and poorer scores on the MoCA (Spearman's rho = -0.27, p =

0.06). Results are represented graphically in Figure 6.8. No correlation was seen between mRNA expression and MoCA score for IL-1β or IL-6.

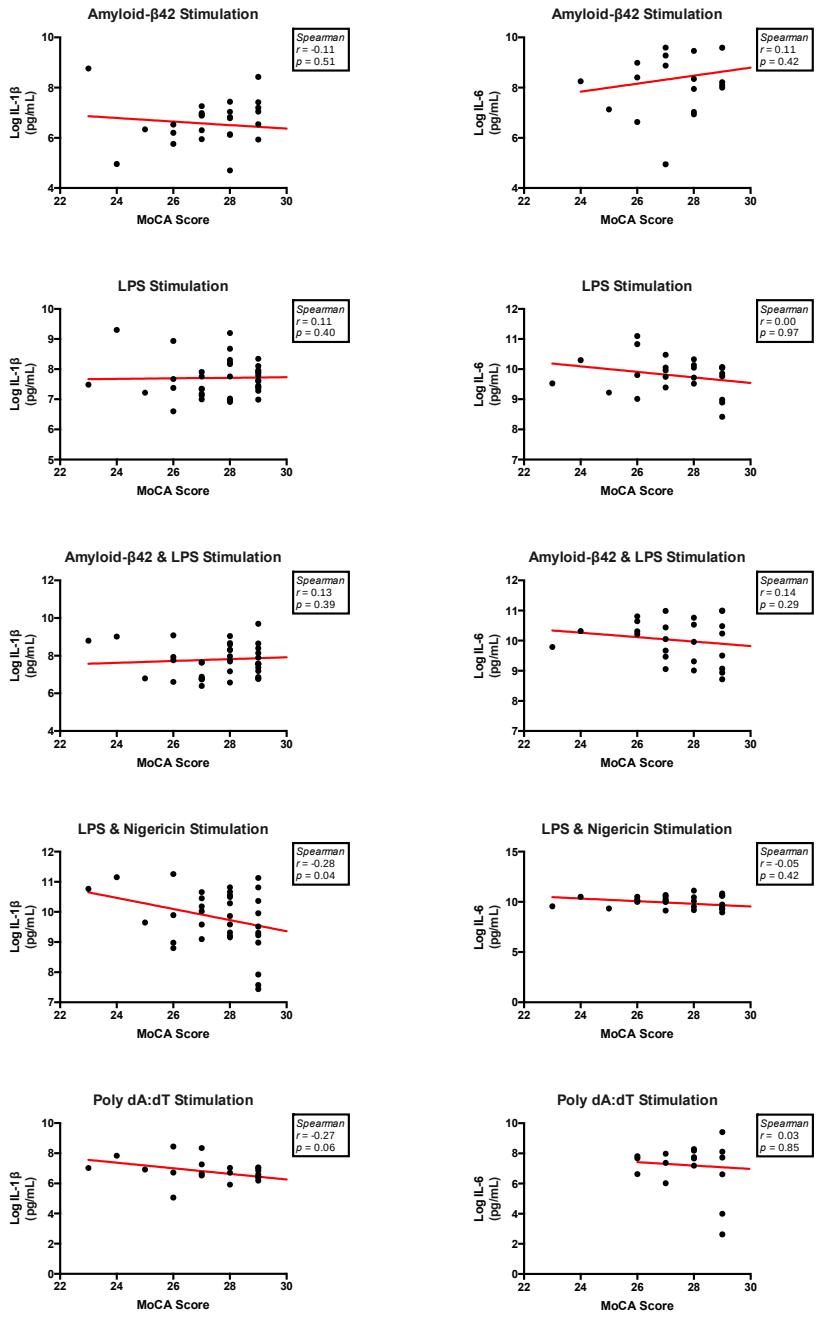

Figure 6.8. Correlational analysis between PBMC cytokine production on 18 hour stimulations and MoCA scores. *Cytokine production was measured following 18 hour incubations with standard commercially available ELISA kits. Data are expressed as the log of cytokine production and scores on Montreal Cognitive Assessment. Spearmann's rho statistics were used to perform non-parametric correlation analysis*

Using poisson regression with log transformed cytokine production as the dependent variable, greater IL-1β production in response to both Aβ-42 alone and also LPS and Nigericin was associated with a significantly increased likelihood of error on the MoCA (p = 0.01 for Aβ-42; Nigericin & LPS = 0.004). Both associations remained significant after adjusting for the above mentioned covariates (p = 0.02 for Aβ-42, p = 0.004 for Nigericin & LPS). Whilst no T2DM-specific effect was seen for the Nigericin & LPS condition, there was a trend for a T2DM-specific effect (modelled as a T2DM*cytokine production interaction coefficient) for greater likelihood of error on the MoCA and greater IL-1β production in response to Aβ-42 alone (p = 0.08). There were no associations seen for IL-6 production. The results of the unadjusted poisson models are presented in Table 6.1

	IL-1β Release (pg/mL)		**IL-6 Release (pg/mL)**	
Condition	**IRR (95% CI)**	***P-Value***	**IRR (95% CI)**	***P-Value***
LPS	1.00 (1.00-1.00)	0.933	1.00 (1.00-1.00)	0.290
Aβ-42	1.00 (1.00-1.00)	**0.010**	1.00 (1.00-1.00)	0.207
Aβ-42 & LPS	1.00 (1.00-1.00)	0.298	1.00 (1.00-1.00)	0.102
Nigericin & LPS	1.00 (1.00-1.00)	**0.004**	1.00 (1.00-1.00)	0.606
Poly dA: dT	1.00 (1.00-1.00)	0.815	1.00 (1.00-1.00)	0.214

Table 6.1. Likelihood of Error on the MoCA and Cytokine Production by PBMCs in response to Various Stimuli. *Poisson models were constructed in the first instance to test this association unadjusted, followed by the construction of a fully adjusted Poisson model accounting for age, gender, family history of dementia/cognitive impairment in addition to years of formal education. Cytokine release was log transformed and included in regression models. Reults of fully adjusted models are presented.*

The same models were then run in order to assess the relationship between inflammatory gene expression changes and likelihood of MoCA error. These were tested for the log transformed baseline gene expression levels of IL-1β and IL-6 in the first instance, followed by the gene expression in response to LPS stimulation and also by the ratio of LPS:Unstimulated (fold change of gene expression in stimulated condition relative to own unstimulated control). No associations were seen either unadjusted or following

adjustment. Exploratory analyses with interaction terms revealed no T2DM-specific effects (See Table 6.2).

	IL-1β mRNA (Ratio to 18S)		**IL-6 mRNA (Ratio to 18S)**	
	IRR (95% CI)	***P***	**IRR (95% CI)**	***P***
Unstimulated	0.97 (0.97 – 1.02)	0.801	0.94 (0.68 – 1.30)	0.703
LPS	1.01 (0.97 – 1.04)	0.702	0.97 (0.87 – 1.04)	0.620
LPS: Unstim Ratio	1.01 (0.98 – 1.05)	0.543	1.00 (0.99 – 1.00)	0.090

Table 6.2. Likelihood of Error on the MoCA and Pro-Inflammatory Gene Expression by PBMCs in response to Various Stimuli. *Absolute gene expression data (relative to a housekeeping gene, 18S), was log transformed and analysed as a dependent variable in a poisson model predicting the likelihood of MoCA error. Results of the fully adjusted models (for age, sex, years of education and family history of cognitive impairment) are presented.*

6.4 PBMC Immune Responses and Neuropsychological Test Scores

6.4.1 Examining the link between PMBC immune responses and Neuropsychological Test Performance

We examined the relationship between PBMC immune responses to our panel of immune activators and the twelve outcomes on the CANTAB assessment battery, detailed in chapters 2 & 3. The aim of this analysis was to examine if PBMC immune responses were significantly associated with differing domains cognitive function. For this analysis, we performed a Z-transformation of the twelve neuropsychological outcome parameters and performed generalised linear regressions with standardised score as the independent variable. Again, we used cytokine production and gene expression values as dependent variable. We tested each association unadjusted, followed by adjustment for age, gender, level of education and family history. We then included interaction analysis with T2DM status to examine any T2DM-specific effects.

6.4.2 No association between cytokine production by PBMCs and cognitive function on the CANTAB battery

We examined the ability of cytokine production (log transformed values) to predict outcomes on the various neuropsychological parameters as detailed above. There were no associations between IL-1β or IL-6 production and the outcome parameters under any model, either adjusted or unadjusted. Exploratory analysis examining for T2DM-specific effects yielded no significant results. Results of unadjusted models for each stimulus-outcome combination are given below with coefficients presented with 95% confidence intervals (Tables 6.3 & 6.4) .

IL1β Release	LPS		Aβ-42		Aβ-42 & LPS		LPS & Nigericin		Poly dA: dT	
	β Coef. (95% CI)	P	β -Coef. (95% CI)	P	β -Coef. (95% CI)	P	β -Coef. (95% CI)	P	β -Coef. (95% CI)	P
RTI										
RTIF-MDRT	0.05 (-0.32 – 0.41)	0.807	-0.06 (-0.41 – 0.29)	0.736	-0.03 (-0.35 – 0.29)	0.851	0.13 (-0.42 – 0.13)	0.323	0.03 (-0.19 – 0.25)	0.792
RTIF-MDMT	0.17 (-0.21 – 0.55)	0.361	0.07 (-0.37 – 0.51)	0.745	-0.01 (-034 – 0.32)	0.938	0.18 (-0.48 – 0.12)	0.231	0.09 (-0.17 – 0.35)	0.491
PAL										
PAL-TEA	-0.17 (-0.51 – 0.16)	0.302	-0.07 (-0.45 – 0.30)	0.693	-0.07 (-0.36 – 0.21)	0.207	0.05 (-0.32 – 0.22)	0.724	0.04 (-0.27 – 0.18)	0.697
PAL-FAMS	-0.17 (-0.51 – 0.16)	0.302	-0.07 (-0.45 – 0.30)	0.693	-0.07 (-0.36 – 0.21)	0.207	0.05 (-0.32 – 0.22)	0.724	0.04 (-0.27 – 0.18)	0.697
SWM										
SWM-BE468	0.00 (-0.32 – 0.31)	0.984	0.02 (-0.37 – 0.32)	0.891	0.11 (-0.16 – 0.39)	0.376	0.01 (-0.24 – 0.25)	0.952	0.18 (-0.04 – 0.40)	0.101
SWM-Strategy	-0.03 (-0.37 – 0.31)	0.848	-0.09 (-0.45 – 0.25)	0.916	-0.03 (-0.32 – 0.26)	0.855	0.10 (-0.36 – 0.16)	0.445	0.13 (-0.10 – 0.37)	0.245
PRM										
PRM-PCI	0.19 (-0.36 – 0.32)	0.914	0.06 (-0.34 – 0.46)	0.763	0.03 (-0.32 – 0.26)	0.844	0.02 (-0.02 – 0.01)	0.623	-0.06 (-0.29 – 0.17)	0.599
PRM-PCD	-0.10 (-0.45 – 0.25)	0.552	-0.23 (-0.57 – 0.10)	0.170	-0.15 (-0.46 – 0.15)	0.316	0.07 (-0.34 – 0.21)	0.604	-0.03(-0.23 – 0.19)	0.838
OTSOC										
OTS-PCFS	0.26 (-0.06 – 0.58)	0.114	0.11 (-0.23 – 0.45)	0.523	0.05 (-0.24 – 0.33)	0.751	0.32 (-0.08 – 0.57)	0.062	0.02 (-0.21 – 0.24)	0.875
OTS-MLFC	0.01 (-0.39 – 0.41)	0.959	0.01 (-0.44 – 0.42)	0.965	0.01 (-0.33 – 0.36)	0.942	0.15 (-0.16 – 0.47)	0.342	0.00 (-0.00 – 0.00)	0.590
RVP										
RVP-AP	0.02 (-0.27 – 0.32)	0.869	0.02 (-0.31 – 0.35)	0.922	-0.18 (-0.43 – 0.08)	0.173	0.11 (-0.12 – 0.34)	0.343	0.11 (-0.08 – 0.29)	0.262
RVP-PFA	-0.11 (-0.29 – 0.06)	0.203	-0.18 (-0.39 – 0.03)	0.088	-0.05 (-0.20 – 0.11)	0.528	-0.1 (-0.24 – 0.04)	0.160	0.09 (-0.10 – 0.50)	0.145

Table 6.3. IL-1β production by PBMCs in response to various pro-inflammatory stimuli and scores on the detailed Neuropsychological Assessment Battery. We used generalised linear regression to examine the association between log of IL-1β production in response to various stimuli and scores on neuropsychological tests (Z-scores).

IL6 Release	**LPS**		**Aβ-42**		**Aβ-42 & LPS**		**LPS & Nigericin**		**Poly dA: dT**	
	β Coef. (95% CI)	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
RTI										
RTIF-MDRT	1.55 (-10.74- 13.84)	0.800	-0.38 (13.6 – 12.85)	0.954	-5.34 (-24.0 – 13.3)	0.568	-5.41 (-20.5 – 9.7)	0.476	8.79 (-3.43 – 21.80)	0.154
RTIF-MDMT	14.71 (-3.95 – 33.4)	0.119	5.60 (-27.36 – 16.2)	0.607	6.82 (-23.5 – 37.2)	0.654	13.54 (10.8 – 37.9)	0.270	14.72 (-3.95 – 33.3)	0.119
PAL										
PAL-TEA	0.44 (-2.96 - 3.86)	0.296	-2.28 (-5.86 – 1.31)	0.207	-2.86 (-7.90 – 2.18)	0.260	-1.51 (-5.64 – 2.61)	0.466	-1.05 (-4.15 – 2.05)	0.497
PAL-FAMS	0.44 (-2.96 - 3.86)	0.296	-2.28 (-5.86 – 1.31)	0.207	-2.86 (-7.90 – 2.18)	0.260	-1.51 (-5.64 – 2.61)	0.466	-1.05 (-4.15 – 2.05)	0.497
SWM										
SWM-BE468	-0.16 (-0.35 – 0.02)	0.086	0.13 (-0.08 – 0.34)	0.214	-0.21 (-0.49 – 0.07)	0.136	0.01 (-0.24 – 0.25)	0.952	-0.11 (-0.29 – 0.72)	0.230
SWM-Strategy	-0.15 (-0.34 – 0.05)	0.139	0.09 (-0.14 – 0.31)	0.437	-0.11 (-0.41 – 0.20)	0.481	0.10 (-0.36 – 0.16)	0.445	-0.07 (-0.25 – 0.13)	0.489
PRM										
PRM-PCI	0.03 (-0.18 – 0.23)	0.803	-0.19 (-0.41 – 0.17)	0.072	-0.15 (-0.46 – 0.15)	0.312	-0.10 (-0.35 – 0.14)	0.404	0.14 (0.01 – 0.29)	0.052
PRM-PCD	0.13 (-0.08 – 0.34)	0.209	-0.23 (-0.48 – 0.02)	0.065	-0.15 (-0.47 – 0.17)	0.343	0.11 (-0.27 – 0.24)	0.927	-05 (-0.25 – 0.15)	0.615
OTSOC										
OTS-PCFS	0.03 (-0.16 – 0.22)	0.780	0.15 (-0.09 – 0.38)	0.210	-0.12 (0.41 – 0.16)	0.387	0.15 (-0.09 – 0.38)	0.210	0.03 (0.14 – 0.20)	0.738
OTS-MLFC	0.05 (-0.16 – 0.26)	0.655	-0.10 (-0.34 – 0.14)	0.422	0.12 (-0.23 – 0.48)	0.506	0.00 (-0.30 – 0.29)	0.977	0.19 (-0.02 – 0.40)	0.075
RVP										
RVP-AP	0.00 (-0.17 – 0.18)	0.989	-0.09 (-0.28 – 0.11)	0.374	-0.13 (-0.40 – 0.13)	0.326	0.14 (-0.07 – 0.35)	0.197	-0.06 (-0.23 – 0.12)	0.523

RVP-PFA	-0.08 (-0.18 – 0.30)	0.135	0.11 (-0.12 – 0.14)	0.860	-0.05 (-0.21 – 0.11)	0.561	-0.10 (-0.22 – 0.03)	0.140	-0.01 (0.12 – 0.11)	0.928

Table 6.4. IL6 production by PBMCs in response to various pro-inflammatory stimuli and scores on the detailed Neuropsychological Assessment Battery. We used generalised linear regression to examine the association between IL6 production in response to various stimuli and scores on neuropsychological tests (Z-scores). We found no associations in the unadjusted models (presented). Further we observed no association in the adjusted model and no T2DM specific associations.

6.4.3 Association between mRNA expression in Unstimulated and LPS Stimulated PBMCs and cognitive outcomes

Analysis was repeated in a similar fashion to above using the log of IL-1β and IL-6 mRNA in both unstimulated (baseline) and LPS stimulated PBMCs. Log mRNA (Ratio to 18S) was used as the dependent variable and the neuropsychological test outcomes (Z-scores) as the independent variable. We observed an association between better performance on the pattern-recognition immediate stimulus condition and greater IL-1β mRNA expression at baseline (β = -0.20, 0.06 - 0.34, p<0.001) (Figure 6.9). This association persisted after controlling for age, gender, family history and years of formal education (p<0.001). Whilst an association was seen for poorer reaction time and significant less LPS induced mRNA expression, the ratio of LPS to Unstimulated mRNA was not significantly associated with poorer reaction time. No T2DM-specific associations were seen for either task (p = 0.572 for interaction for pattern recognition; p = 0.138 for reaction time). Full results of the analysis are detailed in Tables 6.5 and 6.6.

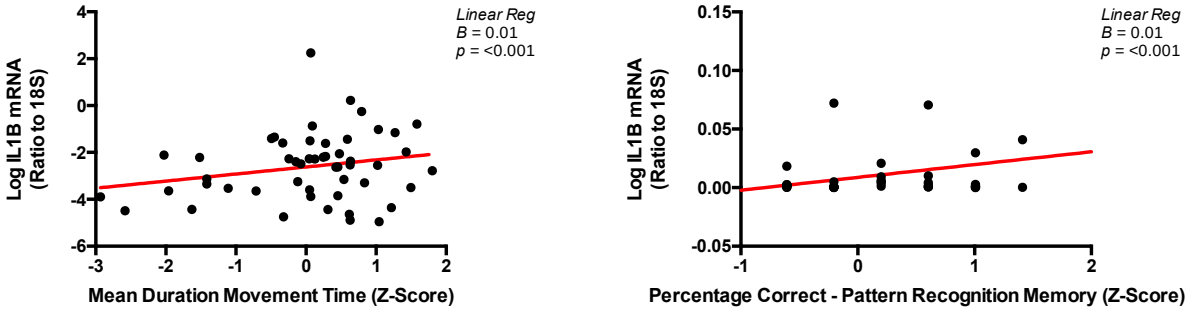

Figure 6.9 Correlational Analysis between baseline IL-1β mRNA expression and performance on the pattern recognition task and LPS stimulated IL-1β mRNA baseline expression and reaction time performance. *Linear models were used to explore the relationship between mRNA expression and the 12 neuropsychological outcomes from the CANTAB assessment battery. Gene expression data was expressed relative to a*

housekeeping gene, 18s and subsequently log transformed prior to inclusion in linear regression model

IL1β mRNA	**Unstimulated (US)**		**LPS**		**US: LPS Ratio**	
	β Coef. (95% CI)	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
RTI						
RTIF-MDRT	-0.08 (-0.24 – 0.07)	0.434	-0.34 (-0.54 - -0.15)	**0.001*** **	0.00 (-0.00 – 0.00)	0.645
RTIF-MDMT	0.11 (-0.05 – 0.27)	0.532	0.04 (-0.20 – 0.26)	0.762	-0.00 (-0.00 – 0.00)	0.912
PAL						
PAL-TEA	-0.11 (-0.25 – 0.03)	0.127	-0.02 (-0.22 – 0.19)	0.877	-0.00 (-0.00 – 0.00)	0.977
PAL-FAMS	-0.11 (-0.25 – 0.03)	0.127	-0.02 (-0.22 – 0.19)	0.877	-0.00 (-0.00 – 0.00)	0.977
SWM						
SWM-BE468	-0.31 (-0.18 – 0.12)	0.626	-0.08 (-0.28 – 0.13)	0.466	-0.00 (-0.00 – 0.00)	0.462
SWM-Strategy	-0.06 (-0.18 – 0.12)	0.481	-0.05 (-0.26 – 0.16)	0.658	-0.00 (-0.00 – 0.01)	0.623
PRM						
PRM-PCI	0.20 (0.06 – 0.34)	**<0.001*** **	0.04 (-0.18 – 0.25)	0.745	-0.00 (-0.00. 0.00)	0.103
PRM-PCD	0.03 (-0.12 – 0.18)	0.868	0.06 (-0.15 – 0.27)	0.577	-0.00 (-0.00 – 0.00)	0.746
OTSOC						
OTS-PCFS	0.13 (-0.01 – 0.27)	0.067	0.07 (-0.14 – 0.27)	0.514	-0.00 (-0.00 – 0.00)	0.452
OTS-MLFC	-0.08 (-0.24 – 0.09)	0.355	0.08 (-0.16 – 0.32)	0.520	0.00 (-0.00 – 0.00)	0.667
RVP						
RVP-AP	0.07 (-0.05 – 0.20)	0.244	-0.01 (-0.19 – 0.17)	0.916	-0.00 (-0.00 – 0.00)	0.932
RVP-PFA	-0.05 (-0.12 – 0.29)	0.215	-0.00 (-0.11 – 0.11)	0.974	0.00 (-0.00 – 0.00)	0.775

Table 6.5 Examining the links between IL-1β mRNA expression and neuropsychological test scores. *Linear models were used to assess the relationship between neuropsychological test scores (independent variable) and the log of the baseline and LPS stimulated RNA for IL-1β.*

IL6 mRNA						
	Unstimulated (US)		**LPS**		**US: LPS Ratio**	
	β Coef. (95% CI)	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
RTI						
RTIF-MDRT	0.08 (-0.23 – 0.39)	0.609	0.03 (-0.07 – 0.13)	0.589	-0.00 (-0.00 – 0.00)	0.326
RTIF-MDMT	0.24 (-0.13 – 0.62)	0.199	0.08 (-0.03 – 0.20)	0.159	-0.00 (-0.00 – 0.00)	0.110
PAL						
PAL-TEA	0.11 (-0.23 – 0.44)	0.530	0.04 (-0.07 – 0.15)	0.450	-0.00 (-0.00 – 0.00)	0.448
PAL-FAMS	0.11 (-0.23 – 0.44)	0.530	0.04 (-0.07 – 0.15)	0.450	-0.00 (-0.00 – 0.00)	0.448
SWM						
SWM-BE468	0.05 (-0.27 – 0.35)	0.769	0.02 (-0.09 – 0.12)	0.701	-0.00 (-0.00 – 0.00)	0.053
SWM-Strategy	0.15 (-0.20 – 0.49)	0.390	0.05 (-0.05 – 0.15)	0.317	-0.00 (-0.00 – 0.00)	0.220
PRM						
PRM-PCI	0.04 (-0.31 – 0.38)	0.829	0.01 (-0.11 – 0.11)	0.976	-0.00 (-0.00 – 0.00)	0.793
PRM-PCD	0.03 (-0.31 – 0.37)	0.868	0.02 (-0.10 – 0.11)	0.969	-0.00 (-0.00 – 0.00)	0.092
OTSOC						
OTS-PCFS	-0.11 (-0.43 – 0.21)	0.506	-0.05 (-0.15 – 0.06)	0.383	-0.00 (-0.00 – 0.00)	0.733
OTS-MLFC	-0.16 (-0.21 – 0.53)	0.398	0.05 (-0.07 – 0.16)	0.433	0.00 (-0.00 – 0.00)	0.576
RVP						
RVP-AP	-0.16 (-0.45 – 0.13)	0.271	-0.06 (-0.15 – 0.04)	0.232	0.00 (-0.00 – 0.00)	0.829
RVP-PFA	0.03 (-0.10 – 0.17)	0.621	0.01 (-0.03 – 0.06)	0.614	-0.00 (-0.00 – 0.00)	0.265

Table 6.6 Examining the links between IL-6 mRNA expression and neuropsychological test scores. *Linear models were used to assess the relationship between neuropsychological test scores (independent variable) and the log of the baseline and LPS stimulated RNA for IL-6.*

6.5 Activation of inflammatory responses and Gait Parameters

6.5.1 Exploring the association between activation of inflammatory responses and Gait Speed/Performance

We sought to assess whether peripheral blood immune cell responses showed any association with gait parameters known to reflect cognitive function. For this analysis, as data was normally distributed as detailed in Chapter 4, we performed generalised linear regressions on the four key gait outcomes (self-selected gait speed, maximal gait speed, dual-task cognitive gait speed and dual task cost or DTC). We initially ran the analysis unadjusted and then adjusted for age, gender, Body Mass Index (BMI) and number of medications (these significantly predicted gait speed in Chapter 4)

6.5.1 IL-1β and IL-6 production by stimulated PBMCs and Gait Parameters

Linear models demonstrated several significant associations between dual task cost (or DTC – representing the change in gait speed on addition of the cognitive task expressed as a percentage of self-selected speed with greater cost indicating poorer performance) and IL-1β production by PBMCs in response to various pro-inflammatory stimuli. Namely, in the Aβ-42 & LPS stimulated cells, greater production of IL-1β was associated with poorer dual task performance (β = 0.31, 0.04 – 0.58, 0.023). Similarly, greater IL-1β production in response to LPS & Nigericin was associated with greater DTC (β = 0.29, 0.05 – 0.54, 0.019). Finally, greater production of IL-1β in the poly dA:dT stimulation was associated with significantly greater IL-1β production (β = 0.22, 0.01 – 0.43, 0.019). Two of the three associations persisted in the adjusted models (β = 0.03, 0.01 – 0.06, p = 0.023 for the Aβ-42 & LPS condition; 0.03, 0.01 – 0.06, p = 0.023 for Nigericin & LPS; β = 0.02, -0.00 – 0.04, p = 0.051). These associations were not T2DM-specific however. Results of unadjusted regressions are presented for all five stimulation conditions and four gait parameters in Table 6.7. The three significant results are graphed in Figure 6.10. There were no significant

associations seen when the log of IL-6 concentration across the various stimulations was included as the dependent variable

IL1β Release	**LPS**		**Aβ-42**		**Aβ-42 & LPS**		**LPS & Nigericin**		**Poly dA: dT**	
Gait Speed	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Self-Selected	0.01 (-0.05 – 0.07)	0.659	0.02 (-0.04 – 0.08)	0.534	0.03 (-0.18 – 0.08)	0.193	-0.02 (-0.06 – 0.03)	0.564	0.00 (-0.04 – 0.04)	0.979
Maximal	0.02 (-0.07 – 0.11)	0.855	0.00 (-0.09 – 0.09)	0.965	0.03 (-0.04 – 0.11)	0.408	-0.04 (-0.11 – 0.03)	0.259	0.02 (-0.34 – 0.08)	0.443
Dual Task	0.02 (-0.05 – 0.08)	0.653	-0.00 (-0.06 – 0.06)	1.00	-0.00 (-0.06 – 0.55)	0.890	-0.05 (-0.10 – 0.01)	0.082	-0.02 (-0.07 – 0.02)	0.259
Dual Cost	0.03 (-0.00 – 0.01)	0.614	0.02 (0.01 – 0.04)	0.277	0.31 (0.04 – 0.58)	**0.023**	0.29 (0.05 – 0.54)	**0.019**	0.22 (0.01 - 0.43)	**0.040**
IL6 Release	**LPS**		**Aβ-42**		**Aβ-42 & LPS**		**LPS & Nigericin**		**Poly dA: dT**	
Gait Speed										
Self-Selected	0.00 (-0.03 – 0.04)	0.824	0.00 (0.03 – 0.04)	0.859	0.04 (-0.01 – 0.09)	0.133	0.00 (-0.04 – 0.03)	0.917	0.01 (-0.02 – 0.03)	0.761
Maximal	0.00 (-0.05 – 0.05)	0.416	0.05 (-0.00 – 0.10)	0.059	0.04 (-0.03 – 0.12)	0.251	0.02 (-0.04 – 0.08)	0.532	-0.00 (-0.05 – 0.05)	0.869
Dual Task	0.01 (-0.04 – 0.05)	0.131	0.01 (-0.03 – 0.05)	0.829	0.04 (-0.03 – 0.10)	0.250	-0.01 (-0.06 – 0.04)	0.668	-0.01 (-0.05 – 0.03)	0.664
Dual Cost	0.00 (-0.19 – 0.19)	0.999	0.03 (-0.17 – 0.23)	0.933	-0.06 (-0.35 – 0.24)	0.716	-0.12 (-0.37 – 0.12)	0.308	-0.11 (-0.28 – 0.07)	0.219

Table 6.7 Associations between PBMC cytokine production in response to various stimulations and Gait Parameters. *Gait parameters were modelled as the independent variable with the dependent variable being the log of cytokine concentration. There were significant associations between greater IL-1β production and performance on the dual task gait assessment, expressed as Dual Cost (DTC).*

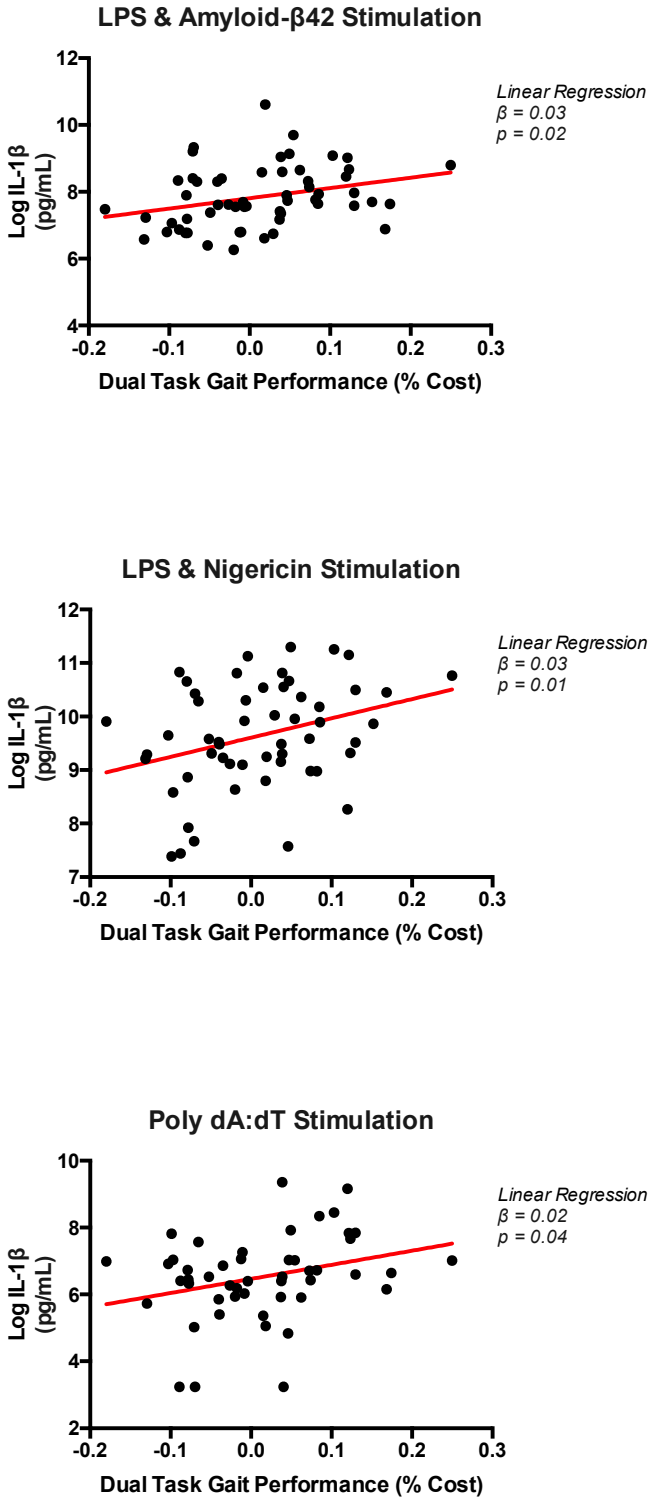

Figure 6.10 Performance on the Dual Task Gait Paradigm and production of IL-1β by PBMCs in response to Various Inflammatory Agonists. *Dual task cost, the difference between self-selected gait speed alone and on addition of a cognitive task (expressed as*

percentage change of self-selected gait speed) was modelled as the dependent variable with the log of cytokine production (protein expression) as the independent variable. Linear regression models were used to model assocaitions.

Finally, the association between gene expression for IL-1β was tested using the same modelling structure as above. We observed no significant associations either in the unadjusted or adjusted models. There were no T2DM-specific associations. Full results of unadjusted coeffieicnts are presented in Table 6.8

IL1β mRNA						
	Unstimulated (US)		**LPS**		**US: LPS Ratio**	
Gait Speed	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Self-Selected	1.30 (-0.81 – 3.45)	0.221	0.00 (-0.03 – 0.04)	0.656	0.00 (-0.00 – 0.00)	0.065
Maximal	0.93 (2.24 – 4.10)	0.588	-0.01 (-0.06 – 0.04)	0.683	0.00 (-0.00 – 0.00)	0.858
Dual Task	-0.61 (-3.14 – 1.91)	0.628	-0.00 (-0.05 – 0.03)	0.630	0.00 (-0.00 – 0.00)	0.060
Dual Cost	-0.53 (-0.24 – 0.27)	0.116	0.02 (-0.00 – 0.03)	0.092	-0.00 (-0.00 – 0.00)	0.550
IL6 mRNA						
	Unstimulated (US)		**LPS**		**US: LPS Ratio**	
Gait Speed	**β Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**	**β -Coef. (95% CI)**	**P**
Self-Selected	0.05 (-0.01 – 0.10)	0.070	0.02 (-0.00 – 0.03)	0.047*	0.00 (0.00 – 0.00)	0.017*
Maximal	0.04 (-0.04 – 0.11)	0.335	0.01 (-0.01 – 0.04)	0.312	0.00 (-0.00 – 0.00)	0.871
Dual Task	0.04 (-0.03 – 0.11)	0.240	0.02 (-0.00 – 0.04)	0.187	0.00 (-0.00 – 0.01)	0.010*
Dual Cost	0.01 (-0.02 – 0.04)	0.589	0.00 (-0.01 – 0.01)	0.639	-0.00 (-0.00 – 0.00)	0.574

Table 6.8 Associations between unstimulated/LPS induced PBMC gene expression Gait Parameters. *Gait parameters were modelled as the independent variable with the dependent variable being the log of mRNA expression. There were no significant associations for IL-1β or IL-6 mRNA and gait performance either on single (self/maximal) or dual tasks.*

6.6 Discussion

In the current analysis, we found no difference in PBMC responsiveness to innate immune inflammatory stimuli in those with midlife T2DM and healthy controls. However, we report significant associations between NLRP3 inflammasome activation by stimuli such as Aβ-42 and Nigericin when added to LPS stimulated cells and measures of general cognitive function in addition to dual task gait performance. Our study is the first to analyse this in a midlife T2DM population and also the first to examine the relationship of NLRP3 inflammasome stimulation to gait characteristics in a T2DM cohort.

The lack of between group differences to all stimuli in those with midlife T2DM vs healthy controls is noteworthy. Whilst previous evidence has demonstrated exaggerated innate immune responses and NLRP3 inflammasome activation in T2DM (Lee et al. 2013), these differences were only seen for untreated participants with a new diagnosis of T2DM. In fact, in the main study demonstrating this effect in human subjects with T2DM, Lee et al. (2013) reported that 8 weeks of metformin treatment normalised PBMC responses to innate immune stimuli in T2DM. In the ENBIND study, nearly all participants were on treatment for T2DM and included metformin and GLP-1 agonists amongst others, which have beneficial immunomodulatory effects on the innate immune system (Hogan et al. 2013, Lee et al. 2013). Nonetheless, the increased risk of dementia and cognitive impairment is seen in T2DM regardless of specific treatment. However, the role of the innate immune system may be too subtle to detect in those on treatment. It must also be recognised that a lack of association may be a result of Type II error, and the current study may be underpowered to detect subtle differences between those with and without midlife T2DM.

The most exciting finding from the current analysis centres around the increased production of IL-1β, an NLRP3 inflammasome dependent cytokine, from cells treated with LPS and Aβ-42 in comparison to LPS alone. No effect was seen for mRNA expression and this is consistent with activation of the innate immune inflammasome after priming with LPS.

This is particularly exciting in that the amount of IL-1β produced by cells showed a strong association with likelihood of error on the MoCA, a measure of general cognition. Thus, our findings directly implicate NLRP3 inflammasome dependent cytokine production in response to Aβ-42, the putative pathogenic protein in Alzheimer Disease, in determining overall cognitive performance in midlife. Our findings are further supported by the fact that an association was also seen between greater IL-1β production in response to treatment of LPS-primed cells with Nigericin, which is known to be a particularly potent activator of the innate immune inflammasome response. Further, these effects were not seen for IL-6 production, which is not an NLRP3 inflammasome dependent cytokine. Again this lends further support to our hypothesis that the NLRP3 inflammasome may have a role to play in cognitive impairments in midlife T2DM.

We also found a significant association between IL-1β production in response to Aβ-42 mediated NLRP3 inflammasome activation and performance on the dual-task gait paradigm, a putative marker of executive function and attention. This is the first assessment of PBMC immune cell responses with other potential clinical biomarkers which may have a role to play in detecting cognitive impairment and later cognitive risk in those with T2DM. Our findings, however, were not specific to those with T2DM. It must be remembered that our cohort in the ENBIND study was a cognitively normal cohort and that no participants actually demonstrated cognitive impairment. Thus, our findings may be thought of as a measure of cognitive vulnerability. To implicate activation of the innate immune NLRP3 inflammasome by Aβ-42 and LPS in this cognitive vulnerability (with increasing likelihood of error on the MoCA and poor dual task cost) is one of the principal findings of this study.

Conclusion

In conclusion, we provide the first evidence implicating the NRLP3 inflammasome is midlife cognitive performance and the first analysis in a T2DM population. We also report the first analysis of NLRP3 activation and clinical biomarkers (gait speed) and report a novel association between NLRP3 activation and performance on the dual task

gait paradigm. The particularly exciting part of the current project is the longitudinal component of the study. Our analysis forms part of the baseline characterisation of the ENBIND cohort and subsequent follow up will determine the role of NLRP3 activation in predicting cognitive trajectories in T2DM. Such analysis will have important implications in understanding the role of the NLRP3 inflammasome in T2DM related cognitive impairment and later dementia risk.

Chapter 7: Discussion, Conclusions and Future Directions

7.1 Main Findings

In the current study, we carried out a baseline cross-sectional evaluation of cognitive function in midlife Type 2 Diabetes Mellitus (T2DM). We assessed both general cognitive function and specific neuropsychological domains using both standardised and computerised cognitive testing. Participants also carried out assessment of gait (walking) speed and the relationship between gait and cognition investigated. Further, we investigated the relationship between peripheral blood pro-inflammatory cytokines and cognitive/gait function in midlife T2DM. Finally, we used Peripheral Blood Mononuclear Cells (PBMCs) isolated from otherwise healthy participants with midlife T2DM and healthy controls to assess immune cell reactivity to stimulation with several innate immune stimuli, particularly those targeting the innate immune NRLP3 inflammasome. Our results represent the first analysis of midlife markers of cognitive impairment and implicate the relationship between gait and cognition in midlife which has been poorly assessed to date. We provide the first evidence for the role of the NLRP3 inflammasome in midlife cognitive deficits. Each of our main findings is discussed here with reference to the relevant literature and future directions.

7.1.2 Midlife Cognitive Function in Type 2 Diabetes

In our cohort, we demonstrated a significantly greater likelihood of error on general cognitive testing (on the Montreal Cognitive Assessment: MoCA) in addition to poorer overall scores on the MoCA in those with midlife T2DM. Such a difference is particularly striking given the young age and matched demographic characteristics between the T2DM and healthy control groups. The finding for greater likelihood of error on the MoCA even persisted after adjusting for age, sex, years of formal education and family history of cognitive impairment/dementia. Despite the poorer scores seen on general cognitive testing, we failed to demonstrate a significant effect of T2DM on performance on our custom-designed neuropsychological assessment battery (CANTAB) aimed to probe working memory, attention and executive function, designed to probe the

earliest domains effected in T2DM related cognitive impairment as well as prodromal AD.

The lack of between group differences observed on the CANTAB assessment battery is noteworthy. Whilst the CANTAB assessment has been well validated (Egerhazi et al. 2007; Saunders & Summers, 2011), the tests are mainly designed to assess for pAD and MCI. It may be that in the current, non-impaired cohort which did demonstrate cognitive deficits/decrements on general testing (MoCA), the CANTAB assessment battery was not able to detect subtle cognitive differences between groups. Another possibility is that the group as a whole, given their young nature and lack of other risk factors for cognitive impairment, simply have too much cognitive reserve to detect differences.

Our results for general cognition (MoCA) are largely consistent with previous literature (Biessels et al. 2001, Garcia-Casares et al. 2014, Kinga & Anett, 2016, Kumar et al. 2015, Mehrabian et al. 2012, Naseer et al. 2014, Nazaribadie et al. 2013, Ryan et al. 2000, Solanki et al. 2009, Yau et al. 2009). However, the results for the neuropsychological testing did not demonstrate a significant effect of T2DM (albeit there were strong trends). Our analysis is the first to assess performance on a computerised neuropsychological assessment battery. Such methods have great advantages in terms of allowing analysis of parameters which would be unobtainable from simple cognitive assessments (for instance, time to make decisions, reaction time, detailed attentional and executive function testing). Such testing should be seen as complimentary to traditional cognitive testing on tools such as the MoCA, but not a replacement.

Due to the nature of outcome parameters on the CANTAB assessment, there is wide variation in the responses seen within groups of participants. Whilst such data distributions make finding between-group differences difficult, they serve as an excellent baseline assessment of detailed domains of cognitive/neuropsychological function. The real value of the data collected as part of the baseline wave of the ENBIND

study may only be realised once follow up waves using the same participants are completed in five years' time in order to examine changes over time. Such assessment will readily demonstrate whether those with T2DM really experience greater cognitive decline than healthy controls over time. In such a design, each participant, tested at multiple time points under identical conditions (allowed by the computerised standardized assessment). Analysis of such measures, nested within participant, allows the greatest determinant of cognitive function over time. Apart from sub-studies of larger cohorts such as the Atherosclerosis Risk in Communities Cohort (ARIC) or data from the Whitehall II sub-study, longitudinal analysis of cognitive trajectories in midlife T2DM has not taken place. Further, no study to specifically assess cognitive function over time in T2DM has ever been conducted. Analysis to date has been limited to cross sectional studies, which have multiple sources of bias in making inferences about cognitive function and cognitive trajectories.

7.1.3 Gait and Cognitive Function in Midlife Type 2 Diabetes

In the current study, we demonstrated a significant slowing of gait across all three gait tasks in those with midlife T2DM. Of note, these findings persisted on controlling for numerous covariates including age, BMI, medication use and other factors known to impact on gait performance in midlife cohorts. Across all tasks, T2DM was associated with a significant slowing effect, both on raw gait speed and on gait performance in terms of gait quartile in the overall study sample. Such findings are consistent with previous findings such as those from the Rotterdam Study. In the Rotterdam Study, just over 300 older adults (mean age = 70) were studied and T2DM was associated with slower gait speed (Maksimovic et al. 2016). Our study is the first study demonstrate this effect in a younger cohort, with a mean age nearly 20 years younger than the Rotterdam Study. Such a finding has important implications for longitudinal analysis of gait and cognitive trajectories in midlife T2DM.

Explaining such a difference may be more difficult however. In the first instance, all participants in our study were screened by clinical examination and a symptom score for assessment of peripheral neuropathy. Thus, we can exclude a contribution to slower gait speed by peripheral neuropathy as an explanatory factor. Further, we adjusted for BMI and antidepressant use in our analysis, factors which have known impact on midlife gait performance. Such slowing of gait may represent an underlying vulnerability in those with T2DM and may herald the onset on later peripheral neuropathy or even cognitive impairment. It will only become evident on longitudinal analysis. For instance, gait trajectories have never been explored in T2DM in detail. Subsequent waves of this study will analyse for both the development of peripheral neuropathy as well as the relationship between cognitive and gait parameters longitudinally in this high risk group.

We demonstrated a significant association between gait speeds across all three gait tasks and performance on measures of general cognition (the MoCA). Interesting, poorer gait speed in terms of gait speed quartile was predictive of likelihood of error on the MoCA across all three tasks in an unadjusted model. In the fully adjusted models, only the association for dual-task cost remained significant. This is particularly given the previous evidence from longitudinal studies which has demonstrated the association between dual-task cost and executive function, processing speed and attention (Killane et al. 2015). In this manner, the lack of association between scores on the neuropsychological tests of executive function, attention and reaction time in the present study are surprising. Again, there may be numerous reasons for this. In the first instance, it may be taken in the context of the lack of between group differences on comparing those with T2DM to healthy controls. In this context, it may be that due to the lack of demonstrable deficits in this analysis in those with T2DM on domain-specific testing, the dual task paradigm is not highlighting any particular deficits. However, we did observe a strong association between poorer performance on the dual-task paradigm and likelihood of error on the MoCA.

Another important observation from these results is that the effects we observed did not demonstrate T2DM specific effects. We analysed whether effects were specific to T2DM throughout the analysis by creating interaction terms between diabetes status and the independent variable of interest. Such an approach reports an interaction estimate, which we used to determine if something was T2DM specific. This technique fits two regression models, one for T2DM and one for healthy controls and the interaction estimate informs us whether the coefficients of these regressions are significantly different. Such a technique is much more robust and powerful than simply testing the association in one strata and not another (i.e fitting a model for T2DM and for HC and reporting something as group specific if $p<0.05$ in one but not the other). However, it may be overly conservative in estimating T2DM effects, particularly given the much greater number of T2DM participants than healthy controls in the current study. Again, it will be the longitudinal piece which may demonstrate the specific trajectories of cognitive and gait parameters in midlife T2DM.

Future work in this cohort will focus on the use of Inertial Motion Unit (IMU) or similar wearable accelerometry to measure not only gait speed but other parameters of gait. For instance, in the Rotterdam Study, not only was T2DM associated with slower gait overall, but also poorer performance in terms of gait pace and gait tandem, defined using an electronic walkway (Maksimovic et al. 2016). Detailed analysis of these parameters, particularly over time, may provide more useful insight in the longitudinal relationship between gait and cognitive parameters in T2DM and further knowledge of the potential of gait as an important non-invasive biomarker in T2DM related cognitive decline.

7.1.4 Peripheral Immune Cytokines

As part of the current study, we analysed differences in the serum levels of six peripheral inflammatory markers in order to investigate the role of such immune activity as a potential marker of cognitive and gait decrements in midlife T2DM. No difference in the

amount of circulating peripheral pro-inflammatory cytokines were demonstrated between those with T2DM and healthy controls.

On analysing the relationship between these inflammatory markers and cognitive performance, we found that, higher levels of both MCP-1 and CXCL10 were associated with greater likelihood of error on the MoCA. This finding is particularly interesting given the recent evidence linking MCP-1 and both Mild Cognitive Impairment (MCI) and Alzheimer's Disease (Lee et al. 2018, Morgan et al. 2019). MCP-1 is also elevated in T2DM serum (deFronzo et al. 2016) and may represent a particularly interesting marker for later cognitive decline in those with T2DM. Future longitudinal analysis of this cohort will assess this relationship in detail. On assessment of neuropsychological higher levels of pro-inflammatory cytokines were associated with poorer scores on reaction time (for IL-6 levels) and greater probability of error (with increasing MCP-1 levels) on the visual processing task. Again, these findings are particularly interesting given the role of MCP-1 and general cognition above in the current study as well as the previous literature as discussed.

Despite exciting findings for MCP-1 and its relationship to performance on the various cognitive variables, associations didn't persist when age, sex and other covariates with a known impact of cognitive function were adjusted for. Similarly, none of the effects observed were specific for T2DM and the relationship between cognitive function and MCP-1 was not observed to significantly differ between groups. Potentially, such a relationship reflects an association between MCP-1 and cognitive function more generally. Further, whether analysis using interaction terms is overly-conservative for the current analysis cold potentially introduce a false negative finding, however using such an approach reduces the probability of Type I error substantially in the current study.

7.1.5 PBMC Activity, T2DM, Cognitive and Gait Function

We present the first analysis of peripheral immune cell responsiveness to Aβ-42 and other immune cell stimulants in midlife T2DM with the specific aim of examining whether these responses demonstrate any association with midlife cognitive performance and gait performance in T2DM. In the first instance, we demonstrate the ability of Aβ-42, alone or in combination with LPS to activate pro-inflammatory pathways with the production of IL-1β (a cytokine dependent on activation of the NLRP3 inflammasome).

In our between-group analysis, we demonstrated no difference in PBMC responsiveness to Aβ-42 on comparing those with T2DM to healthy controls. Previous studies using different NLRP3 inflammasome stimuli have demonstrated significantly upregulated NRLP3 activity in those with T2DM (Lee et al. 2013). One of the biggest difference between the current analysis and that of Lee et al. 2013 is that we analysed PBMC responsiveness is that our cohort of participants with T2DM were on treated in comparison to the aforementioned study which considered newly diagnosed individuals not on treatment. In the same study, authors demonstrated that treatment with metformin normalised PBMC immune responses in T2DM and thus this may be the reason for lack of replication of this finding in the current paper.

Interestingly, we demonstrated that greater production of IL-1β (the NLRP3 dependent cytokine) under both NLRP3 inflammasome stimulated conditions (Nigericin & LPS and Aβ-42 & LPS) was significantly associated with greater likelihood of error on the MoCA, which persisted after covariate adjustment. A trend towards a T2DM-specific effect was observed for these findings. This is a particularly noteworthy finding and suggests that individuals whom demonstrate a greater pro-inflammatory reaction to Aβ-42 in terms of IL-1β production perform poorer on tests of general cognitive function. This is the first time this has been demonstrated in such a young cohort and also the first time it has been studied in those with T2DM. Longitudinal analysis of cognitive trajectories in this study will be particularly important and provide novel insight into the potential relationships between

PBMC responsiveness and cognitive trajectories in those with midlife T2DM and healthy controls.

Further, IL-1β production in response to the same two conditions (Nigericin & LPS and Aβ-42 & LPS) was significantly associated with performance on the dual-task gait paradigm (in terms of dual task cost). This is an exciting result. Dual task gait performance, and in particular dual task cost is known to be strongly associated with executive function, attention, working memory and reaction time. Thus, dual task performance appears to correlate with underlying cognitive vulnerability. In this context, the association between greater IL-1β production in response to two separate known NLRP3 and poorer gait performance is fascinating. Again, analysis of these cognitive and gait parameters over time in subsequent studies will enable further analysis of the longitudinal relationship between PBMC reactivity and cognitive/gait trajectories.

7.2 Future Directions

The current analysis demonstrated significantly poorer general cognition, but not domain specific cognitive function in midlife T2DM. We demonstrated a significant association between dual task gait performance and general cognitive function in midlife in addition to several associations between gait speed and tests of executive function. We also report significant associations between immune cell reactivity and cognitive/gait function in midlife T2DM, and this is the first report to do so.

One of the open questions in the field of research linking T2DM appears to be when exactly differences between T2DM and age-matched healthy controls occurs. Studies examining differences at 5 years have shown no difference but cohorts such as the Whitehall II and ARIC mentioned above appear to show significant differences after 10-20 years. By the time cognitive deficits are demonstrable, much of the damage may have already occurred. The current study, the ENBIND study will follow participants at 5 yearly intervals in order to examine for changes in cognitive, gait and immunological

parameters over time. We aim, using this purpose designed study, to assess when exactly the differences between T2DM and healthy controls begin to emerge, and what the earliest signs are.

Particularly interesting is the analysis of gait parameters such as gait speed. Previous studies have demonstrated that change in gait speed over time may be more important in predicting risk of later cognitive decline and dementia as opposed to raw measures of gait speed (Montero-Odasso et al. 2018; Hackett et al. 2018). The addition of a dual task and analysis of dual task-performance over time in future waves of the ENBIND study will be important. Changes in dual-task over time have been rarely performed and most of the data linking dual-task performance and cognitive function is based on cross-sectional analysis. Thus the data reported in this thesis represents an exciting start point to further disentangle this complex relationship.

Analysis of parameters such as gait as a potential biomarker of later cognitive decline is particularly attractive due to its non-invasive and cost-efficiency. Most smartphone and smartwatch technologies at present have inbuilt accelerometers that are capable of measuring gait speed. The development of new technological advances such as application development to enable ecologically-valid measurements of gait speed and eventually even other spatio-temporal gait parameters is an exciting prospect for future research. Such developments may be incorporated into future waves of the ENBIND study.

More research is needed into the basic mechanisms underlying cognitive impairment in T2DM. Whilst a great deal of research has focused on clinically relevant parameters (such as looking at the influence of hyperglycaemia and insulin resistance for instance (Stachan et al. 2008, Jan Biessels et al. 2018), more exploratory analysis is needed to uncover, repute or confirm the putative pathophysiological hypotheses. In the current analysis we have supported the potential role of the innate immune NLRP3

inflammasome, however only longitudinal analysis over the next 5 and 10 years will fully confirm this exciting results.

On foot of recent developments in the field of “inflamaging”, we need further work assessing the role of chronic low grade “sterile” inflammation and age-related diseases such as T2DM and dementia (Franchesci et al. 2018). Given the rate of progress in the understanding of how our body responds to danger signals, it is no surprise that application of such new knowledge and hypothesis may yield insightful results in examining the relationship between T2DM and cognitive impairment.

Ultimately, the aim of this body of work is to enable clinicians to adequately select-out individuals affected by midlife T2DM who may be at the greatest risk of later cognitive decline. Given the prevalence of both conditions and projected increases based on population demography, such an ability is an important unmet clinical need. If we can adequately select-out those with greatest risk at midlife, multi-domain interventions such as those in the FINGER study (Ngandu et al. 2015) may produce cognitive benefit in this group where interventions that have been trialled are often performed at too late a stage (Dyer et al. 2019). Ultimately, we need multi-domain interventions in midlife specifically aimed at those at highest risk.

7.3 Conclusion

In conclusion, we demonstrate a significantly poorer general cognitive performance and poorer self-selected, maximal and dual-task gait speed in midlife T2DM. A significant relationship between performance across all three gait tasks and general cognition was demonstrated in addition to several associations between self-selected and maximal gait speeds and neuropsychological test scores in midlife T2DM. Whilst we observed no between differences were observed in peripheral pro-inflammatory cytokines, we observed associations between peripheral levels of MCP-1 and likelihood of error on the MoCA in the combined cohort. We demonstrated a significant association between

NLRP3 inflammasome dependent cytokine production and performance on the MoCA as well as dual-task gait performance. Such findings have important implicates for future research and demonstrate the potential utility of gait as a novel biomarker of cognitive function worthy of future study in addition to providing evidence for an association between NLRP3 activation and cognitive/gait performance in a midlife cohort. The current study (ENBIND) will follow participants at five yearly intervals and uncover the earliest potential signs of later cognitive decline in midlife T2DM with the ultimate aim of selecting out individuals with T2DM whom are particularly at risk.

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