Molecular and Genetic Studies of the Cerebral Folate System in Human Aging & Alzheimer's Brain

By

Syeda Farwa Naqvi

Human Genetics Lab, Department of Zoology,

Faculty of Biological Sciences

Quaid-i-Azam University, Islamabad,

Pakistan

2022

Molecular and Genetic Studies of the Cerebral Folate System in Human Aging & Alzheimer's Brain

A dissertation submitted in partial fulfilment of requirements for degree of Doctor of Philosophy in Human Genetics and Neuroscience

Doctoral Supervisor: **Dr. Sajid Malik (QAU)** Doctoral Co-Supervisor: **Dr. Jaleel Miyan (UoM)**

Conducted by:

Syeda Farwa Naqvi

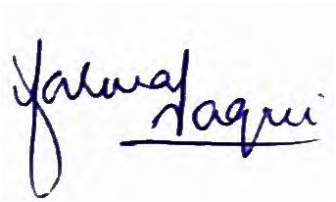
Human Genetics Lab, Department of Zoology,
Faculty of Biological Sciences
Quaid-i-Azam University, Islamabad,
Pakistan

2022

بسم الله الرحمن الرحيم

DECLARATION

I **Ms. Syeda Farwa Naqvi** hereby declare that I have worked on my thesis "Molecular and Genetic Studies of the Cerebral Folate System in Human Aging & Alzheimer's Brain" independently and that the work presented here is original. This thesis has not been submitted in the current or any similar form to any other university.



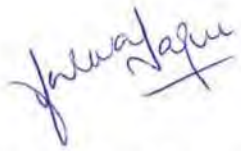
Syeda Farwa Naqvi

January 2022

Author's Declaration

I **Ms. Syeda Farwa Naqvi** hereby state that my PhD thesis titled "Molecular and Genetic Studies of the Cerebral Folate System in Human Aging & Alzheimer's Brain" is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after my Graduate the University has the right to withdraw my Ph.D. degree.



Ms. Syeda Farwa Naqvi

Date: 10-11-2022

Plagiarism Undertaking

I solemnly declare that research work presented in the thesis titled "Molecular and Genetic Studies of the Cerebral Folate System in Human Aging & Alzheimer's Brain" is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature: _____

Name: **Ms. Syeda Farwa Naqvi**

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled "Molecular and Genetic Studies of the Cerebral Folate System in Human Aging & Alzheimer's Brain" was conducted by **Ms. Syeda Farwa Naqvi** under the supervision of **Prof. Dr. Sajid Malik**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Zoology of Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Human Genetics.

Student Name: **Ms. Syeda Farwa Naqvi** Signature:_____

Examination Committee:

a) External Examiner 1: Signature:_____

Dr. Mazhar Qayyum
Professor/Dean
Department of Zoology
Arid Agriculture University
Rawalpindi

b) External Examiner 2: Signature:_____

Dr. Jahangir Arshad Khan
(Ex Chief Research Officer)
House No. 68, Street No.51,
F-11/3, Islamabad

Supervisor Name: **Prof. Dr. Sajid Malik** Signature:_____

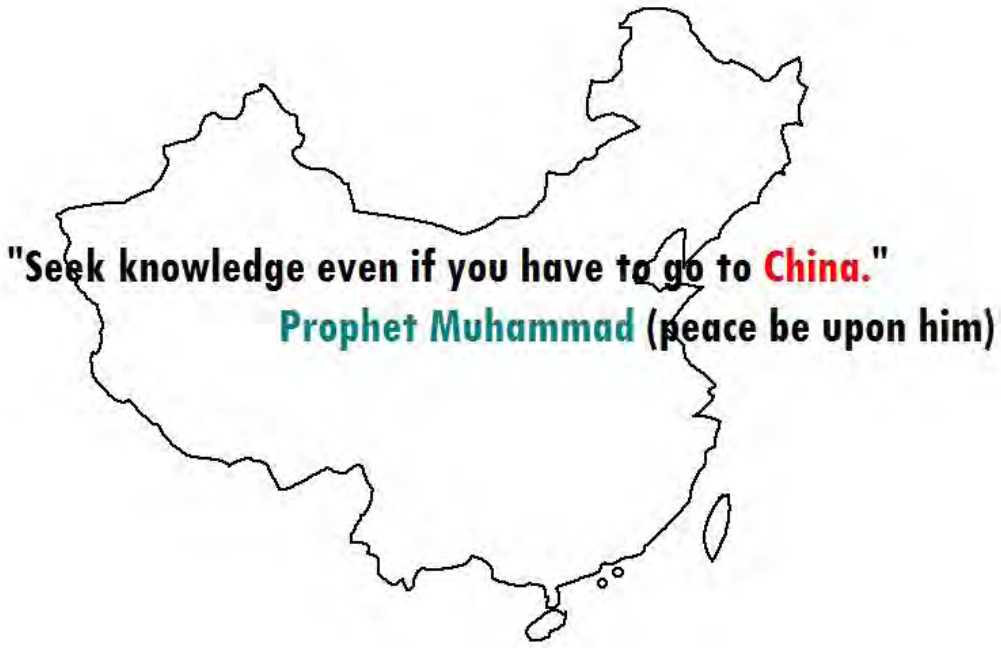
Name of HOD: **Prof. Dr. Amina Zuberi** Signature:_____

Date: 10-11-2022

CHAIRPERSON
Department of Zoology
Quaid-i-Azam University
Islamabad.

DEPARTMENT OF ZOOLOGY
ISLAMABAD
DATE 10-11-22


قُل لَّوۡ كَانَ ٱلۡبَحۡرُ مِدَادٗا لِّكَلِمَٰتِ رَبِّي
لَنَفِدَ ٱلۡبَحۡرُ قَبۡلَ أَن تَنفَدَ كَلِمَٰتُ رَبِّي
وَلَوۡ جِئۡنَا بِمِثۡلِهِۦ مَدَدٗا
IF THE SEA WERE INK FOR [WRITING] THE WORDS OF MY LORD, THE SEA WOULD BE EXHAUSTED BEFORE THE WORDS OF MY LORD WERE EXHAUSTED, EVEN IF WE BROUGHT THE LIKE OF IT IN [CONTINUAL] SUPPLEMENT.
Quran 18 : 109


"Seek knowledge even if you have to go to China."
Prophet Muhammad (peace be upon him)


بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ
اللَّهُ نُورُ السَّمَاوَاتِ وَالْأَرْضِ مَثَلُ نُورِهِ كَمِشْكَاةٍ
فِيهَا مِصْبَاحٌ الْمِصْبَاحُ فِي زُجَاجَةٍ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ
دُرِّيٌّ يُوقَدُ مِن شَجَرَةٍ مُبَارَكَةٍ زَيْتُونَةٍ لَا شَرْقِيَّةٍ وَلَا غَرْبِيَّةٍ يَكَادُ
زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ نُّورٌ عَلَىٰ نُورٍ يَهْدِي اللَّهُ
لِنُورِهِ مَن يَشَاءُ وَيَضْرِبُ اللَّهُ الْأَمْثَالَ لِلنَّاسِ
وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ ٣٥

Allah is the Light of the heavens and the earth. The example of His light is like a niche within which is a lamp, the lamp is within glass, the glass as if it were a pearly [white] star lit from [the oil of] a blessed olive tree, neither of the east nor of the west, whose oil would almost glow even if untouched by fire. Light upon light. Allah guides to His light whom He wills. And Allah Presents examples for the people, and Allah Knows all things (The Holy Quran, 24:35).

Acknowledgment

All praise belongs to Allah Alone Who Created the Heavens and Earth and granted us His Permission to explore and understand His Creation and, in that, to find Him. Reflecting the Light of the Heavens and Earth, The Holy Prophet Muhammad ﷺ instructed all to explore the Creation of Allah Almighty stating that "an hour of contemplation and study of God's Creation is better than a year of adoration". Thus, the door was opened for Islamic science, which we continue in the modern world at a much more detailed, microscopic level. May Allah Almighty Allow us the wisdom to understand what He Reveals to us. I am thankful to Allah Almighty, Holy Prophet Muhammad ﷺ and his family for providing me spiritual strength on each step.

I am grateful to my supervisor, Dr Sajid Malik, for his support throughout my research at QAU. I also thank the Dean of the Faculty of Biological Sciences; Prof. Dr Sarwat Jahan and the Chairman of the Department of Zoology; Prof. Dr Amina Zuberi, for hosting my doctoral studies and work. I must also thank Mujahid bhai for technical assistance, Mr Naeem and Mr Samiullah for clerical assistance.

I am thankful to my co-supervisor Dr Jaleel Miyan and his team at the University of Manchester, UK, for the intensive training I received during my two years there under the prestigious Commonwealth scholarship.

I really enjoyed working with my lab fellows at University of Manchester. I am very thankful to Alicia (late) for teaching me technical skills. Very much obliged to take help from Jocelyn Glazier, John Gig and Chris Murgatroyd. I am thankful to all my lab fellows, Claire, Lucy, Aldo, Annabel, Charlotte, Ellie and Mark. It was the best time of life so far. Working with them was a great experience combined with a lot of fun.

I would like to extend my thanks to the Commonwealth Scholarship Commission UK, for providing me the opportunity for this visit through a split-site scholarship, UK. It was an honour to be a part of this prestigious and generous scholarship. I am especially thankful to my program officer Mr. Nadim Khan for his support throughout my stay in the UK. I

am thankful to all my Commonwealth fellows specially Mr. Naveed Hussain. I am thankful to Zahra for her guidance in my application for the Commonwealth scholarship.

I am highly thankful to Manchester Brain bank and to brain donors as without their donation this study would never have been possible. I am also thankful to Dr. Federico Roncaroli and Dr. Andrew C. Robinson, who helped and facilitated me to get the samples. Special thanks to all brain donors at Manchester Brain bank.

I am thankful to my family friends Gulmeena, Imran and Nargis who made my stay memorable in UK. Grateful to my uncle Mr. Khalid for his extra ordinary support.

I also want to pay special thanks to my PhD lab fellows/juniors at my home university (QAU) Fizza, Sara, Afzal, Kamran, Rehana, Qandeel, and Anisa.

I feel proud that I have friends like, Hizran, Saima, Taqveem, Sadaf, Sidra, Sehrish, Ruqia, Riffat, Madeeha, Tayyaba, Sheila and Sonia. I always remember them in my prayers.

No deeds can return, and no words can express the affection, love, amiable sacrifices, attitude, unceasing prayers, advices, inspiration and support that my parents, Syed Ali Anjum Naqvi and Fakhra Ali, brothers, Shahbaz, Mustafa, Moosa, Ali, Haider and Hassan Abbas. I am thankful to my father and mother-in-law for their prayers. I owe my deepest gratitude to my elder sisters Salma (Dede) and Dr. Laila for their guidance and help in various aspects of my life and studies. I am thankful to my younger sisters, Zainab, Ana and Zahra, my nieces, Abeeha, Sakeena, Aleeha, Haya, Zainab and Aaima, Shahnoor and my nephews, Mahad and Muhsin, for all their love.

I am thankful to my loving husband Rabi Rizvi and all family for their prayers and good wishes.

Finally, any errors or omissions that remain are mine alone.

Syeda Farwa Naqvi

CONTENTS

List of Acronyms

Abbreviations	Elaborations
5-HMC	5-Hydroxymethylcytosine
5-MC	5-Methylcytosine
5-mTHF	5-methyl tetrahydrofolate
AD	Alzheimer's disease
ADAS	Alzheimer's disease assessment scale
ADHD	Attention deficit hyperactivity disorder
APOE4	Apolipoprotein epsilon 4
APP	Amyloid precursor protein
AQP4	Aquaporin 4
asp	Abnormal spindle' gene
ASPM	Abnormal spindle-like microcephaly-associated
Aβ	Amyloid beta
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BCSFB	Blood-cerebrospinal fluid-barrier
BH2-BH4	Dihydrobioptrein-tetrahydrobiopterin
BHMT	Betaine hydroxymethyl transferase
BSA	Bovine serum albumin
CFD	Cerebral folate deficiency
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CP	Choroid plexus
CSF	Cerebrospinal fluid
CT	Computerised tomography
DAB	Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DB	Dot blot
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid

ECL	Enhanced chemiluminescence
FDH	10-formyl tetrahydrofolate dehydrogenase
Fe	Iron
FOLR1	Folate receptor alpha 1
FRα	Folate receptor alpha
FTD	Frontotemporal dementia
GFAP	Glial fibrillary acidic protein
GWAS	Genome-wide association studies
HRP	Horseradish peroxidase
HTx	Hydrocephalic Texas
I	Iodine
ID	Intellectual disability
IgG	Immunoglobulin G
IHC	Immunohistochemistry
K	Kaleidoscope
kD	Kilo dalton
LBD	Lewy body dementia
MCI	Mild cognitive impairment
MCPH	Autosomal recessive primary microcephaly
MMSE	Mini mental state examination
MoCA	Montreal cognitive assessment
MRC	Medical research council
MRI	Magnetic resonance imaging
MTHFD1	Methylenetetrahydrofolate dehydrogenase 1
MTHFR	Methylenetetrahydrofolate reductase
MTR/MS	Methionine synthase
MTRr	Methionine synthase reductase
NAP	Non-animal protein
Neu-N	Neuronal nuclei
NHS	National health service
NMD	Nonsense-mediated RNA decay
NPH	Normal pressure hydrocephalus
pAB	Polyclonal antibody

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCFT	Proton-coupled folate transporter
PCR	Polymerase chain reaction
PET	Positron emission tomography
PMD	Post-mortem delay
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RFC	Reduced folate carrier
RNA	Ribonucleic acid
RPM	Revolution per minute
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SCO	Subcommissural organ
SDS	Sodium dodecyl sulphate
SDs	Standard deviations
SEM	Standard error of the mean
SHMT	Serine hydroxymethyl transferase
SMA	Smooth muscle actin
SNPs	Single nucleotide polymorphism
THF	Tetrahydrofolate
TS	Thymidylate synthetase
TWAS	Transcriptome-wide association studies
VD	Vascular dementia
VDR	Vitamin D nuclear receptor
WB	Western blot
WHO	World health organization

List of Tables

List of Figures

Abstract

Background: Recent publications highlight the need for new directions in the search for cause, aetiology and effective treatments for Alzheimer's disease (AD). Metabolic disorders, and specifically folate metabolism, have been identified as areas of potential interest in neurological conditions, and an investigation of the cerebral folate system in normal and AD human brain tissues was therefore carried out.

Methods: Post-mortem human brain tissue and matched cerebrospinal fluid (CSF) samples were provided by the Manchester Brain Bank. Western and dot blots, to measure folate-related proteins and metabolites were performed on CSF and tissue lysates. Immunohistochemistry (IHC) for folate-related proteins and metabolites was performed on formalin-fixed, cryoprotected frozen sections of cerebral cortex. Nutrigenomic analysis of folate related genes was carried out to identify single nucleotide polymorphisms (SNPs) and correlate to physiological changes in folate metabolism.

Results: A decrease in CSF folate metabolism was measured including in 10-formyl tetrahydrofolate dehydrogenase (FDH, *ALDH1L1*), a critical folate enzyme. In tissue, a switch in pathway of folate supply was found in AD compared to normal. The main folate carrier, folate receptor alpha (FOLR1), switched from FDH-positive astrocytes in normal, to glial fibrillary acidic protein (GFAP)-positive astrocytes in the AD cortex which was correlated with hypermethylation of neurones. All folate enzymes were reduced in the cortex, reflecting changes in the CSF except FDH which, although the most reduced of the proteins in the CSF, was raised in the tissue. In addition, a novel SNP in methylene tetrahydrofolate dehydrogenase 1 (*MTHFD1*) was correlated with AD. This was found to be correlated with an increase in glutathione in tissue, while in individuals without this SNP, there was an increase in *MTHFD1*.

Conclusions: These results suggest that in the AD brain, FOLR1 enters the cortex from the CSF via GFAP-positive astrocytes, rather than FDH-positive astrocytes seen in normal brain. Folate is then delivered directly to neurones for hypermethylation. Moreover, there is a significant association of an SNP in *MTHFD1* with AD that is reflected in a change in folate metabolism with an increase in tissue glutathione, while in normal there is no increase in this metabolite but there is an increase in *MTHFD1*.

Chapter 1

Introduction

Chapter 1

Introduction

1.1. Neurological conditions: a worldwide cause of morbidity and mortality

According to the National Health Service (NHS) in the UK there are more than 600 conditions that can be classified as neurological and have been further sub- classified by the NHS: (https://www.england.nhs.uk/ourwork/clinical-policy/ltc/our-work-on-long-term-conditions/neurological):

1. Sudden onset conditions (e.g., acquired brain injury or spinal cord injury)
2. Intermittent and unpredictable conditions (e.g., epilepsy, myalgic encephalomyelitis or chronic fatigue syndrome, certain types of headaches, or the early stages of multiple sclerosis)
3. Progressive conditions (e.g., motor neuron disease, Parkinson's disease, or later stages of multiple sclerosis, dementia and Alzheimer's disease (AD).
4. Stable neurological conditions (e.g., post-polio syndrome, or cerebral palsy in adults)

Referring to some of the neurological conditions listed by World Health Organisation (WHO), the global impact can be appreciated in the numbers of people affected. For example, schizophrenia typically begins in late adolescence or early adulthood, and has been estimated to affect more than 21 million (0.26%) people worldwide (GBD 2016 Neurology Collaborators, 2019) (Chen et al., 2020). Epilepsy affecting more than 50 million (0.64%) people of all ages, genders, ethnic backgrounds and geographic locations worldwide (Guekht et al., 2021). Autism Spectrum Disorder is a heterogenous neurodevelopmental condition affecting more than 1% of children globally and as high as 2% in some countries (Fischi-Gomez et al., 2021). Bipolar disorder is a severe psychiatric disorder that affects approximately 2-5% of the population worldwide (Jann, 2014; Zou et al., 2021). Attention Deficit Hyperactivity Disorder (ADHD) affects approximately 5% of the child and youth population worldwide and is characterized by symptoms of hyperactivity-impulsivity and inattention (Papadopoulos et al., 2021).

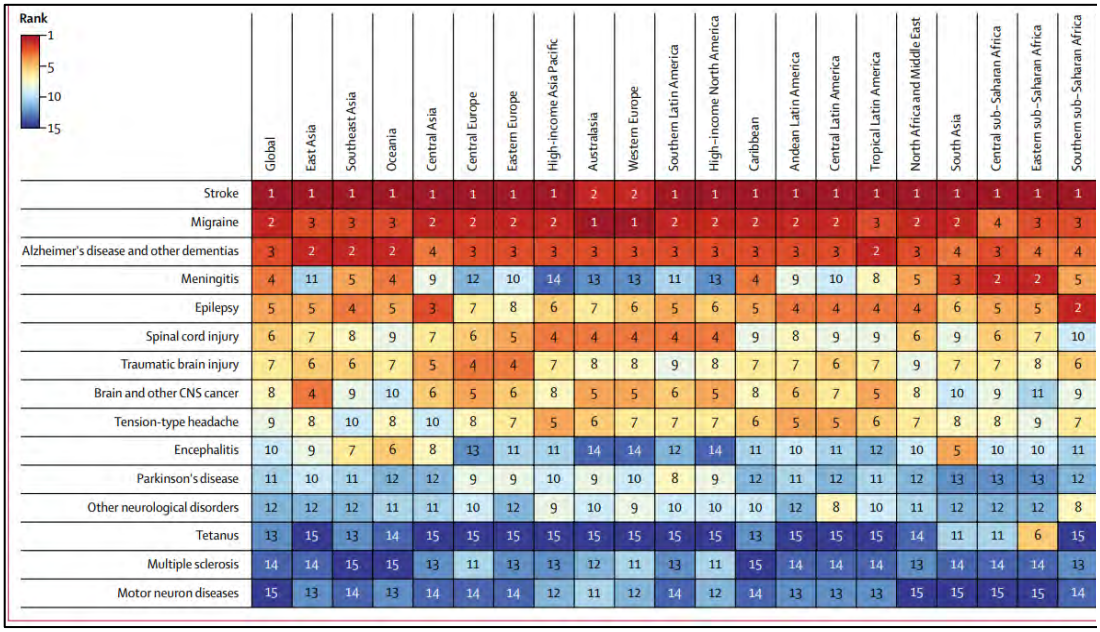


Table 1.1. Incidence of different neurological conditions globally (column 1) and in different regions of the world. Stroke and migraine are the most prevalent conditions with dementia and AD third globally and in all regions. Other conditions differ by region in the world (GBD 2016 Neurology Collaborators, 2019; Grzybowski et al., 2006).

Generally, all neurological conditions can result in severe morbidity and mortality, accounting for at least 16.5% of total deaths globally as well as huge lifetime healthcare cost (GBD 2016 Neurology Collaborators, 2019). The potential impact ranges from physical impairments of motor, sensory, cognitive and communication impairments to psychosocial ones (Olaleye et al., 2021). A recent systematic review shows the impact of the major neurological conditions across the world (GBD 2016 Neurology Collaborators, 2019).

From this table stroke and migraine result in the most severe forms of neurological problems with dementia and AD third in the list at the global level. This makes dementia and AD major health concerns as they are progressive and result in severe morbidity and death with huge health costs and with little effective treatments in drugs or behavioural therapies (Grzybowski et al., 2006). Many strategies, e.g., brain training, increased brain use, fish oils or vitamin supplements have failed to slow down these degenerative conditions although higher educational levels appear to prevent or delay onset of these conditions. There is thus a very urgent need to understand the cause and aetiology of these conditions to give some hope for a treatment to halt or prevent them at early stages.

Although this thesis is focused on dementia and particularly AD, some elements potentially common to neurological conditions will be highlighted here.

1.2. Dementia and AD

Dementia is an umbrella term used to describe a clinical syndrome of progressive cognitive decline, but its subtypes are classified according to the cause of dementia. The 4 common types of dementia: AD, vascular dementia (VD), Lewy body dementia (LBD) and frontotemporal dementia (FTD). AD is the most common neurodegenerative disease responsible for dementia, comprising 60% to 80% of cases. VD is the second most prevalent form of dementia (20%). Also called multi-infarct dementia, vascular dementia results from neuronal deprivation of oxygen caused by conditions that either block or reduce blood flow to the brain. Stroke is the most common cause of VD along with high cholesterol associated with cardiovascular problems. LBD is a form of dementia caused by abnormal deposits of alpha-synuclein protein (Lewy bodies) inside neurons. It accounts for 5% to 15% of all dementias. FTD is a general term used to describe disorders, such as Pick's disease, that affect the frontal and temporal lobes of the brain. FTD tends to occur at a younger age (40-75 years) than does AD (Duong et al., 2017).

Dementia results in deterioration in memory, thinking, behaviour and the ability to perform everyday activities. Symptoms appear gradually over time, are progressive and persistent. Individuals suffering from dementia experience a decline in cognition, sensory and motor functions and behaviour. The clinical presentation varies greatly among individuals, with cognitive deficits presenting variably as memory loss, communication and language impairments, agnosia (inability to recognize objects), apraxia (inability to perform previously learned tasks) and impaired executive function (reasoning, judgement and planning). Cognitive impairment stems from injury to the cerebral cortex caused by synaptic failure, inflammation and change in cerebral metabolism (Duong et al., 2017). There were approximately 46.8 million (0.6%) dementia patients worldwide in 2015, and this figure is expected to steadily increase to 74.7 million (0.95%) in 2030 and 131.5 million (1.68%) in 2050. In addition, statistics indicate an estimate of one new case per 3.2 seconds or 9.9 million per year, distributed worldwide as follows: 4.9 million in Asia, 2.5 million in Europe, 1.7 million in Americas and 0.8 million in Africa (Maryam et al., 2021)

Patients with mild deficits who do not meet all the criteria for dementia are considered to have mild cognitive impairment (MCI). As AD is a progressive condition, in its early stages, individuals may present with MCI and then dementia and finally all the signs and symptoms of AD. Thus, individuals with MCI are at higher risk of developing AD and other dementias than those without MCI (Duong et al., 2017).

AD is currently an irreversible neurodegenerative condition affecting around 40 million people over the age of 60 worldwide, with numbers reportedly doubling every 20 years (Ferri et al., 2005). More than 2000 clinical trials aimed to slow or halt the disease, and most recently to target amyloid clearance have failed (P. P. Liu et al., 2019; Mantile and Prisco, 2020; Oberman et al., 2020; Stoiljkovic et al., 2021). These have been based on several different theories of disease aetiology and progression but without positive benefits, suggesting that the targets are too late in the disease process and or are a consequence of deeper, higher-level processes. With increasing incidence of dementia and AD, there is an urgent need to identify new directions to approach this condition. In the century since Alois Alzheimer discovered AD, scientists have made remarkable strides in understanding the illness although it was not until the 1980s that two key molecular culprits in disease pathophysiology, amyloid beta (Aβ) and Tau proteins, were identified. AD can be categorized into 4 stages on the basis of severity, i.e., MCI, mild AD, moderate AD and severe AD (Dubois et al., 2007; Vellas et al., 2011). Currently, AD can be confirmed only through post-mortem findings or, rarely, in life by brain biopsy or certain kinds of specialist imaging, including positron emission tomography (PET) imaging (Aisen et al., 2017). While the onset of AD is usually undetectable, short-term memory loss is most commonly the first sign. Gradual deficits in cognitive function occur progressively over time, affecting one or more of the 6 recognised cognitive domains (Sachdev et al., 2014) (Figure 1.1.). AD symptoms are classified as cognitive and non-cognitive. While the former usually present throughout the illness, the latter are less predictable through the course of the disorder. More specifically, cognitive symptoms include memory loss (poor recall, losing items), aphasia, agnosia, apraxia, disorientation (impaired perception of time, unable to recognize familiar people) and impaired visuospatial function and executive function. Patients with AD may also present non-cognitive symptoms such as depression, psychotic symptoms (hallucinations, delusions)

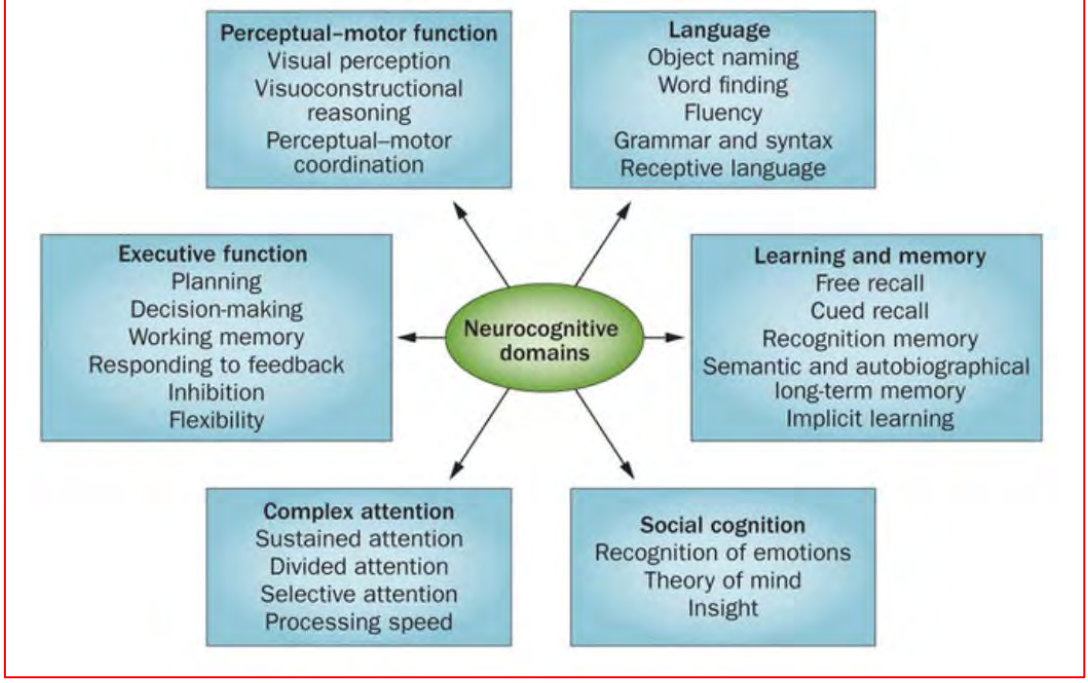


Figure 1.1. Diagram illustrating the 6 recognised cognitive domains that are variably affected in dementia and AD (Sachdev et al., 2014).

and behavioural symptoms (such as physical and verbal aggression, motor hyperactivity, uncooperativeness, wandering, repetitive mannerisms and activities and combativeness). AD is usually characterized by early problems in memory and visuospatial abilities (e.g., becoming lost in a familiar environment). Personality changes and behavioural difficulties may develop as the disease progresses. Hallucinations may occur in moderate to severe dementia. At the end stage, patients may present with near mutism, lacking the ability to sit up, hold up their head or track objects with their eyes (Duong et al., 2017). Death usually occurs through loss of physiological control pathways and susceptibility to infections for example.

In clinical practice, the diagnosis of dementia and its subtype is based on a detailed patient history, physical examination, cognitive assessment, and laboratory testing. Neuroimaging tools, such as magnetic resonance imaging (MRI) or computed tomography (CT) scans, establish the diagnosis. Since cognitive impairment is usually multifactorial, a detailed history is essential. The clinician gathers information from the patient and collateral history from a reliable informant about the history of present illness (details, timing and progression of complaints), functional status (basic activities of daily living), safety (driving, finances, ability to use appliances), medical history

(cardiovascular disease, neurologic disease, history of head trauma or concussions) and social history (current living arrangement, support network). Risk factors for dementia include a positive family history, repetitive head trauma, cardiometabolic factors (diabetes, hypertension, obesity and dyslipidaemia), atrial fibrillation, sleep apnoea and previous depression. The mini mental state examination (MMSE) is the most commonly used cognitive screening tool worldwide and remains the most thoroughly studied/used instrument to date. The Montreal cognitive assessment (MoCA) was created as a rapid screening instrument for MCI with Alzheimer's disease assessment scale (ADAS) used specifically for AD assessment.

1.3. The amyloid hypothesis

Amyloid precursor protein (*APP*) gene produces (Aβ) by two enzymes β and γ-secretase (Murphy and LeVine, 2010), the action of α-secretase prevents Aβ formation and thus prevents plaques. The APP protein is produced in healthy individuals and is broken down and removed by the action of microglia and astrocytes (Gonzalez et al., 2018). The protein becomes harmful when it accumulates in the brain in large amounts over a period of time (Kametani and Hasegawa, 2018). The *APP* gene is located on chromosome 21 and due to various mutations to the gene, increased Aβ production have been identified in dementia patients. The early onset of autosomal dominant AD could be due to mutation in this gene (O'Brien and Wong, 2011). Trisomy 21, responsible for Down's syndrome, shows the symptoms of dementia thought to be a result of genetic linkage resulting in increased Aβ production via the *APP* gene (Weggen and Beher, 2012) and causing severe early onset neurodegeneration in the brains of affected individuals.

Current thinking after the failure of clinical trials targeting amyloid plaques is that the plaques are a physiological response to rising soluble, therefore toxic amyloid to sequester it into an insoluble, non-toxic form. Thus, current thinking is focused on tau and neurofibrillary tangles rather than amyloid although the amyloid hypothesis remains and is likely to explain at least parts of the neurodegenerative processes, particularly in early-onset AD.

1.4. Genetic factors in early and late-onset AD

Metabolic disorders have recently been highlighted as a potential cause and target for treating dementia, including AD. Indeed, insulin signalling dysfunction and brain glucose

metabolic disturbances have been suggested as hallmarks of AD and underlie a proposition that AD should be regarded as type III diabetes, specifically affecting the brain. However, early-onset, familial AD, with a prevalence of around 1%, is known to be associated with high-penetrance mutations in the genes coding for APP, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) (Nikolac Perkovic and Pivac, 2019). *PSEN1* and 2 cause an impairment in γ-secretase activity and lead to an increase in the ratio of the 2 forms of Aβ, Aβ1-42: Aβ1-40. It is not known whether this is due to overproduction of Aβ1-42 or underproduction of Aβ1-40, but the consequence is early onset AD with an average age of onset of 43 years and spread from 25 to 65 years of age. *APP* mutations also result in early onset disease between 35 and 65 years of age. By contrast, late-onset AD is multifactorial with many genetic risk factors, including apolipoprotein epsilon 4 (*APOE4*), the highest risk factor for AD, as well as environmental, nutritional, metabolic and lifestyle factors. No causative genes have been identified for late onset AD but non-coding genetic errors have been suggested (Novikova et al., 2021) as well as heritable and non-heritable epigenetic changes as potential disease onset mechanisms (Nikolac Perkovic et al., 2021). These can be linked to environmental and nutritional toxins with indications that these, as well as other susceptibilities, may be offset through nutrition and diet (Agnihotri and Aruoma, 2020; Norwitz et al., 2021).

Late-onset AD has no genes identified that cause the disease. Rather, genes have been identified through genome-wide association studies (GWAS) as well as by deduced candidate genes. These genes are from many different pathways including lipid metabolism (*APOE*), sortilin-related receptor-1 (*SORL1*), ATP-binding cassette subfamily A member 7 (*ABCA7*), clusterin (*CLU*)), immune system and inflammation, including genes coding for complement C3b/C4b receptor 1 (*CR1*), CD33 antigen, membrane-spanning 4-domains, subfamily A member (*MS4A*), triggering receptor expressed on myeloid cells 2 (*TREM2*), member of the major histocompatibility complex class II HLA-DRB5/HLA-DRB1, SH2-containing inositol 5-phosphatase 1 (*INPP5D*), and/or endosome cycling (genes coding for bridging integrator protein-1 (*BIN1*), CD2-associated protein (CD2AP), phosphatidylinositol binding clathrin assembly protein (PICALM), ephrin type-A receptor 1 (EPHA1) (Nikolac Perkovic and Pivac, 2019).

Using a slightly different approach, Novikova et al (Novikova et al., 2021) found that many genes identified by GWAS were associated with more than a single pathway and

identified myeloid cell function (i.e. innate immunity), endocytosis and phagocytosis as well as lipid metabolism affected by the same genes associated with AD. These functional associations, they argue, are more informative when the multifactorial nature of the disease is appreciated, as well as highlighting potential higher level, primary faults, in this case myeloid cell function that would impact the microglia of the brain. They go on to show how transcriptome-wide association studies (TWAS) can add to GWAS to identify potential causality of disease. Using this approach, they mapped a pathway from myeloid cell-affecting genes to candidate causal genes including *BIN1, SPI1, ZYX, RABEP1* and *SPPL2A* (Novikova et al., 2021). This is a powerful new approach that may indeed identify genetic risk factors more accurately as well as potential genetic causes for AD. However, apart from gene associations being proposed as risk factors, including most notably *APOE4*, no gene has been demonstrated to cause late-onset AD in humans or animals. Animal models for AD are based on transgenes that overexpress the proteins involved in the neuropathology of the condition to understand the consequences of this to brain function as well as the processes of neurodegeneration. The models do not allow studies of cause, particularly given the recent moves away from the amyloid hypothesis. Thus, new approaches are needed to understand cause and aetiology to find effective preventatives or treatments. One of these may be functional genomics, as described above, if this disease is truly genetic in origin.

1.5. Potential epigenetic factors in AD

Epigenetics modifications are induced by environmental factors that impact the methylation/acetylation pathway and thus gene expression (Cao et al., 2020; Lemche, 2018; Nikolac Perkovic et al., 2021; Stoccoro and Coppede, 2018). Trauma related early life stress effects in late on-set AD specially *FKBP5* and *EGR1* mediated and early life stress effects through glucocorticoid converter *HSD11B1*. This suggests coupling of the glucocorticoid receptor to the *MAPT* gene so it is plausible that early tau neuronal mechanism could be affected. The interaction of neuroinflammation, cerebral lipid metabolism, brain insulin resistance and myelin disintegration is suggested by the genomic and epigenomic findings (Lemche, 2018). This would be useful together with GWAS and TWAS as levels of RNA may indicate levels of protein synthesis but do not account for methylation that is needed by many proteins and lipids in their functional states. DNA methylation is critical in retaining basic cellular processes and synaptic

plasticity in central nervous system, affecting cognitive functions. Likewise, DNA hydroxymethylation signifies an important factor during brain neurodevelopment and shows increased levels in central nervous system, suggesting the importance of its degeneration as well. The disturbance in both DNA methylation and DNA hydroxymethylation patterns has been associated with numerous disease states including neuropathologies including AD (Nikolac Perkovic et al., 2021).

1.6. Physiological causes

In addition, for late onset AD, we considered potential physiological causes that might be operating, to precipitate the condition in genetically, or otherwise susceptible individuals. In AD there is reported raised intracranial pressure (Silverberg et al., 2006) as well as enlarged ventricles (MacFarlane et al., 2011), suggesting a cerebrospinal fluid (CSF) drainage issue. Although CSF output from the choroid plexus has been reported to decrease with age and dementia (Silverberg et al., 2001), CSF drainage, through surgical implantation of a shunt, has produced promising benefits to patients (MacFarlane et al., 2011), supporting the view that a CSF drainage obstruction may be operating in these patients and that shunting, commonly used in dementia due to normal pressure hydrocephalus, in restoring drainage, improves outcomes. From our hypothesis, CSF drainage obstruction may also produce a cerebral folate issue that may add to the pathophysiology associated with AD. These physiological effects would be greatly exacerbated by loss of function in key folate enzymes.

1.7. Common features in neurological conditions

Remarkably, these findings of CSF accumulation, ventricular enlargement and severity of symptoms are found in many conditions affecting the cerebral cortex including psychosis (Harvey et al., 1990; Jones et al., 1994), schizophrenia (Saijo et al., 2001), bipolar (Strakowski et al., 2002), and autism (Movsas et al., 2013; Shen, 2018; Shen et al., 2013) and also perhaps hypo-myelination (Mercimek-Mahmutoglu and Stockler-Ipsiroglu, 2007) and epilepsy. Disease severity has also been associated with increased ventricular enlargement in AD (Chou et al., 2009; Delmelle et al., 2016; Madsen et al., 2013; Nestor et al., 2008) indicating the potential operation of a common mechanism in conditions affecting the cerebral cortex. It is interesting, therefore, to speculate that

severity of fluid drainage obstruction and ventricular enlargement may also be associated with a cerebral folate issue as we found in the extreme case of hydrocephalus.

1.8. Cerebrospinal fluid (CSF)

The vital role of CSF in development and function of the cerebral cortex has been discussed in recent literature (Fame et al., 2020; Gato et al., 2020; Miyan et al., 2020) highlighting the importance of CSF flow through the ventricles, subarachnoid spaces and drainage from the head to ensure optimal development and function of the cortex (Figure 1.2, (Miyan et al., 2020; Miyan et al., 2003)). Glymphatic pathways involving CSF have received focussed attention recently as transporters of amyloid, tau and other toxins and as potential causes of various conditions, including AD, when they suffer failures or reduced capacity, which can occur in sleep deprivation (Bidla et al., 2020; Braun and Iliff, 2020; Harrison et al., 2020; Iliff et al., 2014; Peng et al., 2016; Rasmussen et al., 2018; Reddy and van der Werf, 2020; Reeves et al., 2020) (Figure 1.3, (Ng Kee Kwong et al., 2020)). CSF is secreted by the choroid plexus (CP) which is located in the lateral, third and fourth ventricles of the brain ventricular system (Cushing, 1914). The fluid flows from ventricles of the brain to the subarachnoid space from where it drains. CSF drainage was thought to be only by arachnoid villi and granulations and released into the superior sagittal sinus for disposal (Weed, 1914). Further analysis and research revealed other pathways involving facial lymphatics, more specifically the cribiform plate located under the olfactory bulb (Rammling et al., 2008) and recently discovered glymphatic system (Benveniste et al., 2017; Iliff and Simon, 2019; Iliff et al., 2014; Jessen et al., 2015; Rasmussen et al., 2018; Zhang et al., 2019). Latest research showed that, the newly discovered brain lymphatic system is critical in the clearance of metabolic macromolecules from the brain. Meningeal lymphatic vessels located in the dura mater, drain the fluid, macromolecules, and immune cells from CSF and transport them, as lymph, to the deep cervical lymph nodes. The glymphatic system provides the perivascular exchange of CSF with interstitial fluid (ISF) and ensures homeostasis of neuronal interstitial space (Chachaj, Gasiorowski et al. 2022). Physiologically, the CNS lymphatic drainage system with the glymphatic system and meningeal lymphatics as the core which efficiently helps in the clearance of Aβ (Zhou, Zhang et al. 2022).

1.9. CSF drainage link to AD

Alteration in the clearance routes could be a cause of Aβ accumulation. Ott and colleagues (Ott et al., 2010) found that an increase in ventricular CSF is directly related to the increase of Aβ in the CSF of AD patients. The experiments performed on rodents also suggest that CSF drainage problems are linked to Aβ accumulation in brain (DeMattos et al., 2002; Iliff et al., 2012). Calcification and fibrosis of CP during ageing and AD could result in 50% reduction of CSF production (May et al., 1990). It has been shown that the patients with AD and normal pressure hydrocephalus (NPH) have decreased CSF turnover from an average of 4 volumes per day in healthy patients to 1.5 volume per day in the patients (Silverberg, 2004; Silverberg et al., 2003). The dramatic reduction in CSF production causes a disrupted clearance pathway and could be a reason of Aβ accumulation in the brain (de Leon et al., 2017). According to recent research a unique CSF folate transportation and metabolic system is identified which found to be disrupted in many neurological conditions (Jimenez et al., 2019). Reduced folate in the CSF may be related to the disruption in CSF clearance causing accumulation of Aβ. Folate deficiency could have drastic effects on brain. Folate is very important in some vital functions including DNA synthesis, neurotransmitter synthesis, methylation, metabolism and nitric oxide synthesis (Fowler, 2001; Kronenberg et al., 2008). CSF flow, production and drainage may be linked to folate delivery and likely a cause of AD.

Thus, it shows that CSF drainage insufficiency, resulting from multiple different causes, would leading to cerebral folate deficiency/imbalance, while at the same time result in failure to remove toxic molecules including amyloid. It is also possible that rising amyloid levels could themselves result in CSF drainage compromise through toxic effects on draining cells in the subarachnoid spaces. In either case the situation would be greatly exacerbated. These possibilities should be investigated as a potential mechanism for various conditions of cerebral cortical malfunction as well as neurodegeneration leading to dementias including AD.

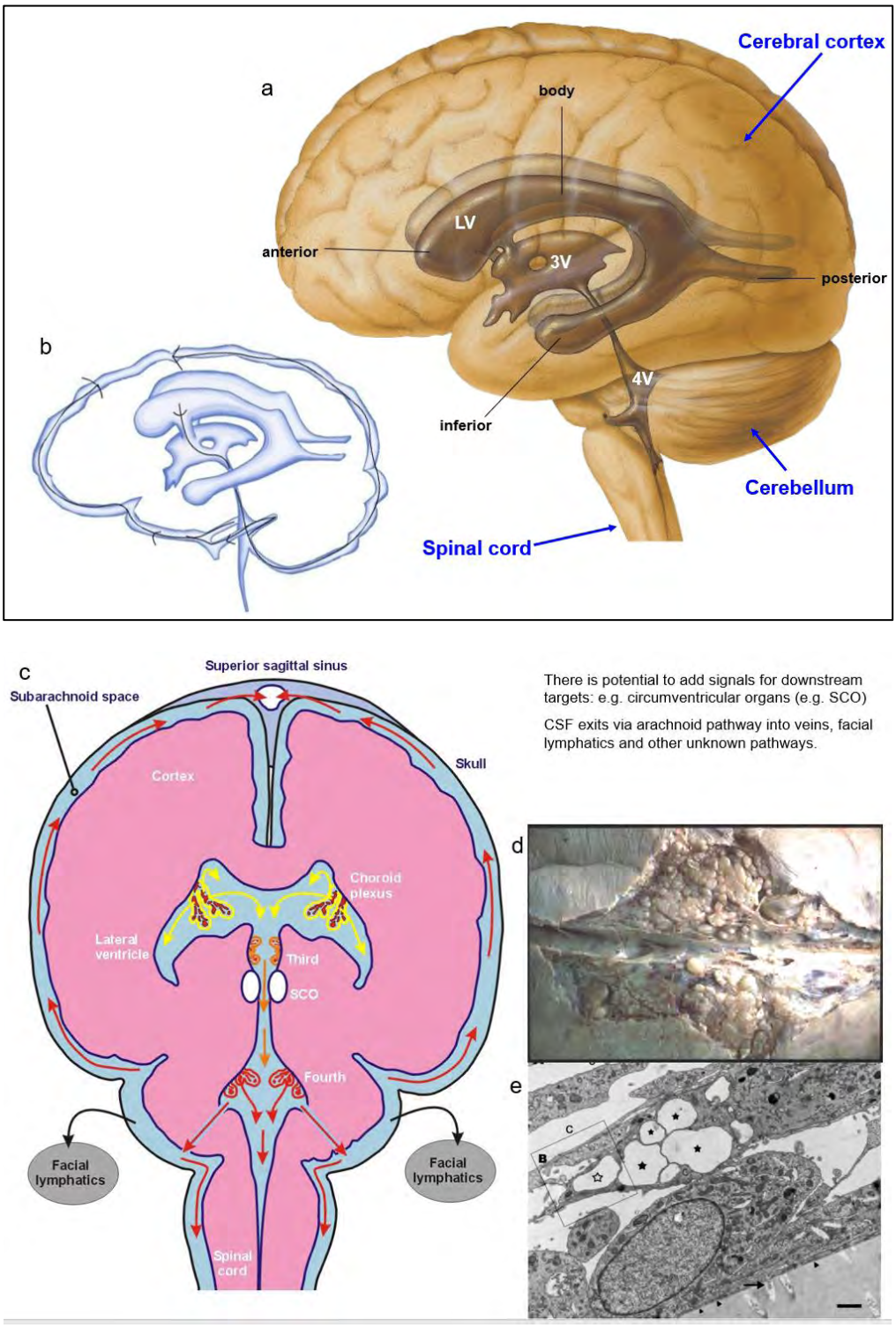


Figure 1.2. Diagrams illustrating the ventricular system of the adult brain (a) and the connection of this to the external subarachnoid CSF space (b). The diagram in (c) shows the CSF flow pathway from the lateral ventricles (LV), where 70% of CSF is secreted from the choroid plexus, through the third ventricle (3V), adding a further 20% CSF, through the cerebral aqueduct where additional components are added by the circumventricular organs (subcommissural organ, SCO) and then into the fourth ventricle (4V) adding a further 10% CSF which then exits into the subarachnoid space from where it drains into the superior sagittal sinus (d) and facial lymphatics. The active transport of CSF across the arachnoid cells into the sinus is illustrated in the micrograph in (e) (Grzybowski et al., 2006) showing vesicles of fluid being transported through the cells Modified from (Miyan et al., 2020; Miyan et al., 2003).

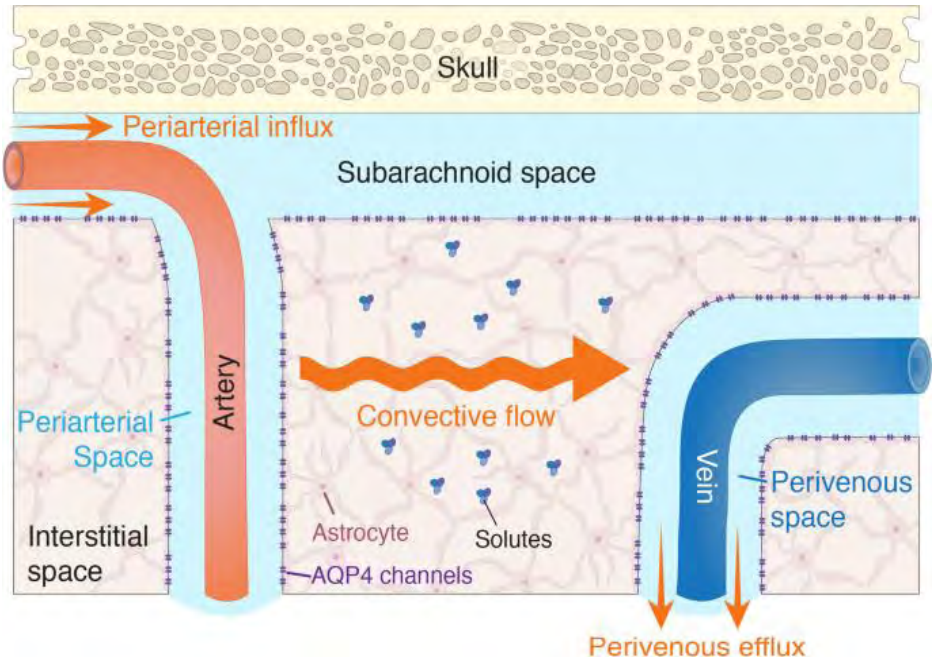


Figure 1.3. Diagram illustrating glymphatic fluid movement mediated by astrocytes pumping water through aquaporin 4 (AQP4) transporters on arterioles into the interstitial fluid space in the brain and this then flows through the interstitial spaces and is removed by astrocytes on the venules. This pathway has been shown to expand during sleep and is thus thought to be important in the removal of toxins including amyloid (Ng Kee Kwong et al., 2020).

1.10. Metabolic links to dementia and AD

In a major review of the literature, Liu et al (X. Liu et al., 2019) highlighted metabolic deficiencies as one of the most likely potential causes of the disease. Supporting this review, recent studies document an association between deficits in cerebral folate and neurological conditions, including for schizophrenia that also has an association with folate receptor autoantibodies and cerebral folate deficiency (Ho et al., 2010; Ramaekers et al., 2014). Cerebral folate issues are remarkably associated with many conditions affecting the cerebral cortex including psychosis (Harvey et al., 1990; Jones et al., 1994), schizophrenia (Saijo et al., 2001), bipolar (Strakowski et al., 2002), dementia and autism (Movsas et al., 2013; Shen, 2018; Shen et al., 2013). These conditions also have another association of disease severity with increased ventricular enlargement, which is also seen in AD (Chou et al., 2009; Madsen et al., 2013; Nestor et al., 2008; Ye et al., 2016) indicating the potential operation of a common mechanism in conditions affecting the cerebral cortex.

1.11. Examples of single elements associated with neurological conditions

1.11.1. Iron (Fe)

Lack of sufficient iron leads to deficiencies in overall brain performance at any stage of life (Campos-Escamilla, 2021). However, there is also increasing evidence that iron overload may become common in people of older age, and this has therefore been linked to neurodegenerative diseases although this link is disputed. In neuronal cells, iron is necessary for neurotransmitter synthesis, myelination of axons and signalling through neurotransmission, as it acts as a cofactor for proteins such as phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase as well as being involved in synaptic transmission. Myelination is also negatively affected by iron deficiency, and it is significant in this regard to know that CSF has a high concentration of transferritin, the main carrier of ferritin from blood into CSF and thus acts as a sink of both iron and oxygen in the healthy brain. Iron overload can lead to a variety of detrimental consequences, such as oxidative stress, cell death, and neurodegeneration. In addition, iron has the ability to induce the aggregation of some intrinsically disordered proteins including neurofibrillary tangles and amyloid plaques which are associated with iron deposits with the metal-binding sites of Aβ, catalysing its aggregation. Furthermore, transferrin had already been proposed as a biomarker for identifying AD, as it was found that in this pathologic condition, transferrin glycosylations were altered and thus lost binding activity (Campos-Escamilla, 2021).

1.11.2. Iodine (I)

Alterations of thyroid function during human development are known to produce extensive damage to the central nervous system (CNS) including severe mental retardation. The most severe brain damage associated with thyroid dysfunction during development is observed in neurological cretins from areas with marked iodine deficiency and sever neurological consequences throughout life (Martinez-Galan et al., 1997). Thyroid hormone is known to be normal in most of these cretinous children by the time of birth and postnatal treatment is not effective. (DeLong et al., 1985). Cretinism was removed from the world by introducing iodine into table salt and by switching to sea or rock salt rather than using table salt (pure sodium chloride).

1.12. Folate and the folate metabolic cycle

Folate (vitamin B9) is very important in many biological pathways. Its main role is in 1-carbon metabolism involved in purine and pyrimidine synthesis for generation of nucleic acids, methylation of homocysteine to methionine and the latter into the methylation pathway, synthesis of neurotransmitters and nitric oxide via the BH2-BH4 cycle and amino acid, as well as repair and methylation and in a number of other biosynthetic pathways. The recommended daily intake of folate is 400-600 μg/day and its deficiency are a global health concern (Steinfeld et al., 2009). Humans cannot synthesis folate and need to acquire it from dietary sources. Bacteria, fungi and plants generally can synthesize folate (Gorelova et al., 2017).

Folic acid is a synthetic form of folates that requires additional steps before entering the 1-carbon metabolic cycle as it enters via dihydrofolate (DHF), is converted to tetrahydrofolate (THF), and then has to acquire a 1-carbon element (methyl, formyl etc) to become useful. Thus, it shows that high dose folic acid would actually dilute 1-carbon availability. Free folic acid in blood causes disruption in cerebral folate system due to folate deficiency and blocking folate receptor alpha (FOLR1), the main folate transporter across the blood-CSF barrier (BCSFB) and can causes hydrocephalus in susceptible rats (Cains et al., 2009). Deficiency of folates is linked to a number of neurological disorders such as Parkinson's disease, dementia and depression where patients shows reduced levels of folate and increased levels of homocysteine (Bottiglieri, 2005). Many studies have reported positive responses to supplements with natural folates, including folinic acid (5-formyl THF) in comparison to folic acid where only few studies provide evidence of any benefits to neurological conditions.

1.13. Clues from neurodevelopmental conditions

There are developmental conditions that may give some clues as to cause and aetiology of later onset conditions including dementia and AD. The findings described above also fit with our investigations of neonatal hydrocephalus and its associated cerebral folate imbalance. Specifically, we described a unique cerebral folate handling system which utilises the CSF system to deliver this, and other key metabolites to the developing and adult brain (Cains et al., 2009; Jimenez et al., 2019; Naz et al., 2016) which has also been reported in other studies (Grapp et al., 2013; Mangold et al., 2011). These findings of a

cerebral folate imbalance have demonstrated a direct link to the aetiology of congenital hydrocephalus and, furthermore, have shown that folate supplements, NOT including folic acid, can prevent this devastating condition and indeed, maximise development of the brain (Cains et al., 2009). For example, the large head size associated with autism has been found to be associated with CSF accumulation both outside the brain, in the subarachnoid space (Shen, 2018; Shen et al., 2017; Shen et al., 2018) but also inside the brain associated with enlarged ventricles (Movsas et al., 2013; Shen, 2018). In a small study, autistic children have been found to respond positively to high dose folate treatment (Frye et al., 2020; Frye et al., 2018; Frye et al., 2017) indicating a cerebral folate deficiency or imbalance. A particular category of autism, as well as a related severe neurological condition, has been found to be caused by maternal autoantibodies to FOLR1 that block transfer of folate from fetal blood into fetal brain causing a folate deficiency, consequential poor development and increasingly sever neurological signs and symptoms after birth (Bonkowsky et al., 2008; Frye et al., 2016; Frye et al., 2013; Hasselmann et al., 2010; V. Ramaekers et al., 2013; Ramaekers et al., 2007a; Ramaekers et al., 2005; Ramaekers et al., 2018). In the absence of an autism phenotype this condition is recognised as cerebral folate deficiency syndrome (CFD) that is associated with various severe neurological conditions including spasticity (Bonkowsky et al., 2008; Duarte et al., 2020; Hasselmann et al., 2010; V. Ramaekers et al., 2013; Ramaekers et al., 2007a; Ramaekers et al., 2005; Ramaekers et al., 2018), epilepsy (Mafi et al., 2020), extreme behavioural abnormalities (Leuzzi et al., 2012), encephalopathy (Bonkowsky et al., 2008) and others. CFD is common in children but has also been recognised in adults indicating that it may develop later in life (Ferreira et al., 2016; Sadighi et al., 2012; Thome et al., 2016). CFD has various other causes, including genetic changes (Cao et al., 2020; Cario et al., 2011; Grapp et al., 2012; Krsicka et al., 2017; Ramaekers et al., 2018; Serrano et al., 2012; Steinfeld et al., 2009; Zhang et al., 2020a), and potential blockade of FRα/FOLR1 by high dose folic acid (Zhao et al., 2011), or autoimmunity (Ramaekers et al., 2018). Thus, it shows that cerebral folate issues are significantly associated with CSF drainage insufficiency, fluid accumulation and enlarged ventricles or extra axial fluid spaces. Understanding the role of the CSF fluid system may therefore give clues to what may go wrong later in life. A recent report also detailed changes in the methylation and polyamine pathways that are intimately linked to folate metabolism (Mahajan et al.,

2020) further reinforcing the idea that folate imbalance may be a key part of the cause and aetiology of dementia and AD.

1.14. Folate transport

The unique nature of the cerebral folate system was first described in detail in the hydrocephalic Texas (HTx) rat that was found to be disrupted in early stages of hydrocephalus (Naz et al., 2016). CSF act as a medium in delivering folates in the form of 5-mTHF and is transported to brain by different metabolic enzymes and carrier proteins (Cains et al., 2009; Naz et al., 2016). The system comprised of two main proteins 10-Formyl tetrahydrofolate dehydrogenase (FDH) and FOLR1. FDH is folate binding protein enzyme and is abundantly present in kidney and liver (Strickland et al., 2011). There are many folate transporters and carriers but FDH is a predominant one in metabolizing 5mTHF in the folate metabolic pathway (Berrios-Rivera et al., 2002). FDH deficiency is linked to number of neurological conditions due to its role in folate dependent metabolic pathways. FOLR1 has high affinity to folate and predominantly transports folate to the brain. Its high concentrations are in kidney, choroid plexus and ovarian epithelial cells. FOLR1 is the main folate transport protein carrying folate from the blood across the CP into the CSF (Frye et al., 2016; Steinfeld et al., 2009). In an important paper Alam et al (Alam et al., 2019) demonstrated that where FOLR1 is not present then reduced folate carrier (RFC) is active in the transport of folate. Where FOLR1 is present, then blocking it, e.g. with autoantibodies, results in a severe cerebral folate deficiency (Bonkowsky et al., 2008; V. Ramaekers et al., 2013; Ramaekers and Blau, 2004; Ramaekers et al., 2005; Ramaekers et al., 2007b) as RFC does not seem to take over this function. Proton coupled folate transporter (PCFT) is also present on the basal (blood) side of the choroid plexus while RFC is present on the apical side. These seem to unimportant in transport of folate into the CSF as they do not compensate for loss of FOLR1 through autoantibody blockade for example.

1.15. The cerebral folate system

Folate is absorbed across the gut and transported to the whole body including the BCSFB where after crossing the CP it is released into the CSF bound to FOLR1. The vesicles in the CSF containing FDH and FOLR1 bound to folates independently as well as vesicles containing FOLR1 and FDH both bound to folates in lateral ventricles of both normal

and hydrocephalic rat brains(Naz et al., 2016). This provides evidence that these proteins could play both independent and co-dependent roles in transferring folate to the brain. The relationship between the two is unknown. Immunolocalization of the FDH and FOLR1 has been reported for the CP as well but this may reflect transfer of folate from FOLR1 to FDH adjacent to this secretory structure (Jimenez et al., 2019). However, other studies suggest that they come from radial glial stem cells which are highly positive for FDH (Naz et al., 2016).

Some severe neurological conditions have been found to be caused by autoantibodies to FOLR1 (see above) that block transfer of folate from blood into brain causing a folate deficiency. This is associated with poor brain development in autism and increasingly severe neurological signs and symptoms after birth (Bonkowsky et al., 2008; Frye et al., 2016; Frye et al., 2013; Hasselmann et al., 2010; V. Ramaekers et al., 2013; Ramaekers et al., 2007a; Ramaekers et al., 2005; Ramaekers et al., 2018). Similar findings have been reported for schizophrenia including CSF accumulation with enlarged brain ventricle (Andreasen et al., 1982; Chance et al., 2003; Horga et al., 2011; Jayaswal et al., 1988) and folate receptor autoantibodies with cerebral folate deficiency (2009; Ramaekers et al., 2014).

1.16. Conclusions and hypothesis

Most studies of dementia and AD have been focused on late-stage pathological processes associated with severe symptoms. Clues from other neurological conditions and from neurodevelopmental conditions suggest some common elements that could explain cause and aetiology in dementia. Head traumas, mini stokes, infections, cardiovascular disease, and metabolic disorders could cause loss of CSF drainage capacity. The physiological consequences would reflect the severity of drainage obstruction which is seen in the association of ventricular enlargement with severity of neurological conditions. Furthermore, CSF drainage issues have been directly linked to cerebral folate deficiency or imbalance with the extreme case of hydrocephalus showing a blockade of available folate in the CSF by withdrawal of the folate binding protein, FDH. Hydrocephalus can occur at any time in life through infection, stroke, particularly subarachnoid haemorrhage, injury or head trauma. In the extreme case these would lead to death unless treated with a CSF shunt. In milder form they would lead to drop in CSF folate availability and a consequential effect on cerebral folate metabolism.

1.16.1. Hypothesis

Folate is absolutely critical to cerebral functions, including:

a. DNA and RNA synthesis
b. production of new cells, critical to memory formation in the hippocampus,
c. synaptogenesis, important for association memory in frontal and temporal lobes, methylation
d. gene expression and protein and lipid function, and
e. synthesis of the neurotransmitters, serotonin, dopamine, adrenaline, nor-adrenaline, and melatonin.

Folates deficiency plays a critical role in the pathogenesis of AD. This is the major cause of dementia and AD, explaining most cases where genetics may have less of an impact in many late onset conditions. In addition, due to the link of ventricular enlargement with severity of condition, this also explain cause and aetiology of other neurological conditions. Significantly, most cases of childhood epilepsy seem to be caused by cerebral folate deficiency, while other conditions, including dementia and AD show positive responses to folate supplements.

1.16.2. Aims

Thus, in this thesis the objective is to examine the cerebral folate system in dementia and AD brains and compare these to normal ageing brains having no neurological condition in life.

1.16.3. Objectives

CSF and fresh frozen tissue homogenates will be examined by Western and dot blot for folate related enzymes, proteins, and metabolites.

Fixed brain tissue will be examined by immunohistochemistry (IHC) using antibodies directed against folate related enzymes, proteins, and metabolites along with their association with specific cell types and different parts of the cortex.

To examine the genes related to folate metabolism, methylation, and neurotransmitter synthesis, as well as *APOE* gene type to test for any genetic error in these folate-related metabolic pathways.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1. Human tissue samples

All brain tissues were supplied from the Manchester Brain Bank under their ethical approval (09/H0906/52+5 and 19/NE/0242). All experiments were compliant with the Human Tissues Act requirements as well as passed by Newcastle NHS Research Ethics Committee for the Manchester Brain Bank. The specific use of human tissues in our experiments were sanctioned by Manchester University Research Ethics Committee. CSF and brain tissue from the cortex of ageing and demented humans was obtained from the Manchester Brain Bank. All CSF was taken post-mortem. The samples were either collected during the brain extraction procedure by mortuary technicians or were collected at the Manchester brain bank by pipetting CSF direct from the lateral ventricles during dissection. Table 2.1. gives details of the 30 brains used in this study including 10 from each of 3 categories: 1. normal ageing (Braak 0-II), 2. Moderate AD (Braak III-IV) and 3. severe AD (Braak V-VI). CSF was obtained from the same brains as the tissues used and was collected at the time the brains were removed from the bodies of the deceased and transferred to the Manchester Brain Bank. At the Brain Bank, brains were cut in the mid-sagittal plane. One hemisphere was placed in formalin for fixation and stored at 4°C while the other hemisphere was frozen to -80°C. CSF samples were frozen to -80°C for long term storage.

3 sets of tissues were obtained from the Brain Bank: 1. Formalin fixed 2. Fresh frozen temporal lobe plus CSF; 3. Fresh frozen tissue from occipital lobe plus CSF samples. The first set of brain tissue was used for cyrosectioning and staining and the CSF from this set was used for the major study of CSF composition changes. It was used for DNA extraction and a pilot study for gene single nucleotide polymorphism (SNPs) in the folate pathway as well as for tissue lysate experiments. The second set was used for a large gene SNP analysis as well as the major tissue lysate experiments.

No.	Case No.	Neuropathology Grading/Braak group	Neuropathological diagnosis 1	Neuropathological diagnosis 2	Clinical Grading	Clinical diagnosis	MRC ID	Gender	Age at death	APOE
Normal										
1	DPM16/23	0-II	Mild tau pathology in temporal lobe	Possible PART	2	Bulbar palsy	BBN005.28711	F	86	33
2	DPM16/30	0-II	Normal for age/incipient AD	Moderate AD changes in temporal lobe	1	Control	BBN005.29167	F	95	33
3	DPM17/36	0-II	Age changes only		1	Control	BBN005.32382	F	94	33
4	DPM17/23	0-II	Normal for age		1	Control	BBN005.30844	M	91	34
5	DPM17/09	0-II	Normal for age	ARTAG, possible PART	1	Control	BBN005.30100	F	88	23
6	DPM16/35	0-II	Normal ageing	Mild to moderate SVD	1	Control	BBN005.29398	M	84	33
7	DPM16/15	0-II	Moderate SVD with ischaemic lesions	Mild AD changes in temporal lobe	2	Dementia	BBN005.28546	F	92	34
8	DPM17/38	0-II	Normal for age		1	Control	BBN005.32526	F	101	23
9	DPM16/29	0-II	Normal for age		1	Control	BBN005.29063	M	69	24
10	DPM16/31	0-II	Normal for age	Mild SVD	1	Control	BBN005.29168	M	90	33
Moderate										
11	DPM17/28	III-IV	Alzheimer's disease		2	FTD	BBN005.30898	M	80	34
12	DPM16/36	III-IV	Small vessel disease	Moderate Alzheimer changes	2	FTD	BBN005.29413	M	75	33
13	DPM15/46	III-IV	Alzheimer's disease		2	Progressive Apraxia	BBN005.26177	M	76	34
14	DPM14/35	III-IV	Early /Incipient AD	Melanosis of dentate nucleus	1	Control	BBN_24213	F	83	33
15	DPM14/18	III-IV	Incipient Alzheimer's disease	Mild SVD	1	Normal	BBN_21001	M	77	34
16	DPM13/30	III-IV	Alzheimer's disease		3	FTD/PNFA	BBN_15257	M	77	44
17	DPM10/18	III-IV	Alzheimer's Disease	Rare TDP-43 inclusions, mild CAA, mild SVD	3	Dementia	BBN_5767	F	97	33
18	DPM11/09	III-IV	Moderate Alzheimer's disease	TDP-43 positive, severe hippocampal sclerosis	3	Alzheimer's disease	BBN_3449	M	91	34
19	DPM16/37	III-IV	Possible Alzheimer's disease/PART	CAA, Secondary TDP-43	3	Vascular dementia	BBN005.29414	F	86	33
20	DPM12/34	III-IV	Probable Alzheimer's disease	Mild SVD	2	Dementia	BBN_9482	F	80	33
Alzheimer's										
21	DPM14/30	V-VI	Alzheimer's disease		3	dementia, learning difficulty	BBN_23794	F	70	44
22	DPM15/29	V-VI	Alzheimer's disease	Mild SVD	3	AD and Vascular Dementia	BBN_25921	M	81	34
23	DPM16/10	V-VI	Alzheimer's disease		3	AD	BBN005.28400	F	59	24
24	DPM15/02	V-VI	Alzheimer's disease	sec TDP-43 proteinopathy	3	Alzheimer's Disease	BBN_24373	M	78	44
25	DPM18/27	V-VI	Alzheimer's disease		3	AD	BBN005.33712	M	75	34
26	DPM14/50	V-VI	Alzheimer's disease	Moderate SVD	3	Alzheimer's Disease	BBN_24361	F	63	44
27	DPM14/07	V-VI	Probable Alzheimer's disease	Moderate SVD	1	Normal	BBN_19704	F	95	33
28	DPM13/10	V-VI	Alzheimer's disease	Mild CAA	2	Dementia	BBN_11028	F	85	34
29	DPM12/25	V-VI	Alzheimer's disease		2	Semantic Dementia	BBN_6076	M	62	34
30	DPM12/01	V-VI	Alzheimer's disease	Mild SVD	2	Dementia	BBN_3469	M	67	34

Table 2.1. Details of the 30 human brains from set 1 used in the study. 10 brains from normal ageing, 10 from moderate and 10 from AD. Both neuropathology (I-II, III-IV, V-VI) and clinical gradings (1,2, 3) are given where higher number shows maximum severity. We used neuropathological gradings /Braak grading as the recognised method in the literature. However, the green highlighted cases (with red numbers) demonstrate some contradictions between clinical and neuropathological that led to a comparison in the analysis of data (see later). *APOE* refers to genotypes for two alleles where 1 is for normal and 4 is for severe.

2.2. Western and dot blotting

2.2.1. Western blotting

1-dimensional sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to separate proteins before Western blotting and probing with antibodies for semi-quantitative analysis of specific proteins. Lammeli sample buffer and 2-Mercaptoethanol were mixed to a ratio of 95:5%. Sample buffer mixed with sample CSF in equal amount i.e., 6 µl of CSF was added to 6 µl of the sample buffer. Vortex briefly then centrifuged at 13 RPM for 2 minutes. Placed it in heat block for 10 minutes at 70°C then spun at 13 RPM for 2 minutes. 9 µl of this solution was added to each well of a readymade gel/precast gel (NuPAGE, 12 well gels, 4-12% gradient – Invitrogen, stacking gel and running gel) and the proteins separated by molecular weight using a constant 150V applied to the gel through the running buffer. 7 µl Kaleidoscope was used as a ladder/marker. Running buffer (NuPAGE SDS 20X, ddH2O used for dilution) could be MES or MOP (MES for small and medium proteins and MOP for bigger proteins) depending upon the size of the protein of interest. The electrophoresis was stopped when the sample front (indicated by the blue colour of the sample buffer) reached the bottom of the gel. This usually took 40 to 60 mins after which the gel was carefully extracted from its plastic holder and placed onto the nitrocellulose membrane of an iBLOT (Invitrogen™ iBlot™ 2 Transfer Stacks) semi-dry blotting system, and the proteins transferred to the membrane from the gel using a 7 min runtime in P3 program settings of the iBLOT (iBlot® Gel Transfer Device, Invitrogen by life technologies). Gels were stained with Coomasie blue to check for transfer of protein, but this was usually clearly seen by transfer of the pre-stained molecular weight protein standard bands.

Membranes were placed into blocking solution for an hour made in 1:2:0.1% combination of blocker (Non-Animal Protein, NAP): phosphate buffered saline (PBS) and fish gelatine, respectively. Alternatively, 5% bovine serum albumin (BSA, Thermo Scientific™ Blocker™ BSA (10X) in PBS) in 1% tween-20 in PBS could also be used as blocker and for diluting primary and secondary antibodies. Blocking is a flexible step with no strict time except for the minimum 1 hour (or longer) at room temperature. It can also be done overnight at 4°C on a slow shaker. Membranes were then washed with PBS-tween 20 (0.1%) three times for 5 minutes (3 x 5). After washing, membranes were

incubated overnight in primary antibody which was diluted in the same blocker. The dilutions of primary antibody are listed in Table 2.2.

Primary Antibodies				**Dilution**			
Target	**Species**	**Source**	**Ref. No**	**WB CSF**	**DB**	**IHC**	**Tissue lysate WB**
FDH	Rb anti Rat	Krupenko	Gift	1:3000	---	1:1000	---
FDH	Rb anti Human	Krupenko	Gift	---	---	1:1000	---
FDH	Monoclonal Anti-ALDH1L1 in Ms	Sigma Life Science	SAB 4100141	---	---	1:1000	---
FDH	Ms anti Human	UC Davis/NINDS /NIMH NeuroMab Facility Antibodies Incorporated	Anti-ALDH1L1, Clone N103/39 73-140	---	---	---	1:200
FRα/FOLR1	Anti-hFOLR1 Goat IgG	R & D System	Cat No. AF5646	1:3000	---	1:1000	1:1000
Folates	Ms anti Human	Joe	Gifted	---	---	---	1:200
MTR	Anti Human Polyclonal Rb	Atlas Ab	HPA054915	1:3000	---	1:1000	---
MTRR	MTRR Rb Poly Ab	Proteintech	Cat No. 26944-1-AP	1:3000	---	1:1000	---
MTHFR	Ms Anti MTHFR mAb	Novus a biotech brand	NBP2-37607	1:3000	---	1:1000	---
MTHFD1	Rb Poly Ab	Proteintech	Cat No. 10794-1-AP	1:3000	---	1:1000	---
Tau	Rb pAb to Tau	Abcam	Ab74391	1:3000	---	---	---

	Ms Anti Tau C-terminal	Invitrogen	Ref 136400				
PCFT	SLC46A1 Rb pAb	ABclonal	WH152945 Cat No. A7397	1:3000	---	1:1000	---
DHFR	Rb PolyAb	Proteintech	Cat No. 15194-1-AP	---	1:3000	1:1000	---
Folates	Ms Anti folic acid	Bio-rad Ab	MCA2870	---	1:3000	1:1000	
Homocysteine	Rb pAb to Homocysteine	Abcam	Ab 15154	---	1:9000	1:1000	---
Glutathione	Ms mAb to Glutathione	Abcam	Ab19534	---	1:3000	---	---
B12	Rb pAb to Vit B12 (whole antiserum)	Abcam	Ab31510-200	---	1:5000	---	---
B6	Rat pAb to Vit B6 Pyridoxine IgG	Abcam	Ab37012	---	1:5000	---	---
GFAP	Chkn Polyclonal Antibody to GFAP	Encor Biotechnology Inc	CPCA-GFAP	---	---	1:3000	---
S100	Anti S100 B Anti Rb	Atlas Ab	HPA015768	---	---	1:1000	---
RFC	X Human SLC19A1 [647] Ms mAb	R & D System	FAB8450R	---	---	1:1000	---
5-Hydroxymethylcytosine (5-HMC)	Rat mAb to (5-HMC)	Abcam	Ab106918	---	---	1:1000	---
5-Methylcytosine (5-MC)	Rb mAb	Cell Signalling technology	D3S2Z Ref: 10/2018	---	---	1:1000	---

Neu-N	Rb Poly	Fede,	Gifted	---	---	1:1000	---
CD31	Ms Mono	Fede,	Gifted	---	---	1:1000	---
SMA	Ms Mono	Fede	Gifted	---	---	1:1000	---
Neurofilament	Ms Mono	Fede	Gifted	---	---	1:1000	---
FDH	Monoclonal Anti-ALDH1L1 in Ms	Sigma Life Science	SAB 4100141	---	---	1:1000	---

Table 2.2. Details of primary antibodies used in Western blot, dot blots, tissue lysate and for Immunohistochemistry. Their source and dilutions used are also given. Gt: goat, Ms: mouse, Dnky: donkey, Chkn: chicken, Rb: rabbit, mAB: monoclonal antibody, pAB: polyclonal antibody, IgG: immunoglobulin G.

After the primary antibody incubation step, membranes were washed with PBS-tween-20 (0.1%) three times for 5 minutes. Secondary antibodies (horse radish peroxidase (HRP)-conjugated) were diluted in the same blocker with combination of 1: 2: 0.1% NAP blocker:PBS:fish gelatine, respectively. 6ml of solution is enough to cover the blot. Secondary antibody incubation time was 2 hours. The dilutions of secondary antibody are listed in Table 2.3. After the secondary antibody incubation step, membranes were again washed with PBS-tween-20 (0.1%) three times for 5 minutes. Enhanced chemiluminescence (ECL) substrates were used in equal ratio 1:1 (reagent A and reagent B) for HRP Western detection. Time is critical during this step and 5 minutes are enough for having maximum chemiluminescence. The blots should be in aluminium foil to avoid any light interference during this step. The last step is to image the chemiluminescent signals on the C-Digit scanner (LI-COR, UK) by using a standard 12-minute exposure. Images were captured from the scanner into Image Studio software for densitometry analysis. Data were transferred to Microsoft Excel for statistical analysis.

Secondary Antibodies			Dilutions		
Species	**Source**	**Ref.No**	**WB (CSF)**	**DB**	**WB (Tissue Lysate)**
Anti Rb IgG (HRP)	Cell Signalling	06/2015	1:3000	1:9000	
Dnky anti Goat HRP)	Invitrogen	A15999	1:3000	---	1:2000 (FR alpha)
Anti Ms IgG HRP-linked	Cell Signalling	12/2016	1:3000	---	---
Gt pAb to Rat (HRP)	Abcam	Ab7097	1:3000	---	---
Rb pAb to Gt IgG HRP	Abcam	Ab6741	1:3000	1:5000	
Goat anti Rat IgA HRP	BioRad	STAR111P149214	1:3000	---	---
Anti Rb IgG	Cell Signalling	08/2009	1:3000	---	---
Anti Sheep IgG	R & D System	HAF016	1:3000	---	---
Anti Ms IgG	Cell Signalling	01/2017	1:3000	---	---
Gt anti Ms IgG	LI-COR WS	926-80010	1:3000	---	1:2000 (Folates and FDH)
Rb anti Sheep IgG	Life Technology	618620	1:3000	---	---

Table 2.3. Details of secondary antibodies used in Western blot, dot blots and tissue lysate. Their source and dilutions are also given. Gt: goat, Ms: mouse, Dnky: donkey, Rb: rabbit, pAB: polyclonal antibody, IgG IgA: immunoglobulin G or A.

2.2.2. Dot blots

5 µl of CSF was pipetted directly onto nitrocellulose transfer membrane, cut to the size for the LI-COR C-digit scanner and with sections drawn on in pencil to define the location of each dot, which spread to form a small dot. Membranes were allowed to air dry for 30 to 60 minutes before being processed as for Western blots above, with incubation in blocking buffer (1:2:0.1%, NAP, PBS and fish gelatine, respectively) followed by overnight incubation in primary antibody diluted in the same blocking buffer. Primary antibodies used and dilutions are given in Table 2.2. above. After primary antibody, washing with PBS tween-20 (0.1%) thrice for 5 minutes (3x5). Secondary antibody (HRP conjugated) diluted in the same blocking buffer incubation was for 2 hours at room temperature followed by washing with PBS tween-20 (0.1%) thrice for 5 minutes (3x5). Secondary antibodies used and dilutions are given in Table 2.3. above. ECL chemiluminescent substrates were used for 5 minutes in equal ratio 1:1 (reagent A and reagent B) for HRP western detection. After this step, the last step was to check it on scanner (LI-COR) by using standard 12 minutes run to check.

Western blots and dot blots were scanned using a luminescence scanner (LICOR C-Digit) after incubation in ECL solution for 5 minutes (wrapped in aluminium foil, to avoid light interference with blots in ECL). Scans were then subjected to densitometry analysis using LICOR Image Studio version 5.3 software. Data were transferred to Microsoft Excel for statistical analysis. All samples were run 3 times and analysed by groups based on Braak grading. Additional analysis was performed on the basis of available clinical diagnoses made during patient lifetimes.

2.3. Immunohistochemistry (IHC)

Brain samples were received as wet formalin fixed tissue in fixative solution. Areas encompassing the full thickness of cortex from pial surface to ventricular zone and ependymal layer were selected and dissected from the full block of tissue. These were washed in PBS and then immersed in 30% sucrose in PBS until they sank in the solution (usually overnight). This provided cryoprotection allowing the tissue to be snap frozen by immersion in isopentane cooled with dry ice and then cut into 30µm to 50µm thick sections. These were collected onto charged microscope slides or collected into citrate buffer, pH 6.0 as free-floating sections. For future use sections were preserved in the

cryoprotectant in -20 freezer. Some sections were collected on slide in PBS and were dried over the slide for future use.

The sections which were in cryoprotectant were washed thrice for 10 minutes with PBS. The sections which were collected in citrate buffer were ready to go for antigen retrieval. Antigen retrieval was carried out by heating the sections in citrate buffer (pH 6.0) in a water bath at 90°C for 20 mins (may increase the time depending upon the thickness of the sections) then a further 20 mins at 40°C and 20 minutes to cool at room temperature before further processing. As folates are heat sensitive, antigen retrieval was carried out at 40°C for 40 minutes followed by cooling at room temperature for 20 minutes. Alternatively, sections were microwaved in citrate buffer for 10 seconds for antigen retrieval. Sections were then allowed to cool down for 10 minutes. The solution was changed for a wash in PBS triton X100 (1%) thrice for 10 minutes and then immersion in blocking buffer which was 1% PBS Triton X100 with 0.5% serum (goat, donkey used mostly, each with 0.5% concentration but any animal could be use in which the secondary antibody is raised) for 1 hour at room temperature with gentle shaking. Primary antibody was diluted in the same blocker for overnight at 4C. The dilutions of primary antibody are listed in Table 2.2., above.

After washing in PBS 1%Tween 20 (3X10), sections were immersed in Alexa Fluor conjugated secondary antibodies diluted in PBS-Tween-20 (1%) (without any blocking serum) for 2 hours. The dilutions of secondary antibodies are listed in Table 2.4.

Sections were then washed in PBS 3x 10 mins and then rinsed with deionised distilled water (ddH20) in a petri dish (free floating) before placing on a microscope slide and manipulated with a plastic pipette to straighten and orientate them before mounting with Vectashield aqueous mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) and a coverslip.

Fluorescent Secondary Antibodies				Dilutions
Species	**Source**	**Ref. No**	**Alexa Fluor**	**For IHC**
Gt pAb to Chkn IgY	Abcam	ab150176	594	1:3000
Gt pAb to Rb IgG	Abcam	ab150077	488	1:1000
Gt pAb to Ms IgG	Abcam	ab150120	594	1:1000
Dnky pAb to Goat IgG	Abcam	ab150132	594	1:1000
Gt pAb to Ms IgG	Abcam	ab150113	488	1:1000
Gt pAb to Chkn IgY	Abcam	ab150169	488	1:3000
Gt pAb to Rat IgG	Abcam	ab150165	488	1:1000
Gt pAb to Rb IgG	Abcam	ab150080	594	1:1000
Dnky pAb to Rb IgG	Abcam	ab150075	647	1:1000
Dnky pAb to Goat IgG	Abcam	ab150129	488	1:1000
Dnky pAb to Ms IgG	Abcam	ab150110	555	1:1000
Dnky pAb to Ms IgG	Abcam	Ab175738	750	1:1000

Table 2.4. Details of secondary antibodies used in tissue sections. Their source and dilutions are also given. Gt: goat, Ms: mouse, Dnky: donkey, Chkn: chicken, Rb: rabbit, pAB: polyclonal antibody, IgG IgY: immunoglobulin G or Y.

Sections were viewed on a Leica DMLB fluorescence microscope and micrographs captured using a Coolsnap digital camera (Princeton Instruments, USA) and Metaview software. Sections were scanned using a 20x objective on a 3D Histech Pannoramic 250 Flash Slide Scanner before viewing on 3D Histech Caseviewer software.

Multi staining was carried out using primary antibodies made in different species and secondaries targeted at the different species. This allowed up to 5 colour merged imaging on the individual sections with the fifth being DAPI for nuclear staining. This technique allowed colocalization analysis to be carried out as colocalised stains mixed colours to give different colours, e.g., red and green colocalised to give a yellow stain and so on.

High resolution confocal micrographs were collected on a Leica TCS SP8 AOBS upright confocal using a 20x / 0.50 Plan Fluotar objective and 1x confocal zoom (figure 2.1). All settings and microscope controls used the Leica LAS X v3.5.2.18963 software. The confocal settings were as follows, pinhole 1 airy unit, scan speed 1000Hz bidirectional, format 1024 x 1024. micrographs were collected using hybrid detectors with the following detection mirror settings; FITC 494-530nm; Texas red 602-665nm; Cy5 640-690nm using the white light laser with 488nm (20%), 594nm (10%) and 633nm (10%) laser lines respectively. When it was not possible to eliminate crosstalk between channels, the micrographs were collected sequentially. When acquiring 3D optical stacks, the confocal software was used to determine the optimal number of Z sections. Only the maximum intensity projections of these 3D stacks are shown in the results. Details of antibodies used are given in table 2.2 and 2.4.

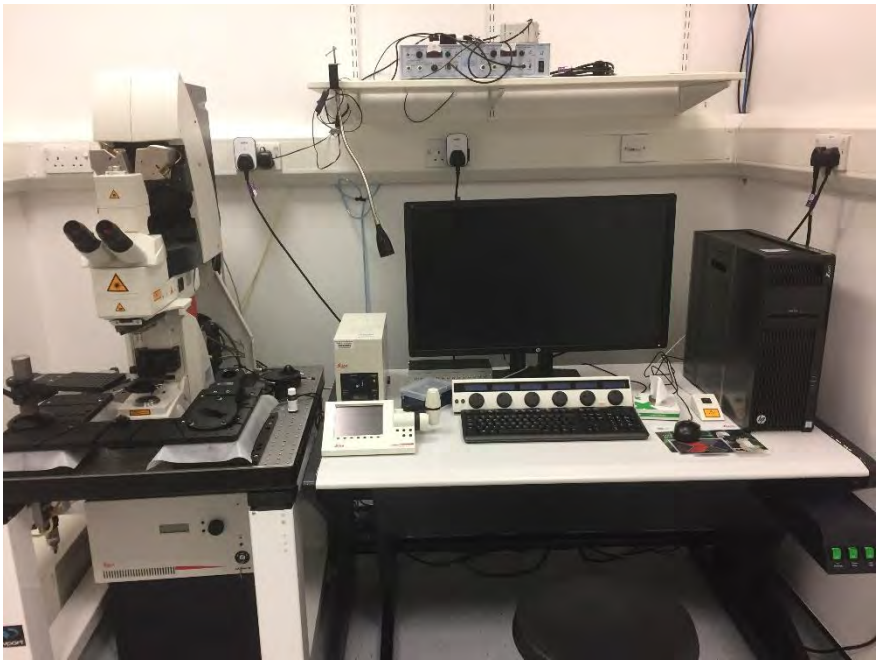


Figure 2.1. Photograph of the Leica SP8 upright confocal microscope and set up in the Bioimaging centre of Manchester used in these studies.

2.4. Fresh frozen tissue lysate analysis

To measure tissue levels of folate and folate-related proteins, fresh frozen cortical plate regions and ventricular zone were homogenised using FastPrep Lysing Matrix D (MP Biomedicals 116913100) and SDS lysing buffer. Approximately 50µg of tissue was

weighed on a microbalance and placed into the tubes with 1ml of SDS buffer and then located into the FastPrep homogeniser. The ceramic beads in the tubes homogenised the tissue as the tubes were shaken at high frequency for 4x30s. Tubes were centrifuged at 13000 RPM and the supernatant decanted into Eppendorf's. Samples were analysed for total protein content using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Rapid Gold BCA Kit). All samples were diluted to give a concentration of 50µg/ml and then frozen at -80 in 50µl aliquots until used. Equal amounts of total protein from the samples were then analysed by Western blotting, using 3mg per sample, and dot blots using 0.5mg.

2.5. DNA extraction and gene single nucleotide polymorphism (SNP) analysis

In this study fresh frozen unfixed human tissue from the occipital cortex, that included the full thickness of the cortex from the pia to the ventricular ependymal, was provided by Manchester Brain Bank. The cortical plate was dissected and used in the genomics study. Information on the cases used are provided in Table 2.5. a, b. Only individuals who were clearly normal, based on both pathology and clinical observations prior to death, or were clearly suffering severe AD were included in this study.

Nutrigenomics is a relatively new field of research looking at individual susceptibilities based on analyses of genes involved in metabolic pathways and how they might respond to diet and environmental factors (Agnihotri and Aruoma, 2020; Brennan and de Roos, 2021). In this study functional genomics method was used, derived from nutrigenomics, to query the genes involved in specific metabolic pathways and to identify SNPs giving negative effects on protein functions. The aim of the method was to identify genes involved in folate and methylation, biogenic amine neurotransmitter and nitric oxide synthesis, glutathione synthesis and the *APOE* genotype. This is different to GWAS and TWAS as it investigates the genes of specific metabolic pathways, identifies potentially defective genes, and thereby highlights metabolic errors in individuals.

2.6. Genetic studies

Fresh frozen, tissues were from 25 clinically and neuropathologically normal ageing individuals and from 25 severe AD individuals (see table below). Samples were from the occipital lobes of brains. The cerebral cortical plate was dissected and then sent to a commercial company, LGC Genomics, for quality checking, further processing and SNP analysis. They extracted DNA from the tissue using their in house LGC Kleargene extraction chemistry. Genotyping was performed by LGC genomics using their in-house competitive allele specific PCR (KASP) technique. Analyses were sent to LifecodeGX Ltd to input into their bespoke software that matches SNPs to specific metabolic pathways. The software also categorised SNPs according to their functional effect based on literature reviews, namely, beneficial, neutral or harmful (see https://www.lifecodegx.com/ for details and references). These data were then tabulated in Microsoft Excel. Heat maps were generated in Excel to give a pictorial representation of the SNP data. The data were analysed using Mann-Whitney U tests comparing ratios of positive, neutral and negative SNPs. Data were also analysed using Chi squared statistics comparing abnormal:normal genes in AD to normal ageing. For this Chi squared analysis two tests were carried out, firstly with abnormal and neutral grouped together and secondly with neutral and normal grouped together. The SNP data suggested neutral SNPs may have the potential to have some negative effects, hence we tested in both directions for comparison.

In order to calculate sample size in current study, we used G*Power software (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower). Two tailed t-test was used to calculate the difference between mean of two independent groups. The effect size was set as lager i.e. d= 0.80 which is considered clinically meaningful. Our alpha level is set default i.e. 0.05. In order to know that how many participants would be required to be able to detect effect size at certain percentage of time is 0.8. Power analysis result indicated that we required 26 participants in each group and 64 in total. In first part of study we used 30 human brain and in second part, we used 50 human brain. So in total we used 80 human brain which is more than our recommended power analysis result.

Table 2.5. Cases used in the study

a. Normal ageing cases

Case No.	MRC ID	Gender	Age at death	Braak stage	PMD (h)	Clinical diagnosis	Pathological diagnosis 1	Pathological diagnosis 2	*APOE*
DPM12/11	BBN_3478	M	54	0	37	control	normal brain		33
DPM14/04	BBN_19634	F	87	0-I	24	Normal	Age changes only		34
DPM14/08	BBN_20005	M	85	0-I	98	Normal	Age changes only	moderate SVD	33
DPM14/20	BBN_21003	F	90	0-I	39	normal	Age changes only		33
DPM14/46	BBN_24316	F	94	0-I	111	control	age changes only	mild SVD	23
DPM16/29	BBN005.29063	M	69	0-I	53	Control	Normal for age		24
DPM18/03	BBN005.32560	M	88	0-I	39	Control	Normal for age		33
DPM14/09	BBN_20006	M	84	I	69.5	Normal	Age changes only	moderate SVD	33
DPM17/09	BBN005.30100	F	88	I	52.5	Control	Normal for age	ARTAG, possible PART	23
DPM17/34	BBN005.31485	M	89	I	125	Control	Normal for age	Incidental Lewy bodies?	23
DPM09/31	BBN_3396	F	94	I-II		cognitively normal /stroke	Age changes only	mild to moderate SVD	33
DPM12/09	BBN_3476	F	87	I-II	87	cognitively normal	mild AD pathology in temporal lobe		33
DPM13/35	BBN_15591	F	76	I-II	47	normal	mild AD changes in temporal lobe	very mild CAA, moderate SVD in BG	33
DPM14/11	BBN_20195	M	91	I-II	43.5	Normal	mild SVD		33
DPM15/01	BBN_24368	M	90	I-II	156	control	Age changes only		33
DPM16/11	BBN005.28403	M	77	I-II	63	Control	Mild temporal tau, possible PART		33
DPM16/31	BBN005.29168	M	90	I-II	155	Control	Normal for age	Mild SVD	33
DPM17/15	BBN005.30170	M	90	I-II	125	Control	Normal for age	Incidental Lewy bodies?	33
DPM17/36	BBN005.32382	F	94	I-II	70	Control	Age changes only		33
DPM18/11	BBN005.32822	F	90	I-II	143	Control	Age changes only	Possible ARTAG	33
DPM11/06	BBN_3446	F	92	II	37	cognitively normal	Age changes only	mild SVD	34
DPM11/25	BBN_3463	M	89	II	27	Control	Age changes only		33
DPM11/29	BBN_3467	M	89	II	123	cognitively normal	Age changes only	mild SVD	33
DPM15/28	BBN_25917	F	91	II	133	Control	Age changes only	Cerebral infarction	23
DPM19/09	BBN005.35435	M	95	II	153	Control	Age related changes (mild)	Severe SVD	33

b. Severe AD cases

Case No.	MRC ID	Gender	Age at death	Braak stage	PMD (h)	Clinical diagnosis	Pathological diagnosis 1	Pathological diagnosis 2	*APOE*
DPM16/16	BBN005.28547	F	81	V	176	AD	AD	secondary TDP-43 proteinopathy. V.severe CAA	34
DPM12/01	BBN_3469	M	67	V-VI	84	Dementia	AD	mild SVD	34
DPM12/32	BBN_9480	M	73	V-VI	36	AD	AD		33
DPM13/09	BBN_11027	F	85	V-VI	73	AD	AD	moderate to severe SVD, v. Mild DLB	34
DPM13/10	BBN_11028	F	85	V-VI	24	dementia	AD	Mild CAA	34
DPM13/45	BBN_19208	M	78	V-VI	138	AD	AD		33
DPM14/10	BBN_20007	F	78	V-VI	70	AD	AD	CAA with capillary involvement	44
DPM14/50	BBN_24361	F	63	V-VI	54	AD	AD	moderate SVD	44
DPM15/02	BBN_24373	M	78	V-VI	173	AD	AD	sec TDP-43 proteinopathy, incidental Lewy bodies?	44
DPM17/37	BBN005.32384	F	90	V-VI	76	AD	AD	Possible AGD	34
DPM11/28	BBN_3466	F	71	VI	64	AD	AD		44
DPM12/03	BBN_3470	M	72	VI	81	AD	AD		34
DPM12/05	BBN_3472	M	73	VI	107	AD	AD	mod SVD	44
DPM14/30	BBN_23794	F	70	VI	89	dementia, learning difficulty	AD		44
DPM14/31	BBN_23803	M	64	VI	98.5	AD	AD	moderate SVD	34
DPM15/48	BBN005.26301	F	81	VI	98	Dementia	AD	Secondary TDP-43	34
DPM16/10	BBN005.28400	F	59	VI	87	AD	AD		24
DPM16/40	BBN005.29461	M	82	VI	25.5	AD	AD	Moderate CAA	34
DPM18/12	BBN005.32823	M	70	VI	120.5	AD?	AD	Moderate SVD	33
DPM18/39	BBN005.35131	F	75	VI	127.5	Dementia	AD		34
DPM19/04	BBN005.35211	M	82	VI	124	AD	AD	Temporal intra-cortical infarct. Secondary TDP-43.	33
DPM19/07	BBN005.35399	F	86	VI	72	Dementia	AD	Severe hippocampal sclerosis. Secondary TDP-43.	34
DPM19/12	BBN005.35441	F	88	VI	96	AD	AD	ARTAG	33
DPM19/29	BBN005.35889	M	56	VI	295	Early onset AD	AD (early onset)		33
DPM19/31	BBN005.35912	M	73	VI	135	Atypical AD	AD		44

Table 2.5. Cases used in the study separated into normal ageing (a) and severe AD cases (b).

Case number are Manchester Brain Bank references while MRC ID refers to the Medical Research Council National register of human brain tissue. PMD is the post-mortem delay before the brain and CSF were taken from the deceased individuals. This is very variable and may impact study results. Clinical diagnoses were recorded prior to death and 2 pathologists reported on brain tissue analysis (diagnosis 1 and 2). *APOE* genotype was recorded for both alleles of each individual as numbers 2-4 referring to the different genotypes of *APOE* found.

Chapter 3

Results I

Analysis of cerebrospinal fluid folate status

Chapter 3

Analysis of cerebrospinal fluid folate status

3.1. Aldehyde dehydrogenase 1L1 (ALDH1L1) in CSF

ALDH1L1, also known as 10-formyl tetrahydrofolate dehydrogenase (FDH) is a folate enzyme also involved in a variety of important pathways, e.g., in cancer and tumour suppression (Krupenko and Krupenko, 2018; Krupenko and Krupenko, 2019). FDH has been found to be significantly reduced, sometimes absent in the CSF of hydrocephalic infants, both rats and humans, suggesting the decrease in FDH may be related to severity of fluid obstruction and accumulation (Cains et al., 2009). As AD also has severity associated with ventricular enlargement (Guptha et al., 2002; Nestor et al., 2008), we investigated levels of this important folate enzyme. As expected, levels of FDH in the CSF of AD patients are decreased compared to patients in the less severe, normal and moderate categories. The decrease is significant comparing normal to AD (p=0.03) but not significantly different between other groups.

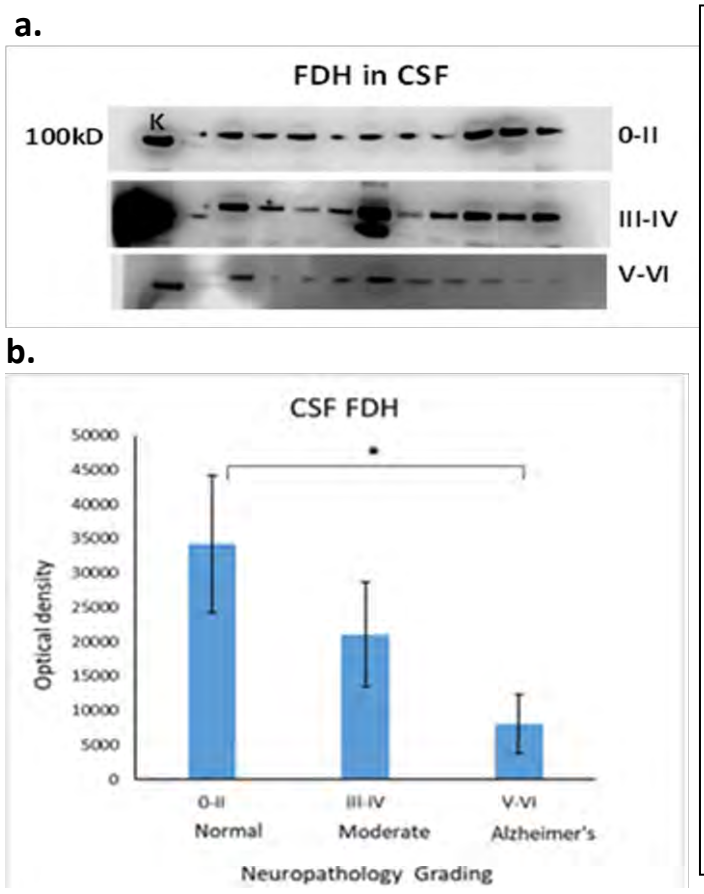


Figure 3.1.

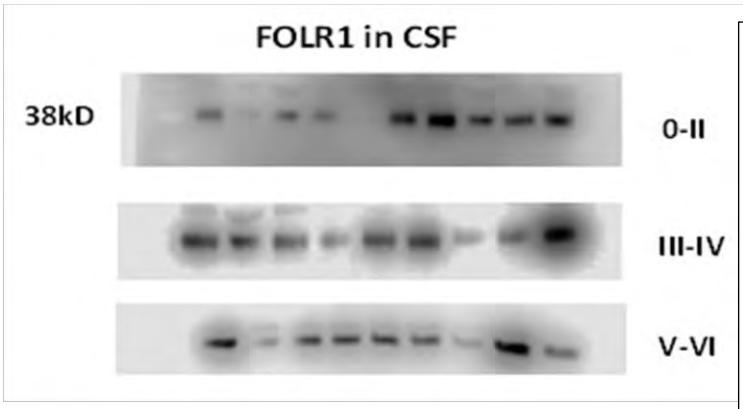
a. Western blots for FDH, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane in each blot is the Kaleidoscope Marker (K) following by 10 samples of normal (0-II), moderate (III-IV) and severe (V-VI). The molecular weight of FDH is 100kD. AD CSF is clearly reduced in FDH content compared to the other two.

b. Western blots were analysed by densitometry. Data show means and standard error of the mean (SEM). Although a decline is seen in moderate and severe cases, this is only significant between normal and severe (p=0.03).

3.2. Folate receptor alpha (FOLR1) in CSF

Folate receptor alpha (FOLR1 or FRα) is the main transporter for folate in the blood and is also the major transporter for folate into the brain. As a membrane bound folate transporter in the CP, FOLR1 binds to folate and internalises in endosomes that then merge with the apical membrane and release FOLR1 bound to folate into the CSF. Abnormal FOLR1 (Grapp et al., 2012; Steinfeld et al., 2009)or blockade of FOLR1 with autoantibodies is now a well-recognised phenomenon that produces severe cerebral folate deficiency with associated neurological problems including epilepsy in children (Ferreira et al., 2016; Frye et al., 2016; Frye et al., 2017). A possibility therefore exists that this important pathway for folate delivery to the brain is upset in dementia and/or AD.

a.



b.

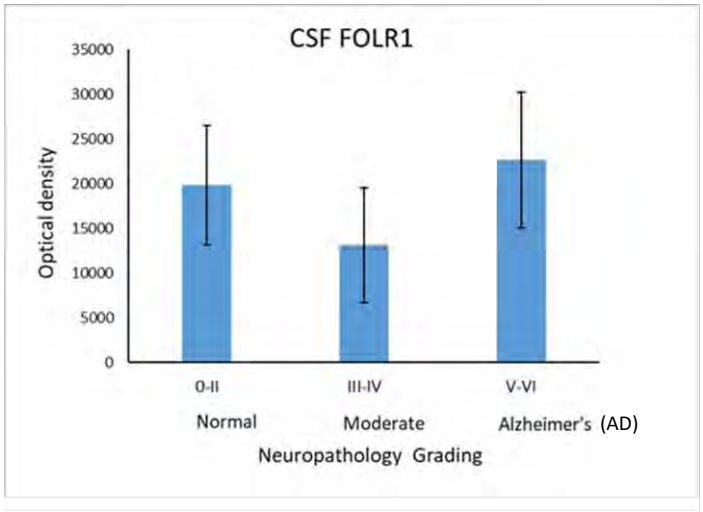


Figure 3.2.

a. Western blots for FOLR1, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane in each blot is Marker/Kaleidoscope (K) following by 10 samples of normal (0-II), moderate (III-IV) and severe (V-VI). The predicted molecular weight of FOLR1 is 29.82kD but the observed was 38kD.

b. Western blots were analysed by densitometry. Data show means and SEM. No significant difference was found in the concentration of FOLR1 between any of the three condition groups but there is non-significant increase in AD CSF.

3.3. Methionine synthase (MTR) in CSF

MTR gene encodes methionine synthase (MS). This is the rate limiting enzyme for folate metabolism converting 5mTHF to THF and involving vitamin B12 and the methylation of homocysteine to methionine. Thus, it is not only vital to folate metabolism, but also involved in supplying methionine to the methylation cycle where it produces s-adenosyl methionine (SAM) from s-adenosyl homocysteine (SAH). SAM is the universal methyl donor for methylation reactions while SAH feeds into choline metabolism and synthesis of acetyl CoA and acetylcholine (Lucock, 2000; Nzila et al., 2005).

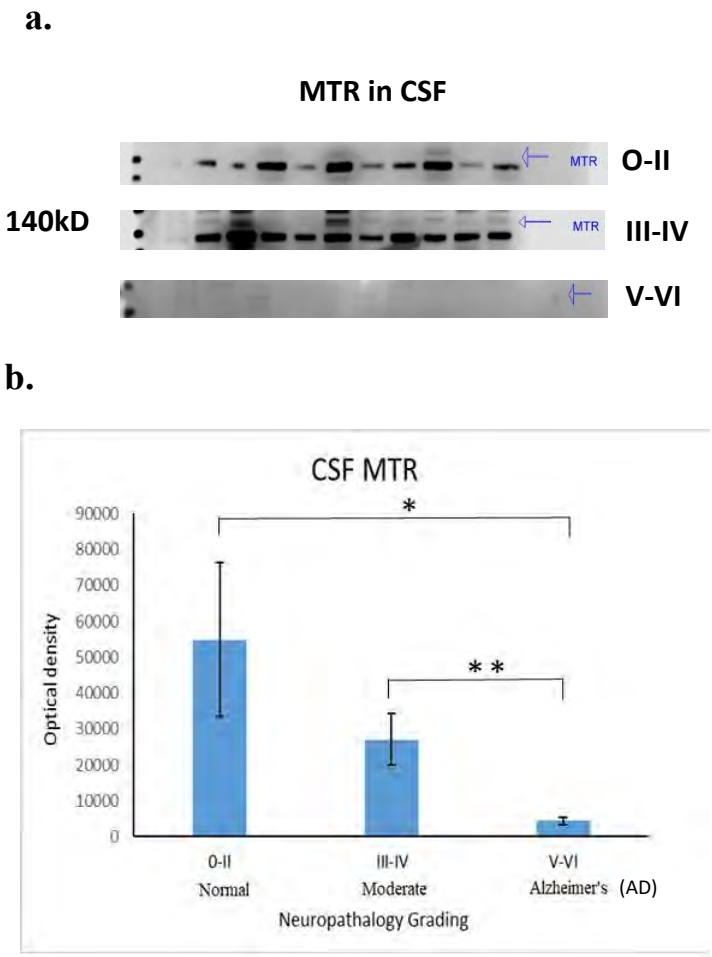


Figure 3.3.

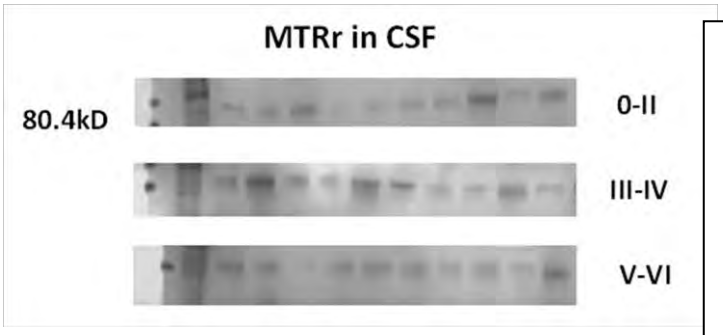
a. Western blots for MTR, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane in each blot is Marker/Kaleidoscope (K) following by 10 samples of normal (0-II), moderate (III-IV) and severe (V-VI). The predicted molecular weight of MTR is 140kD. AD CSF is clearly reduced in MTR content compared to the other two. The molecular weight of MTR is reported as 140kD which is indicated by the arrows on the right side. MTR antibodies commonly give additional protein bands with higher intensities than MTR itself, suggesting common targets on these proteins.

b. Western blots were analysed by densitometry. Data show means and SEM. A significant decline is seen in moderate and severe cases with (p=0.04) between normal and severe and (p=0.01) between moderate and severe.

3.4. Methionine synthase reductase (MTRr) in CSF

MTRr codes for the enzyme methionine synthase reductase which is involved in the rate limiting step in both folate metabolism and the methylation cycle. MTR is involved in the methylation of cobalamin, vitamin B12. Methyl cobalamin is responsible for the methylation of homocysteine to methionine (Lucock, 2000; Nzila et al., 2005).

a.



b.

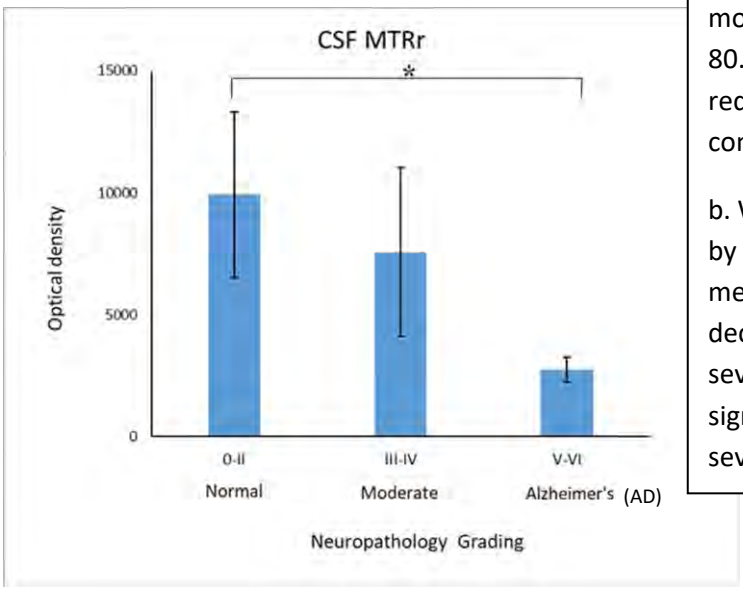


Figure 3.4.
a. Western blots for MTRr, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane in each blot is Marker/Kaleidoscope (K) following by 10 samples of normal (0-II), moderate (III-IV) and severe (V-VI). The molecular weight of MTRr is 80.4kD. AD CSF is clearly reduced in MTRr content compared to the other two.

b. Western blots were analysed by densitometry. Data show means and SEM. Although a decline is seen in moderate and severe cases, this is only significant between normal and severe ($p=0.05$).

3.5. Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) in CSF

MTHFD1 is a key enzyme in the synthesis of, and balance between 5 different folate metabolites and thus the pathways they feed into, including methylation and trans-sulphuration pathways (Field et al., 2013). Its functions are thus critical to the balance of folate metabolism and changes in this enzyme are likely to cause potentially more severe outcomes.

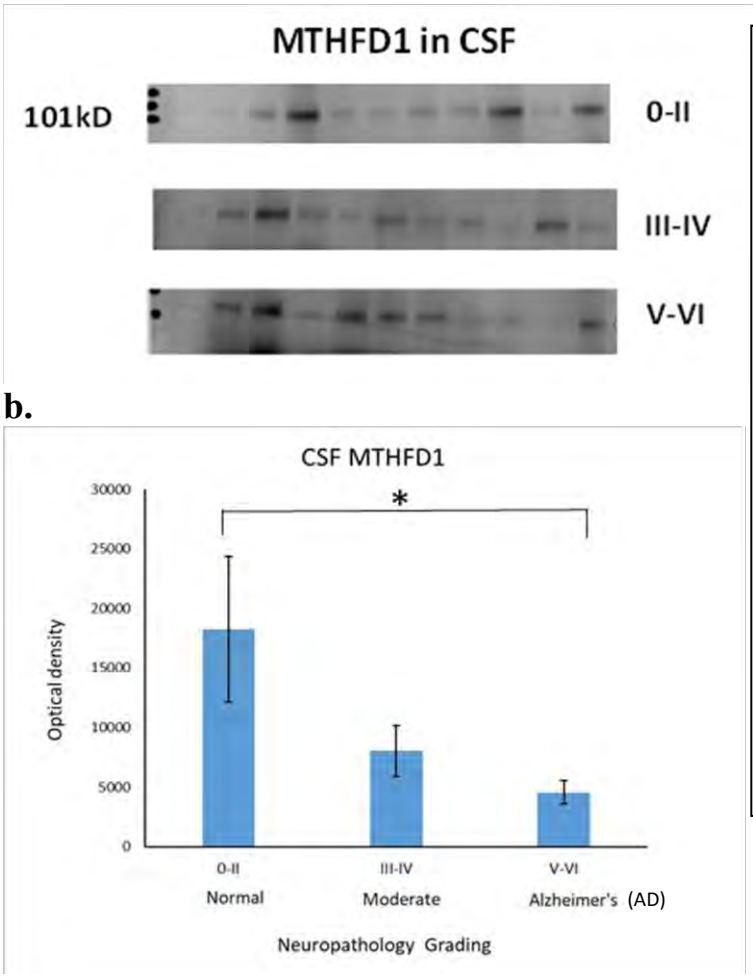


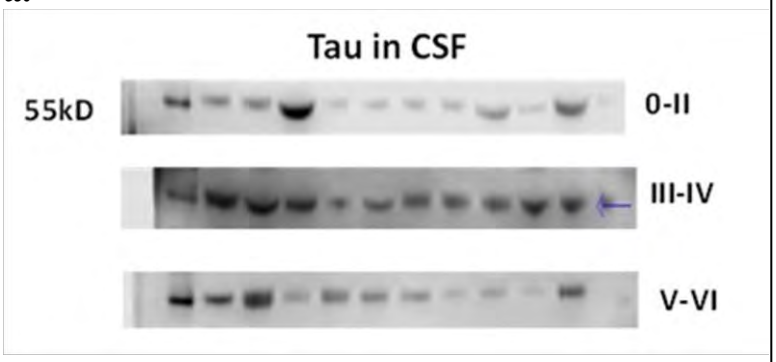
Figure 3.5.
a. Western blots for MTHFD1, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane in each blot is Marker/Kaleidoscope (K) following by 10 samples of normal (0-II), moderate (III-IV) and severe (V-VI). The molecular weight of MTHFD1 is 101kD.

b. Western blots were analysed by densitometry. Data show means and SEM. Although a decline is seen in moderate and severe cases, this is only significant between normal and severe ($p=0.05$).

3.6. Tau in CSF

Tau is one of the neuropathological markers used to diagnose AD in post-mortem brain tissue, as it forms neurofibrillary tangles, a hallmark feature of this condition. Its presence in CSF provides a potential biomarker for dementia (Mielke et al., 2021).

a.



b.

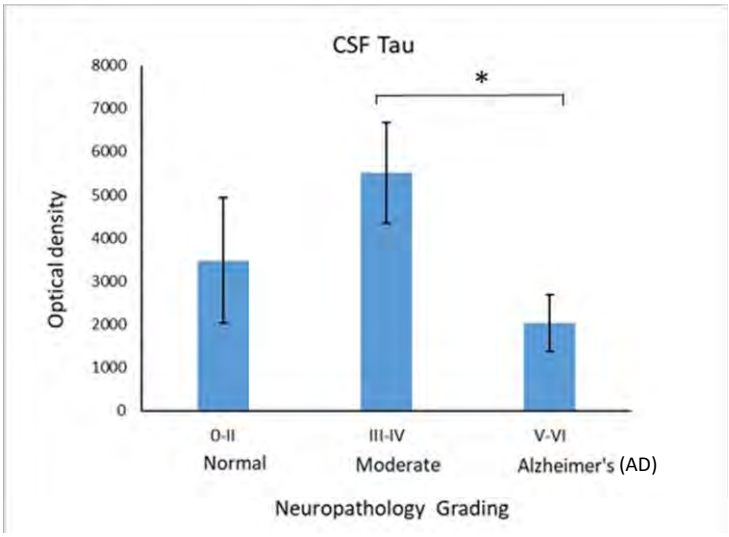


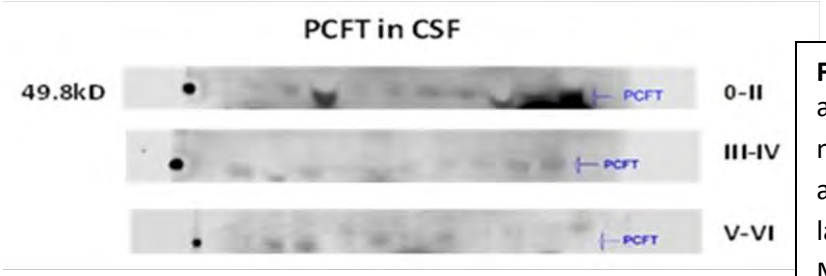
Figure 3.6.
a. Western blots for tau, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane in each blot is Marker/Kaleidoscope (K) following by 10 samples of normal (0-II), moderate (III-IV) and severe (V-VI). The molecular weight of Tau is 55kD.

b. Western blots were analysed by densitometry. Data show means and SEM. No significant difference was found in the concentration of Tau between normal and moderate but there is significant increase of Tau in moderate's CSF compared to AD CSF (p=0.02).

3.7. Proton-coupled folate transporter (PCFT) in CSF

PCFT is the most important transporter for folate from the gut to the blood and also throughout the body for folate transport into tissues via endothelial transport, and brain via endothelial or choroid plexus transport. Abnormalities in this transporter result in severe folate deficiencies including cerebral folate deficiency, the latter even in the presence of high serum folate levels (Torres et al., 2015).

a.



b.

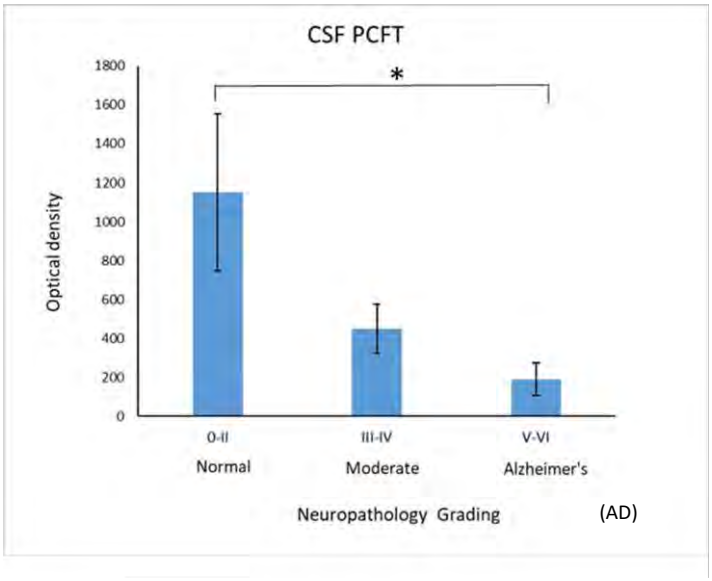


Figure 3.7.
a. Western blots for PCFT, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane in each blot is Marker/Kaleidoscope (K) following by 10 samples of normal (0-II), moderate (III-IV) and severe (V-VI). The molecular weight of PCFT is 49.8kD.

b. Western blots were analysed by densitometry. Data show means and SEM. Although a decline is seen in moderate and severe cases, this is only significant between normal and severe ($p=0.04$).

3.8. Dihydrofolate reductase (DHFR) in CSF

DHFR is a mysterious enzyme as it is useful only in the conversion of synthetic folic acid to THF. However, it is present in the neuropil of the brain (Allen Protein Atlas: https://www.proteinatlas.org/ENSG00000228716-DHFR/tissue/cerebral+cortex#img) indicating an important function. A potential pathway exists from 5,10 methylene THF to dihydrofolate which would then give functional significance to DHFR. Its presence in neuropil would imply a supply of 5,10 methylene THF, most likely from the CSF as that contains MTHFD1 which is involved in the interactive synthesis of 5,10 metheleneTHF and 5,10 methenyl THF (Lucock, 2000; Nzila et al., 2005). DHFR has also been shown to be associated with thymidylate synthase and in the synthesis of pyrimidines so may be involved in DNA synthesis, repair and gene modifications (Yuvaniyama et al., 2003).

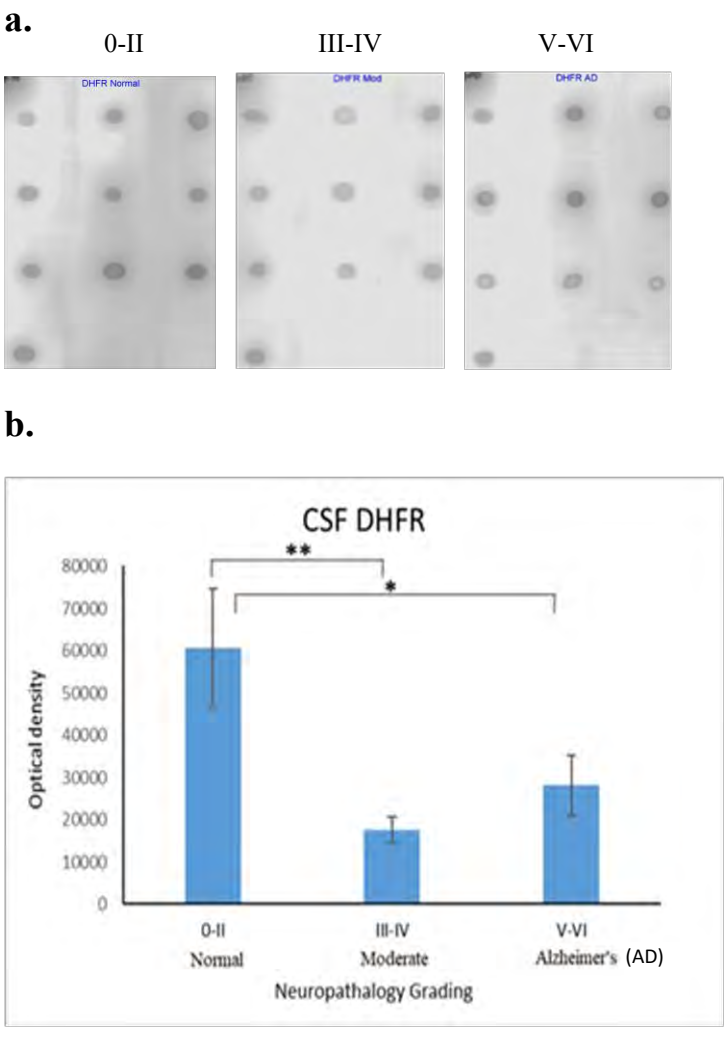


Figure 3.8.

a. Dot blots for DHFR, normal (0-II), moderate (III-IV) and severe AD (V-VI). Total three blots with 10 samples of normal (0-II), 10 samples of moderate (III-IV) and 10 samples of severe (V-VI). The molecular weight of DHFR is 25kD.

b. Dot blots were analysed by densitometry. Data show means and SEM. Significant difference was found in the concentration of DHFR between normal and severe (p=0.05) while most significant change between normal and moderate groups (p=0.01).

3.9. Folates (vitamin B9) in CSF

Folate is vitamin B9 and has many forms through the folate metabolic cycle. Food folate and the major circulating form of folate is 5mTHF. Within the cytoplasm and mitochondria of most cells, folate is transformed through addition or removal of 1-carbon moieties (Lucock, 2000; Nzila et al., 2005). Folic acid is a synthetic form and has no 1-carbon moiety to donate. Cerebral folate deficiency is a serious metabolic disorder and is responsible for a range of related neurological conditions. AD has been associated with cerebral folate deficiency although this is likely to be due to dietary deficiency rather than a specific cerebral problem.

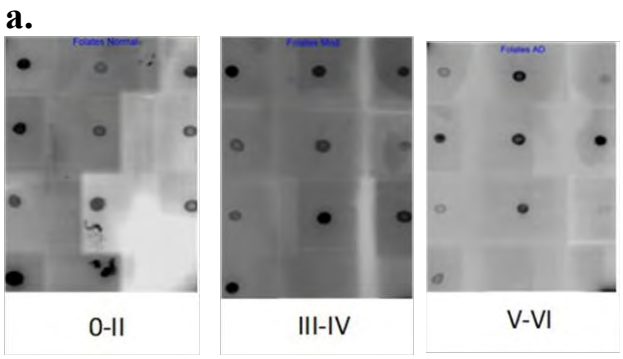


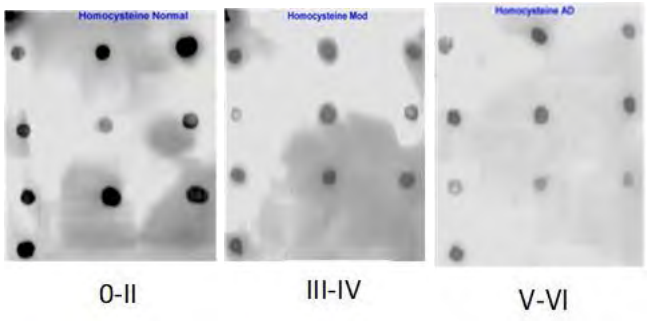
Figure 3.9.
a. Dot blots for folates, normal (0-II), moderate (III-IV) and severe AD (V-VI). Total three blots with 10 samples of normal (0-II), 10 samples of moderate (III-IV) and 10 samples of severe (V-VI). The molecular weight of folates is 400D.

b. Dot blots were analysed by densitometry. Data show means and SEM. No significant difference was found in the concentration of folates between any of the three condition groups but there is non-significant increase in moderate's CSF while the concentration of folates looks similar in normal and AD.

3.10. Homocysteine in CSF

The methyl group in the 5-methyl-THF is transferred to MTRr from where it is transferred to vitamin B12 making it methylated B12. This methylated B12 is used by MTR to methylate homocysteine into methionine (Miller, 2003). Homocysteine is thus a key molecule involved in this rate step in both folate metabolism and the methylation cycle. Raised homocysteine is a toxic, specifically neurotoxic phenomenon that indicates a failure in the methylation process, usually resulting from a folate deficiency. (Miller, 2003).

a.



b.

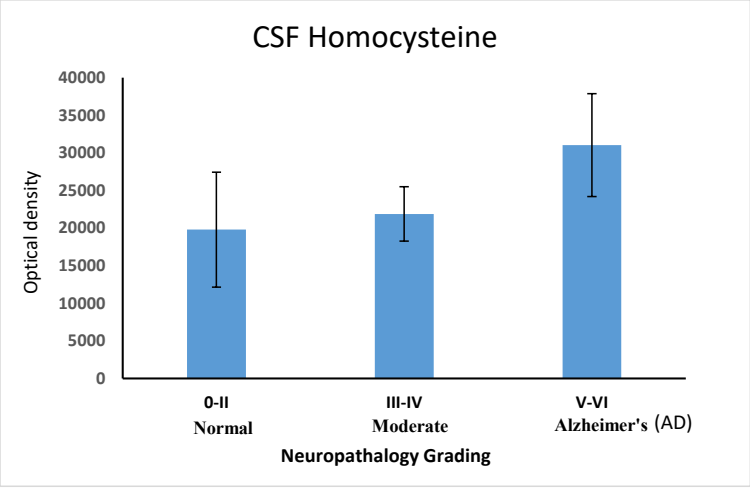


Figure 3.10.

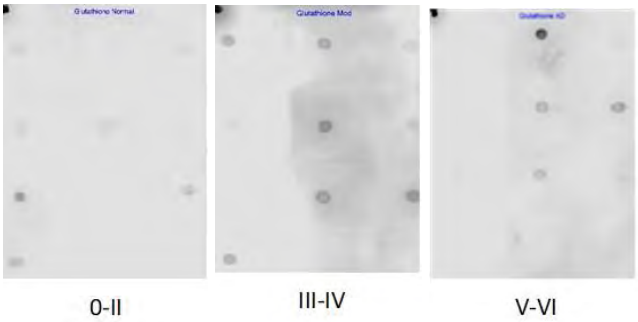
a. Dot blots for homocysteine, normal (0-II), moderate (III-IV) and severe AD (V-VI). Total three blots with 10 samples of normal (0-II), 10 samples of moderate (III-IV) and 10 samples of severe (V-VI). The molecular weight of homocysteine is 135D.

b. Dot blots were analysed by densitometry. Data show means and SEM. No significant difference was found in the concentration of homocysteine between any of the three condition groups but there is non-significant increase in AD CSF following by moderate and normal.

3.11. Glutathione in CSF

There is an inverse relationship between homocysteine levels and glutathione. This is driven by high homocysteine, a reduction in oxidative capacity through folate deficiency and a physiological upregulation of glutathione to compensate for lost oxidative potential. Thus, glutathione is intimately linked to folate metabolism and as a major molecule involved in detoxification pathways it is sensitive to changes in both folate and homocysteine (Chanson et al., 2007; Child et al., 2004).

a.



b.

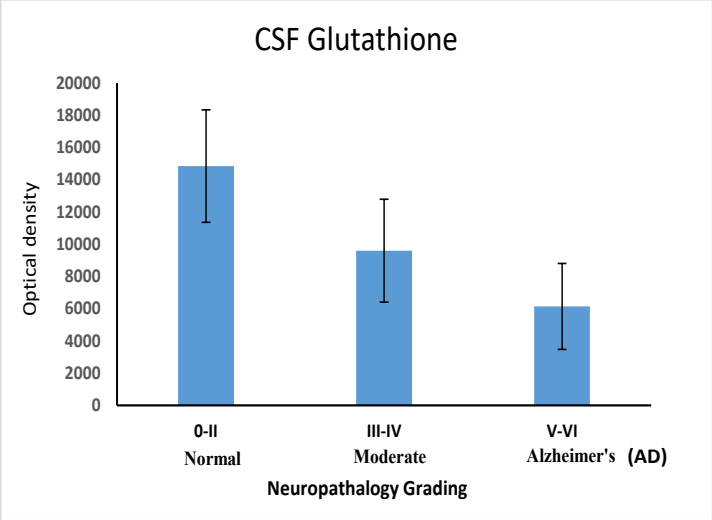


Figure 3.11.

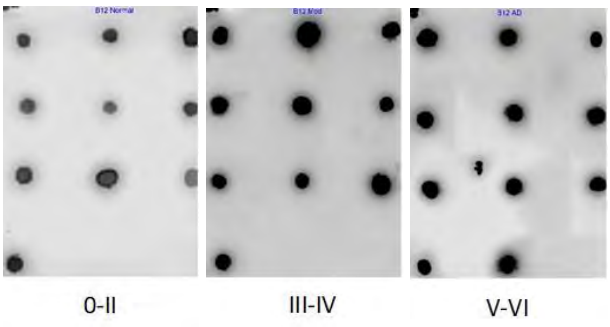
a. Dot blots for glutathione, normal (0-II), moderate (III-IV) and severe AD (V-VI). Total three blots with 10 samples of normal (0-II), 10 samples of moderate (III-IV) and 10 samples of severe (V-VI). The molecular weight of glutathione is 300D.

b. Dot blots were analysed by densitometry. Data show means and SEM. A non-significant decline of glutathione in CSF is seen in moderate and severe cases as compared to normal.

3.12. Cobalamin (vitamin B12) in CSF

The methyl group in the 5-methyl-THF is transferred to MTRr from where it is transferred to B12 making it methylated B12. This methylated B12 is used by MTR to methylate homocysteine into methionine (Miller, 2003). Given its vital role in the rate limiting step in both folate metabolism and methylation, it is not surprising that deficient vitamin B12 status is associated with increased risk of cognitive impairment, depression, and AD (Troen, 2012).

a.



b.

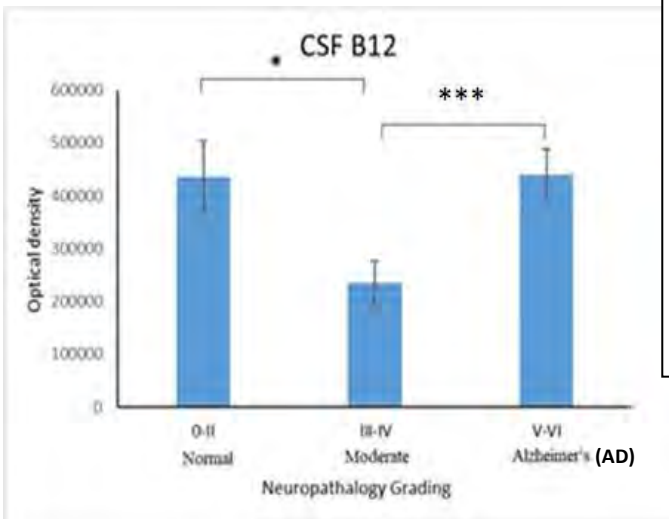


Figure 3.12.

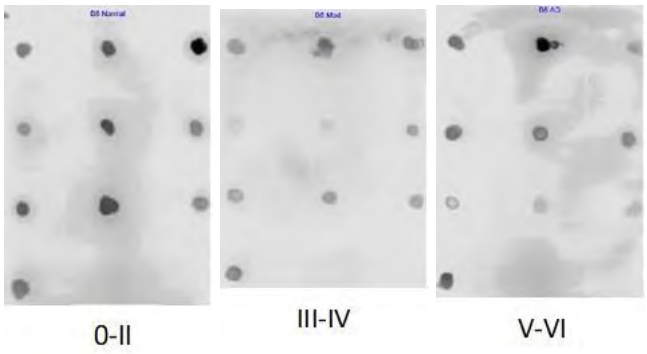
a. Dot blots for B12, normal (0-II), moderate (III-IV) and severe AD (V-VI). Total three blots with 10 samples of normal (0-II), 10 samples of moderate (III-IV) and 10 samples of severe (V-VI). The molecular weight of B12 is 1.35kD.

b. Dot blots were analysed by densitometry. Data show means and SEM. Significant difference was found in the concentration of B12 between normal and moderate (p=0.02). Most significant change was in between moderate and severe groups (p=0.004).

3.13. Pyridoxine (vitamin B6) in CSF

B6 is an important vitamin in folate metabolism in which it transfers a carbon unit from serine or glycine to tetrahydrofolate (THF) to form methylene-THF. This is used in pyrimidine synthesis or can produce formyl-THF which is used in purine synthesis. it can also be reduced to methyl-THF which, as already mentioned, is critical in the rate limiting step of folate metabolism and methylation through methylation of homocysteine to methionine, a reaction which is catalysed by a B12 (Selhub, 2002). B6 deficiency on its own is not noted for adverse effects but in combination with folate and B12 and/or raised methionine, it is implicated in a variety of abnormalities including cognitive impairment, neurodegeneration and dementia (An et al., 2019; Nuru et al., 2018; Wu et al., 2020; Zhang et al., 2020b).

a.



b.

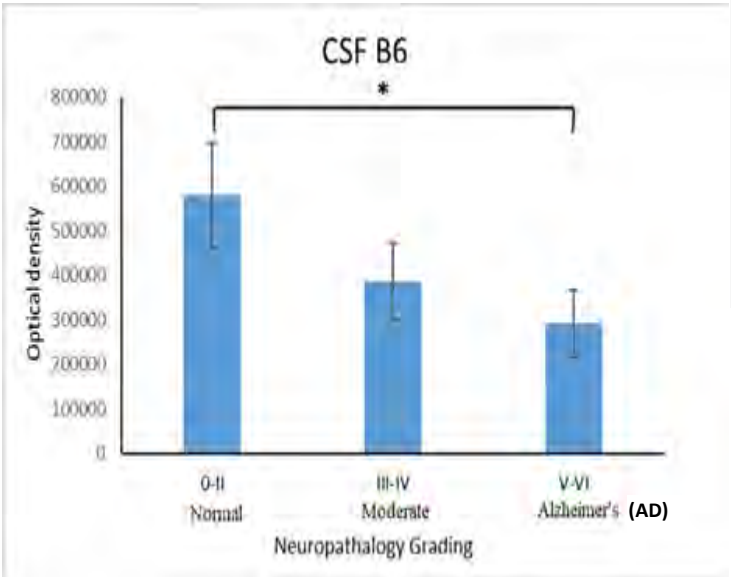


Figure 3.13.

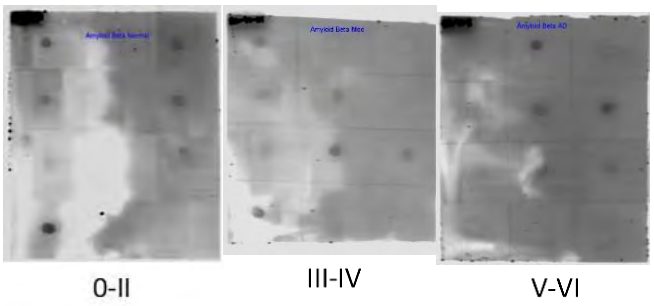
a. Dot blots for B6, normal (0-II), moderate (III-IV) and severe (Alzheimer's) (V-VI). Total three blots with 10 samples of normal (0-II), 10 samples of moderate (III-IV) and 10 samples of severe (V-VI). The molecular weight of B6 is 169D.

b. Dot blots were analysed by densitometry. Data show means and SEM. Although a decline is seen in moderate and severe cases, this is only significant between normal and severe (p=0.05).

3.14. Amyloid beta in CSF

Aβ is the most studied protein in AD and dementia research due to its association with amyloid plaques and presumed link to neuropathology. Number of forms of this protein exists with the one associated with dementia being amyloid 1-42. Amyloid is a normal component of cell membranes and in a monomeric form is neuroprotective while in polymer form becomes neurotoxic (Giuffrida et al., 2009). Recent research indicates that amyloid plaques may also be neuroprotective in the face of a loss of drainage of CSF and/or removal mechanisms for the toxic, soluble polymers (Kokjohn and Roher, 2009).

a.



b.

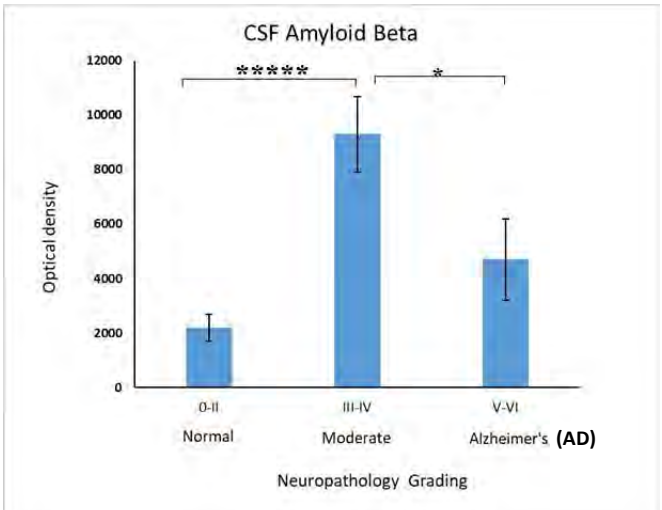


Figure 3.14.

a. Dot blots for Aβ, normal (0-II), moderate (III-IV) and severe AD (V-VI). Total three blots with 10 samples of normal (0-II), 10 samples of moderate (III-IV) and 10 samples of severe (V-VI). The molecular weight of Aβ is 4.5kD.

b. Dot blots were analysed by densitometry. Data show means and SEM. A very significant decline is seen in normal to moderate ($p=0.0005$) and a significant decline in severe to moderate cases with ($p=0.03$). No significant difference between normal to severe although there is a non-significant increase in severe.

3.15. Molecules not detected in CSF

3.15.1. Methylenetetrahydrofolate reductase (MTHFR)

MTHFR is effectively zero in CSF of any of the groups analysed. No significant signal for MTHFR was found in the blots so we assume that MTHFR is not present in CSF. MTHFR is a key enzyme in the re-synthesis of 5mTHF from 5,10 methylene THF. According to known pathways this is the only route for folate metabolism to return to 5mTHF (Lucock, 2000; Nzila et al., 2005).

3.15.2. Reduced folate carrier (RFC)

There are 3 major pathways reported for folate transport in the brain i.e., reduced folate carrier (RFC), proton-coupled folate transporter (PCFT), and FOLR1. Primarily, cerebral folate delivery takes place at the choroid plexus through FOLR1 and PCFT but inactivation of only FOLR1 can result in severe neurodegenerative disorders due to very low folate levels in the CSF. This suggests FOLR1 is the main pathway for delivery of folate to the brain. In knockout mice lacking FOLR1, treatment with vitamin D nuclear receptor (VDR) activating ligand, calcitriol, results in over a 6-fold increase in 5-formyltetrahydrofolate concentration in the brain tissue with levels comparable to wild-type animals. Thus, in the complete, developmental absence of FOLR1, the folate supply system upregulates RFC expression at the Blood Brain Barrier (BBB), providing an alternative route for brain uptake of folate. In the presence of functional FOLR1 that is blocked, for example by autoantibodies, then RFC apparently has no role in compensation and neurodegenerative disorders occur (Alam et al., 2019). Interestingly neither PCFT nor RFC seem able to compensate for lack of FOLR1 transport of folate where FOLR1 is blocked, and we also do not find them upregulated in the dementia or AD CSF or brain compared to normal. In fact, PCFT is significantly decreased in affected CSF compared to normal.

3.16. Summary

3.16.1. Folate enzymes in CSF

Figure 3.15 shows that MTR and DHFR are the most abundant enzymes in normal CSF while they are significantly reduced in AD. MTR is the rate limiting enzyme for folate metabolism converting 5mTHF to THF and involving B12 and the methylation of homocysteine to methionine. Thus, it is not only vital to folate metabolism, but also involved in supplying methionine to the methylation cycle where it produces s-adenosyl methionine (SAM) from s-adenosyl homocysteine (SAH). SAM is the universal methyl donor for methylation reactions while SAH feeds into choline metabolism and synthesis of acetyl CoA and acetylcholine (Lucock, 2000; Nzila et al., 2005).

DHFR is useful in the conversion of synthetic folic acid to THF but, more importantly, is involved in recycling of 5,10, methylene THF to dihydrofolate and tetrahydrofolate. It is present in some of the neuropil of the brain (Allen Protein Atlas: https://www.proteinatlas.org/ENSG00000228716-DHFR/tissue/cerebral+cortex#img) indicating an important role there. Its presence in neuropil would imply a supply of 5,10 methylene THF, most likely from the CSF as that contains MTHFD1 which is involved in the interactive synthesis of 5,10 methelene THF and 5,10 methenyl THF (Lucock, 2000; Nzila et al., 2005). DHFR has also been shown to be associated with thymidylate synthase and in the synthesis of pyrimidines so may be involved in DNA synthesis, repair and gene modifications (Yuvaniyama et al., 2003).

FDH, MTRr and MTHFD1 are significantly reduced in AD while MTHFR is not detected in CSF. MTRr works with MTR in the rate limiting step of folate metabolism and is also reduced in CSF. FDH is an important molecule in CSF as it shows to mediate cellular uptake into the normal brain and its reduction or absence from CSF is associated developmental deficits and cell cycle arrest in neonatal hydrocephalus (Cains et al., 2009; Owen-Lynch et al., 2003). It has important roles in associated formate metabolism in the conversion of THF to 10formyl THF.

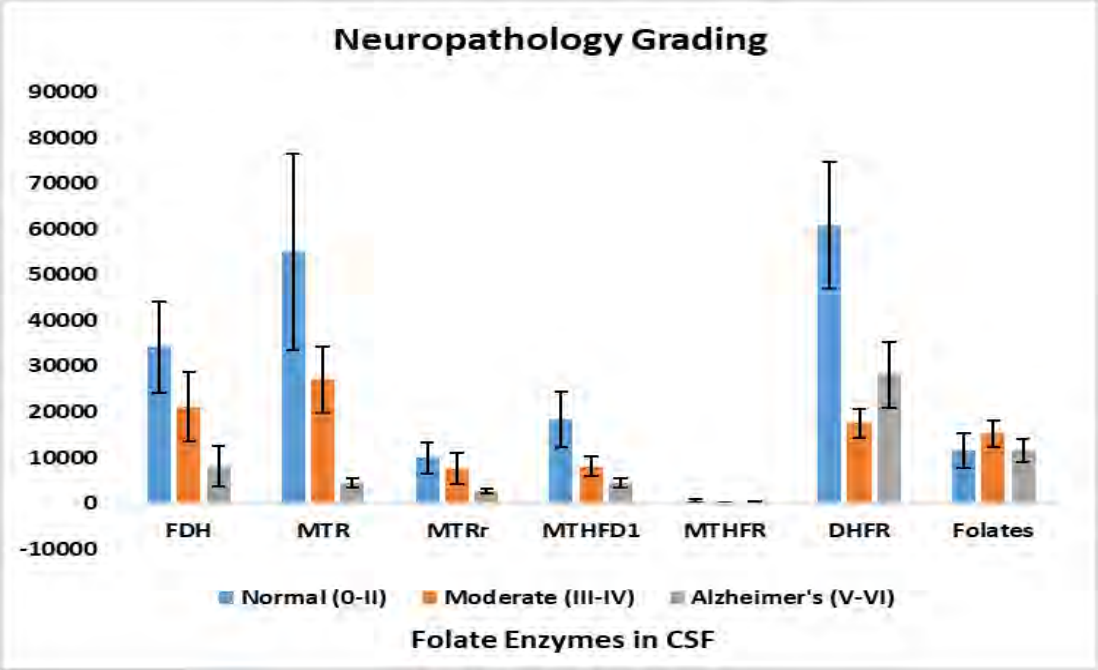


Figure 3.15. Western blots analysis for FDH, MTR, MTRr, MTHFD1, MTHFR and dot blots for DHFR and folates for normal (0-II), moderate (III-IV) and severe Alzheimer's (V-VI). Alzheimer's CSF is significantly reduced in all folate enzymes as compared to normal.

3.16.2. Folate transporters in CSF

There are 3 major transporters for folates from blood into brain. PCFT is poorly expressed in the brain, RFC is expressed in choroid plexus and endothelium, and FOLR1 is mainly expressed in the choroid plexus. Only FOLR1 is expected in CSF as it transports folate from blood across the choroid plexus into CSF (Figure 3.16). RFC and PCFT are involved in transport across the BBB although RFC is also expressed in choroid plexus.

Compared to levels in the normal ageing CSF, FOLR1 is reduced in moderately affected, and not significantly different in severely affected brain CSF. This is reflected in raised folate in moderate brains compared to both normal and severe levels of folate. However, this is not matched by the decreasing levels of FDH in both moderate and severe brains compared to normal. Raised levels of FOLR1 in severe cases may, therefore, be due to low level of FDH that are not able to transfer folate from FOLR1 to the brain. The relationship of FOLR1 to folate levels suggests we are measuring unbound folate and that the folate bound to FOLR1 and/or FDH is not measured using our simple method.

As expected, PCFT and RFC are at negligible levels in CSF since they are thought to remain in their membrane locations and simply transfer folate across the membrane. By contrast FOLR1 is at high levels in CSF as it carried folate across the choroid plexus into the CSF. These results shows that FOLR1 is the major transporter of folates in CSF.

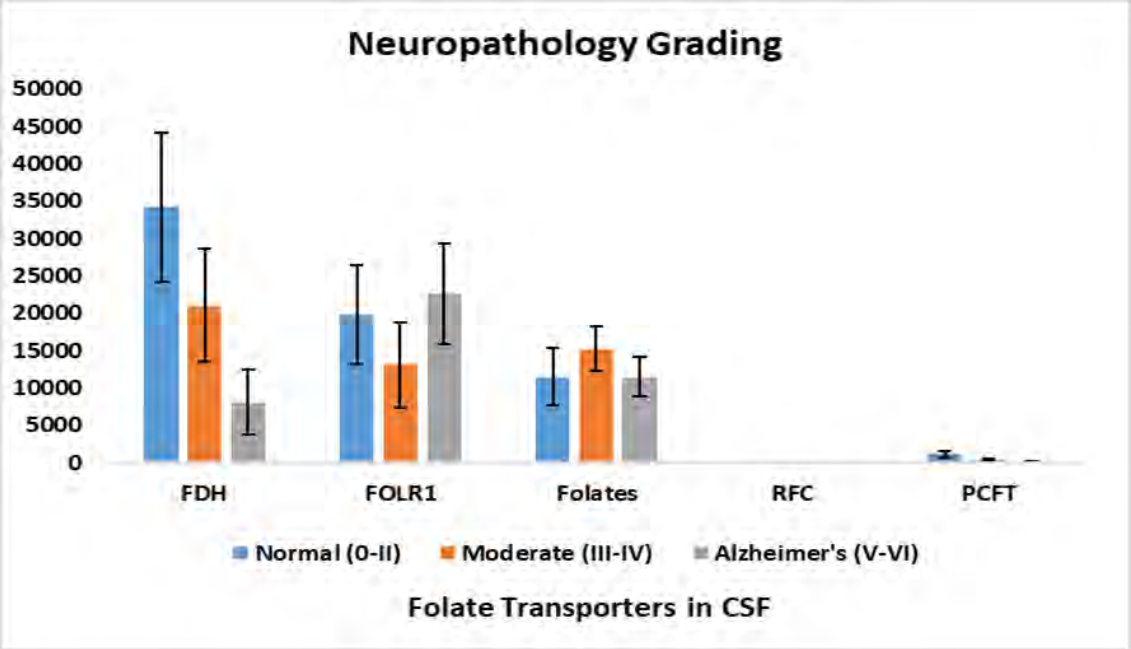


Figure 3.16. Western blots analysis for FDH, FOLR1, RFC, PCFT and dot blots for folates for normal (0-II), moderate (III-IV) and severe Alzheimer's (V-VI). AD CSF has negligible PCFT while FDH is significantly reduced as compared to normal. Compared to levels in the normal ageing CSF, FOLR1 is reduced in moderately affected, and not significantly different in severely affected brain CSF. This is reflected in raised folate in moderate brains compared to both normal and severe levels of folate. RFC is not present in CSF.

3.16.3. Neurodegeneration and folate metabolites

Tau is a microtubule-associated protein, with a strong influence on both the morphology and physiology of neurons. In AD, Tau protein undergoes post-translational modifications, which could play a relevant role in the onset and progression of this disease (Jara et al., 2020). Amyloid is a normal component of cell membranes and in a monomeric form is neuroprotective while in polymer form becomes neurotoxic (Giuffrida et al., 2009). Recent research indicates that amyloid plaques may actually be neuroprotective in the face of a loss of drainage of CSF and/or removal mechanisms for the toxic, soluble polymers (Kokjohn and Roher, 2009).

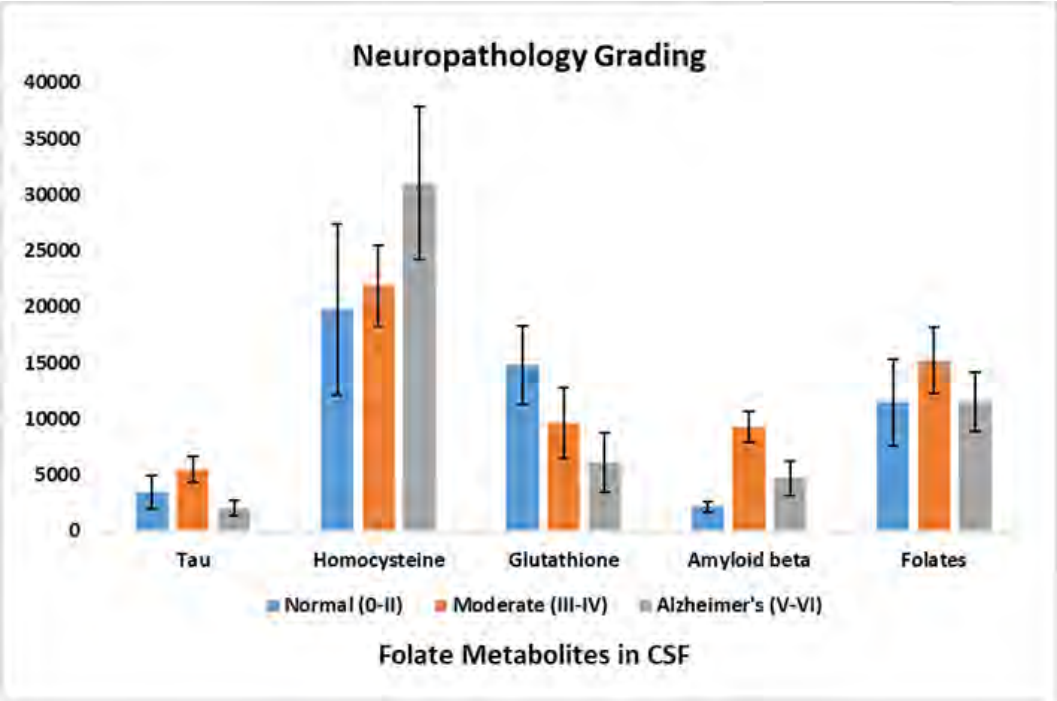


Figure 3.17. Western blots analysis for Tau and dot blots for homocysteine, glutathione, amyloid beta and folates for normal (0-II), moderate (III-IV) and severe Alzheimer's (V-VI). Both tau and amyloid show increase in moderate CSF with decrease in severe cases which is likely to be related to the change from soluble to insoluble forms, the latter remaining the brain. Homocysteine and glutathione show an inverse relationship in the CSF reflecting a failure in methylation of homocysteine to methionine and the onward production of SAM and SAH required to generate glutathione. CSF folate levels show no significant change between the different conditions.

The plaques seem to act to hold an otherwise toxic molecule in a toxic, insoluble form. Aβ is upstream of tau in AD pathogenesis and triggers the conversion of tau from a normal to a pathological state, but there is also evidence that toxic tau enhances Aβ toxicity via a feedback loop (Bloom, 2014). Results in this study show that in moderate and AD CSF, Tau is reduced as compared to Aβ. In addition, there is an inverse relationship between homocysteine levels and glutathione which is clearly evident in our results. B12 is similar in normal and AD CSF though reduced in the moderates. B12 acts in the conversion of homocysteine into methionine together with MTR and MTRr (Figure 3.17). As MTR is reduced in AD so B12 is ineffective to methylate homocysteine into methionine. Reduced levels of B6 halt the conversion of homocysteine into glutathione which is supported by our results. Homocysteine is a toxic, specifically neurotoxic

molecule that when raised also indicates a failure in the methylation process utilising 5-methyl THF (Miller, 2003).

3.17. Comparison of patient clinical data vs post-mortem neuropathology grading

The case details of the brain tissues used in this study are shown in Table 2.1. in the methods chapter. Each brain is scored by two neuropathologists to give a Braak score and cases assigned to Braak 0-II (normal ageing), Braak III-IV (moderate dementia/mild cognitive impairment) and Braak V-VI (severe AD). On examining the additional details provided by the Brain Bank, we found that clinical diagnosis during the lifetime of the individuals conflicted with neuropathology grading. These are highlighted in the table with a dark green colour. For example (Table 2.1), for case number 1 the clinical and neuropathology findings conflict as the patient was clearly suffering with mild dementia but has been assigned to normal ageing. The biggest change can be observed in the moderate group where 6 individuals can be re-assigned based on conflicting clinical and neuropathology observations. Similarly, four in the AD group can be re-assigned including one that can be re-assigned to normal ageing. This highlights the difficulties of a conclusive diagnosis in these cases and also the inherent possibility of error in diagnosis using only a strict, post-mortem, neuropathology grading. In order to consider the effects of the re-assignments on the statistical outcomes, the data were analysed in two ways, a. by neuropathology grading (used throughout this chapter) and b. clinical grading, i.e., based on re-assignment of cases to clinical grading. These are shown in figures 3.18a and 3.18b. Figure 3.18a summarises all the Western blot and dot blot data as percentage changes relative to levels in normal CSF based on the original neuropathological gradings. Generally, there is a decreased concentration of folate enzymes in the affected CSF compared to normal. The folate transporter FOLR1 is reduced in moderate but increased in AD CSF, tau is increased in moderate but reduced in AD while amyloid is increased in both moderate and AD CSF although reduced in AD compared to moderate. Homocysteine is increased in both, but more so in AD indicating a potential folate block. There is no folate deficiency in the affected CSF as it is raised in moderate and not changed in AD compared to normal. Of all the folate enzymes only one is missing from CSF and that is the key enzyme MTHFR, responsible for recycling of folate back to 5methylTHF (see discussion). Figure 3.18b shows the same data but grouped according to the clinical notes that re-assign individuals to different severity groups. There are

significant changes when the data is grouped in this way compared to the neuropathological grading alone. FOLR1, Tau, folates, homocysteine, B12, B6 and Aβ are significantly changed between the two grading systems. Decreased error bars in most cases on the clinical scoring suggests that this is more accurate reflection of the cases. However, the literature does not use clinical grading but exclusively relies on neuropathology, Braak grading. The analysis shown in these figures suggests that clinical grading may be more accurate, and a recommendation would be to at least present data in both ways in future publications.

Figure 3.18a.

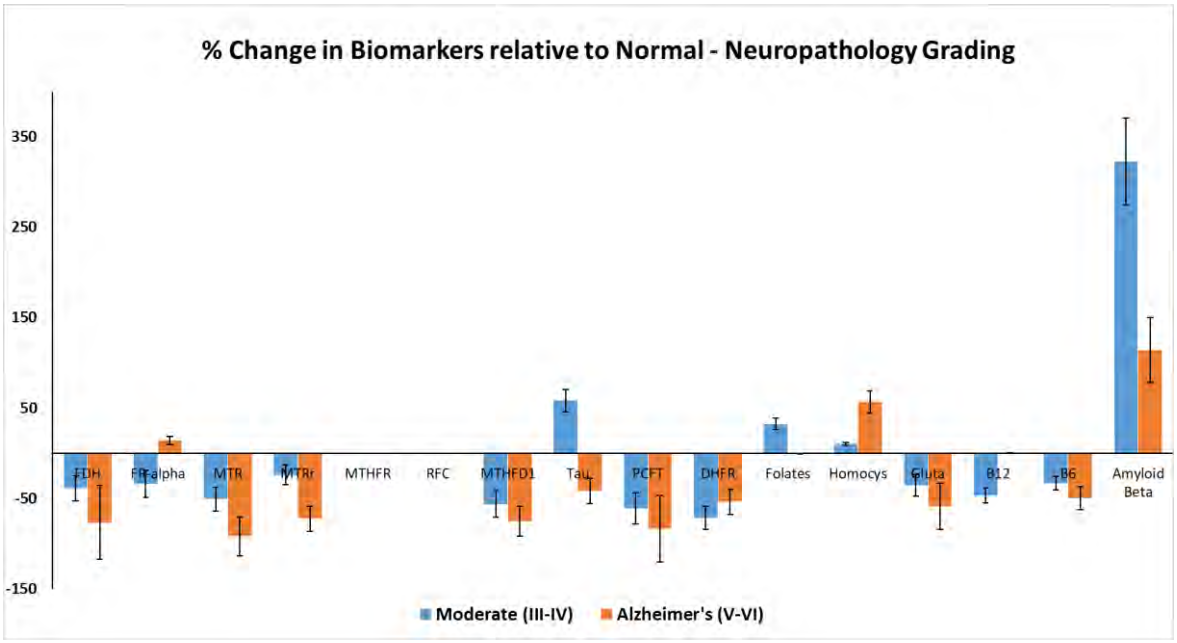


Figure 3.18b.

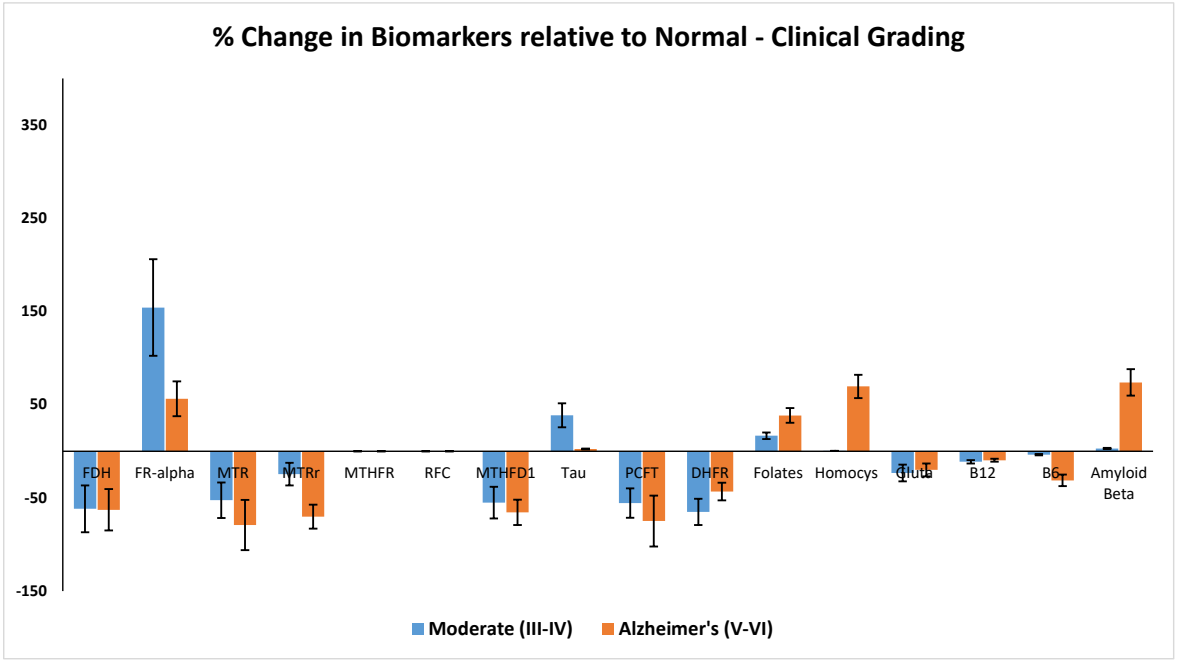


Figure 3.18. Analysis of Western and dot blot data plotted as percentage of normal values using neuropathology grading (a) or clinical grading (b).

3.18. Epidemiological details

3.18.1. Gender vs disease status

Male to female proportion showed that 51.42% were normal males whereas 48.57% were normal females. Similarly, male AD and female AD cases were 51.42% and 48.57%, respectively.

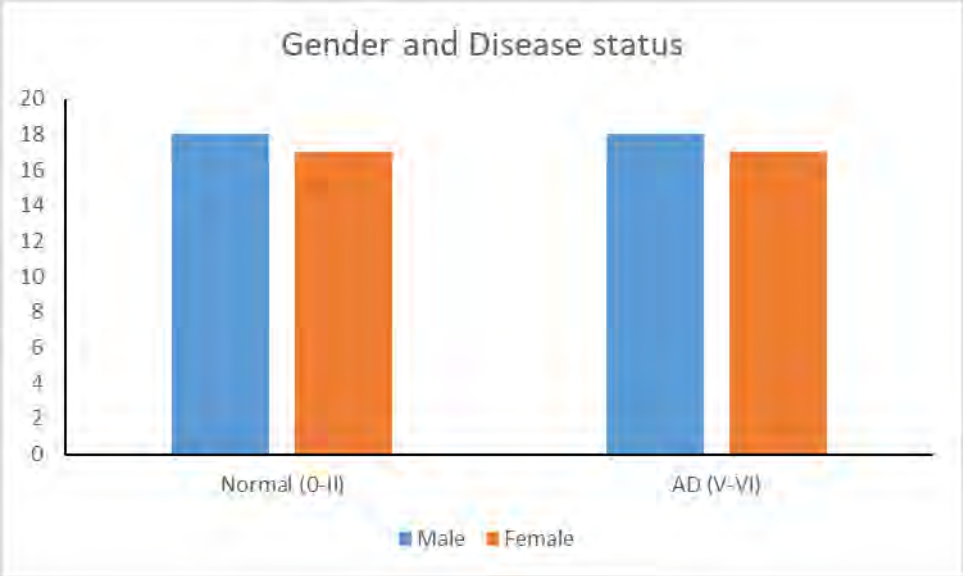


Figure 3.19 (a) Representation of subjects with respect to gender and disease status

3.18.2. Age at death with respect to gender and disease status

Age-wise distribution of subjects revealed that males age at death is less than the females age at death, both in normal and diseased condition.

Figure 3.19 (b). Age at death with respect to gender and disease status

3.19. Discussion

In many publications, folic acid has been used as the "natural" entry point into the folate metabolic pathway. This is incorrect as folic acid is an artificial man-made substance and is missing the one carbon moiety which would make it useful. Taking folic acid would have two immediate consequences, firstly, it would effectively dilute one carbon availability since it needs to acquire a one-carbon moiety to become useful (Lucock, 2000). Secondly it would unbalance folate metabolism by increasing the concentration of dihydrofolate (Bailey and Ayling, 2009). Folic acid also has a reported property of competitive/irreversible binding to FOLR1 specifically in the choroid plexus, decreasing or completely blocking transfer of folates from the blood to CSF and brain (Zhao et al., 2011).

Food folate is 5-mTHF which is shown as an important entry point into the folate cycle as it is involved in the rate limiting step of conversion to THF. This step requires homocysteine which is converted to methionine through the action of MTR and MTRr requiring B12 and the methyl group from the folate. THF is then available for all other aspects of metabolism including DNA synthesis, methylation and formate metabolism. 5mTHF is also directly involved in the BH2-BH4 cycle along with D3and B2. BH4 is directly involved in biogenic amine synthesis and also nitric oxide synthesis. The production of methionine is critical to methylation through the generation of SAM, a vital function in gene expression. Transfer of methyl groups from SAM produces SAH which in turn can be re-methylated to SAM or converted to homocysteine, cysteine and then glutathione. Glutathione is the major mechanism for removal of toxins from the brain and body. These few points highlight some of the critical roles of folates in brain homeostasis, metabolism, and function.

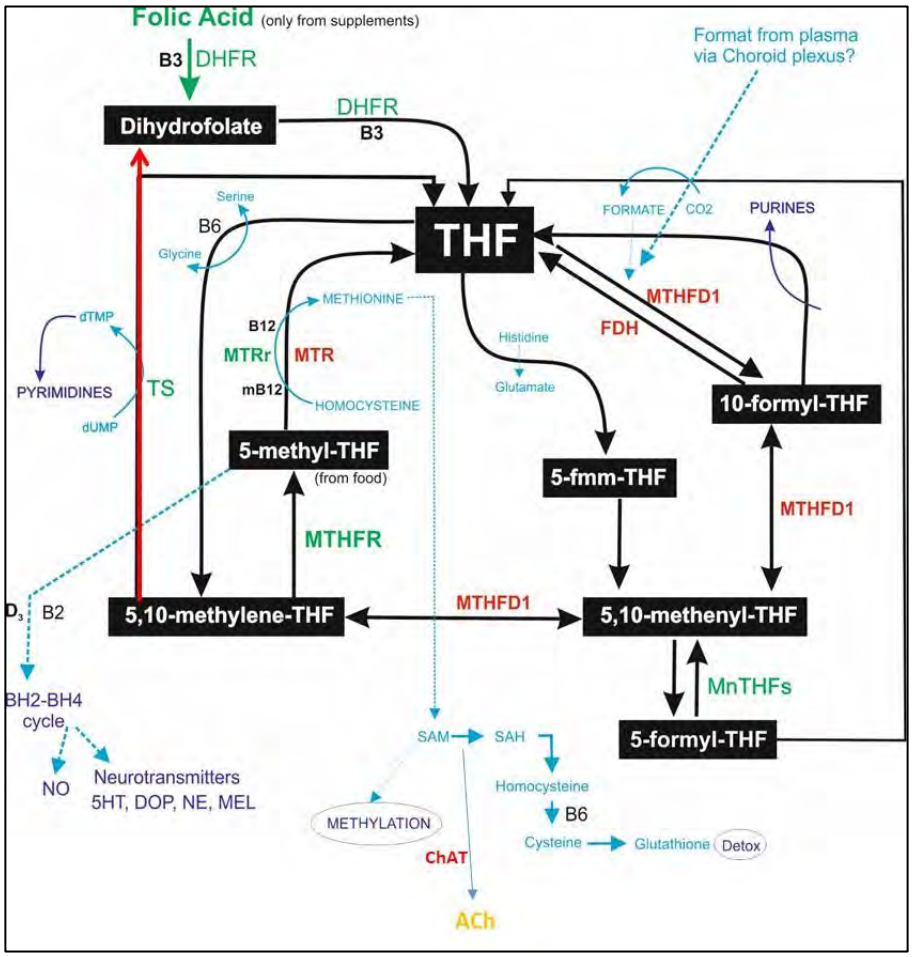


Figure 3.20. This diagram shows the folate metabolic cycle with the entry of natural folate, 5-methylTHF, into the rate limiting step methylating homocysteine to methionine and onward into both halves of the cycle feeding into methylation, neurotransmitter and nitric oxide synthesis as well as nucleotide synthesis. Folic acid enters through dihydrofolate and effectively dilutes the 1-carbon pool.

The results of this study show a global down regulation of folate metabolism through the reduced concentrations of folate metabolising enzymes in the CSF from affected brains, both moderate and AD, compared to normal ageing. This is further indicated by raised homocysteine suggesting that the block is in the rate limiting step where homocysteine is methylated to methionine. In other neurological conditions that show raised homocysteine, treatment with high dose folate reduces homocysteine suggesting a folate deficiency existed. In dementia and AD there is no significant difference in folate concentration in CSF compared to normal ageing indicating that this is not a folate deficiency. More likely, the reduction in FDH may be critical in a failure to deliver folate

to the brain as we have previously found in hydrocephalus (Cains et al., 2009). The slightly raised FOLR1 could also be taken to confirm this as we also found this raised in hydrocephalus. Thus, based on previous findings in neonatal hydrocephalus and the results of the analysis of CSF alone in these ageing brains, with reduced FDH there would be reduced transfer of folate from FOLR1 that then remains in CSF at a slightly higher concentration with little change in total folate.

In the next chapter studies of folate status are presented that shed a different light on adult folate transfer to the brain, which shows big differences to the neonatal system.

Chapter 4

Results II

Western and dot blot analysis of folate status in brain tissue lysate

Chapter 4

Western and dot blot analysis of folate status in brain tissue lysate

4.1. Western and dot blot analysis of folate status in brain lysate

Folate status is significantly changed in the CSF of affected individuals. To test if this is reflected in tissue folate status, we carried out a similar analysis of fresh frozen brain tissue from the same individuals. Tissue was dissected to sample the different zones of the cortex and then homogenised. Total protein was calculated relative to a BCA protein assay kit (Thermo Fisher Rapid Gold BCA Kit). Final protein concentrations were standardised across the samples to allow equal volumes of lysates to be run in Western and dot blots. 3mg of lysate was used in Western blots and 0.5mg in dot blots. High protein concentration was used to optimise detection of the low abundant proteins and metabolites. For details of the function and importance of each target molecule please see the previous chapter.

4.2. ALDH1L1 (FDH) in brain lysates of cortex and ventricular zone

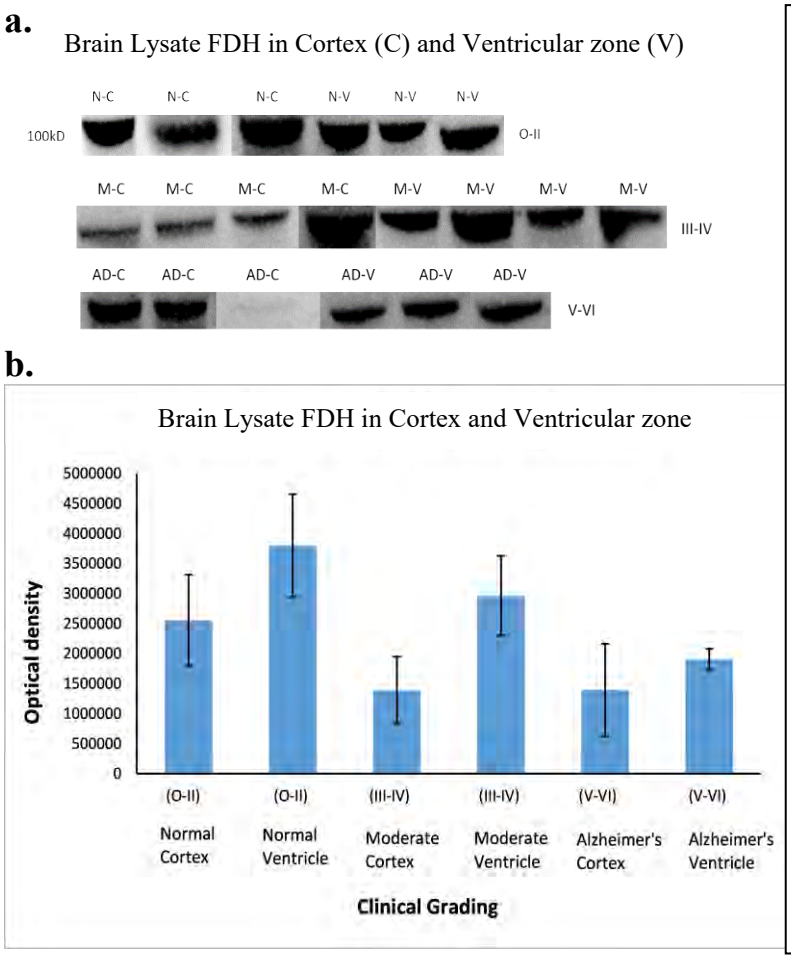


Figure 4.1.
a. Western blots for FDH in brain lysate, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane has 3 samples of normal cortex (N-C) with normal ventricle (N-V) second lane has 4 samples of moderate cortex (M-C) with moderate ventricle (M-V), and third lane has 3 samples of Alzheimer's cortex (AD-C) with Alzheimer's ventricle (AD-V). The molecular weight of FDH is 100kD.

b. Western blots were analysed by densitometry. Data show means and SEM. A non-significant decline is seen in cortex but not in the ventricular zone.

4.3. Folate receptor alpha (FOLR1) in brain lysate of cortex and ventricular zone

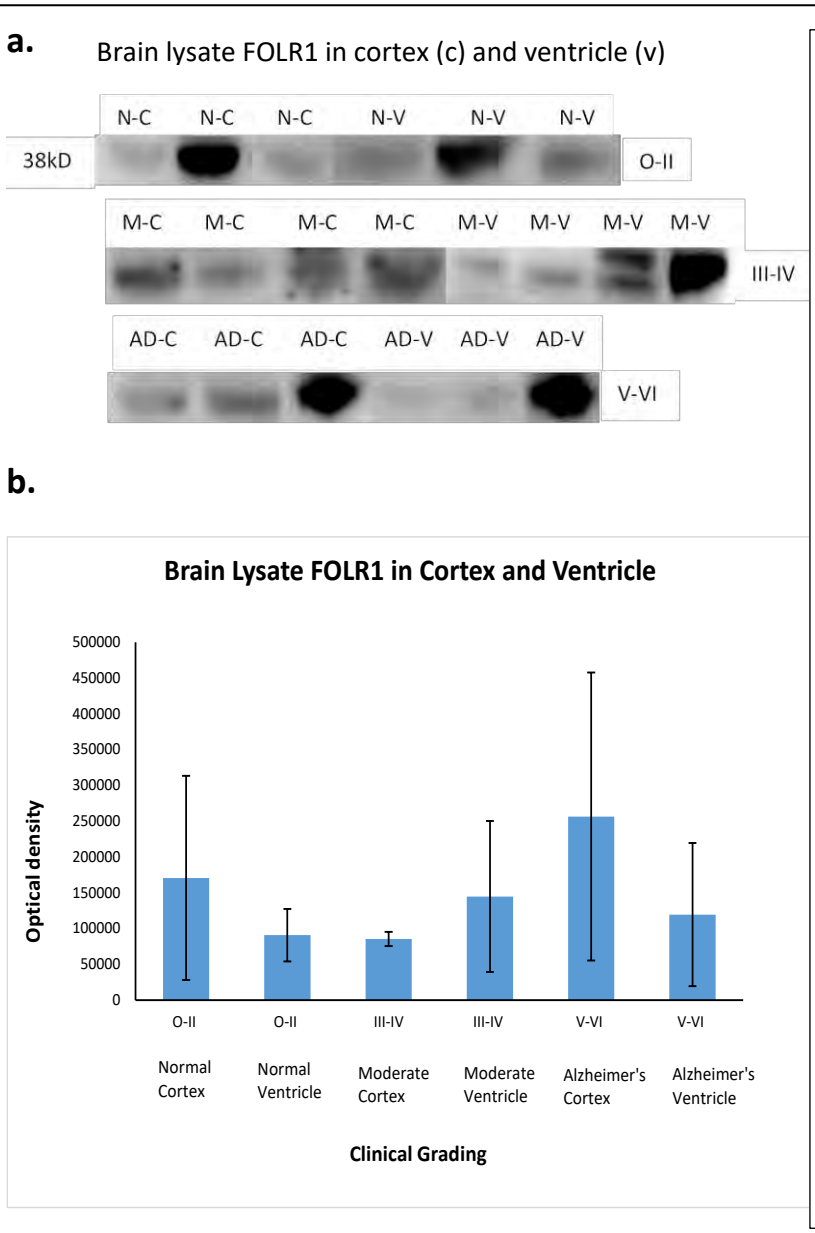


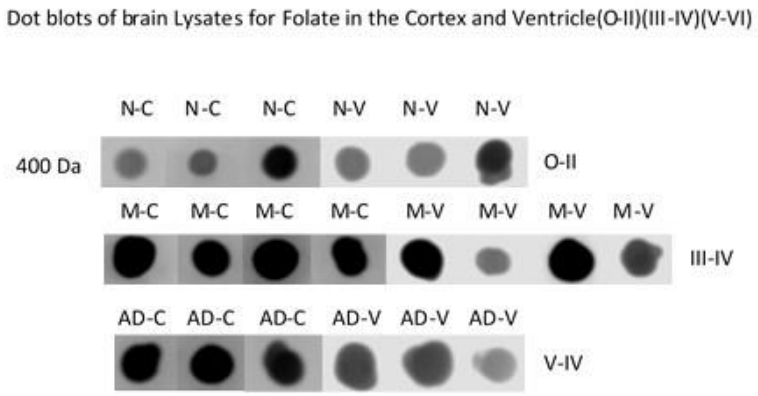
Figure 4.2.

a. Western blots for FOLR1, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane has 3 samples of normal cortex (N-C) with normal ventricle (N-V), second lane has 4 samples of moderate cortex (M-C) with moderate ventricle (M-V), and third lane has 3 samples of Alzheimer's cortex (AD-C) with Alzheimer's ventricle (AD-V). The predicted molecular weight of FOLR1 is 29.82kD but the observed was 38kD. FOLR1 is reduced in ventricles in both normal and AD but increased in moderates tend to protect the brain.

b. Western blots were analysed by densitometry. Data show means and SEM. No significant difference was found in the concentration of FOLR1 between any of the three condition groups but there is non-significant increase in moderate ventricles.

4.4. Folates in brain lysate of cortex and ventricular zone

a.



b.

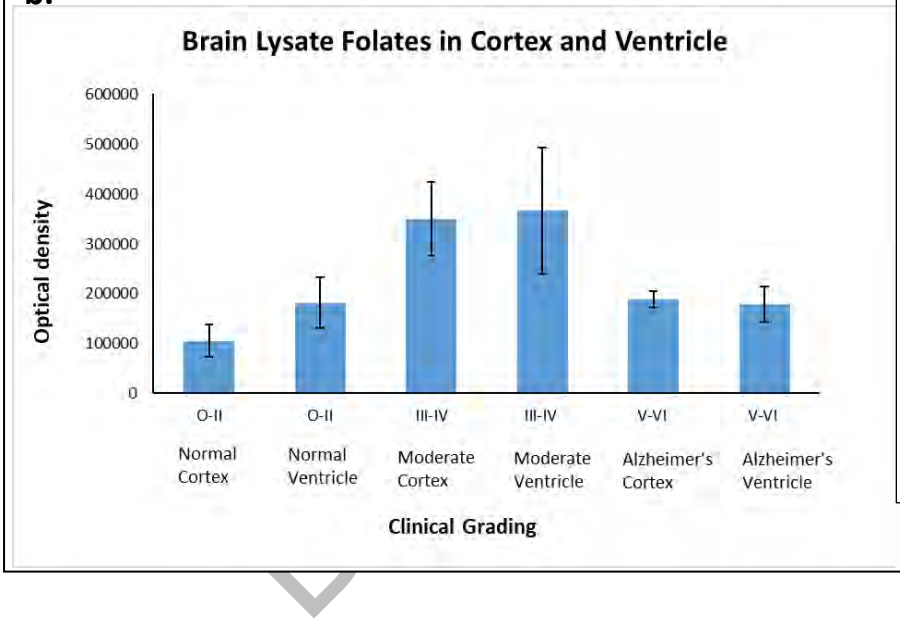


Figure 4.3.

a. Dot blot for folates, normal (0-II), moderate (III-IV) and severe AD (V-VI). The first lane has 3 samples of normal cortex (N-C) with normal ventricle (N-V), second lane has 4 samples of moderate cortex (M-C) with moderate ventricle (M-V), and third lane has 3 samples of Alzheimer's cortex (AD-C) with Alzheime''s ventricle (AD-V). The molecular weight of folates is from 400 to 497Da depending on folate species folate is slightly increased in ventricles in all.

b. Dot blots were analysed by densitometry. Data show means and SEM. No significant difference was found in the concentration of folates between any of the three condition groups.

4.5. Summary

4.5.1. Folate transporters in brain tissue lysate

Figure 4.4. Summary of results plotted as absolute optical densities for comparison of concentrations. Mean levels are compared between cortical plate (C) and ventricular zones (V) of normal, moderate, and severe brain tissue. FDH is the major folate-related protein in the tissue of the brain and appears more abundant in the ventricular zone though this is not significantly different to cortical levels. There is decreased FDH in moderate and severe brains, showing a similar reduction in the cortical plate but a greater reduction in the ventricular zone of sever cases. FOLR1, by contrast appears in lower concentration in the tissues of the brain with a decrease in moderate brains. Folates are generally raised in the tissues of affected brains compared to normal. These are also plotted as percentage changes in the next figure. (Figure 4.5).

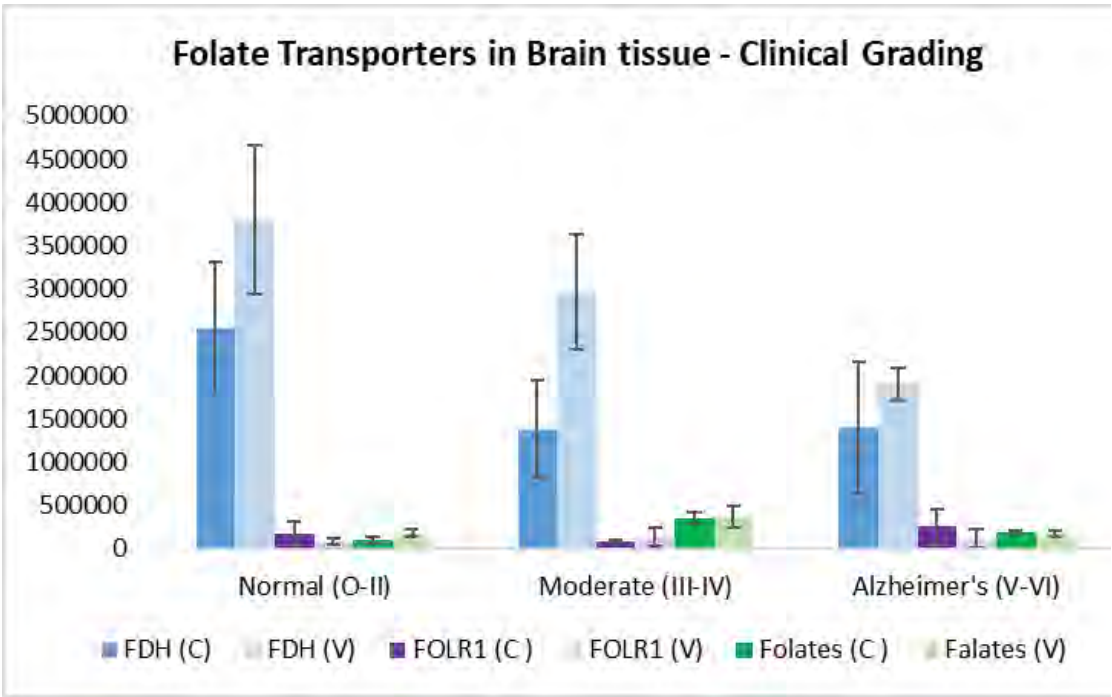


Figure 4.4. Tissue lysate analysis for folate status showing FDH, FOLR1 and folates measured by Western blots (FDH, FOLR1) and dot blots (folate). This graph shows the measurements of absolute optical density (arbitrary units) for comparison of tissue concentrations of these molecules.

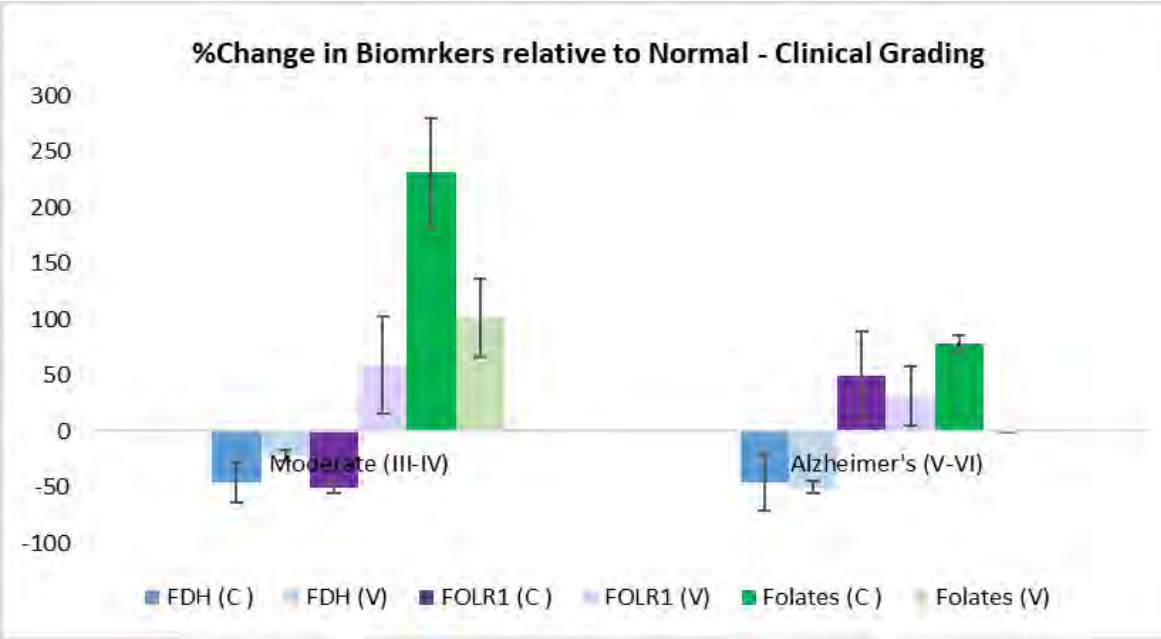


Figure 4.5. Data from Figure 4.4 replotted as percentage change using levels in normal brain tissue as 100%. This shows the similar decrease in tissue FDH compared to normal with a rise in FOLR1 and folate. The folate rise is particularly evident in the moderate cases with a smaller rise in the cortex of severe brains and no change in the ventricular zone of severe cases.

The data in Figure 4.5. shows % changes in FDH, FOLR1 and folate. These show a decrease in FDH in both moderate and severe brains compared to normal. FOLR1 is decreased in moderates but increased in severe brain cerebral cortex while it is increased in both ventricular zones. Folate is increased in both moderate and severe cerebral cortex and in moderate ventricular zone but is not changed in the ventricular zone of severe brains.

FDH is reduced in the CSF of affected brain so is no longer available to bind folate and transport it into the brain. This may also reflect a downregulation of FDH production since it is not present at normal levels in the CSF of affected individuals. FOLR1 also shows a decrease in the cortex of moderates but is increased in severe cortex as well as in both ventricular zones. This can be interpreted together with the IHC results in Chapter 5 that show FOLR1 following a different route of entry into the brain that may increase folate levels as a result.

4.6. Supplementary figures showing full Western blots

4.6.1. Western blots of CSF

Here are shown the full blots from which the specific band pictures were taken for the results chapters (3 and 4).

4.6.1.1. Full blots for FDH (data used in Figure 3.1)

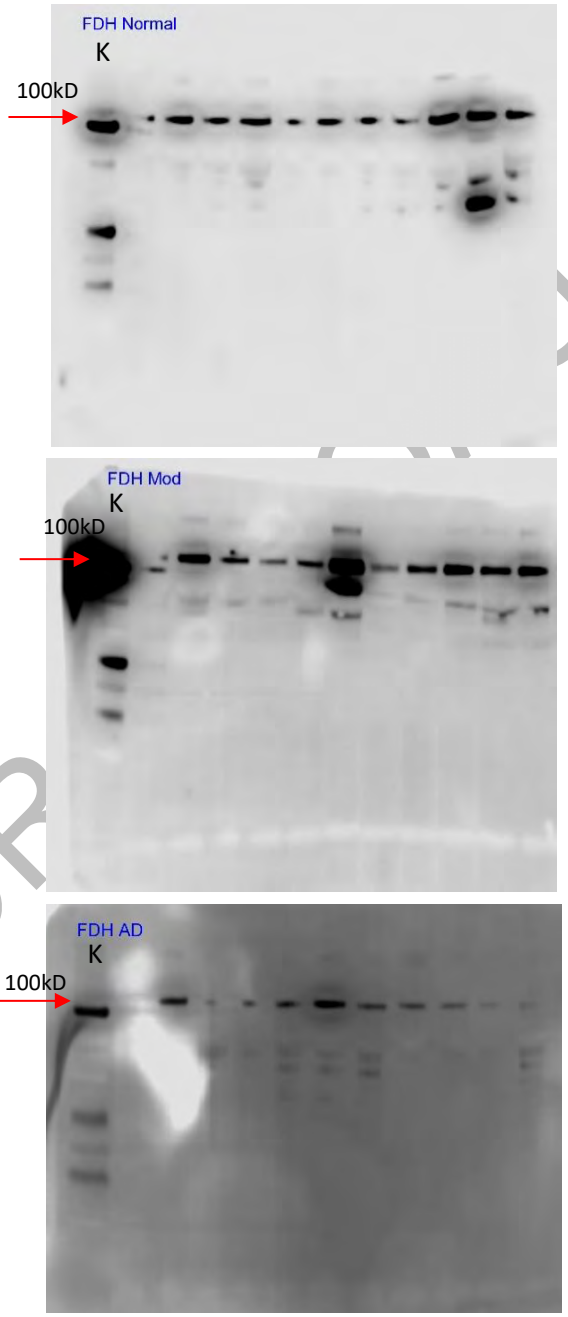


Figure 4.6 Full blots for FDH, normal, moderate (mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is at 100kD for FDH.

4.6.1.2. Full blots for FRalpha/FOLR1 (data used in Figure 3.2)

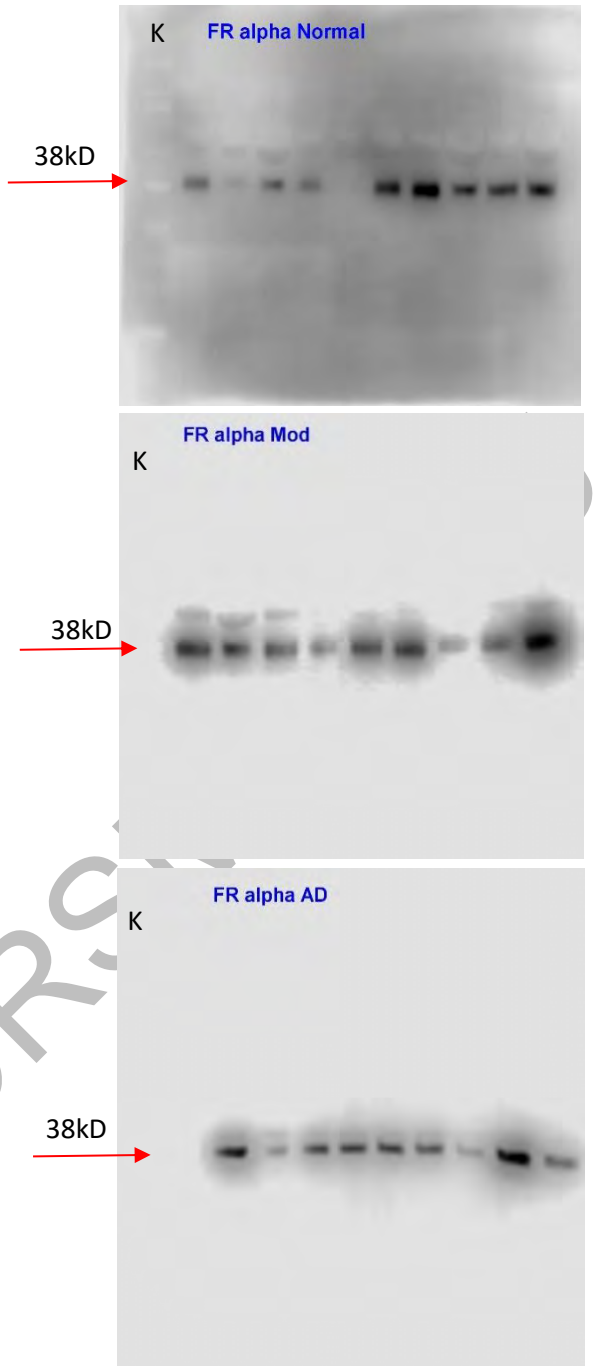


Figure 4.7. Full blots for FOLR1, normal, moderate (mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is at 38kD for FOLR1 (FRα).

4.6.1.3. Full blots of MTR (data used in Figure 3.3)

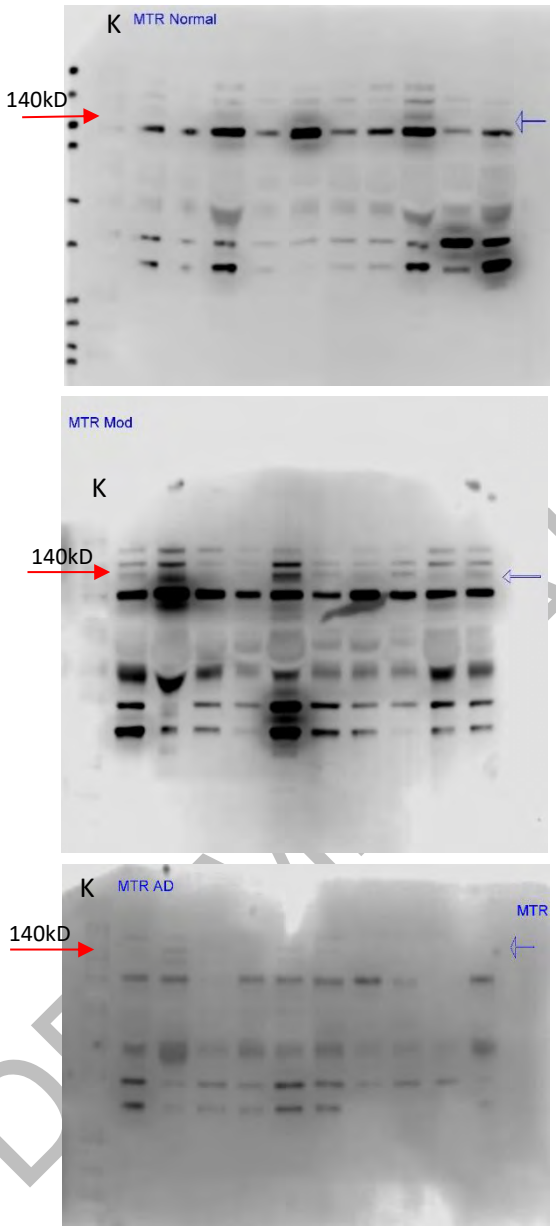


Figure 4.8. Full blots for MTR, normal, moderate (mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is at 140kD for MTR.

4.6.1.4. Full blots for MTRr (data used in Figure 3.4)

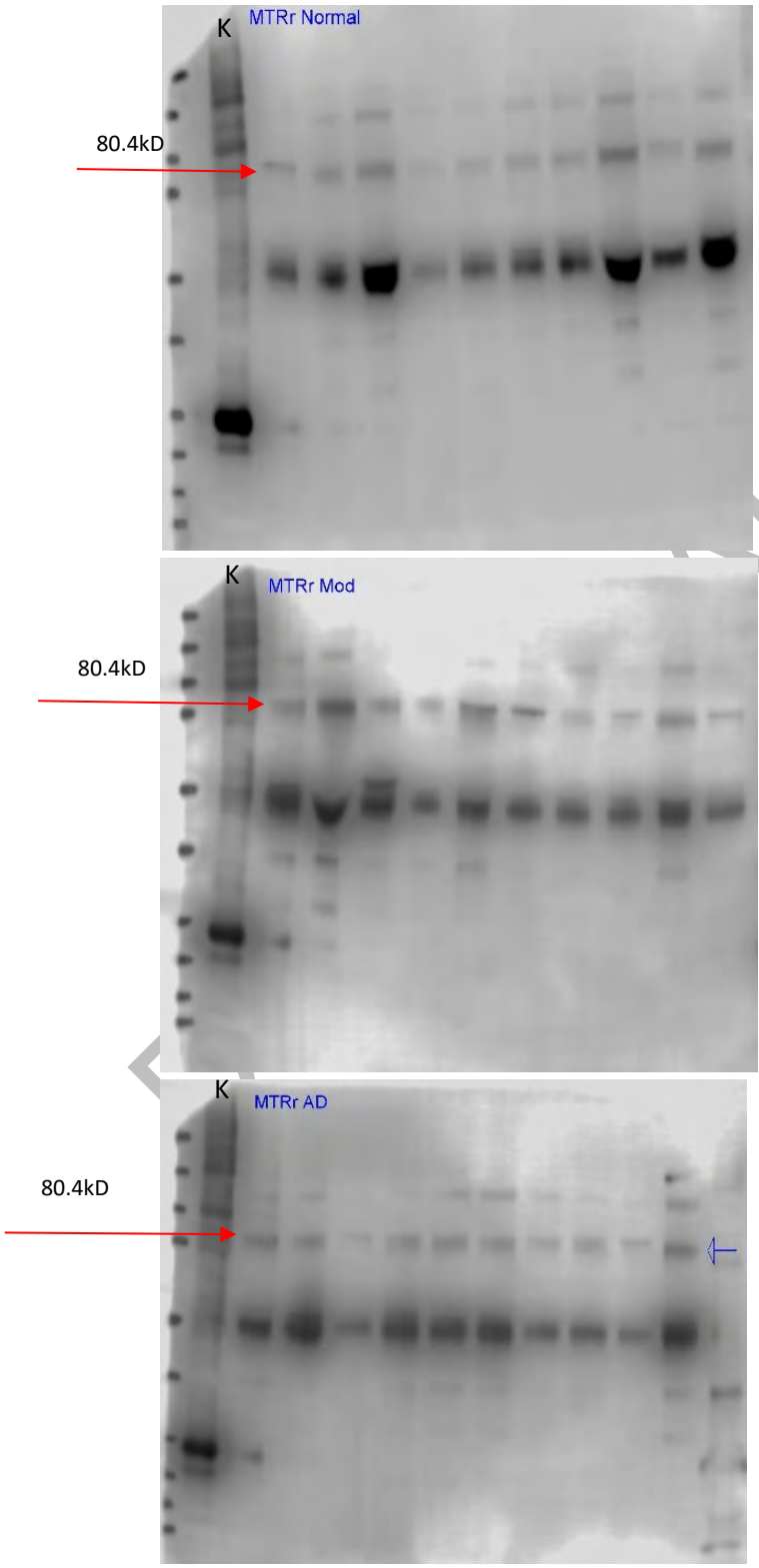


Figure 4.9. Full blots for MTRr, normal, moderate (mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is at 80.4kD for MTRr.

4.6.1.5. Full blots for MTHFD1 (data used in Figure 3.5)

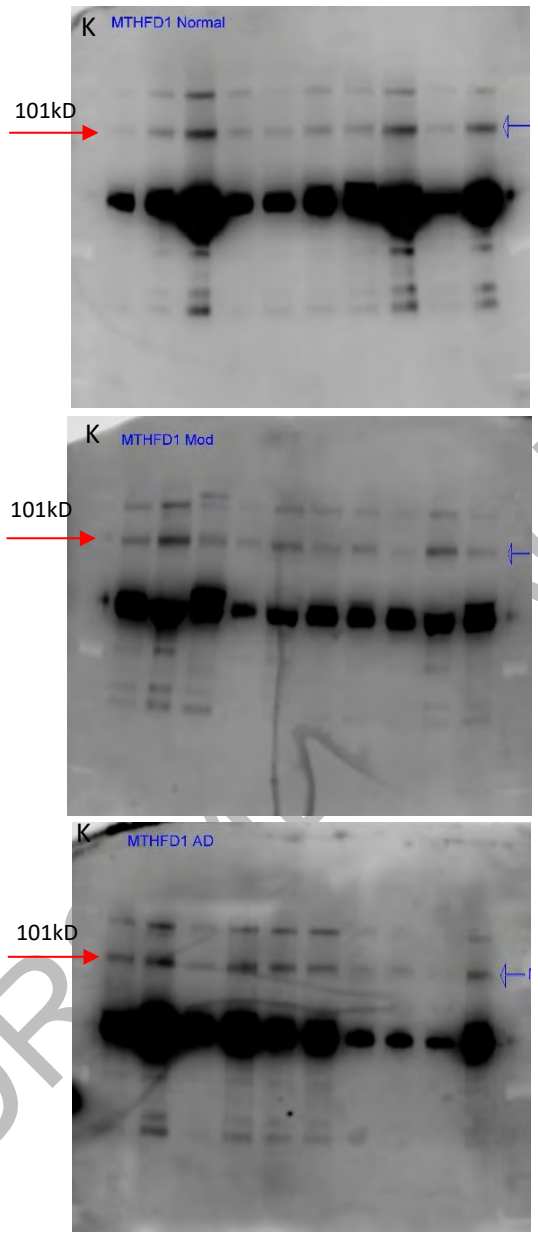


Figure 4.10. Full blots for MTHFD1, normal, moderate (mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is at 101kD for MTHFD1.

4.6.1.6. Full blots for Tau (data used in Figure 3.6)

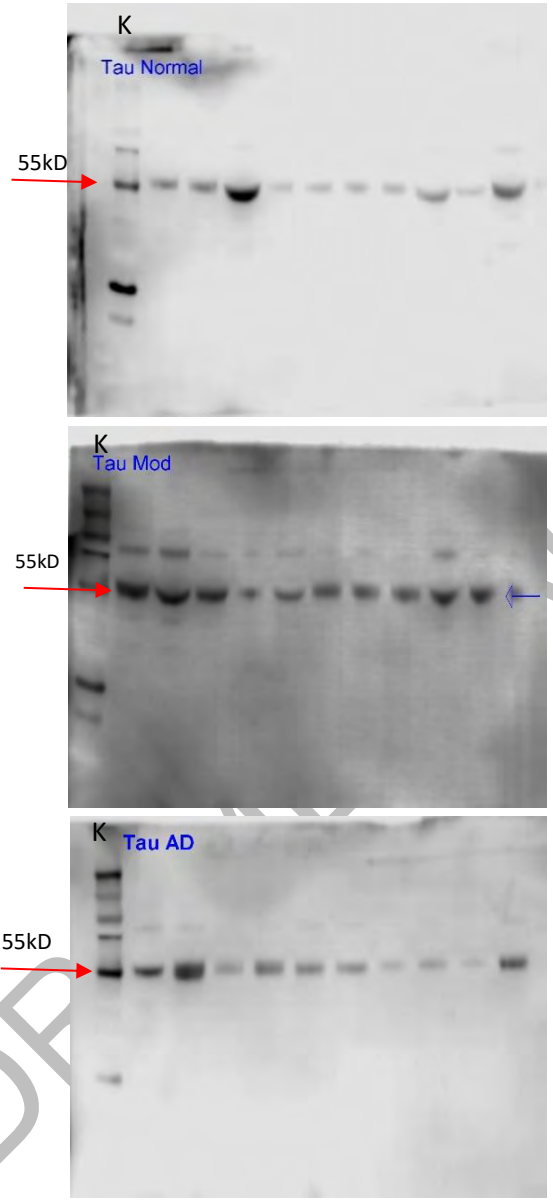


Figure 4.11. Full blots for Tau, normal, moderate (mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is at 55kD for Tau.

4.6.1.7. Full blots for PCFT (data used in Figure 3.7)

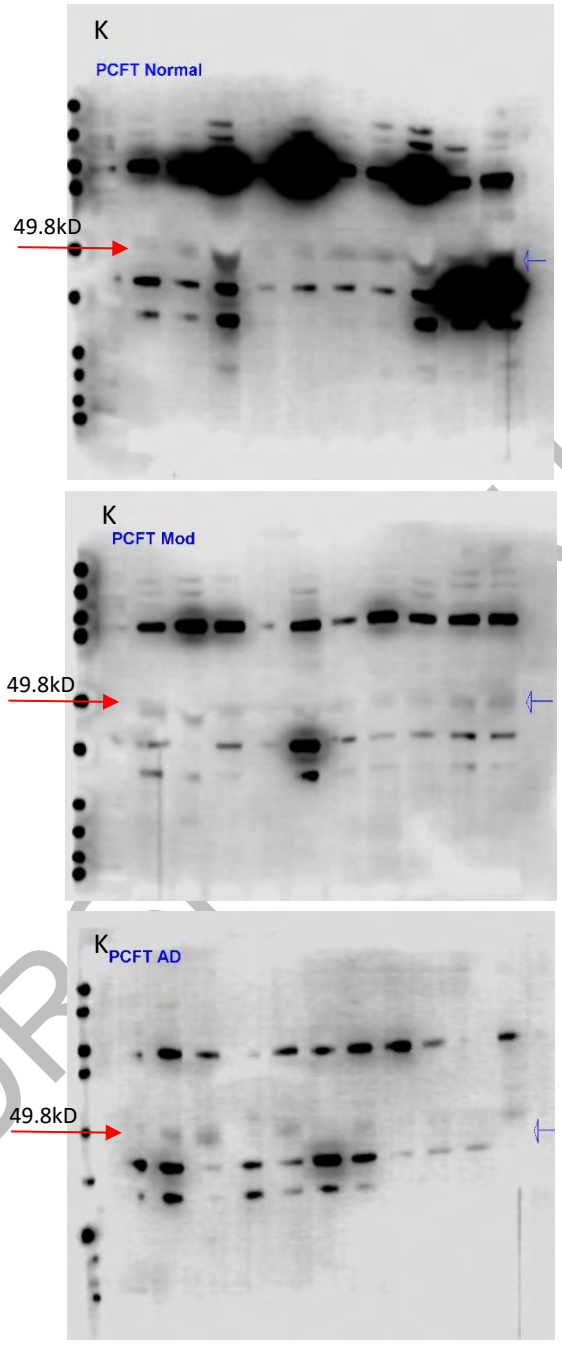


Figure 4.12. Full blots for PCFT, normal, moderate (mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is at 49.8kD for PCFT.

4.6.1.8. MTHFR Western blots (data used in 3.15.1)

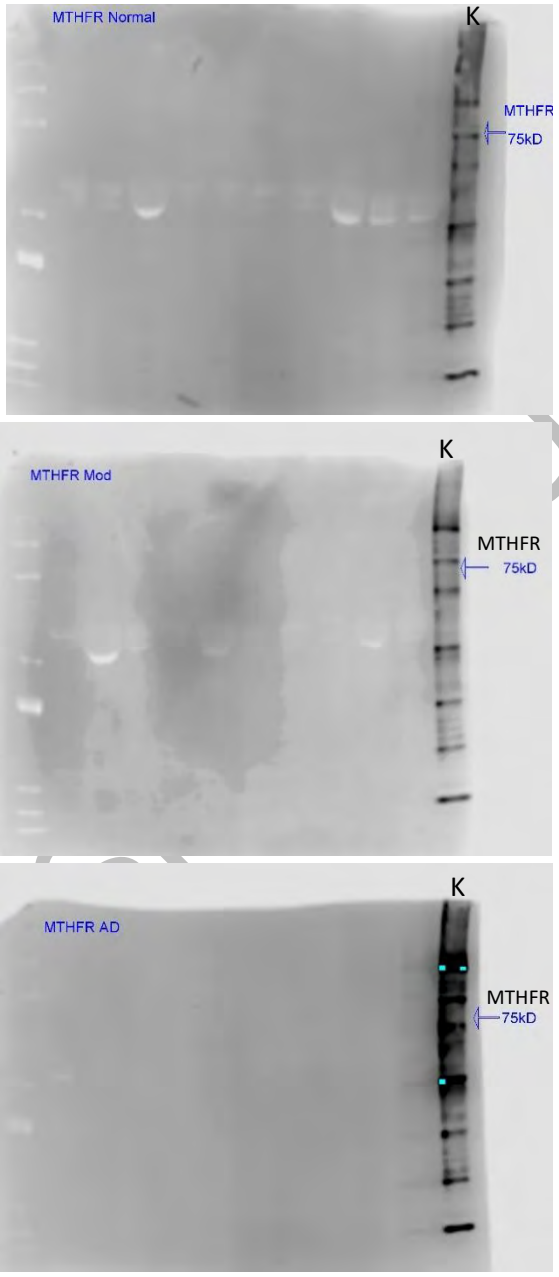


Figure 4.13. Full blots for MTHFR, normal, moderate (mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is at 75kD for MTHFR. MTHFR is not detectable in CSF.

4.6.1.9. RFC Western blots (data used in 3.15.2)

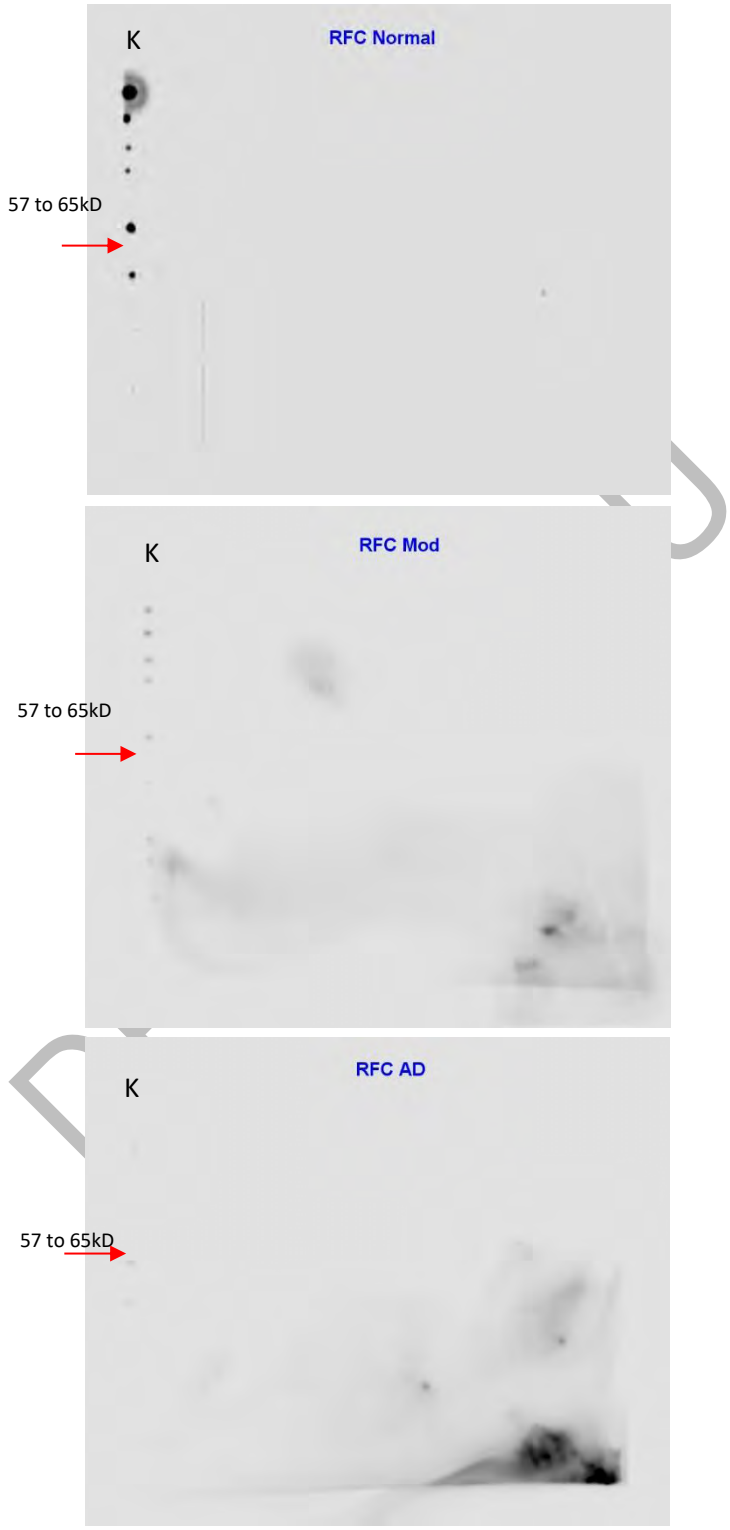


Figure 4.14. Full blots for RFC, normal, moderate, (Mod) and severe (AD). K is Kaleidoscope molecular weight marker. Arrow is midway between 57 to 65kD for RFC. RFC is not detectable in CSF.

4.6.2. Western blots for tissue lysates

4.6.2.1 Full blots for FDH (data used in Figure 4.1)

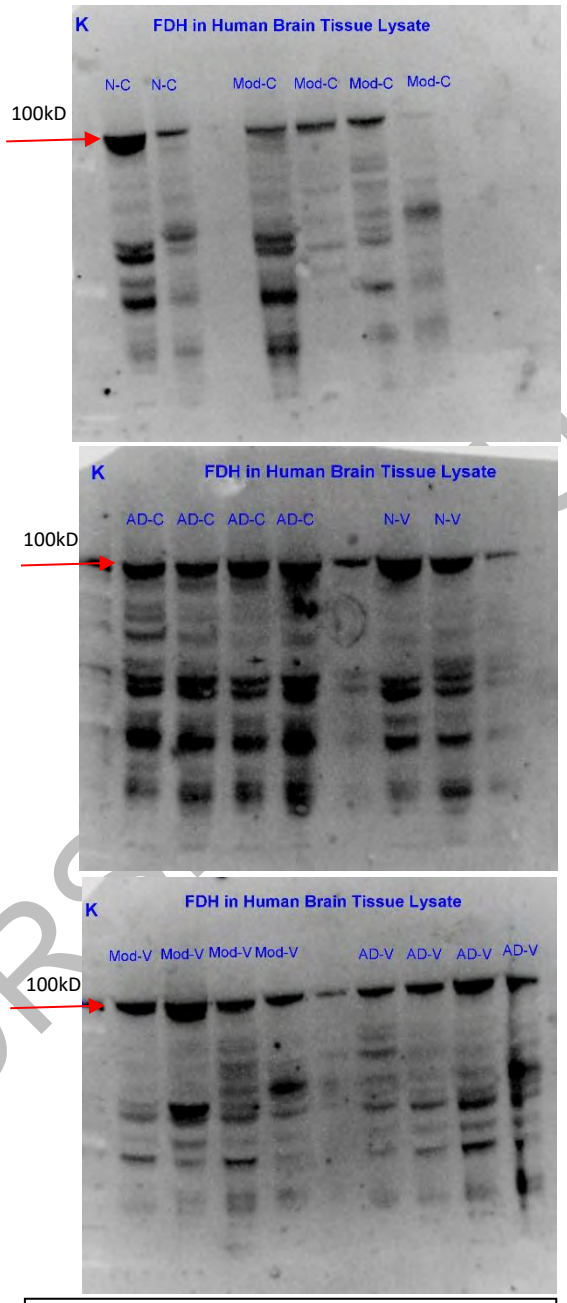


Figure 4.15. Western blots for FDH in brain lysate. Normal cortex (N-C) with normal ventricle (N-V) moderate cortex (M-C) with moderate ventricle (M-V), and AD cortex (AD-C) with AD ventricle (AD-V). K is Kaleidoscope molecular weight marker. Arrow is at 100kD for FDH

4.6.2.2. Full blots for FOLR1/FR alpha (data used in Figure 4.2)

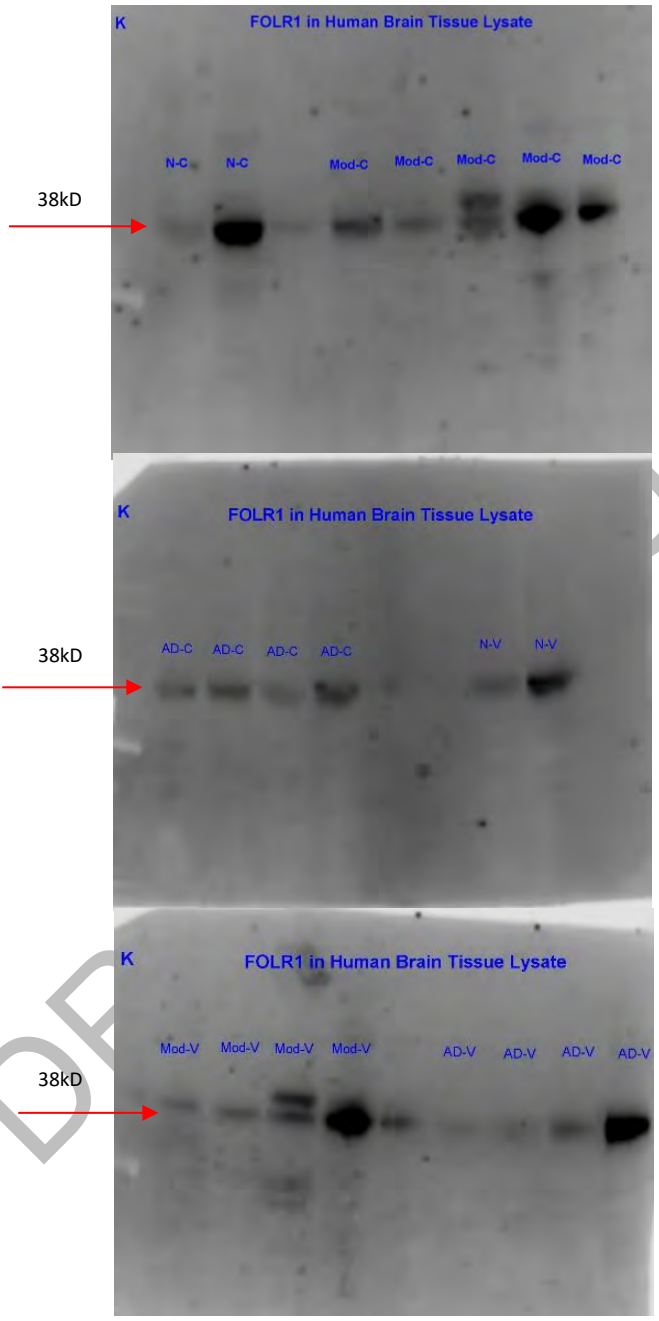


Figure 4.16. Western blots for FOLR1 in brain lysate. Normal cortex (N-C) with normal ventricle (N-V) moderate cortex (M-C) with moderate ventricle (M-V), and AD cortex (AD-C) with AD ventricle (AD-V). K is Kaleidoscope molecular weight marker. Arrow is at 38kD for FOLR1 (FRα).

4.6.2.3. Full dot blots for folates (data used in Figure 4.3)

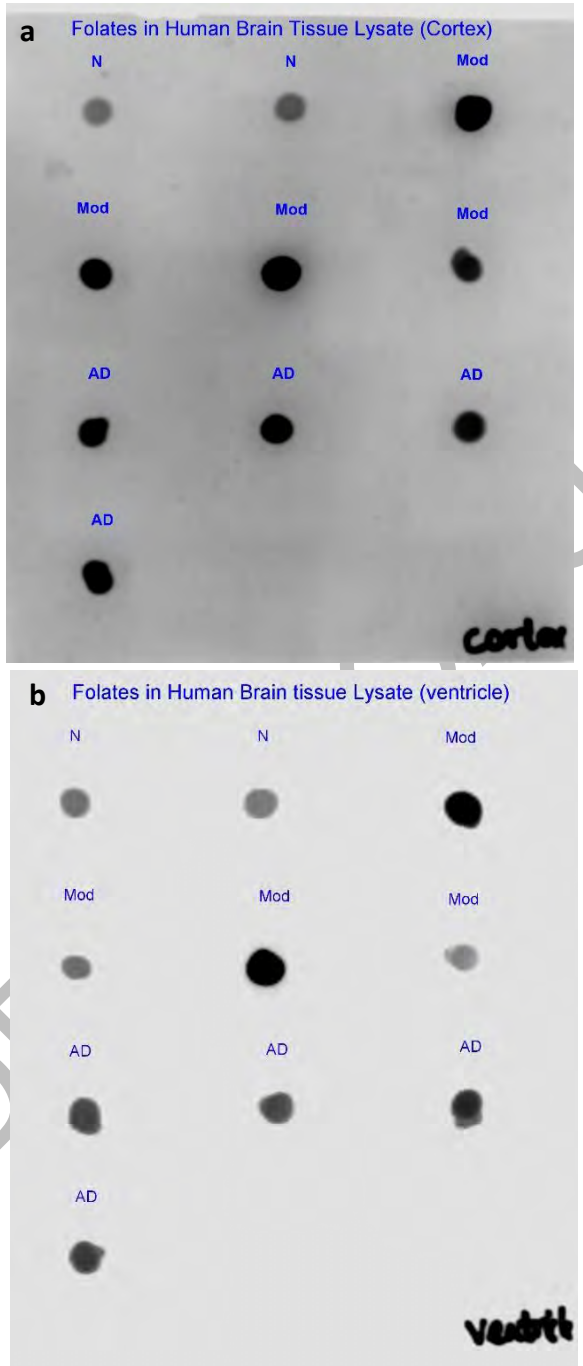


Figure 4.17. Dot blots for folates in brain lysate. a. Cortex and b. Ventricular zone. Normal (N), moderate (mod) and severe (AD). Molecular weight of folates is 400D.

Chapter 5

Results III

Folate in the cerebral cortex of normal ageing and AD

Chapter 5

Folate in the cerebral cortex of normal ageing and AD

5.1. Introduction

The studies reviewed in the introduction support the suggestion of a common cerebral folate metabolic change in dementia and AD. Following a recent report detailing changes in the methylation and polyamine/biogenic amine pathways that are intimately linked to folate metabolism (Mahajan et al., 2020) as well as the critical review of Liu et al (P. P. Liu et al., 2019), this study investigated the cerebral folate system in the post-mortem tissue of normal ageing brains and brains with AD to compare with the CSF analysis already presented in chapter 3.

5.2. Barriers within the central nervous system

Folate is derived from dietary sources and thus must be transported through the blood to all tissues in the body. Substances, including folate, are transported into the brain across the BBB, comprised of the capillary endothelial cells and their tight junctions, and the BCSFB, comprised of the secretary epithelial tissue of the choroid plexus that also generates the CSF and determines its composition.

From the CSF, folate and other substances must cross the CSF-brain barriers that are the ependymal lining of the ventricles and the pial meningeal layer on the outer surface of the brain. A number of folate transporters exist in the body. In the brain reduced folate carrier (RFC) and proton coupled folate transporter (PCFT) are thought to be involved in transporting folate across the BCSFB, while FOLR1 (FRα) transport folate around the body in the blood and is the main transporter for folate across the BCSFB. Little evidence exists for transport of folate across the blood-brain-barrier although some staining for RFC in both endothelium and some neuronal membranes has been reported (e.g., Allen Protein Atlas project: https://www.proteinatlas.org/ENSG00000173638-SLC19A1/tissue/cerebral+cortex#img). In this study, we found no staining for RFC or PCFT in the normal ageing or AD brain but found excellent staining for FRα and other elements of folate metabolism described below.

5.3. Cerebral cortical expression of folate metabolism

Gliogenesis progresses in postnatal development to give rise to several types and forms of astroglia appearing throughout the cortex (Melzer et al., 2021). One type of astrocyte that does not contain glial fibrillary acid protein (GFAP), the most recognised astrocyte marker, contains FDH, a key enzyme in folate metabolism, including nucleotide synthesis, methylation and neurotransmitter synthesis. In the normal adult and aged brain these astrocytes form a network from the top of the cortex, where they connect to the pial meningeal layer (Figure 5.1. and following figures) right through to the ependymal lining of the ventricles where they associate with the ependymal connected to ventricular CSF.

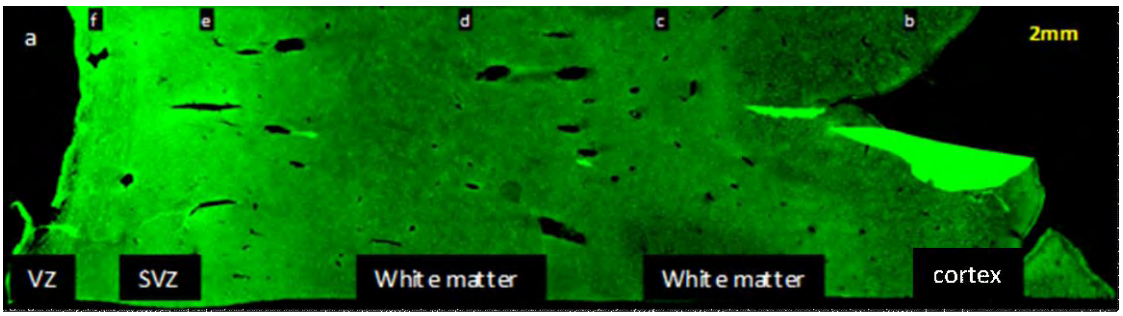


Figure 5.1. Representative micrograph of IHC staining of a 30µm thick section of normal ageing cerebral cortex for FDH (ALDH1L1, 10-formyl tetrahydrofolate dehydrogenase) in a section through normal ageing human cerebral cortex. The entire section is shown at low power to orientate the different regions seen in higher power. The pial surface is at the right with the cortex labelled b, and the ventricular ependymal is at the left with the ventricular zone labelled f and the ventricle labelled a. The different regions labelled b-f are shown in greater detail in the next figures.

These FDH-positive astrocytes have different morphologies in different cortical regions, with classical stellate morphology in the cortex (Figure 5.2 showing area b from Figure 5.1), small and thin with short processes in the white matter (Figure 5.3.), large, more rounded with short processes in the subventricular zone (Figure 5.4.) and with longer processes in the ventricular zone (Figure 5.4, 5.5.) looking more like the classical stellate morphology (Figure 5.5).

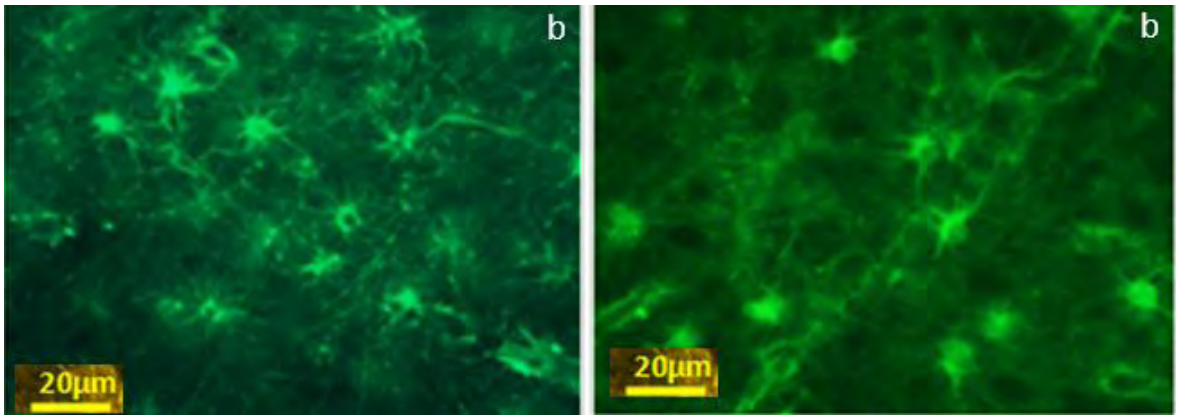


Figure 5.2. Representative micrograph of Stellate morphology of FDH positive astrocytes in the cerebral cortical plate. Letters of each panel refer to the different areas labelled in Figure 5.1. This is true for all subsequent figures in this section.

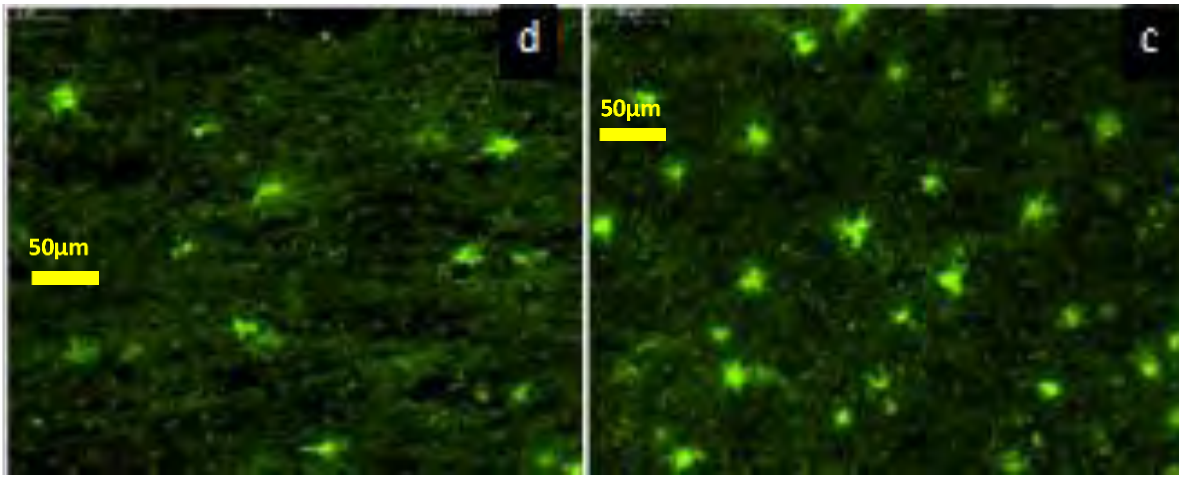


Figure 5.3. Representative micrograph shows the details of morphologies of FDH positive astrocytes in the white matter (c) and fibre tracts (d) of the cerebral cortex.

Although these astrocytes are clearly identifiable using FDH as a marker they can also share other markers including GFAP (Figures 5.5., 5.6.) and S100 (Figure 5.5.) which have variable intensities of co-localisation as well as not sharing total cell localisation, similar to the recent reporting of astrocyte lineages using a number of markers (Melzer et al., 2021). Astrocyte marker differential positivity depends on location across the cortical and ventricular surface (Figure 5.7.) indicating changes between markers potentially depending on localised functional requirements. For example, Figure 5.6. shows a GFAP+FDH positive astrocyte with GFAP+FDH positive end feet over the surface of a capillary. Next to it are FDH positive astrocytes with FDH positive end feet

on the surface of a neighbouring capillary. Figure 5.7.k and l show adjacent areas of cortex with predominantly FDH or GFAP positive astrocytes associated with the pial surface. Figure 5.7.n shows a region of the ventricular zone and ependyma that has adjacent areas exclusively FDH (green arrow) or exclusively GFAP positive (red arrow) glial processes. Figure 5.5.g. and 5.6.m. show areas of ependyma and cortex, respectively, which have very few GAFP or FDH positive astrocytes. Interestingly, in these fields there shows to be a zone specificity for each type of astrocyte, but this is rare and not consistent across the tissue sections.

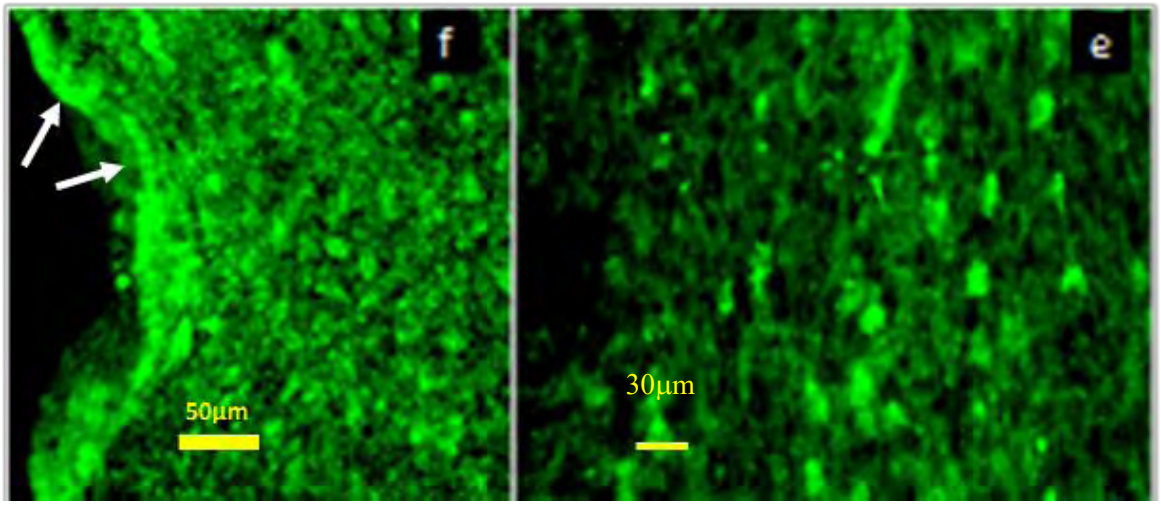


Figure 5.4. Details of morphologies of FDH positive astrocytes in the subventricular zone (e) and ventricular zone (f) of the cerebral cortex. The ependymal layer shows positive stain for FDH in this representative micrograph (arrows in f).

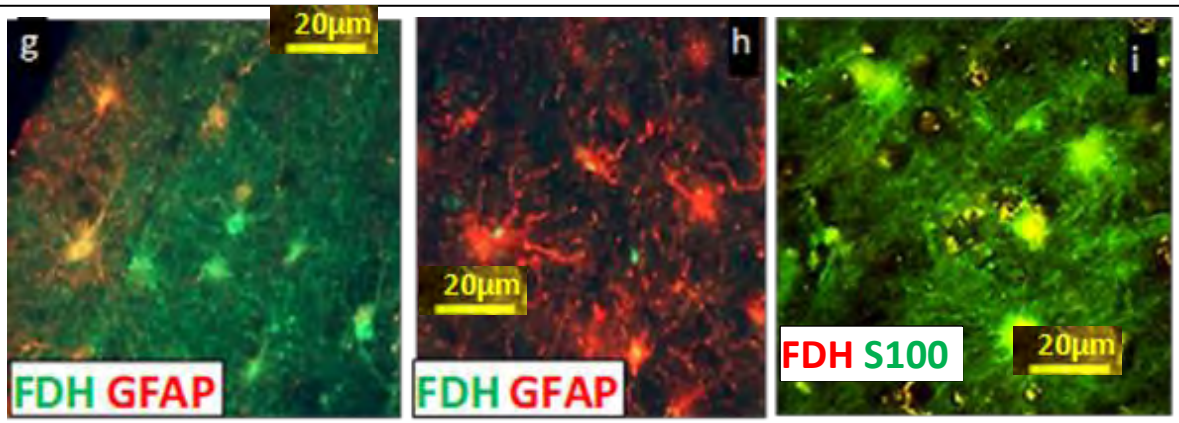


Figure 5.5. Representative micrograph shows dual staining for GFAP (g,h) and S100 (i) with FDH to demonstrate lack of co-localisation of GFAP with FDH but major co-localisation with S100 (red+green=yellow).

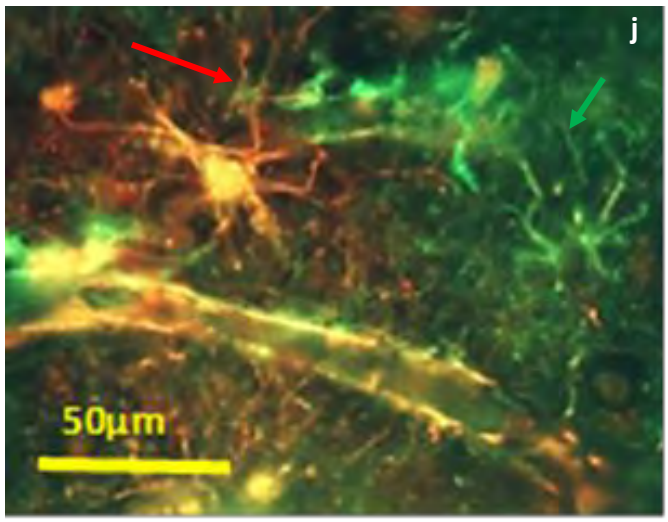
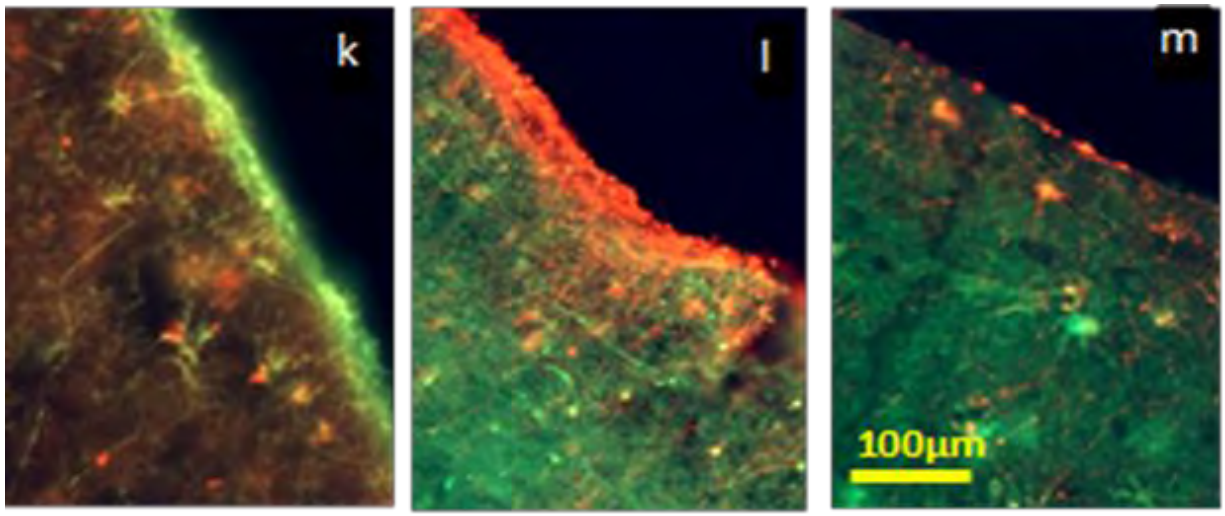


Figure 5.6. Representative micrograph shows dual staining for GFAP and FDH to demonstrate lack of co-localisation of GFAP with FDH. In this micrograph, a GAFP positive astrocytes are seen with extensive processes and end feet on a capillary (red arrow) while FDH positive astrocytes that are negative for GFAP also have extensive processes and end feet on capillaries (green arrow). This makes it possible that folate is transported across the endothelial blood-brain-barrier but this is not supported by other IHC staining so that it shows multi-functions for these astrocytes.



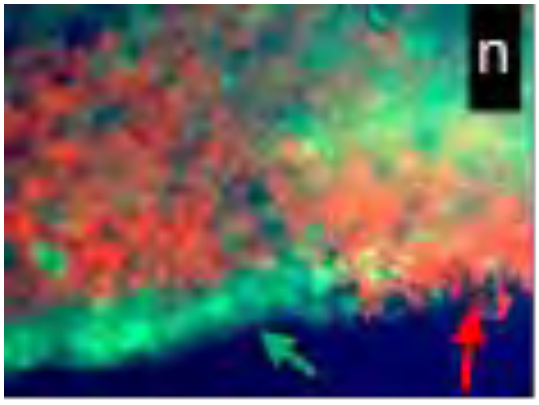


Figure 5.7. Representative micrograph shows dual staining for GFAP and FDH to show areas of the pial and marginal zones that are largely FDH positive (k) or GFAP positive (l). These areas contain both markers and distinct GFAP or FDH astrocytes are seen, sometimes in a sparse pattern as in (m). This is also seen in the ventricular and ependymal zones (n).

Figure 5.8. IHC staining of normal ageing cerebral cortex

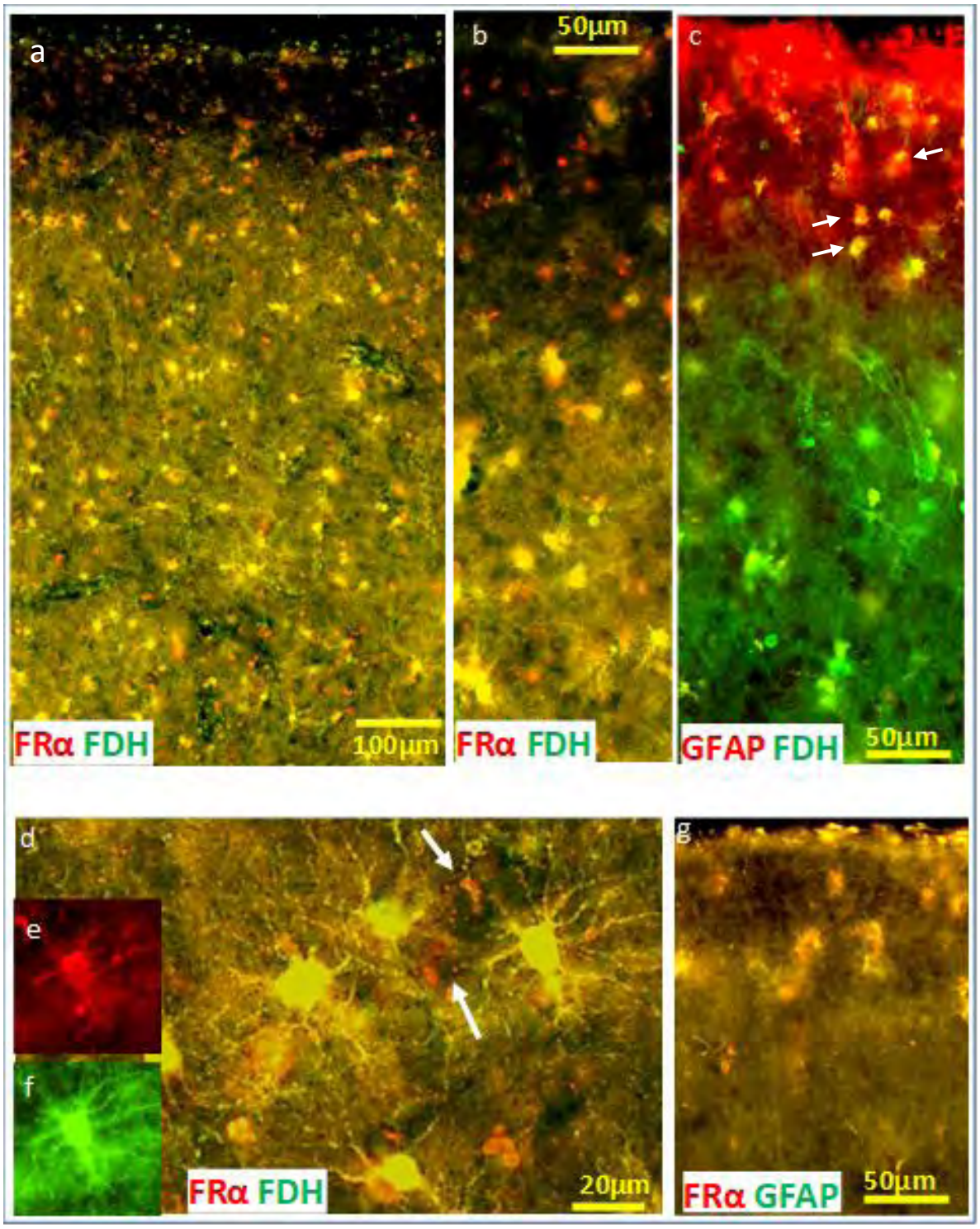


Figure 5.8. Representative micrograph shows IHC staining of normal ageing cerebral cortex for FRα, GFAP and FDH to show the folate delivery pathway in normal ageing. a,b and d show the co-localisation (yellow) of FRα with FDH in the FDH-positive astrocytes. FRα is also seen as speckled red only staining in what appear to be neuronal cell bodies (arrows in d) as well as showing an association with some GFAP positive cells near the pial surface (g). FDH and GFAP are largely separated with some colocalisation in cells within the marginal zone (white arrows in c).

IHC co-staining for FDH (green) and FRα (red) in the normal ageing and AD human brain shows a striking and significant change in distribution of these two folate related proteins (Figure 5.8). FDH is present in the specific population of astrocytes described in Figures 5.1 through 5.7. In the normal ageing brain these astrocytes form the main folate pathway being co-localised with FRα that must have come from the CSF (Alam et al., 2019) along with folate (Figure 5.8 a,b,d-f). These astrocytes are a separate population of astrocytes from the GFAP positive population although there is some possible co-localisation of these markers in specific astrocytes, particularly near the top of the cortex (Figure 5.8.c) but there is much more co-localization of FRα with GFAP (Figure 5.8.g). FRα is also localised in neurons and appears as speckles reflecting its transport through endocytic vesicles (white arrows, Figure 5.8.d) and the FDH positive astrocytes do appear to have close associations with these.

Figure 5.9. IHC staining of severe AD cortex

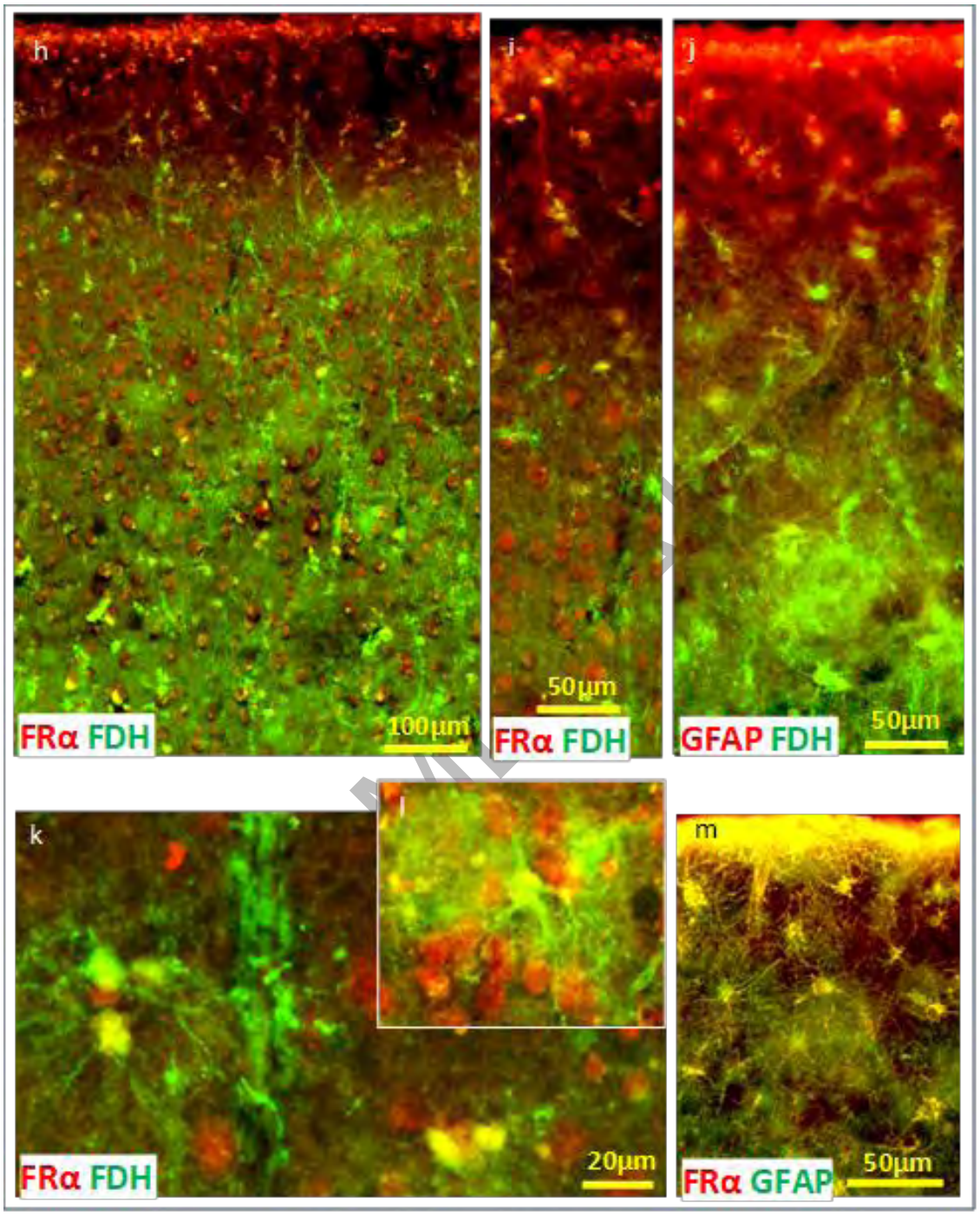


Figure 5.9. Representative micrograph shows IHC staining of sever AD cortex for FRα, GFAP and FDH to show the folate delivery pathway in AD. h,I,k,l show almost complete separation of FRα and FDH staining with FRα positive staining in the pial layer and throughout the cortex completely filling what appear to be neuronal cell bodies. k,l show details of the FDH-positive, FRα-negative astrocytes associated with the FRα-positive neuronal cell bodies. m shows that FRα is now co-localised in GFAP-positive astrocytes while j shows that GFAP and FDH are not co-localised.

In the AD cortex FRα and FDH are almost completely separated except for a few FDH astrocytes that have minimal positive staining for FRα (Figure 5.9.). The FDH astrocytes have more extensive and denser processes than in normal brains (Figure 5.9.j,k,l). FRα is more strongly colocalised in GFAP astrocytes extending to the pial surface (Figure 5.9.m) while FDH remains separate from GFAP (Figure 5.9.j). In addition, FRα is clearly concentrated into neuronal cell bodies throughout the cortex (Figure 5.9.i,k,l), particularly evident when you compare the little neuronal FRα stained in Figure 5.8.b with the complete fill in Figure 5.9.i and 5.9.l. This is matched by localisation of folate in the same cells (Figure 5.10.g) but to the nuclei rather than filling the whole cell (figure 5.10.a. and g.).

In the normal brain folate is in FDH positive astrocytes (Figure 5.10.f,g) and not co-localised with FRα in most of these (Figure 5.10.a-e). By contrast, in the AD brain, folate is not evident in any but a few FDH positive astrocytes even though these seem associated with the folate positive cells (Figure 5.11.k-m). It shows a switch in folate supply from FDH-FRα to the GFAP-FRα pathway (Figure 5.10. and 5.11), which may be a consequence of reduced FDH in the CSF if FDH is required for FRα entry into the brain (Cains et al., 2009; Jimenez et al., 2019; Naz et al., 2016). Moreover, in the AD brain, folate is concentrated in the nuclei (Figure 5.11.) together with FRα compared to the folate presence throughout the cell and processes of FDH positive astrocytes in the normal brain (Figure 5.10).

From IHC staining for 5-methyl cytosine and 5-hydroxymethyl cytosine, the change in folate pathway in AD brain is linked to an apparent change in metabolism towards hypermethylation (Figures 5.12. and 5.13.). In the normal cortex there is co-localization of 5-methyl cytosine and 5-hydroxymethylcytosine, markers of methylation and demethylation, respectively (Figure 5.12.d,e). In the AD cortex there is very little co-localization and essentially all cells are labelled with 5-methyl cytosine alone (Figure 5.13.j,k). This is particularly evident in cells near the pial surface (Figure 5.12.e and 5.13.k) but there are again regional differences as can be seen in Figure 5.12.a.

Figure 5.10. IHC staining in normal ageing cortex

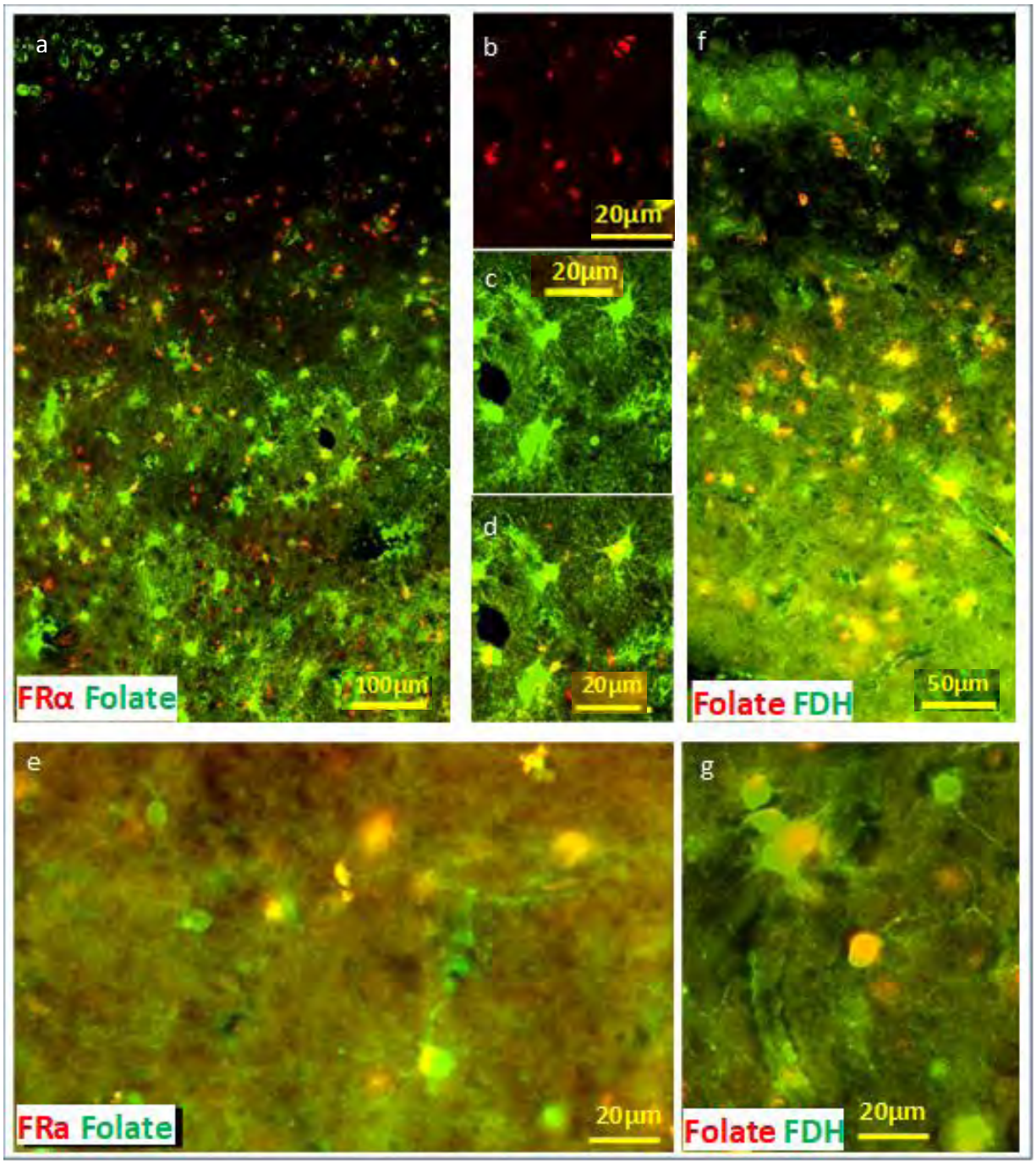


Figure 5.10. Representative micrograph shows IHC staining of normal ageing cerebral cortex for folate, FRalpha and FDH. In normal ageing folate is present in FDH-positive astrocytes (f,g) along with FRα in some (a,b-d,e).

Figure 5.11. IHC staining in AD cortex

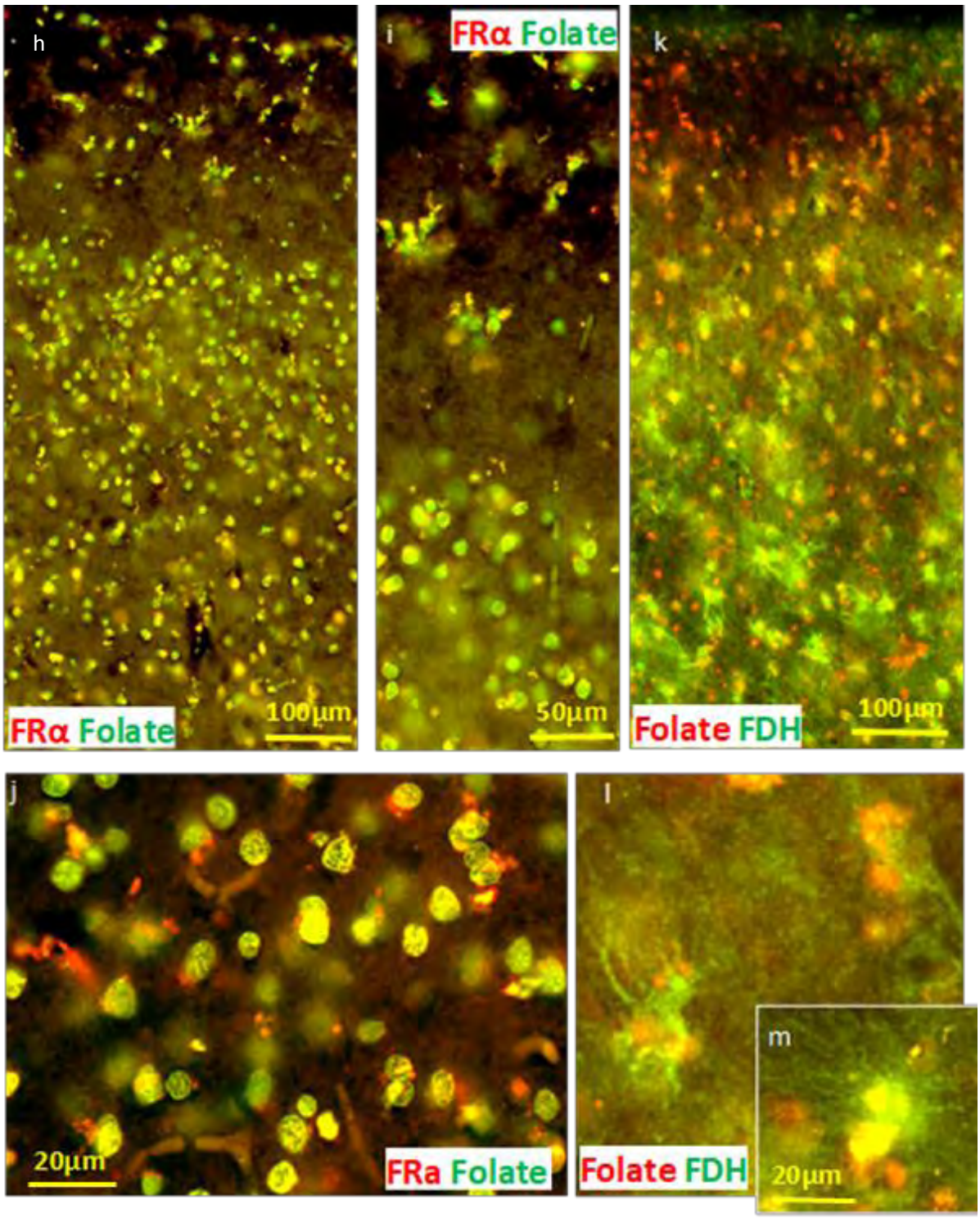


Figure 5.11. Representative micrograph shows IHC staining of AD cerebral cortex for folate, FRalpha and FDH in AD brain. folate is concentrated in nuclei throughout the cortical plate and we assume these are nuclei of cortical neurones (h,I,j). There is some co-localisation of folate in FDH positive astrocytes (k,m) but mostly these are separated (k,l). FRα is seen in FDH-negative astrocyte processes that are associated with folate positive cell nuclei (j) with FDH positive astrocytes wrapped around these (m). This confirms the switch in FRalpha-folate supply route away from FDH-positive astrocytes to GFAP as shown in Figures 5.8. and 5.9.

Figure 5.12. IHC staining of normal ageing cortex

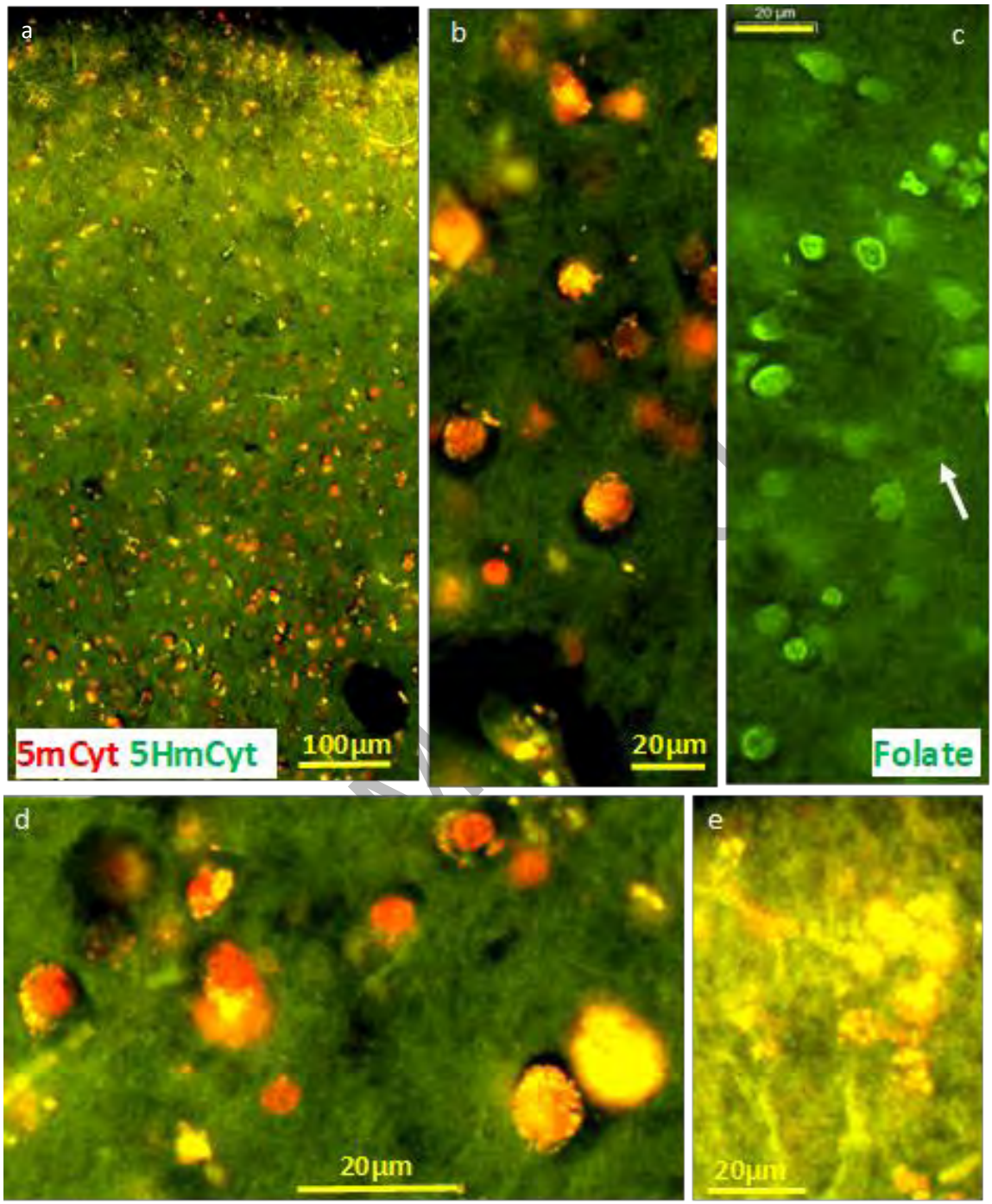


Figure 5.12. Representative micrograph shows IHC staining of normal ageing cerebral cortex for 5-methyl cytosine (marker of methylation-red) and 5-hydroxy methyl cytosine (marker of demethylation-green) which show a balance of methylation and demethylation (seen as yellow staining).

Figure 5.13. IHC staining of AD cortex

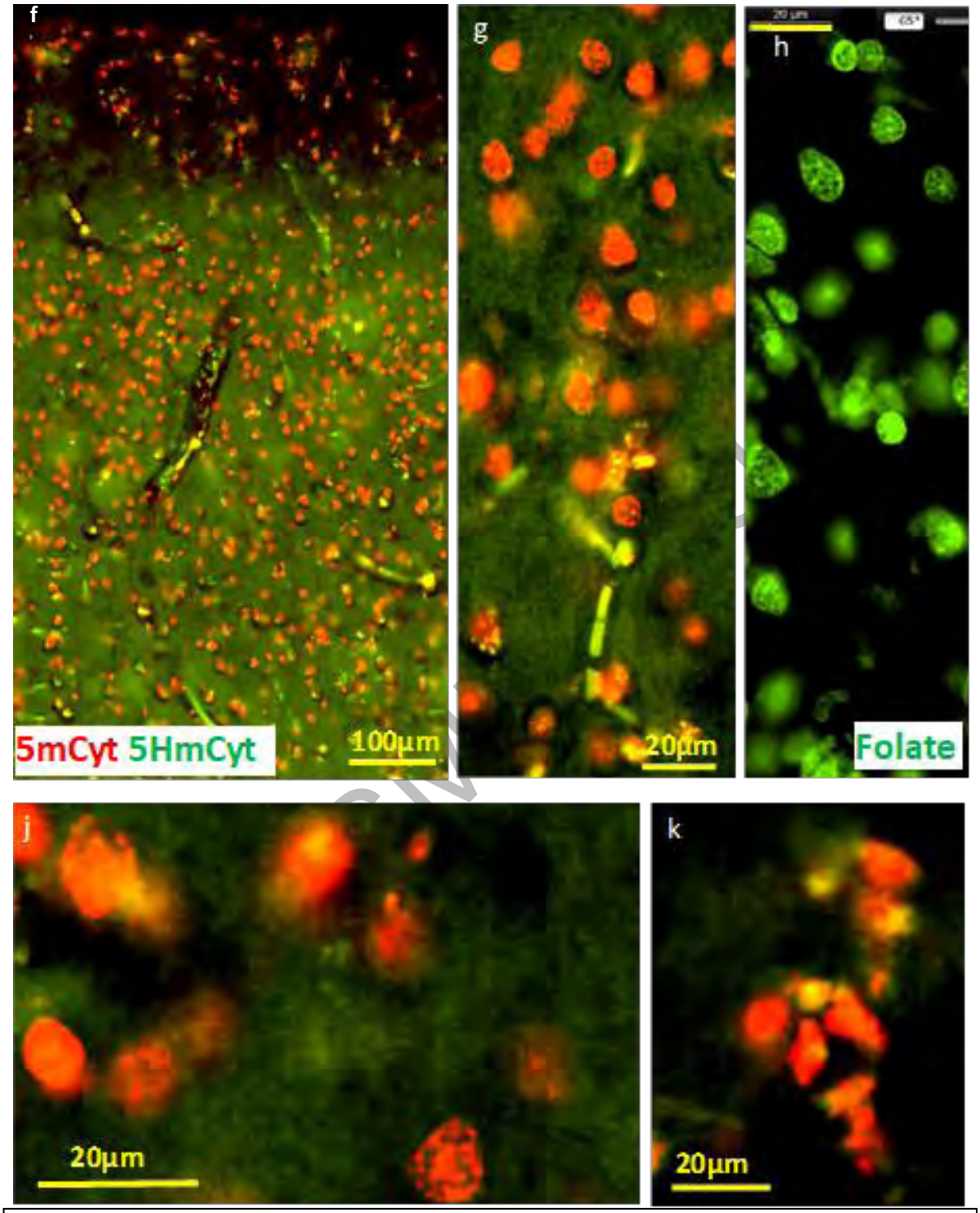


Figure 5.13. Representative micrograph shows IHC staining of AD cortex for 5-methyl cytosine (marker of methylation-red) and 5-hydroxy methyl cytosine (marker of demethylation-green) showing almost exclusive methylation. This is associated with the switch in folate supply with FRα switching from FDH to GFAP positive astrocytes and FRα concentrated in neuronal cells with folate in the nuclei.

5.4. Discussion

Folate supply to the brain is essential for normal development and function. Folate is transported into the brain across the choroid plexus into the CSF by FRα with little, if any, transport across the endothelial blood-brain barrier (Alam et al., 2019) although positive staining for reduced folate carrier in cortical endothelium and neurons has been reported (Human Protein Atlas: https://www.proteinatlas.org/). Interestingly, where FRα is missing completely due to a genetic defect for example, other transporters, including RFC and PCFT are upregulated to compensate (Alam et al., 2019), but this does not seem to happen where FRα is blocked sometime in life (Ramaekers and Blau, 2004; V. T. Ramaekers et al., 2013; Ramaekers et al., 2005; Ramaekers et al., 2014). Folate supply in the developing brain is from the CSF into FDH-positive radial glia (Cains et al., 2009). In the adult brain a network of FDH-positive astrocytes stretching from the pial surface of the cerebral cortex down to the ventricular zone shows a major pathway for folate uptake and delivery throughout the cortex. This is supported by the finding that FRα is colocalised with the FDH-positive astrocyte network in the normal ageing brain together with folate. It shows that both FRα and FDH, perhaps bound together by folate, carry folate into the brain through this pathway. The decrease in FDH in AD CSF would then have the effect of preventing FRα uptake, leading to the changes we observe in the AD brain. This shows more FDH in the AD brain seen in the density of FDH positive astrocyte processes (Figure 5.9. compared to 5.8). This may be a consequence of greater synthesis and expression of the protein by the astrocytes, in response to low CSF-FDH, and/or lack of secretion of FDH into the CSF, perhaps due to the same inhibition to FDH release observed in the hydrocephalic brain (Cains et al., 2009; Frye et al., 2003; Garcia-Cazorla et al., 2008; V. Ramaekers et al., 2013; Ramaekers and Blau, 2004; Ramaekers et al., 2014; Sadighi et al., 2012; Serrano et al., 2010; Willemsen et al., 2005). Moreover, in the AD brain a different pathway opens to FRα-folate through GFAP-positive astrocytes (Figure 5.9.m) which fuels a change in metabolism to hypermethylation, at least in the areas of the brain that were analysed in this study.

A significant finding in the adult brain is that the pial surface is essential for folate transfer from CSF into the cortex giving the subarachnoid CSF a vital function in delivering this critical metabolite (Miyan et al., 2020). In the marginal zone, FDH positive astrocytes are significantly associated with the main blood vessels entering the brain (Figure 5.7., 5.8., 5.9.). These vessels are surrounded by Virchow-Robin spaces which are filled with

subarachnoid CSF so that the astrocytes are connecting with the CSF in these perivascular compartments most recently associated with the glymphatic pathway (Naganawa and Taoka, 2020; Rasmussen et al., 2018; Reddy and van der Werf, 2020). Interestingly, these vessels are also the site for glymphatic fluid transfer into the brain parenchyma so that the FDH astrocytes may be involved in this process as well as other astrocyte functions. It is possible that the loss of FDH in the CSF and associated changes observed in the AD brain may also contribute to glymphatic impairment and build-up of toxins in the brain including tau and amyloid (Harrison et al., 2020; Iliff et al., 2014; Lee et al., 2020; Lou et al., 2018; Peng et al., 2016; Rasmussen et al., 2018; Reeves et al., 2020; Tice et al., 2020).

With ventricular enlargement and/or CSF accumulation outside the brain a hallmark feature of many conditions including dementia and AD, Autism and Schizophrenia, depression and bipolar (Apostolova et al., 2012; Dalaker et al., 2011; Elkis et al., 1995; Erel et al., 1991; Goukasian et al., 2019; Guptha et al., 2002; Hubbard and Anderson, 1981; Jackson et al., 2011; Jakobsen et al., 1989; Jerico et al., 2020; Kempton et al., 2010; Luxenberg et al., 1987; Mak et al., 2017; Martola et al., 2008; Movsas et al., 2013; Muller et al., 2013; Nasrallah et al., 1982; Nestor et al., 2008; Saijo et al., 2001; Sayo et al., 2012; Schenning et al., 2016; Scott et al., 1983; Vita et al., 2000; Wang et al., 1993; Ye et al., 2016; Zhao et al., 2018), a cerebral folate issue may also be present as we have found in early stages of hydrocephalus (Cains et al., 2009). Indeed, some of these conditions have been recorded to respond to folate treatments (Al-Baradie and Chaudhary, 2014; Ferreira et al., 2016; Frye et al., 2003; Frye et al., 2018; Hansen and Blau, 2005; Karin et al., 2017; Mercimek-Mahmutoglu and Stockler-Ipsiroglu, 2007; Moretti et al., 2005; Ramaekers et al., 2014). Even though Silverberg and colleagues suggest a decrease in CSF output in ageing, they also describe raised CSF pressure and accumulation of fluid in AD [67, 68] indicating that CSF drainage is more significant factor as it is also suggested by the reduced FDH found in this study. AD is not associated with raised intracranial pressure or hydrocephalus but does have a reported severity association with ventricular enlargement and this enlargement may be an early marker of the development of this condition [48, 49, 78].

The current study has identified a potentially significant change in folate supply and the metabolic consequence. We surmise that with a decrease in CSF FDH, there is a switch in folate supply from the FRα-FDH pathway to the FRα-GFAP pathway. The

consequence of this switch shows a change in metabolism to hypermethylation where FRα ends up in the neurons of the cortex and the folate is delivered to the nuclei where methylation occurs. We further suggest that this may be a strategy to shut down all but essential activity to safeguard surviving neurons from the toxic effects of AD. This may in turn explain some of the cognitive decline not attributable to loss of neurones alone.

5.5. Conclusion

AD is clearly associated with changes in CSF folate metabolism. Given the importance of CSF, and subarachnoid CSF to cerebral metabolism and function (Miyan et al., 2020), it shows that any shortfall in CSF drainage that, in chronic conditions manifests as enlargement of the ventricles or accumulation of fluid outside the brain, can result in a cerebral folate imbalance leading to severe consequences for brain health. This presents a novel and powerful new insight into changes that may underlie the aetiology of cerebral conditions including dementia and AD. Two pathways may be operating alone or together. Increasing amyloid in the CSF may be a cause of drainage loss through its toxic effects on cells in the drainage pathways. Alternatively, increasing amyloid may be a consequence of a drainage loss and lead to further damage as amyloid levels rise and exacerbate the situation. With sequestration of amyloid into non-toxic but space filling plaques, the levels in CSF drop and so the drainage loss itself would maintain the cerebral folate issue and hypermethylation in AD together with the neurodegeneration. It may also be the case that folate treatment may have benefits in preventing and/or treating AD if this is able to restore normal folate delivery by bypassing the missing FDH, as seen in other cerebral conditions (Al-Baradie and Chaudhary, 2014; Ferreira et al., 2016; Frye et al., 2003; Frye et al., 2018; Hansen and Blau, 2005; Karin et al., 2017; Mercimek-Mahmutoglu and Stockler-Ipsiroglu, 2007; Moretti et al., 2005; Ramaekers et al., 2014).

Chapter 6

Results IV

Folate metabolic enzymes in the cerebral cortex

Chapter 6

Folate metabolic enzymes in the cerebral cortex

6.1. Folate enzymes found in the cortex

Methionine synthase (MTR) with the full name of 5-methyltetrahydrofolate-homocysteine methyltransferase, is the rate limiting enzyme for folate metabolism, converting 5mTHF to THF and releasing the methyl group to cobalamin in which methionine synthase reductase (MTRr) is active (vitamin B12) that then transfers this to homocysteine, methylating it to methionine. THF has a formyl group added through the action of methylene tetrahydrofolate dehydrogenase-1 (MTHFD1) to form 10-formyl

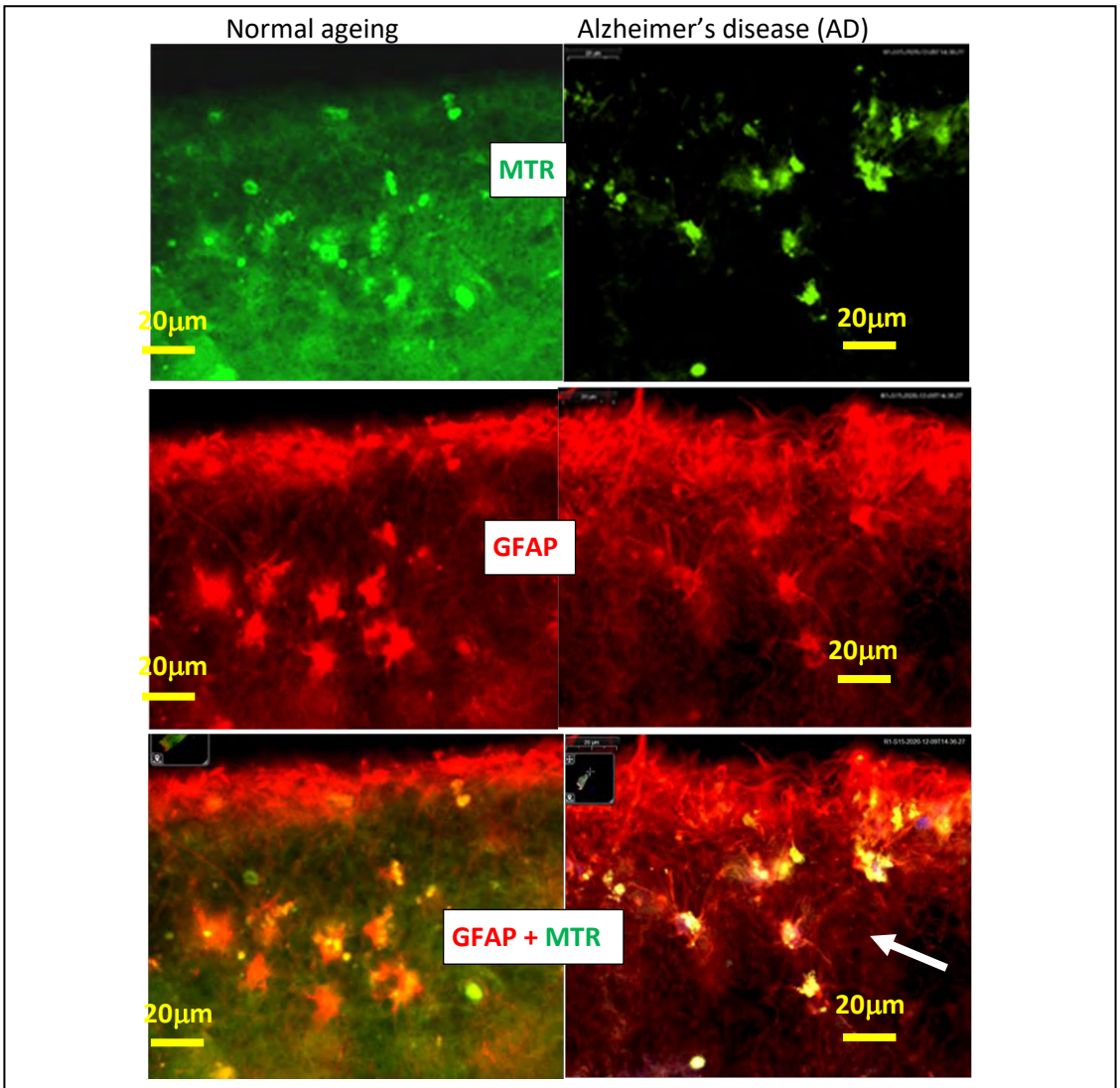


Figure 6.1. Representative micrograph shows that the IHC staining for MTR (green) and GFAP (red) in normal ageing brain cortex (left panels) and AD brain (right panels). There is some staining in GFAP-positive astrocytes and also in GFAP negative cells that are probably neurones in normal cortex. Comparing with AD brain, the green labelled MTR is almost completely missing from the neuropil of the cortex (top right) while MTR is concentrated within the cell bodies of GFAP positive astrocytes (bottom right).

THF and then 5,10 methyenylTHF, also through the action of MHTFD1, and then 5,10 methyleneTHF, again through the action of MTHFD1. THF can also be converted directly to 5,10 methyleneTHF by the action of serine hydroxymethyl transferase (SHMT). These two pathways are known as the long and short pathway from THF back to 5mTHF for which the final step is conversion of 5,10 methyleneTHF to 5mTHF by methyleneTHF reductase (MTHFR). 5,10 methyleneTHF can also be used in pyrimidine synthase through the action of thymidylate synthetase (TS) producing dihydrofolate (DHF). DHF is converted back to THF by dihydrofolate reductase (DHFR). In most somatic cells folate metabolism happens within the cell in the cytoplasm and mitochondria. In the chapter on CSF analysis, we found a general, and significant reduction in folate enzymes, a significant decrease in FDH and a non-significant increase in FRα as well as homocysteine. This suggested a serious deficit in cerebral folate metabolism and led to the investigation reported in this chapter on brain tissue status. In the previous chapter we reported folate transporter changes associated with AD and here we investigated the key enzymes for folate metabolism in the normal ageing and AD brain to establish if any changes had occurred in the diseased brain.

6.2. Methionine synthase (MTR)

MTR, the rate limiting enzyme for entry of dietary 5mTHF into the folate cycle, shows positive staining in some GFAP-positive astrocytes, in the network of neuropil and some non-astrocytic cells, presumed to be neurones (Figure 6.1.). This follows the pattern reported by the protein atlas (https://www.proteinatlas.org/ENSG00000116984-MTR/tissue/cerebral+cortex#img) for MTR although the staining found here is much more extensive than that described in the protein atlas. This may be due to the locations studied being different.

6.3. Methionine synthase reductase (MTRr)

MTRr shows a similar staining pattern to MTR but with much brighter staining (Figure 6.2.) suggesting a higher concentration in cells and neuropil of the cortex. In AD brains there is an almost complete lack of staining except in the cell bodies of a few GFAP positive astrocytes. The pattern of staining is similar to that reported in the protein atlas for normal cerebral cortex (https://www.proteinatlas.org/ENSG00000124275-MTRR/tissue/cerebral+cortex#img) although in the pial region no staining was detected in this study.

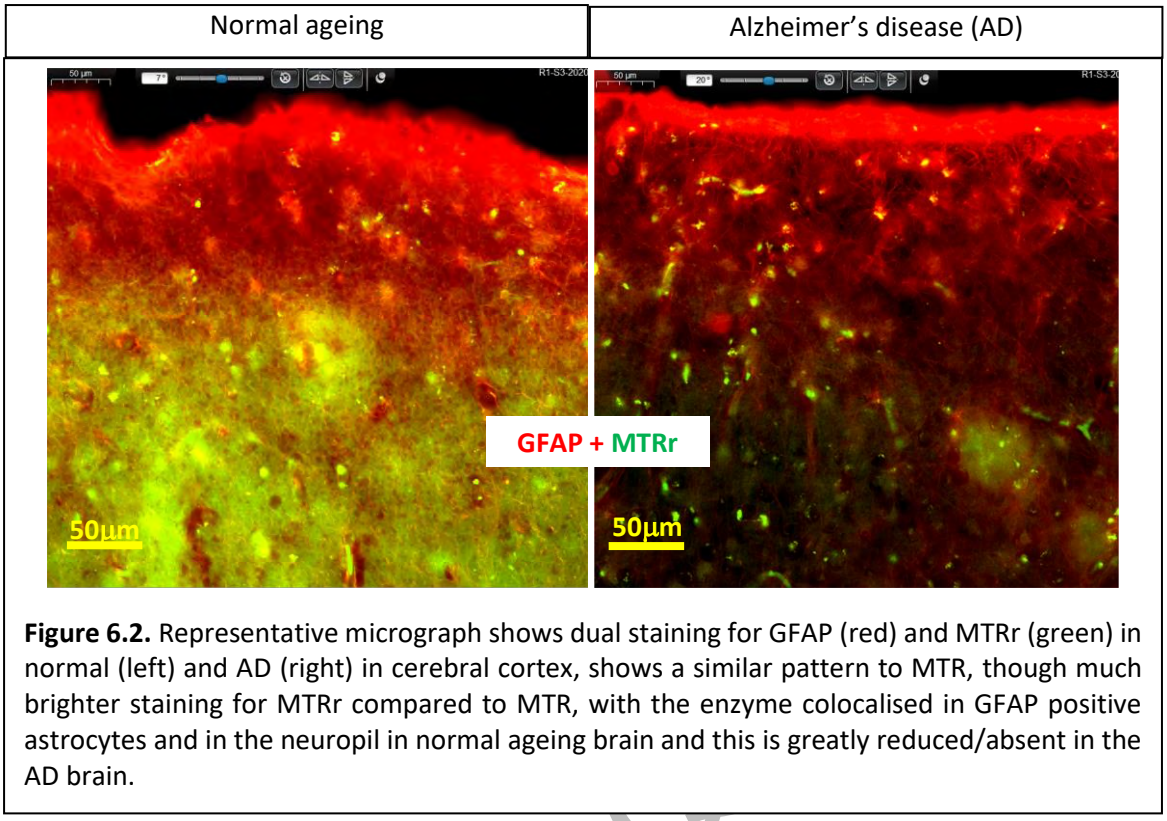


Figure 6.2. Representative micrograph shows dual staining for GFAP (red) and MTRr (green) in normal (left) and AD (right) in cerebral cortex, shows a similar pattern to MTR, though much brighter staining for MTRr compared to MTR, with the enzyme colocalised in GFAP positive astrocytes and in the neuropil in normal ageing brain and this is greatly reduced/absent in the AD brain.

6.4. Methylene tetrahydrofolate dehydrogenase 1 (MTHFD1)

MTHFD1 is a critical enzyme in folate metabolism catalysing 4 reactions including: i. methylenetetrahydrofolate dehydrogenase-1 (NADP+ Dependent), ii. methenyltetrahydrofolate cyclohydrolase, iii. formyltetrahydrofolate synthetase, and iv. C-1-tetrahydrofolate synthase, reflecting its importance to the folate metabolic cycle.

6.5. 10-formyl tetrahydrofolate (ALDH1L1, FDH)

10-formyl tetrahydrofolate (ALDH1L1, FDH) is also a key folate enzyme as well as being the key molecule in CSF that is downregulated in AD and hydrocephalus. Along with its critical role in recycling 10-formyl THF to THF, it is also involved, together with FRα, in folate transport from CSF into the brain as well as transport around the CSF pathways. Micrographs showing changes in this folate enzyme are shown in detail in Chapter 5.

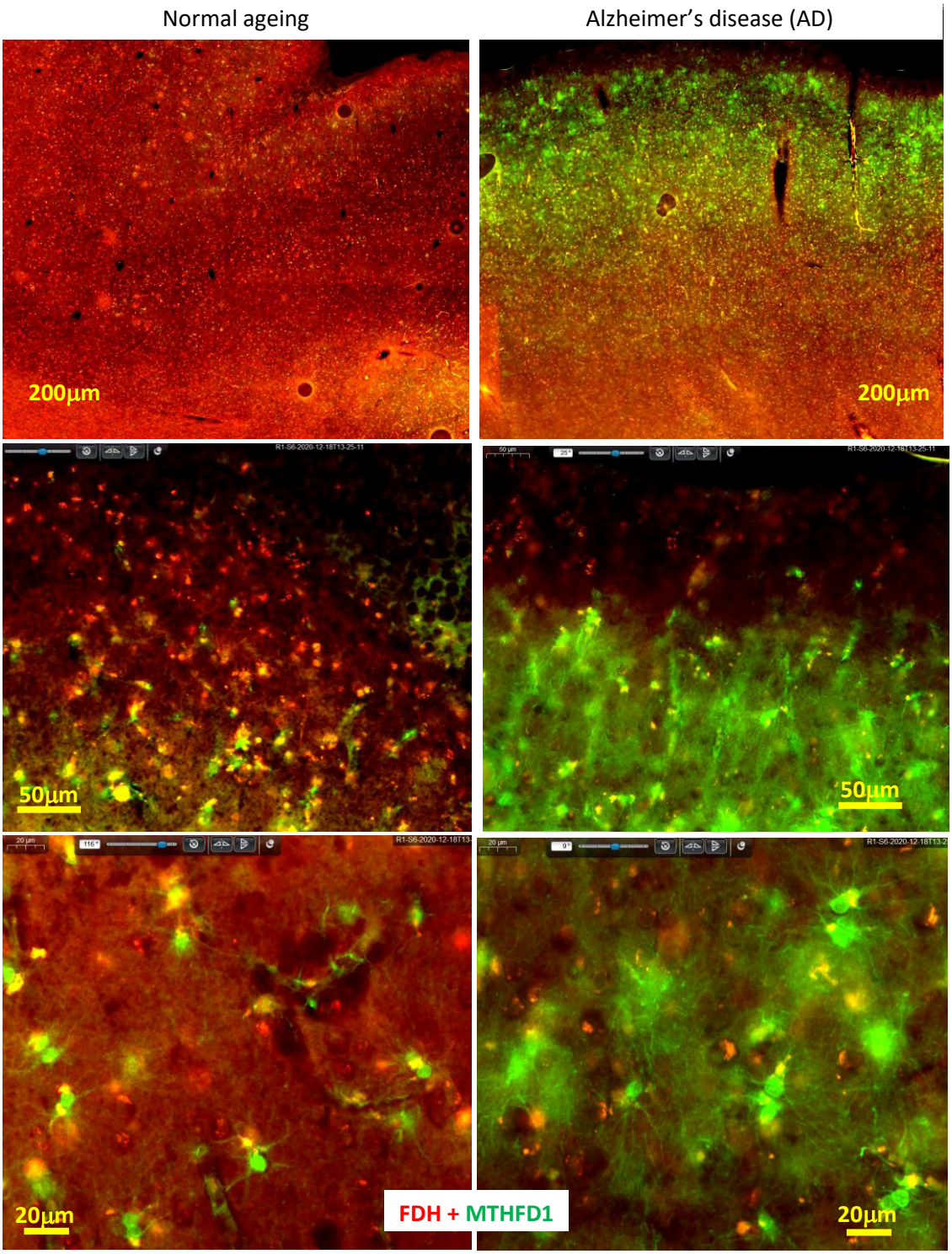


Figure 6.3. MTHFD1 (green) and FDH (red) staining of normal (left) and AD (right) cerebral cortex. There is a clear increase in MTHFD1 staining in the AD cortical section compared to normal ageing. In the low power micrographs at top, the staining for FDH in the subcortical layers and white matter are present but also reduced in AD brain indicating a general decrease in the presence of this important folate enzyme.The high power micrgraphs at the bottom show the increased MTHFD1 positive processes of astrocytes. FDH and MTHFD1 are separate in both normal and AD.

6.6. Dihydrofolate reductase (DHFR)

In the past Dihydrofolate reductase was not thought to have an important function as there was no direct metabolic connection through this enzyme. Subsequently it was found that 5,10 methyleneTHF rather than recycling to THF, was converted to dihydrofolate, in the process supplying elements for pyrimidine synthesis. DHFR then becomes very important in recycling DHF to THF and back into folate metabolism. In addition, DHFR forms the enzymatic route for entry of the synthetic folic acid into the folate metabolic cycle. DHFR is greatly reduced in the AD cortex indicating a potentially reduced recycling of 5,10, methyleneTHF through DHF.

6.7. Methylene tetrahydrofolate reductase (MTHFR)

MethyleneTHFreductase (MTHFR) is the only pathway for return of any folate metabolite to 5-methylTHF. MTHFR is also important as mutations in the gene coding for this enzyme can restrict folate metabolism by up to 70%. Mutations of this gene reportedly affect around 30% of the population ((Suormala et al., 2002). One potential consequence of an error in MTHFR is that folic acid supplements could result in accumulation of homocysteine with associated neurotoxic effects. Such individuals would need to take supplements of 5mTHF to compensate for failures in MTHFR. MTHFR is clearly reduced in AD cortex (Figure 6.4) indicating a potentially serious effect on folate metabolism and cycling.

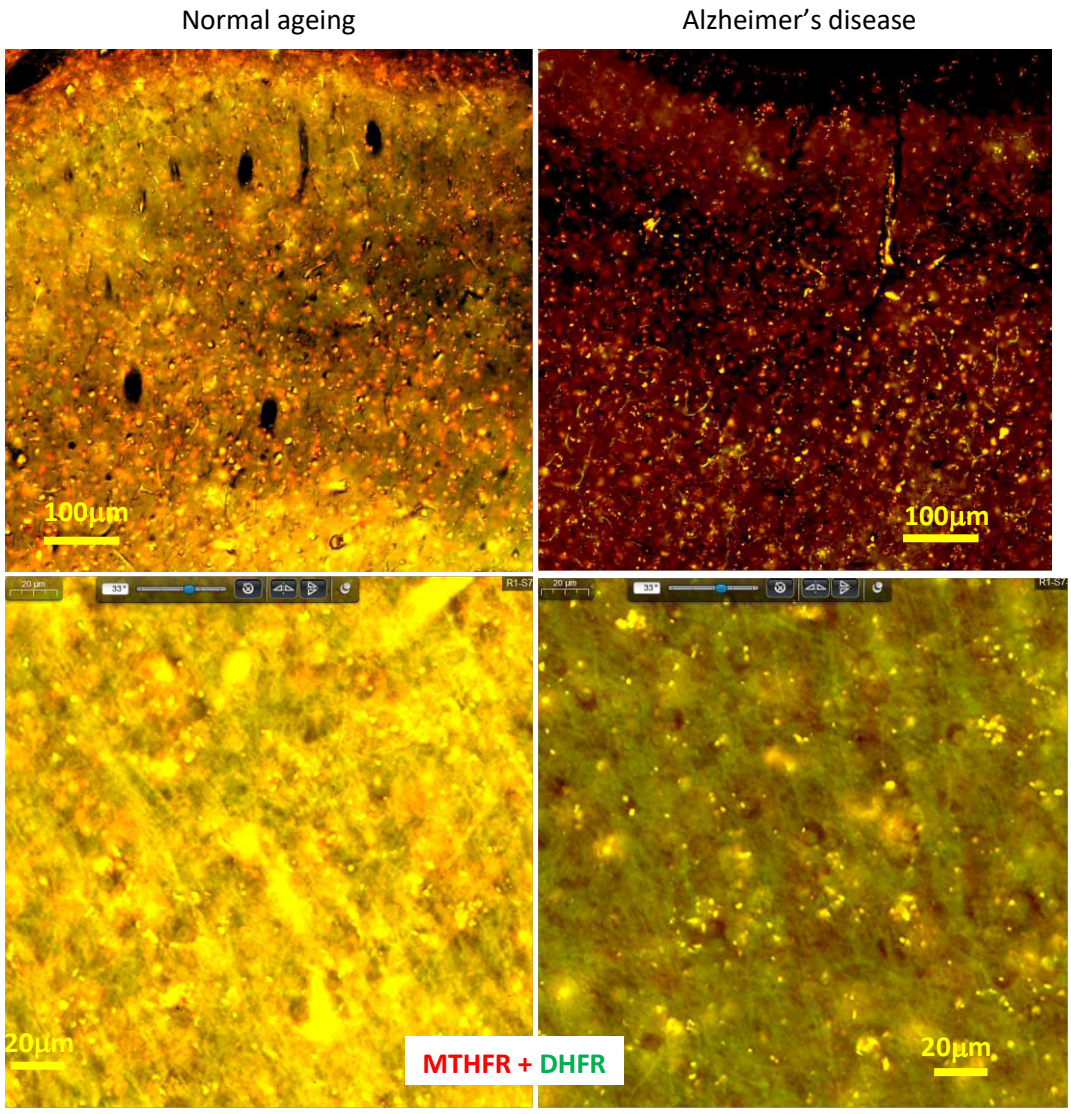


Figure 6.4. MTHFR (red) and DHFR (green) in normal (left) and AD (right) cerebral cortex. The representative micrograph of the AD cortex had to be brightened to see the positive staining so that there is a very significant loss of expression of DHFR with a much-reduced positive stain for MTHFR. In the white matter (bottom micrograph) DHFR is present in both normal and A brain but at a much-reduced level in the latter (bottom right).

6.8. Homocysteine and folate

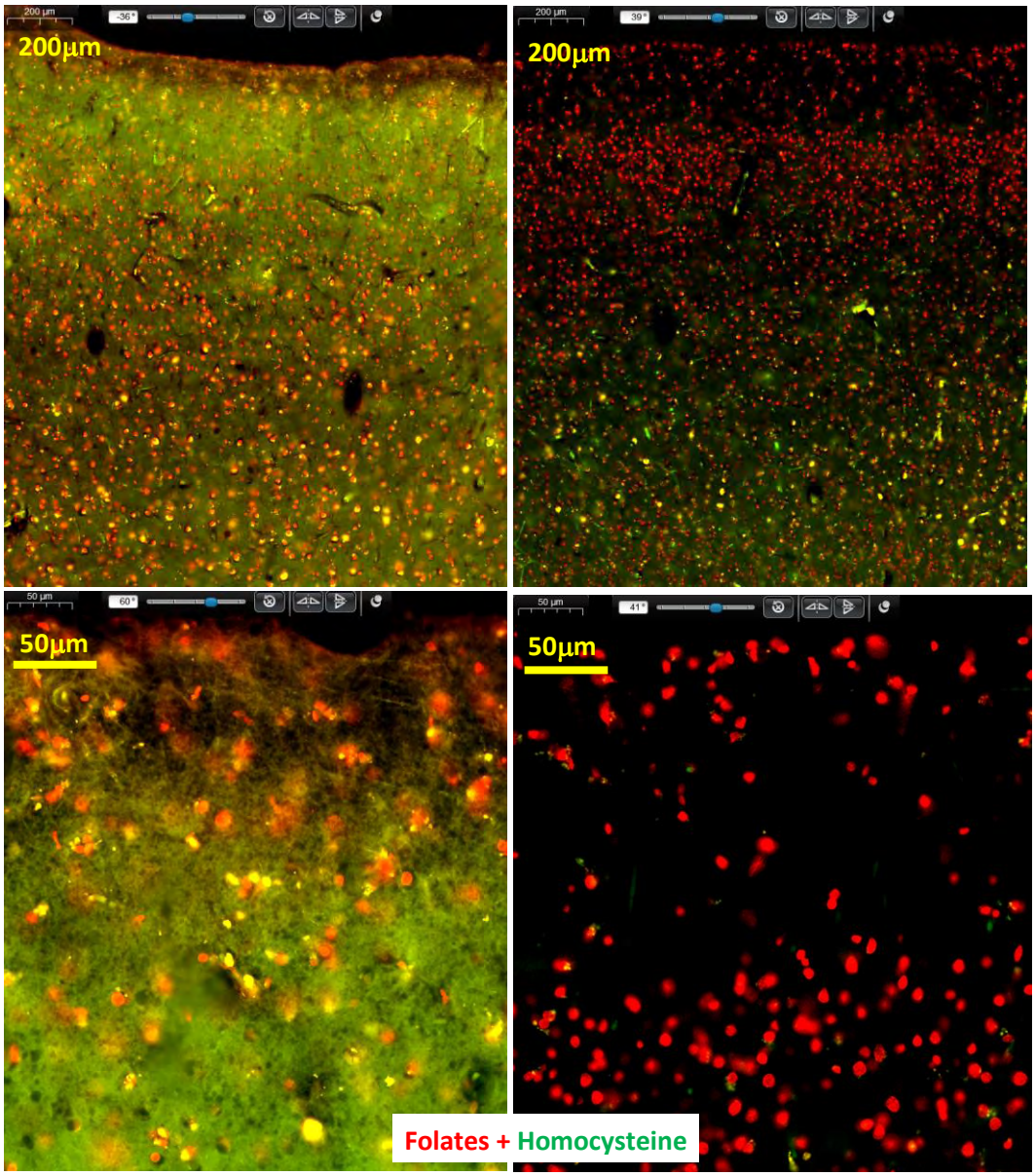


Figure 6.5. Folate (red) and Homocysteine (green) in normal (left) and AD (right) cerebral cortex. Homocysteine is absent from the cortical plate of AD brain (right panels) while it is abundant in the normal cortex (left panels). In a later chapter this is partially explained by shunting of homocysteine to glutathione, a detoxification pathway that may be activated in AD.

Homocysteine is notable by its absence from the AD cortex compared to the intensity of staining seen in normal brains (Figure 6.5). This was discussed in chapter 5 and a hypothesis proposed that homocysteine was being shunted to glutathione to prevent neurotoxic effects. The staining confirms/supports the hypothesis. Figure 6.6. appears to show homocysteine in astrocytic end feet, along with folate, associated with neuronal cell bodies, perhaps removing this toxic molecule from the microenvironment and from the neurones.

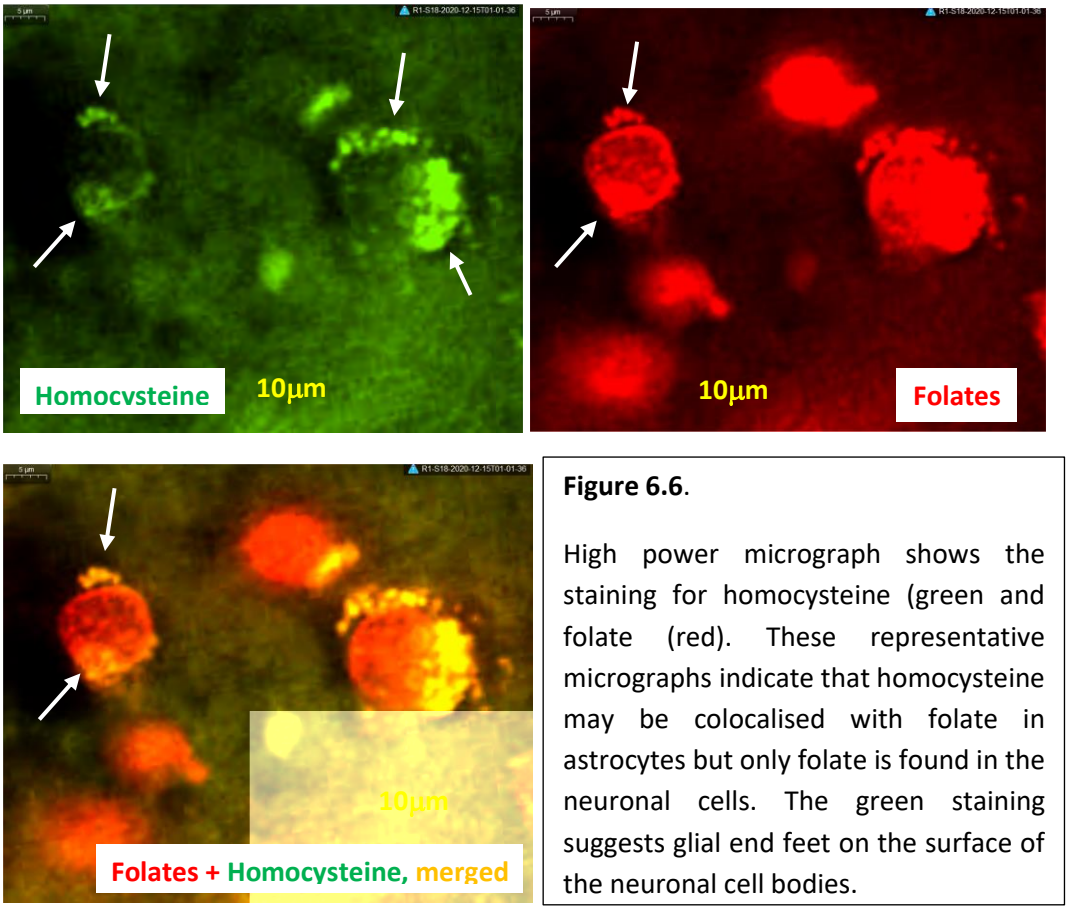


Figure 6.6.

High power micrograph shows the staining for homocysteine (green and folate (red). These representative micrographs indicate that homocysteine may be colocalised with folate in astrocytes but only folate is found in the neuronal cells. The green staining suggests glial end feet on the surface of the neuronal cell bodies.

6.9. Data from the Allen Brain Institute Protein Atlas

As this study was time limited, it has also taken advantage of the free data available from the Allen Brain Institute to map some of the folate related enzymes and metabolites in the normal human brain. The Allen Institute has mapped the localisation of over 2000 proteins in tissue sections of the human brain. They have not carried out co-localisation studies, but the data are high quality and important to the current study. All sections were labelled with antibodies conjugated with HRP and used diaminobenzidine (DAB) to

make the brown reaction product visible as positive stained cells. Counterstaining gives the purple nuclei in the micrographs.

DHFR is present in some neuronal cell bodies as well as being concentrated in the neuropil. The neuropil staining is not consistent with some areas of cortex heavily stained (Figure 6.7.b) and others lacking neuropil staining (Figure 6.7.a).

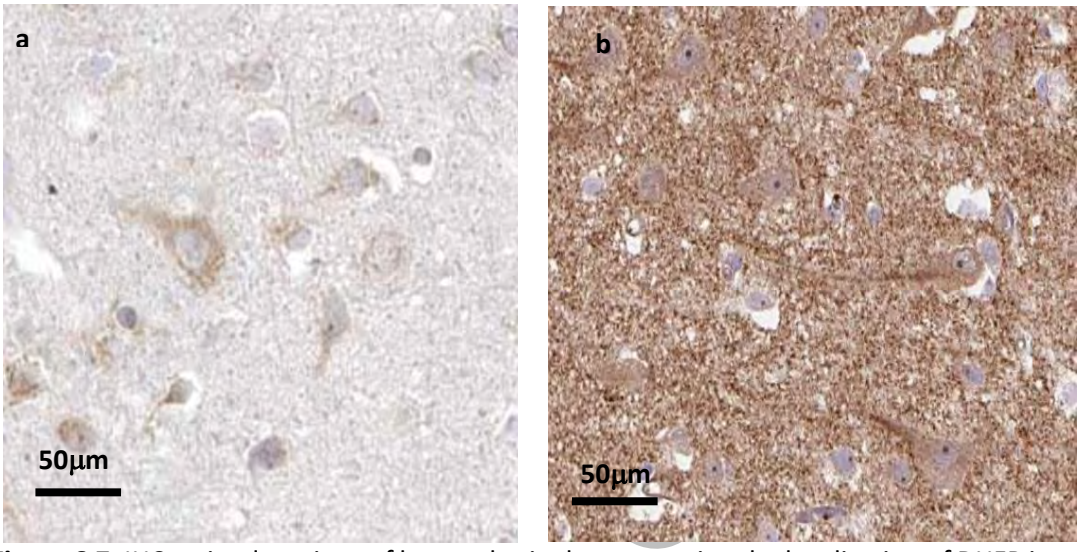


Figure 6.7. IHC stained sections of human brain demonstrating the localisation of DHFR in some neuronal cell bodies (a) and in high concentration in some parts of the neuropil (b).

MTHFR shows similar neuronal staining to DHFR and shows that not all neurones are stained (figure 6.8.). Neuropil staining is not as dark as for DHFR suggesting that not all fibres contain MTHFR.

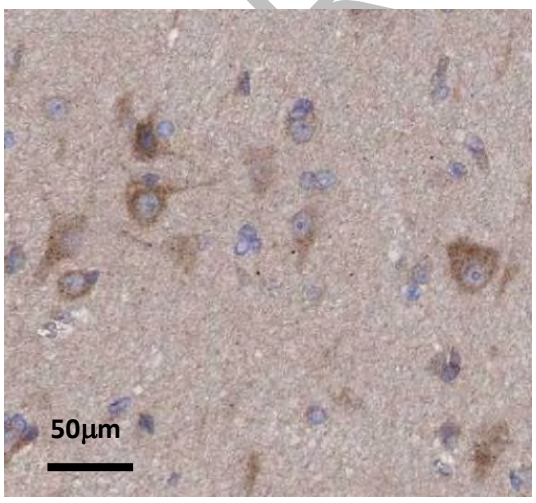


Figure 6.8. IHC staining of human brain sections demonstrating cellular localisation of MTHFR. Possibly all neuronal cells are positive and although the neuropil looks lightly stained, closer examination shows little staining. The smaller nucleated cells are probably glial and show no staining.

MTHFD1 is an important folate enzyme involved in 3 reactions and having three enzymatic effects. MTHFD1 is a key enzyme in the synthesis of, and balance between 5 different folate metabolites and thus the pathways they feed into, including methylation

and trans-sulfuration pathways. Its functions are thus critical to the balance of folate metabolism and changes in this enzyme are likely to cause potentially more severe outcomes. MTHFD1 is a critical folate enzyme involved in 3 parts of folate metabolism (Figure 9.2. in Discussion). It is involved in control of formate levels through the formation of 10-formylTHF from THF, which can then be used in purine biosynthesis. It balances formylTHF with 5,10methenylTHF and also mediates conversion of this to 5,10methyleneTHF which is either converted to 5mTHF by MTHFR, and thus forms the long pathway back to 5mTHF, or to dihydrofolate, fuelling the biosynthesis of pyrimidines in the process. DHFR then converts DHF to THF which picks up one carbon components from the conversion of serine to glycine to form 5,10methyleneTHF that can then be converted to 5mTHF, thus forming the short route to 5mTHF. Thus, for cells involved in folate metabolism DHFR and MTHFR would be important enzymes. In the field shown in Figure 6.8, many neurons show positive staining for MTHFR but there remain some that are not positive for this enzyme as found for the other folate enzymes.

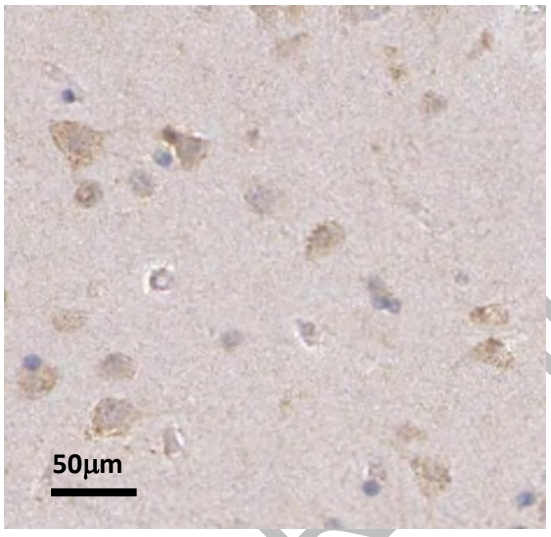


Figure 6.9. IHC staining of human brain sections for MTHFD1. There are many neurons in this field that have positive staining, perhaps all neuronal cells and the neuropil shows only a few fibres with any sign of staining.

MTR and MTRr are key enzymes in the rate limiting step of folate metabolism that takes the methyl group from 5methyl THF, passes it to cobalamin forming methyl cobalamin (MTRr) and then to homocysteine forming methionine (MTR). Thus, these enzymes are intimately linked yet appear to be in different locations or at very different concentrations in similar/different cells. MTR is clearly evident in the neuropil and some large neuronal cell bodies (6.10.a) while MTRr is weakly stained in some cells (6.10.b).

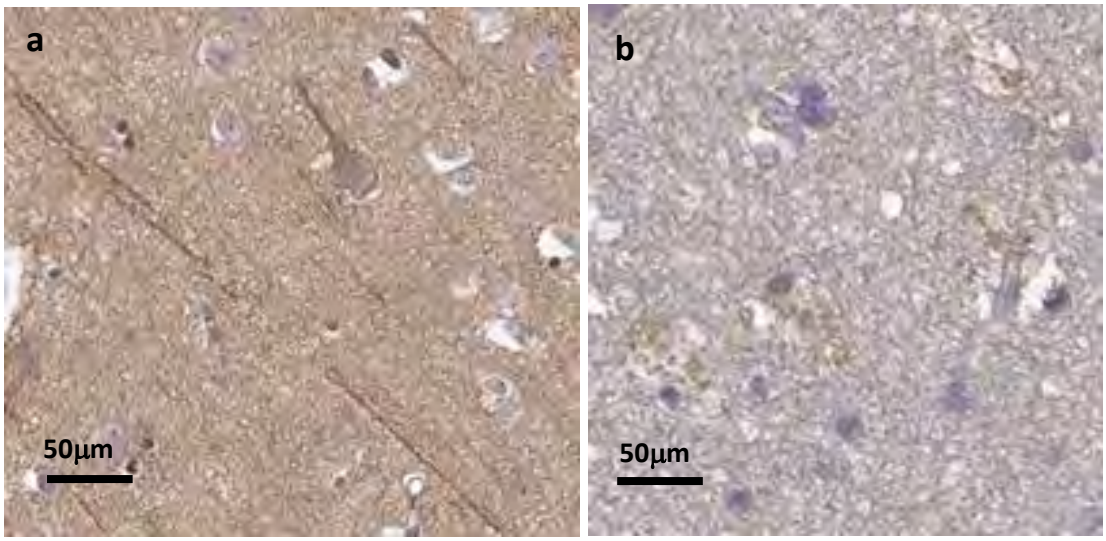


Figure 6.10. IHC staining for MTR (a) and MTRr (b) in human brain

ALDH1L1 (FDH) is an important folate binding protein and enzyme. Its importance in transporting folate into the brain is demonstrated in conditions where it is reduced or missing in CSF resulting in failure of access to available folate by the brain. It is specifically located in astrocytes that are GFAP negative but colocalised in many with S100 (Chapter 5). It is not found in any neurons.

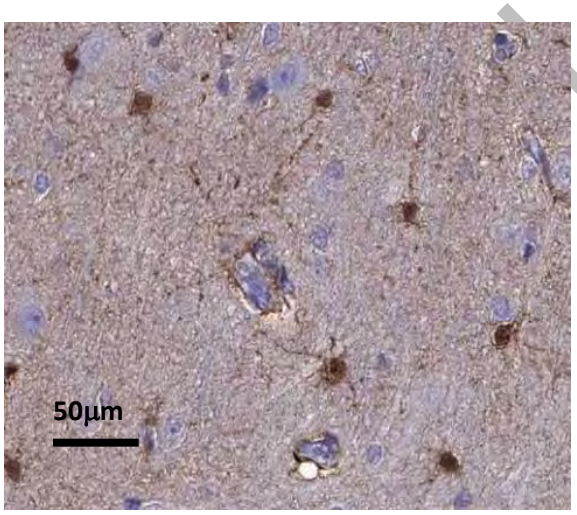


Figure 6.11. IHC staining for ALDH1L1 (FDH) showing very specific localisation in astrocytes and their processes and no staining in other cell type. This enzyme is unique in its localisation to specific astrocytes that are GFAP negative. MTR is also found in some astrocytes, but these are GFAP positive (Figure 6.1) so not co-localised with FDH that is found in GFAP negative astrocytes.

6.10. Discussion

Taken together with the IHC results in the previous chapter, the data here demonstrate that folate metabolism appears to be separated across different cells in the cerebral cortex. Only MTHFR and DHFR were found co-localised in some cells while FDH and MTHFD1 were clearly separated in different cells. In addition, folate and homocysteine show little colocalization even though they are intimately linked in folate metabolism. These findings present a picture of a system unique to the cerebral cortex where cells

contain one or two components of folate metabolism but not all. In the rest of the body, all of folate metabolism is represented in the cytoplasm and mitochondria of cells indicating that the whole of folate metabolism is important to cellular functions.

Two possibilities exist to explain these observations when the results from CSF analysis are considered. The CSF contains all the folate enzymes except MTHFR which is present in all neuronal cells of the cortex but not glial cells. In the cortical cells these enzymes are present in variable numbers of neuronal cells with FDH found in a unique set of astrocytes and MTR in some GFAP positive cells.

6.10.1. Model 1

FDH as the main transporter could deliver 5mTHF, 10 formyl THF, or THF throughout the cortex. 5mTHF must be converted to THF by MTR and MTRr as well as being used in the BH2-BH4 cycle for neurotransmitter and nitric oxide synthesis. Thus, MTR and MTRr in the neurones must be receiving 5mTHF to convert to THF and methylate homocysteine. However, Figure 6.6. indicates that homocysteine may be separated in astrocytes and is not in neurones suggesting that all homocysteine is removed from neurones rapidly to avoid its toxic effects. Presence of other folate enzymes in neurones indicates the ability for limited conversion of folate metabolites for specific metabolic tasks in those cells.

6.10.2. Model 2

In this model the evidence indicates that folate metabolism occurs in the CSF and that FDH positive astrocytes then transport the metabolites throughout the cortex with specific metabolites used by different cells. In addition, it looks likely that some cells, particularly pyramidal cells in the cortex, may be able to take specific metabolites, with bound enzymes from the CSF at the pial surface where their apical processes originate.

In both models, the loss of FDH, observed in AD CSF, would have an effect on folate transported through the FDH-positive astrocyte network as this shows need of FDH bound to folate to extract folate from the CSF. The alternative model would then allow for folate to pass through other pathways and/or directly to neurones connected to the pial surface and/or via GFAP astrocytes.

Chapter 7

Supplementary data

IHC Negative controls

Chapter 7
Negative Controls

In this chapter negative controls are shown for autofluorescence and for each of the antibodies and fluorophores used. The representative micrograph shows similar autofluorescence which is different to the specific staining seen in the previous chapters. This autofluorescence is highly likely to be due to formalin fixation that induces fluorescence from biomolecules including biogenic amines and structural proteins. Although there is a high level of autofluorescence in these control sections, this is not obvious in the specific staining shown in the previous chapters and may have been removed by the extended blocking steps used in the IHC protocols or may be faint in contrast to the specific staining we observed in IHC.

7.1. Negative control without primary or secondary antibody

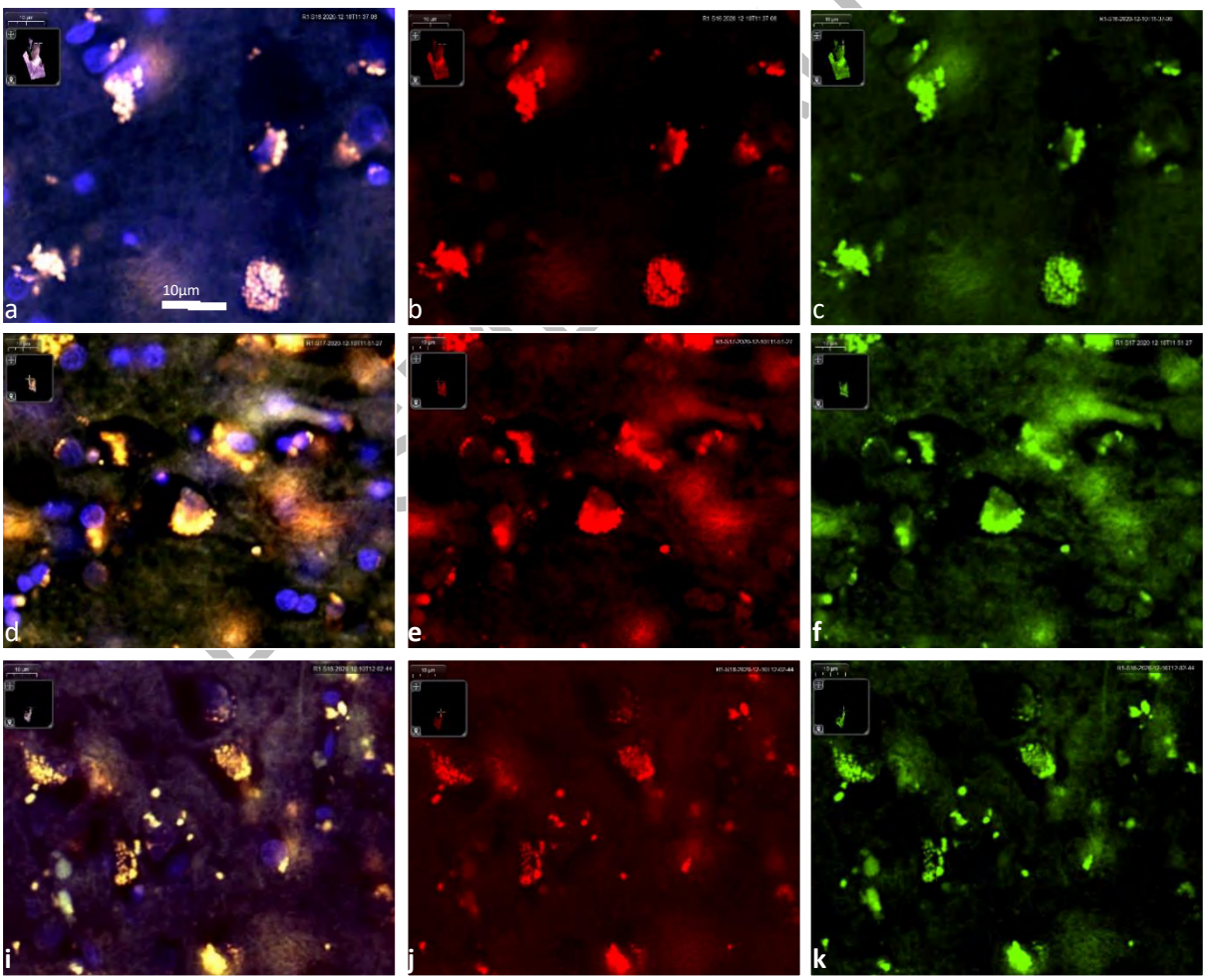


Figure 7.1. Negative control demonstrating auto-fluorescence within the cortical plate in the absence of any primary or secondary antibody under DAPI (blue, a,d,i), 594 (red, b,e,j) and 488 (green, c,f,k) in normal (a,b,c), moderate (d,e,f) and severe (l,j,k) brain tissue. All were taken at the same magnification with the 10µm scale bar in (a) applicable to all. The auto-fluorescence appears as spots within larger structures that may be biogenic amine containing vesicles that become auto-fluorescent after formalin fixation.

7.2. Negative controls without primary antibody but with anti-chicken 594 and anti-rabbit 488 secondary antibodies

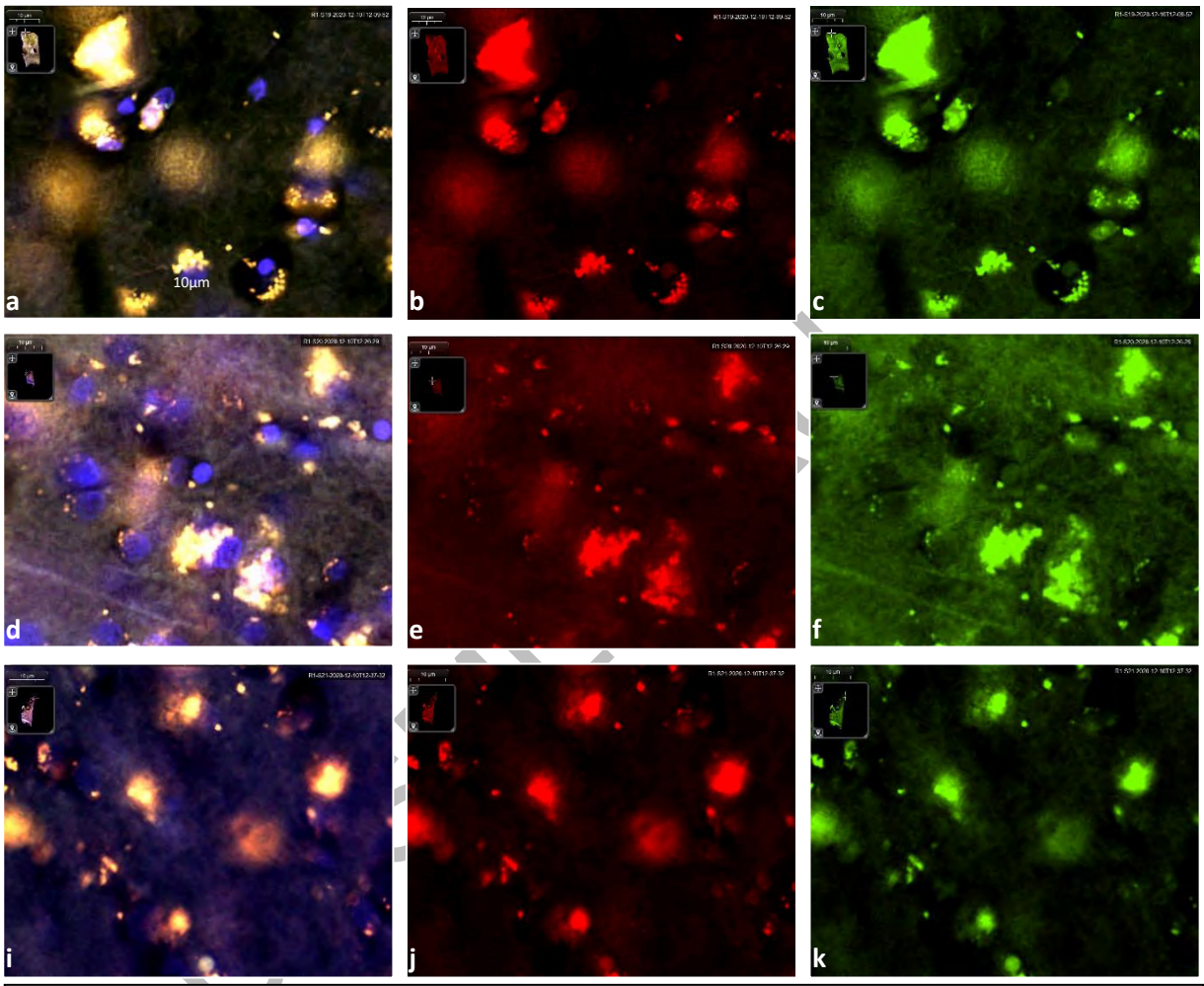


Figure 7.2. Negative control demonstrating auto-fluorescence within the cortical plate in the absence of primary antibody under DAPI (blue, a,d,i). Only secondary antibodies used 594 (red, anti-chicken, b,e,j) and 488 (green, anti-rabbit, c,f,k) in normal (a,b,c), moderate (d,e,f) and severe (i,j,k) brain tissue. All were taken at the same magnification with the 10µm scale bar in (a) applicable to all. The auto-fluorescence appears as spots within larger structures that may be biogenic amine containing vesicles that become auto-fluorescent after formalin fixation.

7.3. Negative control without primary antibody but with anti-mouse 594 and anti-rabbit 488 secondary antibodies

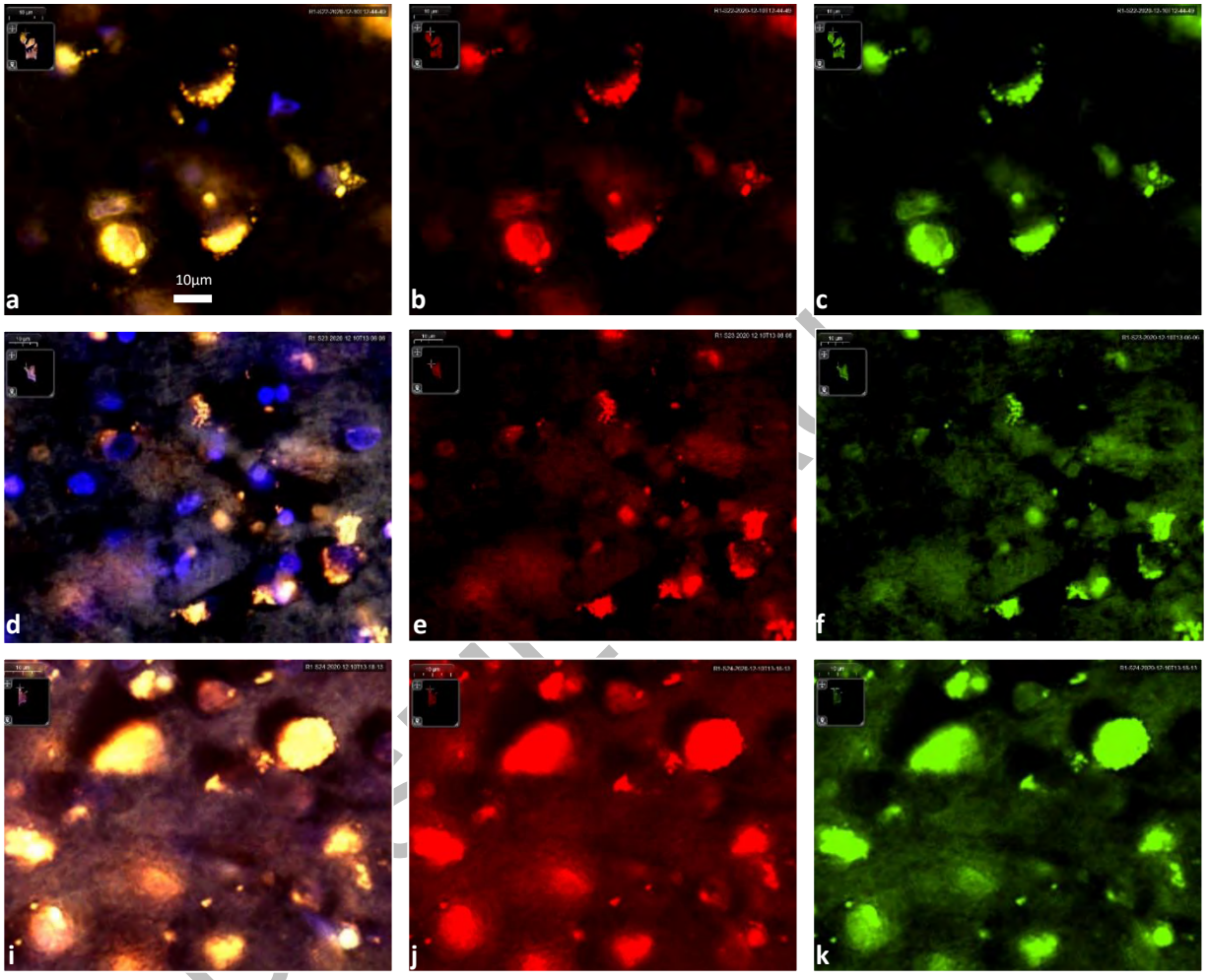


Figure 7.3. Negative control demonstrating auto-fluorescence within the cortical plate in the absence of primary antibody under DAPI (blue, a,d,i). Only secondary antibodies used 594 (red, anti-mouse, b,e,j) and 488 (green, anti-rabbit, c,f,k) in normal (a,b,c), moderate (d,e,f) and severe (i,j,k) brain tissue. All were taken at the same magnification with the 10µm scale bar in (a) applicable to all. The auto-fluorescence appears as spots within larger structures that may be biogenic amine containing vesicles that become auto-fluorescent after formalin fixation.

7.4. Negative controls without primary antibody but with anti-goat 594 and anti-chicken 488 secondary antibodies

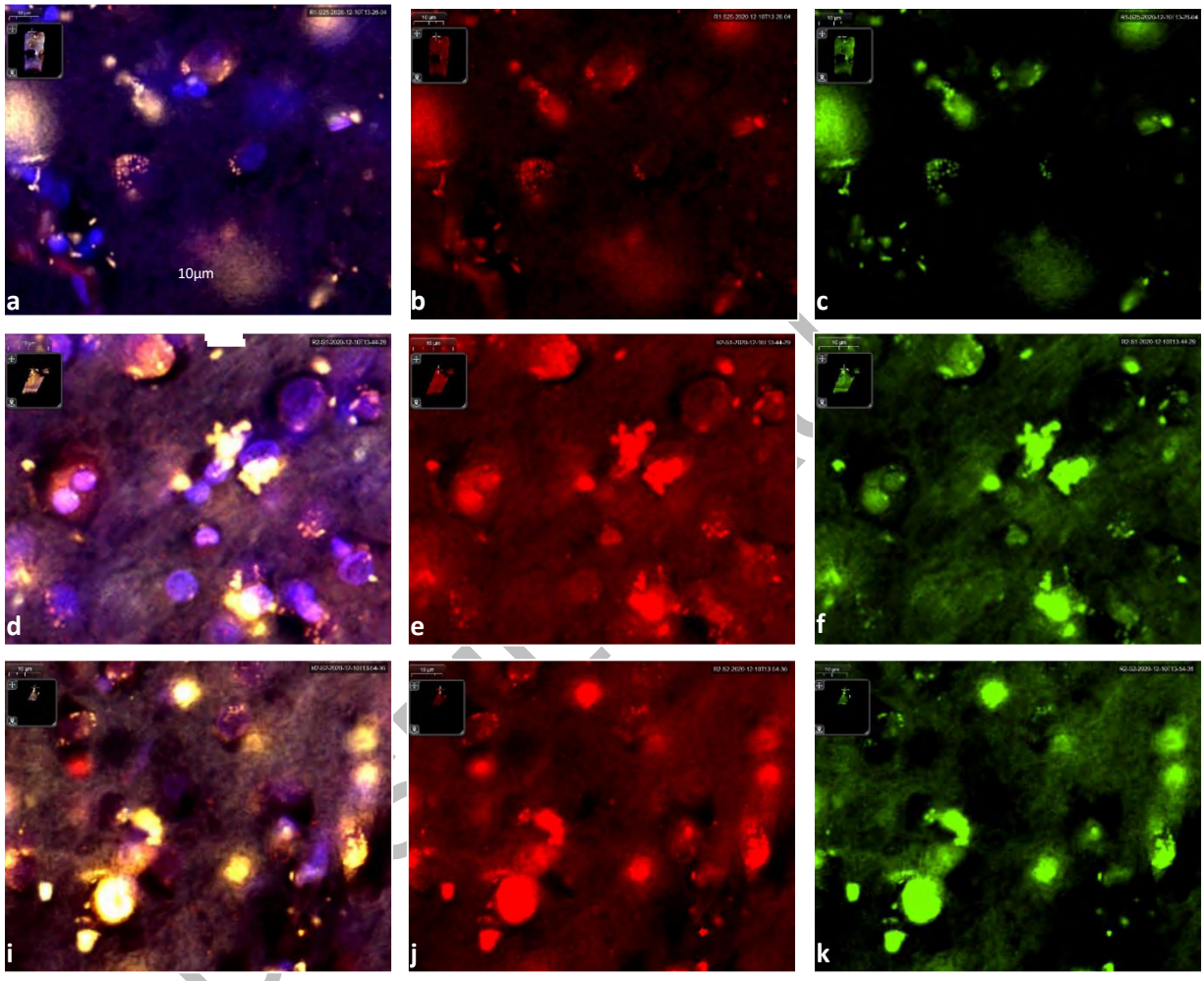


Figure 7.4. Negative control demonstrating auto-fluorescence within the cortical plate in the absence of primary antibody under DAPI (blue, a,d,i). Only secondary antibodies used 594 (red, anti-goat, b,e,j) and 488 (green, anti-chicken, c,f,k) in normal (a,b,c), moderate (d,e,f) and severe (i,j,k) brain tissue. All were taken at the same magnification with the 10µm scale bar in (a) applicable to all. The auto-fluorescence appears as spots within larger structures that may be biogenic amine containing vesicles that become auto-fluorescent after formalin fixation.

7.5. Negative controls without primary antibody but with anti-goat 594 and anti-mouse 488 secondary antibodies

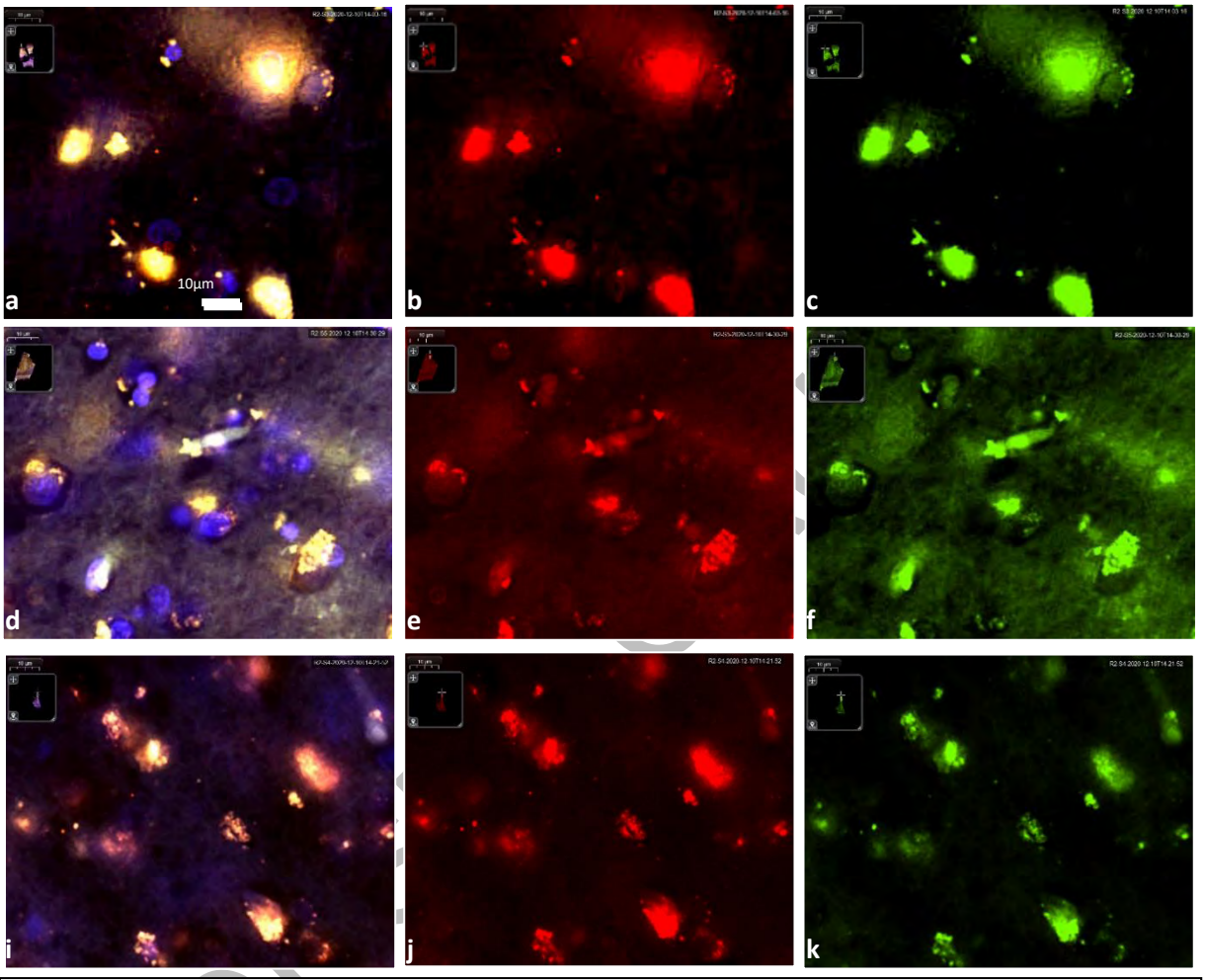


Figure 7.5. Negative control demonstrating auto-fluorescence within the cortical plate in the absence of primary antibody under DAPI (blue, a,d,i). Only secondary antibodies used 594 (red, anti-goat, b,e,j) and 488 (green, anti-mouse, c,f,k) in normal (a,b,c), moderate (d,e,f) and severe (i,j,k) brain tissue. All were taken at the same magnification with the 10µm scale bar in (a) applicable to all. The auto-fluorescence appears as spots within larger structures that may be biogenic amine containing vesicles that become auto-fluorescent after formalin fixation.

7.6. Negative controls without primary antibody but with anti-rabbit 594 and anti-rat 488 secondary antibodies

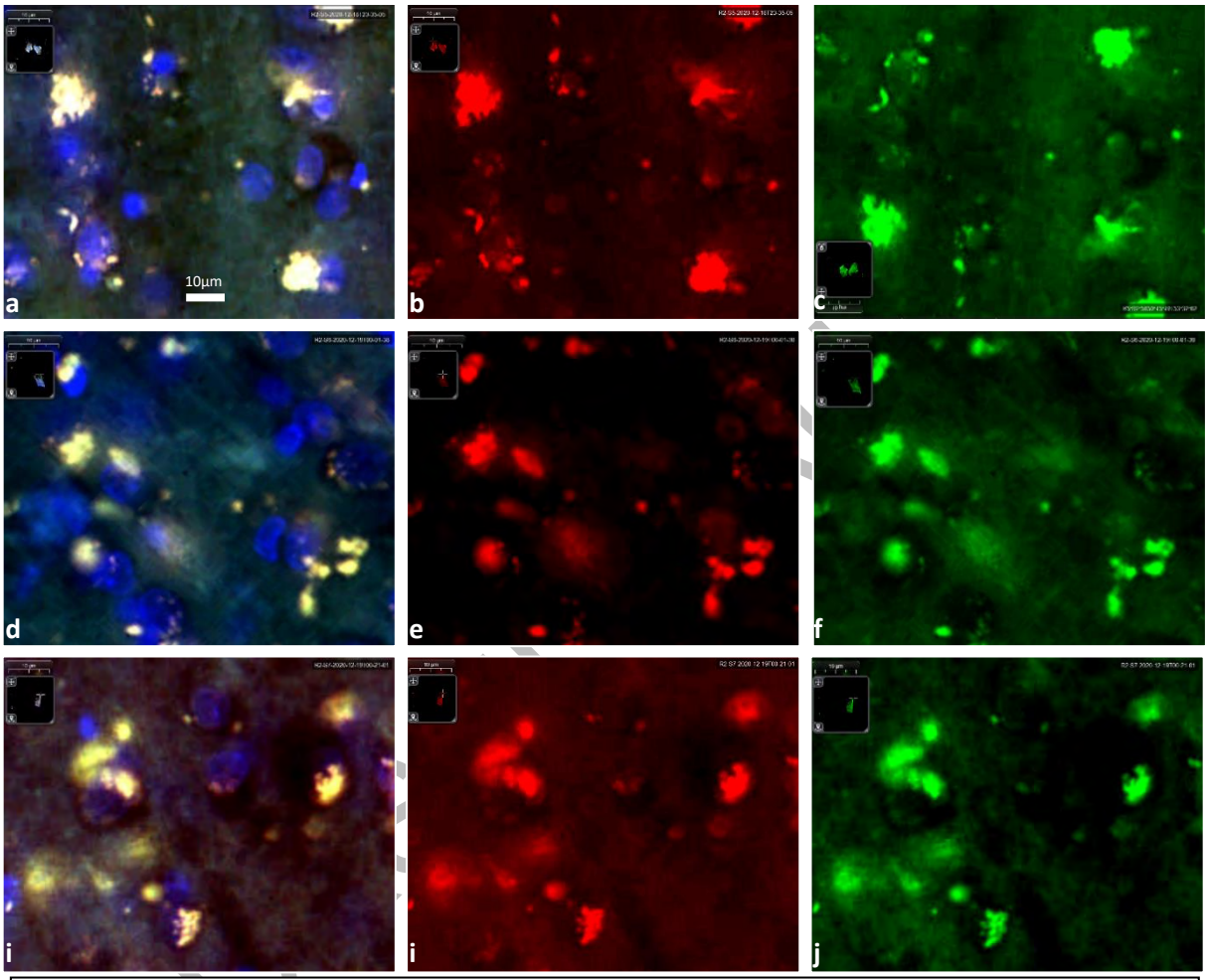


Figure 7.6. Negative control demonstrating auto-fluorescence within the cortical plate in the absence of primary antibody under DAPI (blue, a,d,i). Only secondary antibodies used 594 (red, anti-rabbit, b,e,j) and 488 (green, anti-rat, c,f,k) in normal (a,b,c), moderate (d,e,f) and severe (i,j,k) brain tissue. All were taken at the same magnification with the 10µm scale bar in (a) applicable to all. The auto-fluorescence appears as spots within larger structures that may be biogenic amine containing vesicles that become auto-fluorescent after formalin fixation.

Chapter 8

Results VI

Nutrigenomics: Folate related pathway gene analysis.

Chapter 8

Nutrigenomics: Folate related pathway gene analysis

8.1. Introduction

In this study we used a method of functional genomics, derived from nutrigenomics, to query the genes involved in specific metabolic pathways and to identify single nucleotide polymorphisms (SNPs) giving negative effects on protein functions. This is a different approach to GWAS and TWAS as it investigates the genes of specific metabolic pathways, identifies potentially defective genes, and thereby highlights potential metabolic errors in individuals. We utilised two commercial companies for the analysis, one, LGC Genomics, quality checked extracted DNA and carried out targeted, nutrigenomic gene SNP analysis. The data were transferred to LifecodeGX who converted the information into colour coding the SNPs according to functional effects based on information in the literature (see sample full report at the end of this chapter).

8.2. Results

In this study genes associated with these metabolic processes were analysed. Changes in single nucleotide polymorphisms (SNPs) were identified and presented as heat maps in Tables 8.1. to 8.4. associated with those genes successfully sampled through the methods for neurotransmitter pathways (Table 8.1 and 8.2) and methylation pathways (Table 8.3 and 8.4) as well as *APOE4* genotypes. Gene SNPs are highlighted as normal (green), with potential adverse effects (yellow) and with negative effects (red) on gene/protein function. Using a Mann-Whitney U test we found significant differences in the frequencies of these groupings between normal ageing (Braak 0-II) and AD (Braak V-VI). In addition to Mann-Whitney U, we also carried out Chi Squared tests after grouping positive and neutral SNPs and comparing these with negative SNPs, or by grouping negative and neutral and comparing to positive. Outcomes of all three tests are shown in Table 8.5.

Table 8.1. Normal ageing

	Genes	Variants	09_31	11_06	11_25	11_29	12_09	12_11	13_35	14_04	14_08	14_09	14_11	14_20	14_46	15_01	15_28	16_11	16_29	16_31	17_09	17_15	17_34	17_36	18_03	18_11	19_09
Nervous System	5-HT1A (rs6295)	1019CG	1	1	3	3	2	2	2	2	3	1	2	2	3	1	3	2	3	2	2	3	2	1	2	3	2
	5-HT2A (rs6311)	1438G>A	3	3	2	2	3	2	2	2	2	2	2	3	2	2	3	2	2	2	2	1	2	2	1	1	3
	ASMT (rs4446909)		1	3	3	3	1	3	1	3	3	1	3	1	3	1	1	1	3	1	1	1	3	3	1	3	3
	FKBP5 (rs1360780)		2	3	1	2	2	2	2	3	1	1	1	2	1	2	1	2	1	2	2	2	2	2	1	2	2
	IFN-g (rs2430561)	+874AT	2	2	1	2	3	3	2	2	2	1	1	3	3	2	2	2	1	2	3	1	1	3	1	2	3
	MAOA (rs6323)	R297R	3	2	3	3	3	3	3	3	3	3	3	2	2	1	1	3	3	3	3	3	3	1	3	3	3
	MTNR1B (rs10830963)		1	1	2	1	1	1	1	1	2	1	2	1	1	3	2	1	2	1	2	3	2	2	1	3	1
	QDPR (rs1031326)	690A>G	1	1	2	2	2	3	1	2	1	2	2	1	1	2	2	2	2	3	1	1	2	2	2	1	1
	SLC18A1 (rs1390938)	Thr136Ile	3	2	2	3	1	2	2	3	2	3	3	2	3	3	2	2	2	2	2	2	3	3	2	3	3
	TNF (rs1800629)	-308GA	1	2	2	1	1	1	2	1	2	2	1	2	1	1	2	1	1	2	2	2	1	2	1	1	1
	TPH1 (rs1799913)	A779C	1	1	3	1	2	2	1	1	1	1	3	2	3	2	2	2	1	3	2	1	2	2	2	1	2
	TPH1 (rs1800532)	A218C	1	1	3	1	2	2	1	1	1	1	3	2	3	2	2	2	1	3	2	1	2	2	2	1	2
	TPH2 (rs4570625)	844G>T	1	2	1	1	1	2	1	1	1	2	2	1	1	2	2	1	2	1	1	1	1	1	2	2	1
	VDR (rs1544410)	BsmI	1	1	1	2	1	2	3	2	1	1	2	2	1	2	3	2	3	1	1	3	2	2	1	2	2
	VDR (rs731236)	TaqI	1		1	2	1	2	3	2	1	1	2	2	1	2	3	2	3	1	1	3	2	2	1	2	2
	ADRB1 (rs1801253)	Arg389Gly	3	3	3	2	3	2	3	3	2	3	3	3	2	2	3	2	3	2	3	3	2	3	3	3	3
	ADRB2 (rs1042713)	Arg16Gly	3	2	2	1	2	3	2	1	3	2	2	3	2	2	3	3	2	3	2	3	2	2	1	2	2
	COMT (rs4633)	H62H	3	2	1	3	2	2	2	2	3	3	2	1	2	2	2	3	3		2	2	3	3	2	3	1
	COMT (rs4680)	V158M	3	2	1	3	2	2	2	2	3	3	2	1	2	2	2	3	3	2	2	2	3	3	2	3	1
	DBH (rs1611115)	C-970T	1	2	1	1	1	2	1	1	1	1	1	3	1	1	1	1	2	1	1	3	1	2	3	2	1
	DRD2 (rs1076560)	811-83G>T	1	1	1	1	1	1	2	1	1	2	2	1	1	2	2	1	3	1	1	2	1	2	1	1	1
	DRD2 (rs6277)	957C>T	2	2	1	1	2	3	2	1	2	2	3	1	1	2	3	2	3	3	3	3	3	3	2	2	2
	MAOB (rs1799836)	A644G	1	3	3	1	1	3	2	1	1	3		1	2	3	1	3	3	3	1	1		2	3	2	1
	PNMT (rs876493)	G-161A	3	2	2	3	2	3	2	1	2	2	2	1	2	1	3	2	3	2	2	1	2	3	2	1	3
	SLC6A2 (rs5569)	G1287A	1	1	1	1	3	3	3	3	1	1	1	3	3	1	1	1	3	1	3	1	3	3	1	3	1
	SLC6A3 (rs27072)	328G>A	1	1	1	1	1	1	1	2	1	1	1	1	2	1	1		1	1	3	2	1	2	2	1	3
	SLC6A3 (rs6347)		1	1	1	1	1	1	2	1	1	1	1	1	2	1	1	2	2	2	1	2	2	1	1	1	2
	TH (rs10770141)	C-824T	2	2	2	2	1	1	1	3	2	1	1	1	1	1	1	2	2	2	2	2	3	1	2	1	2
	GABRA2 (rs279858)		2	3	2	3	1	2	2	3	2	1	1	2	3	2	3	2	1	2	3	2	2	2	2	1	3
	ADRB2 (rs1042713)	Arg16Gly	1	2	2	3	2	1	2	3	1	2	2	1	2	2	1	1	2	1	2	1	2	2	3	2	2
	CYP2C19 (rs12248560)	-806C>T	1	1	1	1	1	1	2	2	2	1	1	1	1	1	3	1	1	1	2	1	2	1	1	1	2
	CYP2C19 (rs4244285)	681G>A	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	2	1
	CYP2D6 (rs1135840)	S486T	1	1	1	3	2	1	3	1	3	2	1	1	2	1	1	2	2	2	3	2	2	2	2	1	2
	CYP2D6 (rs16947)	R296C	3	3	2	1	1	3	1	3	1	2	2	2	2	3	1	2	2	2	1	2		2	2	3	1
	CYP2D6 (rs35742686)	2549delA	1	1	1	1	1	1	1	1	2		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CYP2D6 (rs3892097)	1846G>A	1	1	2	1	2	1	1	1	1	1	2	2	1	1	3	1	1	1	1	1	2	1	1	1	2
	CYP3A4 (rs2740574)	-392G>A	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	BDNF (rs6265)	Val66Met	2	2	1	1	1	1	1	2	2	1	1	1	1	1	2	2	2	1	2	1	1	1	1	2	2
	DIO1 (rs2235544)	34C>A	2	1	1	2	3	2	2	1	2	2	3	3	1	1	1	2	2	3	3	1	1	1	2	2	3
	DIO2 (rs12885300)	Gly3Asp	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	DIO2 (rs225014)	Thr92Ala	1	2	2	1	2	1	2	1	1	2	1	2	2	2	3	1	2	1	2	3	1	2	1	1	1
	HNMT (i3000469)	314CT	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	2	1	2	1	1	1	1	1	1
	OPRM1 (rs1799971)	A118G	2	1	2	1	1	1	1	1	1	1		1	1	2	1	1	2	2	1	1		1	2	1	1
	SLCO1C1 (rs10770704)	intron3C>T	2	2	2	2	2	3	3	2	3	2	2	2	2	2	3	2	2	1	3	3	3	3	2	2	2

Genes	Red meaning	Yellow meaning	Green meaning
MTHFD1 (rs1076991) and MTHFD1 (rs2236225)	Reduced gene activity- reduced methyl folate for homocysteine recycling - dependency on the short route via BHMT	Potential reduction in gene activity	Normal genotype- no impact on gene activity or methyl folate availability

Table 8.2. Alzheimer's

	Genes	Variants	11_28	12_01	12_03	12_05	12_32	13_09	13_10	13_45	14_10	14_30	14_31	14_50	15_02	15_48	16_10	16_16	16_40	17_37	18_12	18_39	19_04	19_07	19_12	19_29	19_31
Nervous System	5-HT1A (rs6295)	1019CG	3	2	1	3	2	2	2	2	2	2	2	2	2	3	3	2	1	2	2	3	3	1	2	2	3
	5-HT2A (rs6311)	1438G>A	3	1	3	2	2	2	1	2	3	1	2	2	2	1	2	1	2	2	3	1	1	2	2	1	2
	ASMT (rs4446909)		1	3	1	1	3	1	1	3	3	3	1	1	1	3	3	3	3	3	3	3	3	1	1	3	1
	FKBP5 (rs1360780)		3	2	2	2	1	1	2	1	3	1	1	1	1	2	2	2	1	2	1	1	2	1	2	2	1
	IFN-g (rs2430561)	+874AT	3	2	2	3	3	2	2	3	3	2	2	2	2	3	3	2	1	2	1	2	2	3	2	3	3
	MAOA (rs6323)	R297R	3	3	1	3	1	3	2	1	3	2	1	3	1	3	2	2	1	3	3	2	3	3	3	3	3
	MTNR1B (rs10830963)		2	2	2	1	2	2	1	1	2	2	1	1	1	1	2	1	1	1	1	1	1	1	1	2	2
	QDPR (rs1031326)	690A>G	2	1	2	1	2	1	1	1	2	2	2	3	1	2	1	1	1	3	2	1	3	2	1	2	1
	SLC18A1 (rs1390938)	Thr136Ile	3	2	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3	2	3	2	2	2	1	3	3
	TNF (rs1800629)	-308GA	1	1	1	1	2	2	2	1	2	1	1	2	1	2	2	1	1	1	2	1	2	2	2	1	1
	TPH1 (rs1799913)	A779C	1	2	1	2	3	1	3	1	2	2	1	1	1	1	1	1	1	3	2	1	2	1	3	1	2
	TPH1 (rs1800532)	A218C	1	2	1	2	3	1	3	1	2	2	1	1	1	1	1	1	1	3	2	1	2	1	3	1	2
	TPH2 (rs4570625)	844G>T	1	3	1	1	2	2	1	1	1	1	1	2	1	1	2	2	1	2	2	2	1	1	1	2	2
	VDR (rs1544410)	BsmI	2	2	2	2	2	2	1	2	2	2	1	2	2	1	3	2	3	3	1	2	1	2	2	1	1
	VDR (rs731236)	TaqI	2	2	2	2	2	2	1	2	2	2		2	2	1	3	2	3	3	1	2	1	2	2	1	1
	ADRB1 (rs1801253)	Arg389Gly	3	3	2	3	2	3	3	3	1	2	2	3	3	3	2	2	3	3	2	2	2	2	2	1	2
	ADRB2 (rs1042713)	Arg16Gly	3	3	2	2	2	2	3	2	3	2	2	2	3	1	3	3	2	1	3	2	1	3	3	3	2
	COMT (rs4633)	H62H	2	1	2	1	3	1	2	2	3	2	1	2	1	1	2	2	2	2	1	1	1	2	1	2	2
	COMT (rs4680)	V158M	2	1	2	1	3		2	2	3	2	1	2	1		2	2	2	2	1	1	1	2	1	2	2
	DBH (rs1611115)	C-970T	2	1	1	1	2	1	2	2	1	1	1	1	1	1	2	1	1	2	2	1	2	1	2	1	1
	DRD2 (rs1076560)	811-83G>T	1	1	1	1	1	1	2	2	1	1	3	2	1	1	2	1	1	2	1	1	1	2	1	1	2
	DRD2 (rs6277)	957C>T	3	1	2	1	3	1	3	2	1	1	3	2	1	1	2	2	1	2	2	1	2	3	2	2	2
	MAOB (rs1799836)	A644G	2	3	1	1	3	2	1	1	3	1	1	2	1	1	1	2	1	1	3	2	3	3	1	3	3
	PNMT (rs876493)	G-161A	1	3	3	2	2	2	2	1	2	2	3	2	2	2	3	2	2	2	3	2	2	3	2	3	2
	SLC6A2 (rs5569)	G1287A	3	3	3	3	3	1	3	3	1	3	3	3	1	3	3	3	1	1	3	1	1	3	3	3	1
	SLC6A3 (rs27072)	328G>A	1	1	1	1	1	2	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	2	1	1
	SLC6A3 (rs6347)		1	2	2	1	1	1	1	1	2	1	1	1	1	2	1	2	2	1	2	1	2	1	1	1	1
	TH (rs10770141)	C-824T	2	1	1	2	2	2	1	1	3	1	2	1	2	1	3	1	2	2	2	1	3	1	2	1	1
	GABRA2 (rs279858)		2	3	2	2	2	2	3	2	1	2	3	1	2	2	3	2	1	1	1	2	2	3	2	2	2
	ADRB2 (rs1042713)	Arg16Gly	1	1	2	2	2	2	1	2	1	2	2	2	1	3	1	1	2	3	1	2	3	1	1	1	2
	CYP2C19 (rs12248560)	-806C>T	2	2	2	2	1	1	1	3	1	2	2	1	1	1	1	1	1	1	1	1	2	1	1	1	2
	CYP2C19 (rs4244285)	681G>A	2	1	1	1	1	1	2	1	1	1	1	2	1	2	2	1	1	2	1	2	1	1	1	1	2
	CYP2D6 (rs1135840)	S486T	2	1	3	3	2	2	3	2	3	3	3	3	3	1	2	2	1	2	1	3	2	2	1	3	1
	CYP2D6 (rs16947)	R296C	1	1	1	1	2	1	1	1	1	1	1	1	1	3	2	1	2	2	3	1	1	2	3	1	2
	CYP2D6 (rs35742686)	2549DelA	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	CYP2D6 (rs3892097)	1846G>A	1	3	1	1	1	2	1	2	1	1		1	1	1	1	2	2	1	1	1	1	1	1	1	2
	CYP3A4 (rs2740574)	-392G>A	1	1	2	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1
	BDNF (rs6265)	Val66Met	1	1	1	2	1	1	1	2	2	2	1	2	1	1	1	1	3	1	1	1	2	3	1	1	1
	DIO1 (rs2235544)	34C>A	3	2	2	2	3	2	2	2	2	2	3	1	3	2	3	3	3	2	2	2	2	2	1	2	3
	DIO2 (rs12885300)	Gly3Asp	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	DIO2 (rs225014)	Thr92Ala	1	2	3	2	2	1	3	1	1	2	2	1	3	3	1	2	2	2	2	2	2	1	2	3	2
	HNMT (i3000469)	314CT	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	OPRM1 (rs1799971)	A118G	1	2	1	1	1	2	1	1	1	1	1	1	1	1	2	1	2	1	1	1	1	1	1	1	1
	SLCO1C1 (rs10770704)	intron3C>T	3	2	3	3	2	1	2	1	1	2	2	2	2	2	2	3	2	3	2	2	3	1	2	3	3

Table 8.1 and 8.2. Heat maps of the effects of SNP variants of known genes found in the neurotransmitter pathway in normal (a) and severe Alzheimer's (b) cases. Red is homozygous mutant SNP, Yellow is heterozygous which is either neutral or can have some negative effects, Green is homozygous wild type that is usually positive in function. The clear cells were data points that failed to get SNP data. White cells indicate undetected SNPs in those individuals.

Table 8.3. Normal ageing

	Genes	Variants	09_31	11_06	11_25	11_29	12_09	12_11	13_35	14_04	14_08	14_09	14_11	14_20	14_46	15_01	15_28	16_11	16_29	16_31	17_09	17_15	17_34	17_36	18_03	18_11	19_09
Methylation plus APOE	ALDH2 (rs671)	Glu487Lys	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DHFR (rs70991108)	19bp DEL	1	1	2	2	1	1	1	2	1	2	2	2	3	2	1	1	1	1	2	3	3	1	3	3	1
	FOLH1 (rs202700)	C1561T	1	1	2	2	2	2	2	2	1	1	2	1	2	2	1	2	2	3	2	1	2	2	1	2	2
	MTHFD1 (rs1076991)	C105T	3	2	1	2	2	2	2	2	1	3	3	2	1	1	1	2	3	1	2	2	1	2	2	2	1
	MTHFD1 (rs2236225)	G1958A	2	2	1	2	3	3	3	2	1	3	3	2	2	2	1	1	3	2	1	2	2	2	2	2	2
	MTHFR (rs1801131)	A1298C	2	1	2	1	2	1	1	2	2	2	1	3	1	2	1	1	2	1	3	1	1	1	2	2	2
	MTHFR (rs1801133)	C677T	1	1	2	2	1	3	2	1	2	2	2	1	2	1	3	1	2	2	1	3	1	1	1	1	2
	MTR (rs1805087)	A2756G	1	2	1	1	1	1	1	1	1	1	1	2	3	1	1	3	1	2	1	2	2	2	1	1	1
	RFC1 (rs1051266)	A80G	2	1	2	2	2	1	2	3	3	3	2	3	2	2	1	1	2	2	1	1	3	2	2	2	1
	SHMT1 (rs1979277)	C1420T	1	1	2	3	3	1	1	2	1	1	2	1	1	1	2	2	1	2	2	1	1	2	1	1	2
	TYMS (rs2790)			2	1	1	2	1	1	3	2	1	1	3	2	2	1	1	1	1	2	1	1	1	2	2	1
	AHCY (i5000928)	Tyr143Cys	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	BHMT (rs3733890)	R239Q	2	2	1	3	2	3	2	1	1	2	1	1		1	1	2	1	1	2	1	2	1	1	2	1
	BHMT (rs567754)	BHMT/2	2	2	2	1	1	1	2	1	2	1	1	2	2	2	1	1	1	1	2	2	1	2	3	2	3
	BHMT (rs651852)	BHMT/8	3	1	2	2	1	2	1	3	2	1	1	2	2	3	2	2	2	1	2	3	2	2	3	2	3
	FUT2 (rs1047781)	A385T	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	FUT2 (rs601338)	W143X)	1	3	3	1	2	2	2	2	1	2	3	2	2	3	3	1	2	2	2	2	2	2	2	2	2
	MAT1A (rs1985908)	T1297C	3	2	1	2	1	3	1	1	2	2	1	1	2	2	3	2	2	2	2	1	1	1	3	2	2
	MTRR (rs162036)	K350A	2	3	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
	MTRR (rs1801394)	A66G	2	1	2	2	3	1	2	2	3	2	3	2	2	3	3	3	3	3	3	3	2	2	2	2	1
	PEMT (rs7946)	V175M	3	2	3	3	3	2	2		3	2	2	3	1	3	3	3	1	3	3	2	2	3	3	2	3
	TCN2 (rs1801198)	C776G	2	1	1	2	3	3	1	3	1	3	2	2	1	3	2	2	2	1	1	2	2		1	2	2
	CBS (rs1801181)	C1080T	1	2	2	2	2	1	2	3	1	3	2	1	2	1	1	3	3	1	2	2	2	1	1	1	2
	CBS (rs234706)	C699T	1	2	1	1	1	3	2	1	2	1	1	2	1	2	3	1	1	2	1	1	1	2	2	2	2
	CTH (rs1021737)	G112T)	1	1	1	2	1	2	1	2	1	1	1	1	1	1	2	1	1	1	1	1	2	1	2	1	3
	GSS (rs1801310)	59270A>G	3	2	2	1	2	1	2	1	2	1	2	1	1	1	2	2	1	3	3	2	1	1	1	2	2
	GSS (rs6088659)	A5997G)	2	2	2	1	2	1	1	1	2	1	2	1	1	1	1	1	1	2	1	1	1	1	1	1	2
	MUT (i6060254)	G1595A	3	3	2	1	1	1	1	2	3	1	1	2	1	1	1	2	2	1	2	1	1	2	2	1	1
	SUOX (rs705703)	C5444T	1	1	1	1	1	1	2	1	1	2	1	1	3	1	1	1	2	1	1	2	1	1	1	1	1
	NOS3 (i6015641)	786TC	3	1	2	2	1	1	1	2	1	1	2	2	1	1	2	1	2	3	1	2	2	2	1	1	1
	NOS3 (rs1799983)	G894T	3	1	2	2	1	1	2	2	2	1	3	3	1	1	2	2	1	2	1	2	2	2	1	2	1
	SOD2 (rs2758331)	G816T	1	1	3	3	2	2	3	2	2	3	3	3	2	2	2	1	3	2	2	3	2	2	3	3	2
	SOD2 (rs4880)	A16V	1	1	3	3	2	2	3	2	2	3	3	3	2	2	2	1	3	2	2	3	2	2	3	3	2
	GSTM1 (insert/delete)		3	3	3	1	1	1	3	3		3	3	1	3	3	3	3	1	1	1	3	3	1	3	3	1
	GSTP1 (rs1695)	I105V	1	1	2	1	1	3	2	1	1	2	1	2	2	1	1	2	2	1	1	1	3	1	2	1	1
	GSTT1 (in/del)		1	1	1	1	1		1	1	1	1	1	1	1		1	1		1	1	1	1	1	1	1	1
	APOE		2	3	2	2	2	2	2	3	2	2	2	2	1	2	1	2	2	2	1	2	1	2	2	2	2

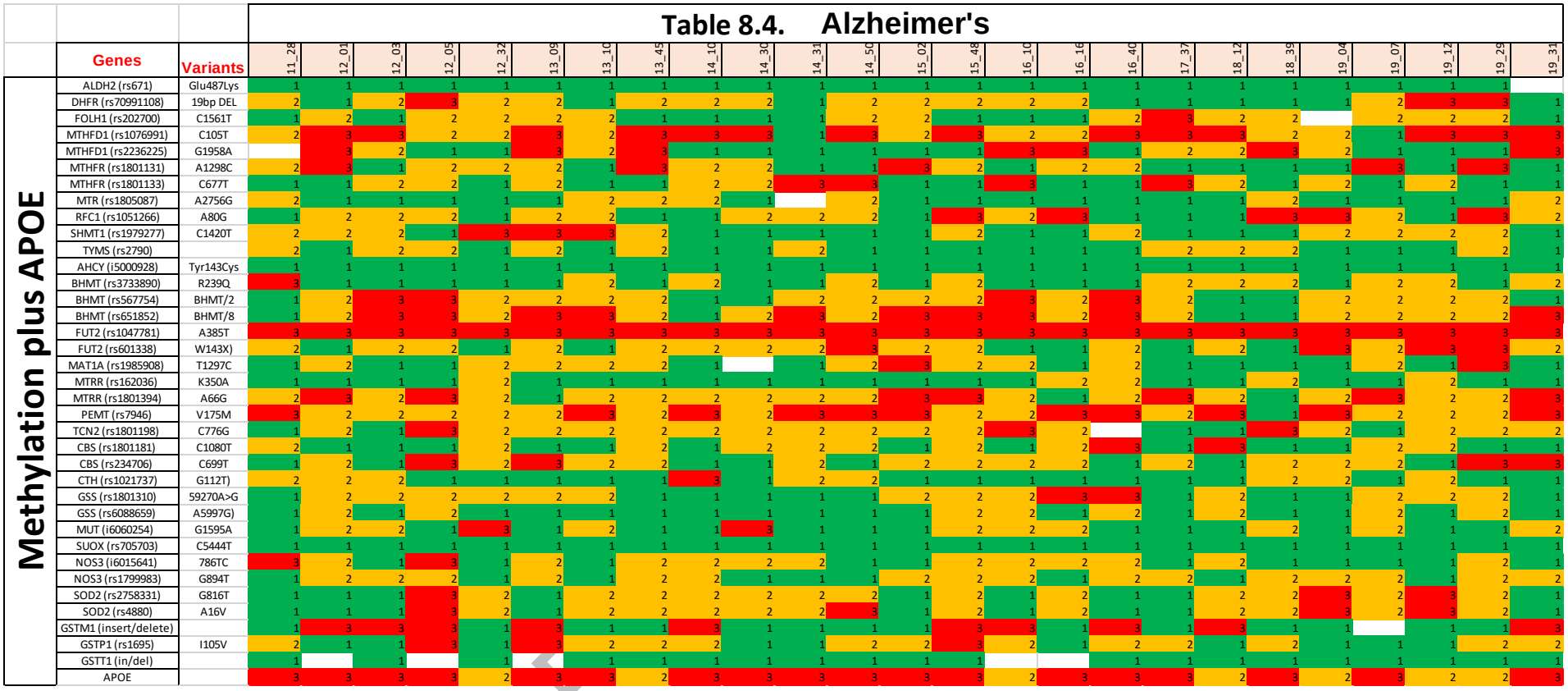


Table 8.3 and 8.4. Heat maps of the effects of SNP variants of known genes found in the folate and methylation pathways in normal (a) and severe Alzheimer's (b) cases. Key as in Table 2. In addition, the *APOE* genotype is given in the final row.

Table 8.5. Gene variants significantly associated with normal ageing and AD

	Mann Whitney, p<0.05 (p≤0.01 in bold)		Chi squared (R+Y), p≤0.05		Chi squared (Y+G), p≤0.05		EFFECT	
Protein	**Genes (variant)**	**p value**	**Genes**	**p value**	**Genes**	**p value**	**AD**	**N**
Apolipoprotein E4 fat metabolism - principle cholesterol carrier in brain supplying neurones via lipoprotein receptors	*APOE4*	**1.61E-06**	*APOE4*	0.029096332	*APOE4*	**4.12E-32**		
MethyleneTHF dehydrogenase long pathway replenishment of 5mTHF	MTHFD1 (rs1076991)	**0.000982**	MTHFD1 (rs1076991)	**0.010097315**	MTHFD1 (rs1076991)	**4.88E-08**		
			MTHFD1 (rs2236225)	**0.000311491**				
MethyleneTHF reductase final step in long and short pathway back to 5mTHF					MTHFR (rs1801131)	0.026992		
synaptic vescile associated monoamine transporter	SLC18A1 (rs1390938)	0.029941			SLC18A1 (rs1390938)	0.004797		
monoamine transporter responsible for reuptake from synapse	SLC6A2 (rs5569)	0.045301	SLC6A2 (rs5569)	0.01562887	SLC6A2 (rs5569)	0.015629		
Cytochrome oxidase involved in metabolism of xenobiotics	CYP2D6 (rs1135840)	0.036104	CYP2C19 (rs4244285)	0.029096332				
Mitochondrial enzyme - sulfite oxidase - detox	SUOX (rs705703)	**0.00987**	SUOX (rs705703)	**0.012419331**				
β-Adrenergic receptor	ADRB1 (rs1801253)	0.032407			ADRB1 (rs1801253)	**0.010097**		
Catechol-O-methyl transferase - degrades monoamines	COMT (rs4633)	**0.002408**	COMT (rs4633)	**2.15E-05**	COMT (rs4633)	**0.001832**		
	COMT (rs4680)	**0.005641**	COMT (rs4680)	**0.001155233**	COMT (rs4680)	**0.008119**		
cytochrome P450 Breakdown of medicines	CYP2D6 (rs16947)	**0.0107**	CYP2D6 (rs16947)	**9.00E-05**	CYP2D6 (rs1135840)	**0.001063**		
Iodothyronine deiodinase activates thyroid hormone			DIO2 (rs225014)	0.045327562	DIO2 (rs225014)	0.026992		
superoxide dismutase - detox from oxidative products	SOD2 (rs2758331)	**0.004987**	SOD2 (rs2758331)	**0.000221847**	SOD2 (rs2758331)	**0.004267**		
	SOD2 (rs4880)	**0.009887**	SOD2 (rs4880)	**0.000221847**	SOD2 (rs4880)	**0.014306**		
Glutathione S-transferase - detox from drugs, environmental toxins, oxidative stress			GSTM1 (insert/delete)	0.035014981	GSTM1 (insert/delete)	0.035015		
Monoamine oxidase					MAOA (rs6323)	0.019208		
5HT receptor 2A			5-HT2A (rs6311)	**0.002088939**				
Dopamine receptor D2			DRD2 (rs6277)	0.045500264				
			IFN-g (rs2430561)	0.025935446				
iodothyronine deiodinase deiodination of T4			DIO1 (rs2235544)	**0.012419331**				

Solute carrier - high affinity transport of organic anions (e.g. T4 and other hormones) may act at BBB					SLCO1C1 (rs10770704)	0.002199647						
Betaine--homocysteine S-methyltransferase 1 required for Hcyst to Methionine					BHMT (rs567754)	0.043951044						
Cystathionine beta-synthase downregulates methionine by converting HCYst to cycsteine					CBS (rs234706)	0.045327562						
Glutathione S-transferase P - conjugates glutathione to wide range of electrophiles/toxins					GSTP1 (rs1695)	0.041226833						

Table 8.5. All the genes variants shown are significantly associated with AD, indicated by the red bar, or with normal ageing, indicated by the blue bar, at p≤0.05 level. P values less than 0.01 are indicated by the darker grey cells. Mann-Whitney U test was used to compare Red:Yellow:green cells between normal ageing and severe AD. 2 separate Chi squared tests are also presented to compare differences when yellow is merged with red, or with green. The most significant association is with *APOE4* with *MTHFD1* next. These are significant on any test while *MTHFR* is only significant in Chi squared where yellow is merged with green. Other details are discussed in the text.

No significant association was found between gender or age for any of these gene SNPs. 25 gene variants were identified that were significant in any of the three tests with all significant at p≤0.05 and many at much higher significance of p≤0.01 or higher (bold p values in Table 8.5). Only 12 were significant using Mann-Whitney U tests while more were significant in either of the Chi Squared tests. Some were significant across all tests (Table 8.5). Even though *APOE4* is known in the literature to give a 40% risk of the disease, the current finding is surprising and significant in showing a 70% association in a small number of individuals picked for disease severity. Importantly for our hypothesis, 2 folate-related genes were found to be significantly associated with AD, methylene tetrahydrofolate dehydrogenase 1 (MTHFD1) and methylene tetrahydrofolate reductase (MTHFR), with MTHFD1 significant on all tests and MTHFR significant only on a Chi Squared test in which positive and neutral variants were grouped together and tested against negative variants. The enzymes derived from these genes are involved in the long and short pathways, respectively, for replenishment of the 5-methyl tetrahydrofolate pool (Figure 8.1).

Figure 8.1. Diagram of the folate metabolic cycle and links to other pathways

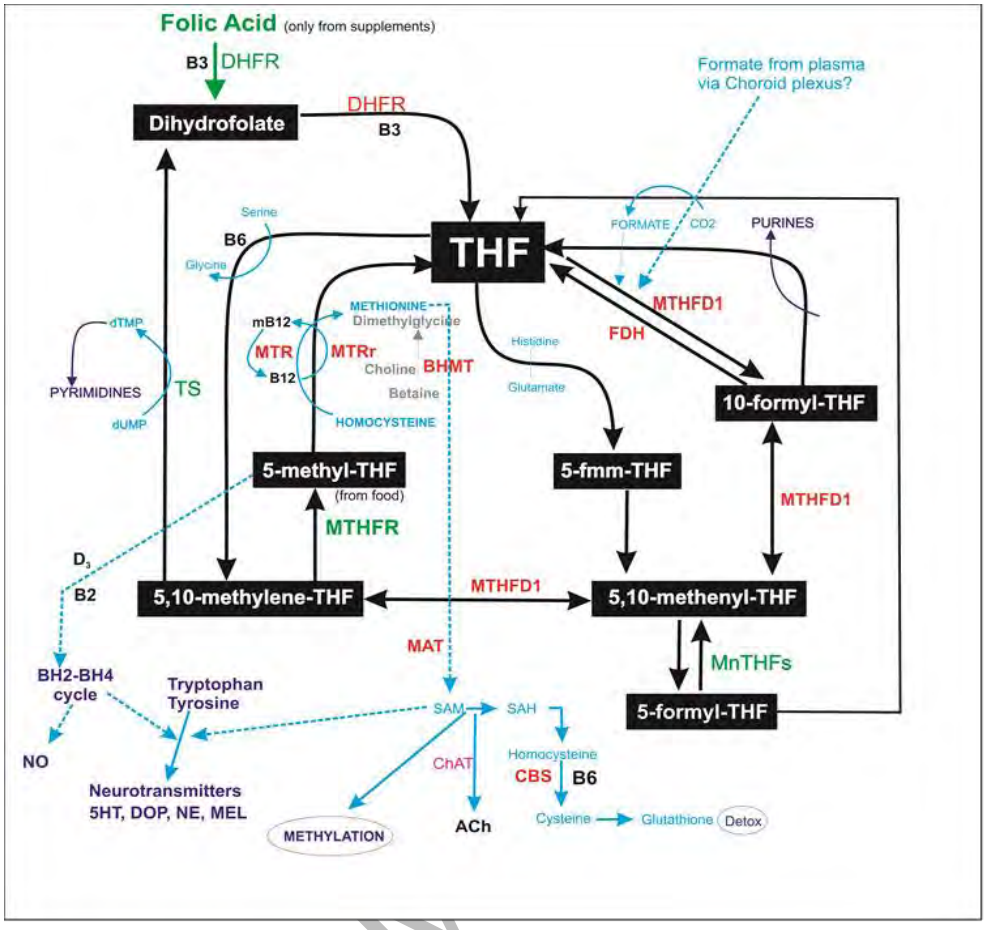


Figure 8.1. Diagram of the folate metabolic cycle and links to other pathways.

5methylTHF is the major species of folate derived from food and forms the recycling point for folate metabolism. It forms the rate limiting step, through the action of methionine synthase (MTR), in the methylation of vitamin B12 and thus the rate limiting step for methylation of homocysteine to methionine. It is thus critical to folate metabolism generally and to production of S-adenosyl methionine (SAM), the universal methyl donor for methylation reactions. MTHFD1 is a multi-role enzyme involved in three reactions in the folate pathway, forming the long route back to 5methylTHF. Tetrahydrofolate (THF) can be recycled back through 5,10 methyleneTHF, forming the short route and requiring B6 and serine to glycine reactions. Both long and short routes require methyleneTHF reductase (MTHFR) for the final step to 5methylTHF. MTHFD1 is further involved in recycling of 10formylTHF to THF, fuelling purine synthesis. 5,10methyleneTHF can also be reduced to dihydrofolate fuelling pyrimidine synthesis. Other pathways include 5mtheylTHF feeding directly into biogenic amine and nitric oxide synthesis through the BH4 cycle, and methionine feeding directly into the methylation pathway as well as acetylcholine synthesis. Folic acid is an artificial substance that enters the folate cycle without any 1 carbon moiety to supply to the metabolic process and so acts to dilute the 1 carbon pool as well as having other negative effects at higher doses (see text).

Thus an error in either or both of these would result in a drop in 5-methyl tetrahydrofolate availability, as well as raised homocysteine and reduced s-methyl-homocysteine (SAM), the universal methyl donor, resulting in reduced methylation as a consequence. Table 8.5 also shows the direction of association, i.e., whether associated with AD or with normal ageing. Gene SNPs associated with normal ageing may be providing some protection from AD. Other SNPs associated with AD are involved in monoamine transport at synapses (SLC18A10, SLC6A2) as well as involved in detoxification of xenobiotics and sulphites (CYP2D6, SUOX)). Those associated with normal ageing and not AD, that may therefore be protective against AD, are involved in monoamine metabolism, methylation and signalling (MAOA, ADRB1, COMT) and detox pathways (CYP2D6, SOD2,GSTM1). There are also SNPs involved in thyroid hormone activation and transport (DIO2, SLC01C1) and neurotransmitter receptors (5-HT2A, DRD2). The remaining SNP's, only significant on Chi Squared tests where negative and neutral SNPs are grouped, are associated with AD and are involved in methylation including betaine homocysteine methyl transferase (BHMT), cysteine beta-synthase (CBS), and glutathione S-transferase P1 (GSTP1).

8.3. Changes in metabolic profile associated with folate gene SNPs

We measured folate metabolites and enzymes in tissue lysates of normal and AD individuals with negative SNPs in *MTHFD1* and/or *MTHFR* and compared these to normal and AD individuals normal or neutral SNPs in these genes. The Individuals, their genotypes and results of analysis are shown in Table 8.6 with the data shown in graphical forms in Figure 8.3. There was no significant difference in tissue folate levels (Figure 8.2.) although the controls had a non-significant reduced level compared to the other groups (Table 8.6.). We therefore used the average folate level to calculate fold levels of the other metabolites and enzymes. In the severe AD cases that have negative SNPs in *MTHFD1*, there is a significant increase in glutathione that is seen in the severe cases without these SNPs. There is no effect of the negative SNPs on the levels of either *MTHFD1* or *MTHFR*. However, in severe cases with normal or neutral SNPs, there is no rise in glutathione but there is a significant rise in MTHFD1. There is also a significant rise in MTR in severe cases both with and without negative SNPs relative to controls and a small but non-significant ($p \leq 0.06$) increased MTR in severe cases without negative SNPs compared to severe cases with negative SNPs.

Table 8.6. Comparison of negative and positive gene SNPs on tissue metabolic profiles

a.

	Normal ageing				Alzheimer's					Controls				Alzheimer Controls			
Genes	12_11	09_31	14_09	14_11	12_01	13_45	14_50	19_29	19_31	11_25	14_08	17_36	17_34	19_04A	12_05B	12_32C	11_28D
MTHFD1 (rs1076991) 48	2	3	3	3	3	3	3	3	3	1	1	2	1	2	2	2	2
MTHFD1 (rs2236225) 49	3	2	3	3	3	3	1	1	3	1	1	2	2	2	1	1	
MTHFR (rs1801131) 50	1	2	2	1	3	3	1	3	1	2	2	1	1	1	2	2	2
MTHFR (rs1801133) 51	3	1	2	2	1	1	3	1	1	2	2	1	1	2	2	1	1
DOT BLOTS																	
Homocysteine	9190	10600	5850	10900	14800	12900	6640	7230	21100	8490	26100	3510	9720	14700	49000	18500	157
SAM	7010	27700	8080	23100	9330	16800	15500	18200	27000	6000	38000	8040	28000	11300	68900	32100	27500
Glutathione	58600	90200	91400	78100	97600	69100	160000	119000	97400	25000	60600	39000	43200	43800	95200	36900	63800
Folates	75800	44100	80800	60400	34800	32800	88700	40400	50600	19600	44700	46200	49700	46000	99200	31400	35100
WESTERN BLOTS																	
MTHFD1	8560	9910	2070	9990	5390	5490	4530	3360	4680	873	3540	6200	7650	19700	9210	36000	28500
MTHFR	NOT USED	NOT USED	4850	15000	2690	3130	5110	6980	6030	4850	4420	5290	5630	14100	3470	7200	6650
MTR	NOT USED	NOT USED	2900	3950	2020	2840	2380	1570	2120	1230	863	717	452	5290	4260	2740	2290

b. t tests relative to control

	p values		
	CvN	**CvA**	**CvAC**
Hcyst	0.554	0.915	0.303
SAM	0.267	0.737	0.139
Glut	**0.005**	**0.008**	0.098
MTHFD1	0.332	0.931	**0.033**
MTHFR	0.530	0.795	0.297
MTR	0.145	**0.002**	**0.013**
Folate	0.140	0.497	0.502

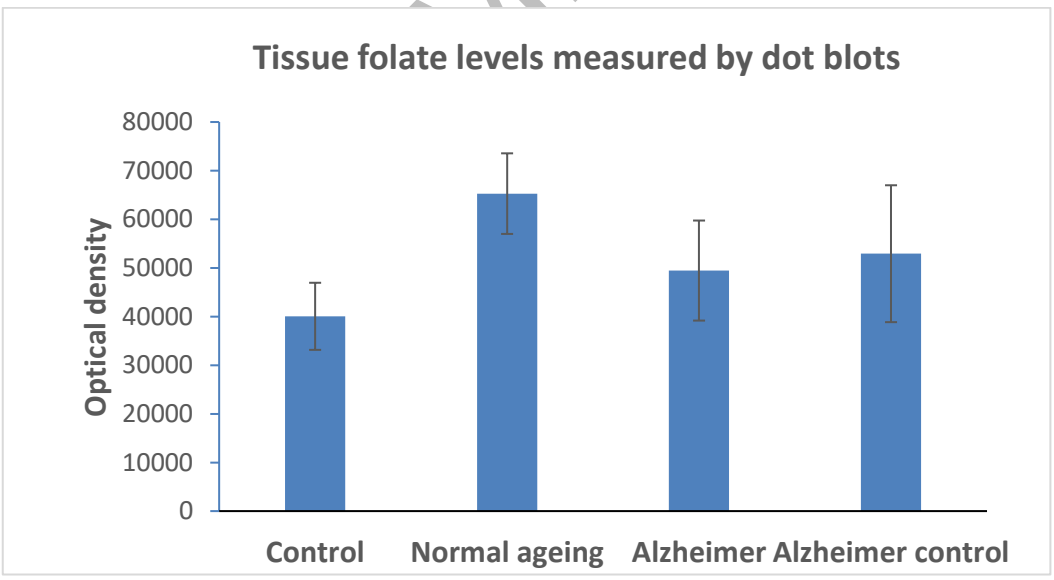


Figure 8.2. Tissue folate levels are not significantly different between controls and other groups with and without mutant SNPs in *MTHFD1* and/or *MTHFR*. The controls have lower level of folate than the other groups although this is not significant.

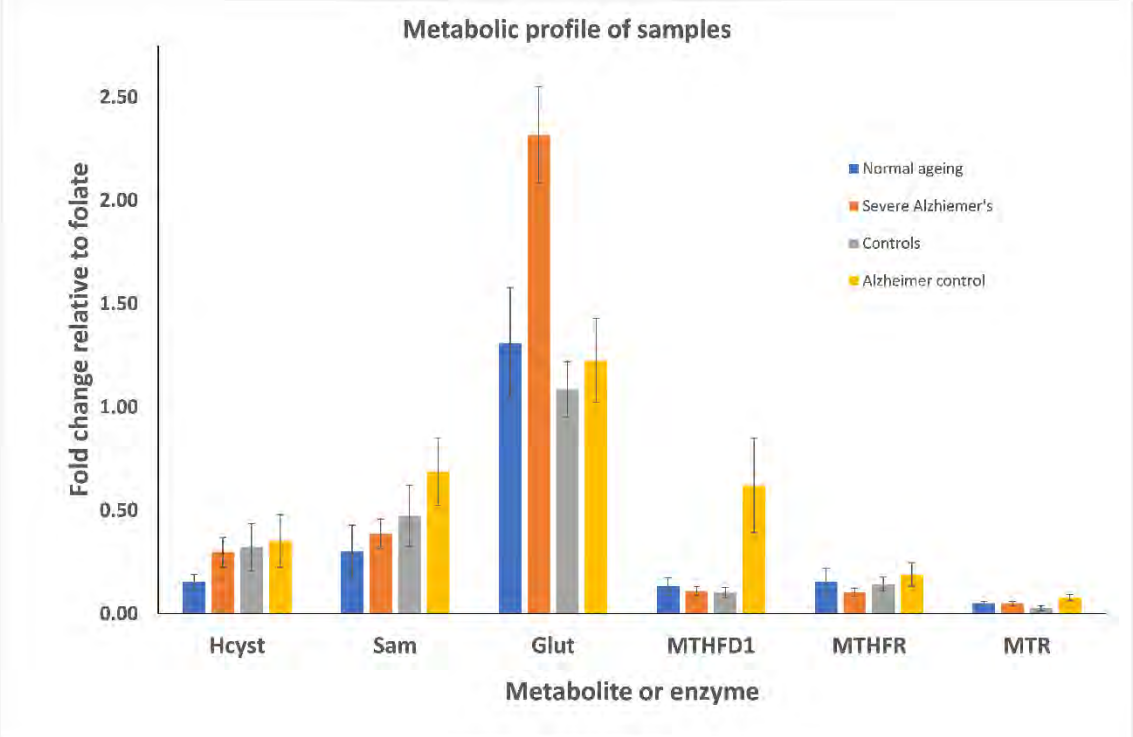


Figure 8.3. Metabolic profiles of samples analysed for key metabolites and enzymes plotted as fold of folate levels since this was the only consistent measure between the cases. Glutathione is significantly raised in Severe Alzheimer's with mutant gene SNPs for *MTHFD1* and *MTHFR*. This is not seen in any other group including Alzheimer's without mutant SNPs. In these latter cases we see significantly raised *MTHFD1* that is not mutant. In both cases we see potential protective mechanisms against raised homocysteine levels which are elevated in all cases except normal ageing although none are significant. MTR is also elevated in both affected and control Alzheimer's cases. This is not mutated so is also involved in metabolising 5methylTHF to methylate homocysteine to methionine and thus feed the methylation pathway. Together the increased glutathione and MTR would act to keep homocysteine levels low.

8.4. Discussion

There is an assumption in genetics and nutrigenomics literature that adverse gene SNPs have a negative effect on protein function and thus a knock on effect on the processes they are involved in. In this study we focused on the genes involved in folate metabolism, methylation and neurotransmitter synthesis, and also included *APOE* genotyping. Surprisingly we found a 70% association of *APOE4*, rather than the predicted 40%, with AD further reinforcing its high risk factor status and also indicating potential direct involvement in the condition in the severe cases used in this study. Several recent studies highlight the role of *APOE4* in a number of critical processes including involvement in amyloid plaque and neurofibrillary tangle formation, insulin resistance, decreased amyloid clearance, mitochondrial dysfunction and in autophagy (Hunsberger et al., 2019; Norwitz et al., 2021). Suggestions have been made for targeted drugs (Hunsberger et al., 2019) and nutritional and lifestyle changes (Norwitz et al., 2021) aimed at *APOE4* processes and pathways to prevent or treat the disease. The second most significant association with AD was *MTHFD1* SNP rs1076991. This was highly significant in all tests and thus we can suggest must be a significant risk factor for late-onset AD. Others have found a weak association of a different *MTHFD1* variant, SNP rs2236225, with early-onset AD (Bi et al., 2010; Dorszewska et al., 2007). We found a highly significant association of this variant with AD only in a Chi Squared test where negative and neutral variants were grouped together and tested against positive variants. There was no significance in a Mann-Whitney U test or the alternative Chi Squared test. So we can agree with the studies demonstrating the weak association of this variant but have found a very significant association with the other variant of *MTHFD1*, which is a novel finding of this study. MTHFD1 is a critical folate enzyme involved in 3 parts of folate metabolism (Figure 9.2.). It is involved in control of formate levels through the formation of 10-formylTHF from THF, which can then be used in purine biosynthesis. It balances formylTHF with 5,10methenylTHF and also mediates conversion of this to 5,10methyleneTHF which is either converted to 5mTHF by MTHFR, and thus forms the long pathway back to 5mTHF, or to dihydrofolate, fuelling the biosynthesis of pyrimidines in the process. DHFR then converts DHF to THF which picks up one carbon components from the conversion of serine to glycine to form 5,10methyleneTHF that can then be converted to 5mTHF, thus forming the short route to 5mTHF. Thus, a negative

variant of *MTHFD1* should have a remarkable effect on folate metabolic balance, decreasing the 5mTHF pool as well as potentially leading to formate toxicity and errors/reduced DNA synthesis and repair. Similarly, a negative variant of *MTHFR* should result in raised homocysteine levels that would exacerbate neurodegeneration and increase the risk to develop AD (Jiang et al., 2021). In our study we found only the *MTHFR* variant rs1801131 associated with AD but only in the Chi squared test putting neutral and positive variants together and tested against negative variants. No significance was found using Mann-Whitney U testing. In the cases studied here MTHFD1 is very significantly associated with AD while *MTHFR* is probably only weakly associated, as already reported for this variant (Liu et al., 2017) by contrast to other studies that found 1-3 abnormal SNPs of *MTHFR* associated with AD (Jiang et al., 2021; Peng et al., 2015; Roman, 2015). The effect of negative variants in *MTHFD1* and/or *MTHFR* were investigated by comparing normal and severe AD cases with and without negative SNPs in these two genes. We found a significant increase in glutathione in severe AD cases with negative variants compared to those with positive or neutral variants (Figure 8.3). We surmise that raised homocysteine, resulting from failure to regenerate 5mTHF, is being shunted to SAM and glutathione to prevent toxic build-up of homocysteine. Interestingly, this is not seen in severe cases with positive or neutral variants in *MTHFD1* and/or *MTHFR*. In these cases we found a significant increase in MTHFD1 and in MTR perhaps in response to raised homocysteine to increase methylation. This would also fuel the hypermethylation seen in the AD cortex and previously reported by us and others (Miyan et al, 2021, in press). Also, or interest is the fact that we found no significant difference in tissue levels of folate indicating that the changes seen are likely to be a response to ineffective gene products and/or to physiological changes in metabolism rather than folate supply.

The other gene variants associated with AD are involved in monoamine neurotransmitter delivery to and reuptake into synapses, and in detoxification from xenobiotics and sulfites. 2 genes involved in the methylation pathway are weakly associated with AD finding significance in only one of the Chi Squared tests. These are involved in the conversion of homocysteine to methionine (BHMT) and in generation of cysteine from homocysteine in the pathway to glutathione (CBS). The remaining significant associations are with normal ageing indicating a possible protective effect of these gene

variants. These are involved in receptors for and breakdown of monoamines as well as in detoxification through breakdown of various drugs and environmental toxins. Interestingly *COMT* variant rs4633 has been associated with AD (Babic Leko et al., 2020) while in the current study it is clearly associated with normal ageing. Other variants of *COMT* have been found to be not associated with AD or other psychiatric conditions (Patel et al., 2018; Zalsman et al., 2005; Zalsman et al., 2008) indicating a possible association with other factors rather than directly to disease aetiology or progression.

8.5. Conclusions

In this initial pathway analysis of genes involved in folate metabolism, methylation and neurotransmitter synthesis, as well as *APOE* genotyping, we found significant associations (summarised in Table 8.5) of a key gene involved in folate metabolism, *MTHFD1*, genes involved in monoamine vesicular transport and reuptake and areas of detoxification, as well as a 70% association of *APOE4* with AD. At the same time, we found significant associations of variants of *COMT* and *SOD2* as well as *CYP2D6* with normal ageing suggesting a protective effect. Other gene SNPs were significant only on a Chi Squared test when neutral SNPs were grouped with positive or negative SNPs (see Table 8.5). Interestingly, these include *MTHFR* which reportedly affects around 30% of the population is implicated in abnormal folate metabolism and raised homocysteine levels (Suormala et al., 2002). In our analysis SNPs of this gene were not associated with AD except when neutral and positive SNPs were grouped and analysed with negative SNPs. Thus, in our analysis the most important gene to be exposed is the *MTHFD1* gene which is critical in a number of folate metabolic reactions and has 4 enzymatic profiles: i. methylenetetrahydrofolate dehydrogenase (NADP+ Dependent) 1, ii. methenyltetrahydrofolate cyclohydrolase, iii. formyltetrahydrofolate synthetase, and iv. C-1-tetrahydrofolate synthase, reflecting its importance to the folate metabolic cycle (Figure 8.1.). However, even this is not 100% associated with AD and is also present in many normal ageing samples. Thus, it can only be added to the increasing number of risk factors for this disease. Finally, we propose that one trigger for the onset and severity of AD may be a physiological change associated with a cerebral CSF drainage issue and associated cerebral folate issue reflecting the fact that severity of this, and other cerebral conditions, is associated with increasing fluid accumulation and ventricular dilation. Life events that decrease drainage capacity might include infection, inflammation, and trauma

or accelerated cell loss in these susceptible ageing individuals. Strategies to maintain drainage, and perhaps increase drainage may therefore present an effective target for prevention and treatment of this condition.

Chapter 9

Discussion

Chapter 9

Discussion

9.1. CSF and folate in the ageing and AD brain

Research into AD has failed to produce effective treatments to prevent, arrest or reverse the condition. Essentially most/all hypotheses regarding this condition are based on pathophysiology during disease progression and end stages of disease resulting in little, if any focus on causation. Published data indicates that severity of the disease can be associated with enlargement of the ventricles (Nestor et al., 2008):

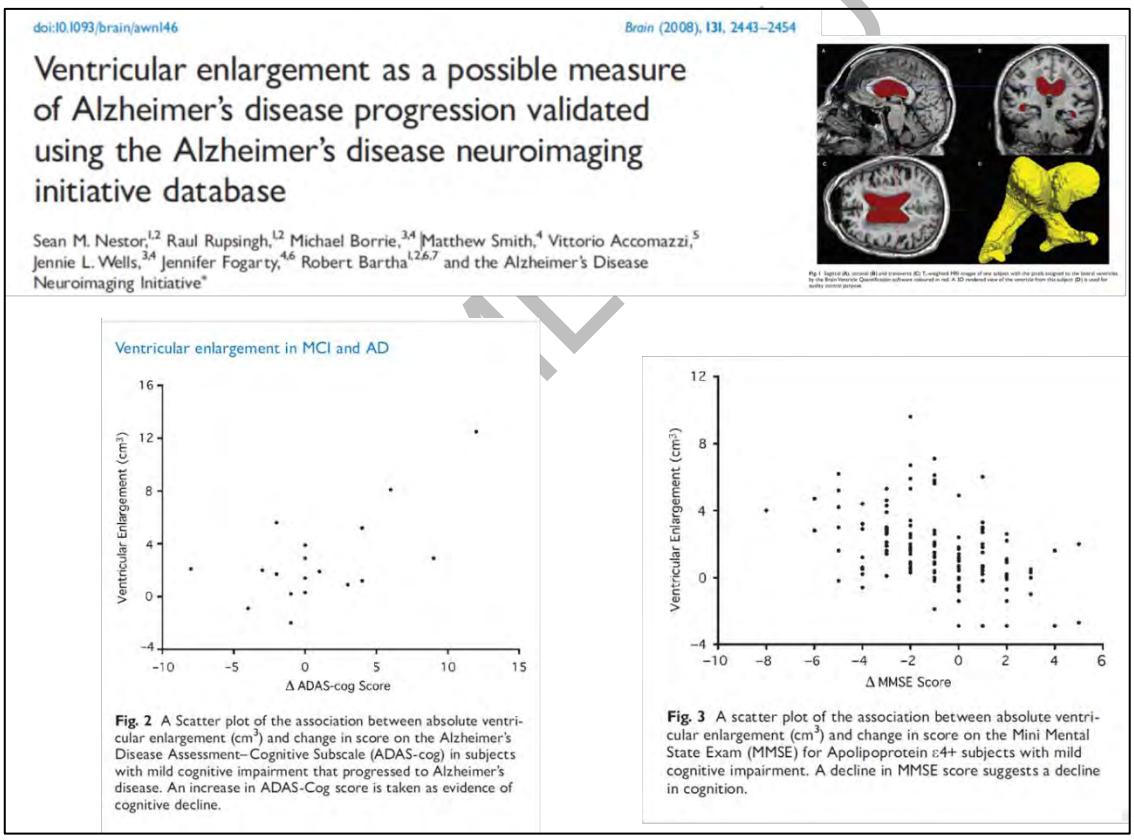


Figure 9.1. Composite figure showing data from Nestor et al (2008) showing enlarged ventricles and graphs demonstrating associations of enlargement of ventricles with severity of the condition using two different psychometric tests, ADAS and MMSE.

Further evidence demonstrates reduced CSF outflow from the choroid plexus at the same time as reporting raised intracranial pressure (Ott et al., 2010; Silverberg et al., 2006; Silverberg et al., 2001; Silverberg et al., 2003). Thus, there must be an imbalance between

production and drainage even in this condition where CSF output has been decreased. These data strongly implicate a CSF drainage insufficiency in the pathophysiology of AD. The extreme version of CSF drainage obstruction is hydrocephalus and in this condition our lab and others have reported a cerebral folate imbalance and a profound effect on the progression of development and function of the cerebral cortex. So this project was designed to test the hypothesis that an early process in the disease may be CSF drainage obstruction, not sufficient for hydrocephalus, but sufficient for a change in folate metabolism and associated effects on cell proliferation, neurotransmitter synthesis, methylation and other pathways involving folate. Evidence in the literature is contradictory on the role of folate in AD as blood folate or CSF folate deficiency was examined but not the details of folate transport and metabolism (Boston et al., 2020; Murdaca et al., 2021; Robinson et al., 2018; Zhang et al., 2021). In this study, CSF and brain tissue was analysed for folate metabolic status and to investigate the pathway for folate delivery comparing normal, moderate and AD brains.

Initially the concentration of FDH was measured and was found to be significantly reduced in the CSF from both moderate and severe cases compared to normal aged brains. This provided initial support for the hypothesis of reduced CSF drainage as reduced FDH has been reported associated with this in hydrocephalus but not in non-hydrocephalic siblings (Cains et al., 2009; Jimenez et al., 2019; Naz et al., 2016). Tissues were then further analysed for changes in folate status. A global reduction in all folate enzymes was found in the CSF of both moderate and severe AD brains compared to normal. The main transporter for folate from blood, FRα, was decreased in moderates but at normal levels in AD, while folate was increased in moderates and at normal levels in AD. This presents a picture of a potential imbalance in folate rather than a deficiency which follows from similar observations in hydrocephalus (Cains et al., 2009).

In the normal ageing brain folate is transported by FRα+FDH into the network of FDH-positive astrocytes. This is different to the neonatal brain in which FDH alone is found in FDH-positive radial glia and FRα remains in the CSF (Cains et al., 2009; Jimenez et al., 2019; Naz et al., 2016). FDH-positive astrocytes have been described in the literature and FDH antibodies are sold as astrocyte markers. However, the significance of these specific FDH-positive, GFAP-negative astrocytes has not been investigated. The data presented here implicates this network as the main pathway for folate delivery from the CSF

throughout the cortex and perhaps the rest of the brain, but this is not the only mechanism. Physiologically, the CNS lymphatic drainage system with the glymphatic system and meningeal lymphatics as the core which efficiently helps in the clearance. In normal brains FRα and folate are co-localised in these cells. This makes the FDH-positive, GFAP-negative astrocytes of huge significance to the function of the normal brain when the role of folate metabolism is fully understood (see below).

In the AD brain, the FDH-positive, GFAP-negative astrocyte network remains intact when observed after IHC staining. However, in the temporal cortex of AD brains analysed in this study, this pathway was NOT transporting folate. There was no co-localisation of FRα or folate with FDH in this pathway. Instead, this study found that FRα and folate were co-localised in the GFAP-positive astrocyte network and that this was associated with folate accumulation in neurons and hypermethylation (5methyl cytosine). Very little demethylation (5-hydroxymethyl cytosine) was observed in the AD brain compared to the widespread staining for this in normal aged.

This study also investigated genes involved in the folate, methylation and neurotransmitter pathway using a nutrigenomics approach. Using this approach, the gene coding for MTHFD1 was found to have an abnormal SNP significantly associated with the AD samples and not present in normal aged brains. In addition, an abnormal SNP in *MTHFR* was also found to have a weak association with the AD samples used in this study. Thus, two very important folate enzymes have an abnormality associated with the small sample of AD brains used in this study. In addition to the gene SNP identification, a change in folate metabolism was detected in tissue lysates from these brains. In AD/normal brains without abnormal genes, there was increased concentrations of MTHFD1 which is presumed to occur to feed 5mTHF, through the long pathway, to maintain low levels of homocysteine as well as provide methionine for the methylation pathway. In individuals with abnormal MTHFD1, there was, by contrast, no change in MTHFD1 but a significant increase in glutathione. This would have the same effect of reducing toxic levels of homocysteine via the methylation pathway and also indicates, significantly decreased recycling of folate back to 5mTHF. Thus, this significant change in folate metabolism compensates for the abnormal MTHFD1 which would not be seen without this detailed analysis of genes and metabolism.

9.2. Folate metabolism

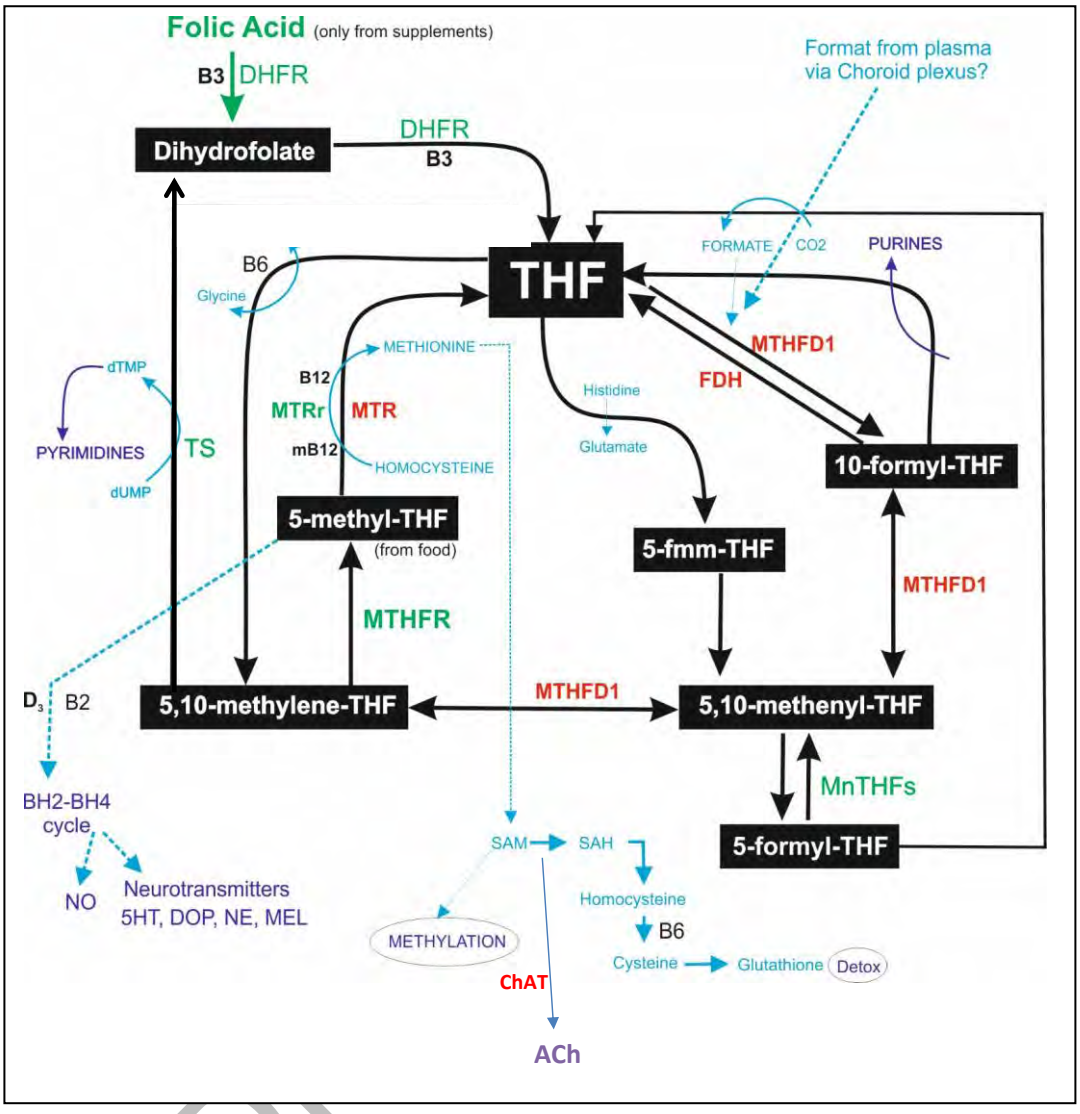


Figure 9.2. Diagram of folate (1-carbon) metabolism demonstrating the links to nucleotide synthesis, neurotransmitter and nitric oxide synthesis, and methylation.

Figure 9.2. shows the inter-relationships between the folate metabolic cycle, DNA synthesis, methylation pathway, and neurotransmitter and nitric oxide synthesis. From this, it is clear that errors or issues with folate metabolism can have severe consequences for brain function, through synthesis of neurotransmitters, including biogenic amines and acetylcholine, the production and maintenance of cells, cardiovascular and neurovascular health through nitric oxide synthesis, etc. 5-methyl tetrahydrofolate (5mTHF) is the main dietary form of folate and is the usual entry point into folate metabolism from where it feeds 2 pathways. The dihydro-tetrahydro biopterin (BH2-BH4) cycle produces tetrahydrobiopterin from 5mTHF that is required for nitric oxide synthesis, linked to

cardiovascular health, and biogenic amine synthesis, producing some of the key neurotransmitters of brain functions including cognition, learning, memory, attention, mood, and sleep. 5mTHF also passes through a rate-limiting step, transferring its methyl group to vitamin B12 that then methylates homocysteine to methionine producing tetrahydrofolate (THF) that feeds into other parts of the metabolic cycle. Methionine is converted to s-adenosyl methionine (SAM), the universal methyl donor involved in most methylation reactions. THF is a central hub for folate metabolism. It can produce 5,10-methylene THF through the conversion of serine to glycine. This can cycle back to 5mTHF or convert to dihydrofolate (DHF) giving up its methylene to thymidylate synthase that produces pyrimidines, key nucleotides in DNA and RNA synthesis. DHF is hydrolysed back to THF. THF can also receive a formyl group through conversion of formate from blood plasma that also exists in CSF (Eells et al., 2000), or through metabolism of 5-formyl-THF. This is mediated by methylene-THF-dehydrogenase 1 (MTHFD1) that also converts the product, 10-formyl THF to 5,10-methenyl THF. MTHFD1 also acts to balance the 2 halves of folate metabolism through interconversion of 5,10-methenyl and 5,10-methylene THF. These 3 reaction steps, mediated by MTHFD1 are known as the long route back to 5mTHF while MTHFR mediates the final step from 5,10-methylene THF back to 5mTHF for both the long route and short route. 10-formyl-THF dehydrogenase (FDH) acts as a buffer to maintain a pool of the reactive THF as it is known to bind tighter to this product than to the substrate 10-formyl-THF (Anguera et al., 2006). This would also have the effect of depleting 1-carbon availability in the presence of high levels of FDH that would decrease 10-formyl-THF levels and prevent downstream conversions including purine synthesis. This would explain cell cycle arrest in cancer and other cells produced by elevated levels of FDH (Krupenko and Oleinik, 2002). In hydrocephalus the inhibition of secretion of FDH from the radial glial cells results in raised concentrations within the cells that may be responsible for the cell cycle slow down/arrest observed in the fetal/neonatal hydrocephalic brain (Owen-Lynch et al., 2003). FDH is known to bond to its product, THF, more than to its substrate, 10fTHF, so that it acts as a buffer for available folate in the form of THF. If true, then a lack of FDH would produce a severe folate deficiency if the system is dependent on supply of THF from CSF metabolised 5mTHF.

The lack of FDH in the CSF means that there is none available for binding to FRα-folate, prohibiting entry to the normal pathway of the FDH-positive radial glial cells. In AD, this loss of FDH in CSF has a similar effect in barring folate uptake into the FDH-positive astrocytes. In hydrocephalus, the unbound (to FDH) folate is then available to cells that can utilise free folate, in particular arachnoid cells that do not need FDH binding and can take the folate directly (Jimenez et al., 2019). This stimulates arachnoid proliferation to generate additional drainage, but which does not balance the continuous outflow of CSF. In AD, in the absence of FDH binding, FRα-folate enters an alternative pathway involving GFAP-positive cells. Thus, in both neonatal hydrocephalus and in ageing AD brains normal pathways for folate uptake, requiring FDH binding of folate are not available and alternate pathways are available to unbound folate. Both pathways may operate in the normal brain but in the AD brains a major switch is observed from one to the other.

The genetic defect found in this study in *MTHFD1* can also now be seen in context of total folate metabolism and its triple role in 3 arms of folate metabolism forming the long pathway back to 5mTHF. Loss of activity in *MTHFD1* would have consequences on formate metabolism, possibly forming formic acid and resulting in acidosis, as well as negatively affecting production of purines. Furthermore, it would reduce the recycling of folate back to 5mTHF and therefore decrease homocysteine methylation, increasing the concentration of this toxic molecule. The alternative pathway for recycling folate would then be via dihydrofolate and to THF but this pathway would bypass the conversion of homocysteine to methionine with consequences on methylation. The same would effectively occur with a mutation in *MTHFR* but the literature suggests this does not happen completely and that even in homozygous negative mutations of *MTHFR*, 30% of normal folate metabolism persists (Suormala et al., 2002). This may explain the weak link to AD that was found in this study. Figure 2 also shows the entry point of folic acid, a synthetic, stable form of folate and thus may also explain some of the negative effects observed with high dose folic acid supplementation. Folic acid has no 1-carbon moiety to donate so practically dilutes the 1-carbon pool becoming useful only after conversion to DHF, then THF and then picking up a 1-carbon in conversion to 5,10 methylene THF, 5 formamido THF or 10-formyl THF (see Figure 9.2).

9.3. Main outcomes of this research

Although the number of brains analysed in this study were small, the study compared normal ageing, with severe AD. Thus, the study compared the extreme cases and the data demonstrate significant differences that could now be applied to all cases to determine if they are involved in the progression of the disease. The findings of an abnormality in *MTHFD1* associated with AD is particularly interesting as a direct genetic route to a folate deficiency that could underlie the progression of the condition, and this clearly needs to be followed up with much larger study to determine the prevalence of this gene SNP in AD, particularly in early diagnoses. It is possible that the disease progression might then be halted with simple folate supplements as has already been described in the literature (Cains et al., 2009). In those individuals with abnormal function in *MTHFR,* they would also suffer a loss of recycling of folate back to 5mTHF so that this also be a risk factor for developing AD although this has clearly not been reported in the literature referring to the 30% of the population that has abnormal *MTHFR* function (Suormala et al., 2002). These genetic findings may explain many cases of AD. In addition, the main hypothesis tested in this body of work was a change in cerebrospinal fluid flow, dynamics and drainage as an underlying cause of cerebral folate imbalance leading to AD. This was originally speculated based on the association between disease severity and ventricular enlargement described in a number of papers in the literature (e.g. Figure 9.1 and 9.3).

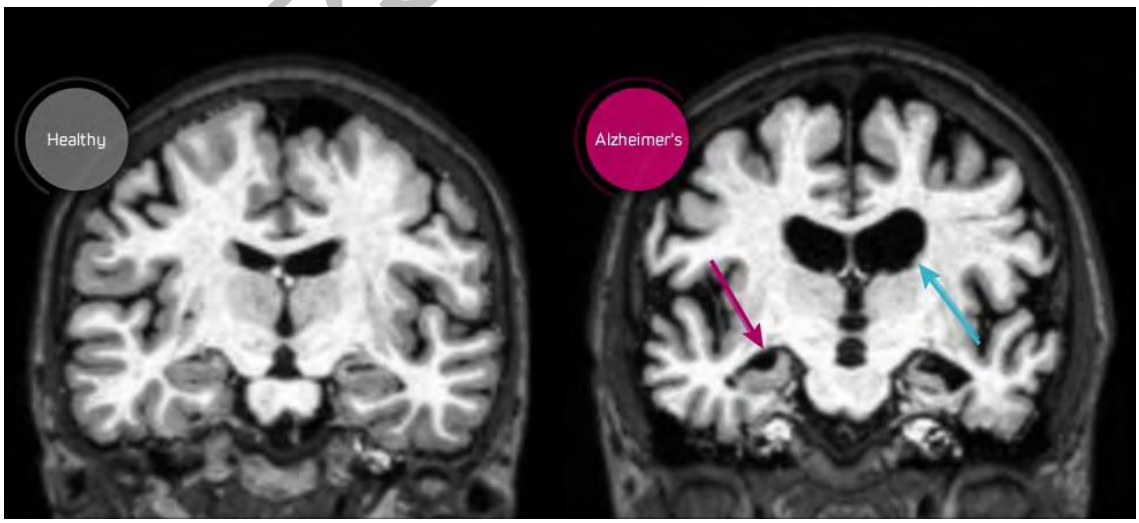


Figure 9.3. MRI images of normal (left) and AD (right) brains showing the significant reduction in brain tissue but also a significant enlargement of the ventricles (blue arrow) in the AD brain.

In studies of hydrocephalus, a profound change in folate metabolism was reported in the Hydrocephalic Texas (HTx) rat in which FDH was significantly reduced or absent from the CSF of affected individuals. In addition, more recent proteomics analysis demonstrate the profound nature of the metabolic change in hydrocephalus (Requena-Jimenez et al., 2021) which also contains top level controllers for the metabolic change. With the previous studies of the UK group as well as those of other groups investigating CSF, it is becoming very clear that CSF is a vital, physiological fluid specifically formulated for cerebral cortical health and function (Bueno et al., 2020; Cains et al., 2009; Gato et al., 2020; Miyan et al., 2020; Miyan et al., 2006). Where it undergoes changes due to fluid drainage obstruction/insufficiency then cortical development and function have been shown to be severely affected. Indeed, hydrocephalus has been shown to cause a developmental arrest in the developing cerebral cortex whereas previous views were that hydrocephalus caused damage to the cortex through fluid accumulation, ventricular enlargement and pressure on the cortical tissue (Owen-Lynch et al., 2003).

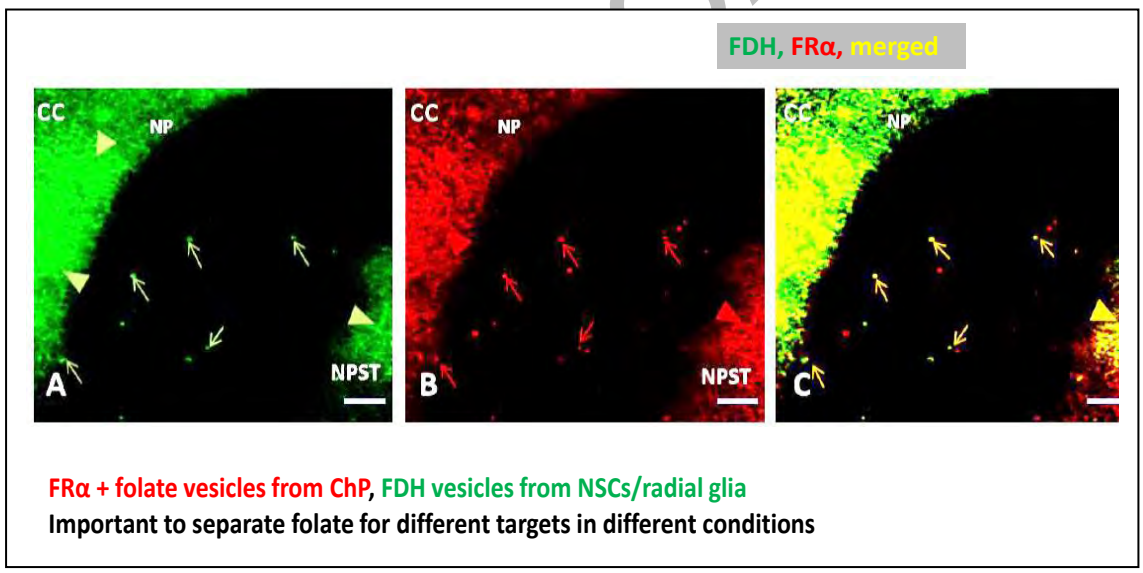


Figure 9.4. from (Jimenez et al., 2019) showing association of FRα and FDH in vesicles in the CSF. These two molecules are found colocalised in some vesicles, presumably with folate, for transfer into radial glia/FDH+ve astrocytes. Vesicles with only FRα transport folate around the CSF pathway for other cells to use unbound to FDH.

In AD then, this study found a similar, and significant reduction in CSF FDH. This supports the hypothesis that ventricular enlargement in AD indicates a fluid drainage problem rather than loss of brain tissue due to pathology which occurs outside the ventricular system with decreasing size of gyri and increasing sulci. The fluid drainage

problem had been described for ageing and dementia in which they found decreased CSF volume production but with raised intracranial pressure and ventricular enlargement. Thus, the observed decreased CSF volume output was not stopping the build-up of fluid, nor the rise in pressure (Ott et al., 2010; Silverberg et al., 2006; Silverberg et al., 2001; Silverberg et al., 2002; Silverberg et al., 2003) indicating a clear problem in drainage. In addition to the decrease in CSF FDH, this study also found a profound change in transport of folate into the brain, a reduction in folate metabolism as well as a change to hypermethylation.

The hypothesis generated from studies of neonatal hydrocephalus was that FRα transported folate into the CSF across the choroid plexus and then FDH also bound to the folate along with FRα. This FDH binding was found to be necessary for normal folate uptake into the brain. In the absence of the FDH, FRα-folate was present in the CSF but not available to the cells of the brain. Instead, the unbound folate passed through the CSF pathway into the subarachnoid space and stimulated proliferation of the arachnoid membrane cells (Jimenez et al., 2019). In neonatal hydrocephalus, the cells containing FDH are radial glial cells that are also the neural stem cells of the developing cortex. In the adult cortex, radial glia are no longer present and FDH is now found in a network of FDH–positive astrocytes. These must be the source of FDH found in the CSF and are also the pathway for FRα-folate-FDH to enter the cortex and supply the cells of the brain. In AD CSF, FDH is not released from the cells and the consequence appears similar to the same loss in hydrocephalus, where cells of the brain can now no longer access available folate. However, in the AD brain we found that a different pathway for folate uptake became activated, which pathway is not present in the developing cortex. This is the GFAP-positive network of astrocytes. These FDH-negative astrocytes, most probably also involved in nutrient supply to the brain, in the AD brain now become the major pathway for folate transport. This may be a consequence of the loss of FDH binding to folate in the CSF that would otherwise restrict transport to the FDH-positive astrocytes. This appears to be an amazing mechanism both in the developing brain, to increase drainage through arachnoid proliferation, and in the adult brain to maintain folate supply to the cortex. However, in the AD brain we observed a change in metabolism associated with the change in pathway for folate delivery to hypermethylation. In this case very little demethylation was observed compared to that seen in normal aged brains. This may

indicate a general shut down of cell activity in response to the on-going pathology perhaps to prevent further loss of neurons. This may also be the basis of some aspects of loss of affect in these patients not directly attributable to loss of neurons alone.

9.4. Folate metabolism in the brain

A significant finding of this study was that, while the CSF contains all the folate enzymes except MTHFR, these enzymes are present in variable numbers of neuronal cells with FDH found in a unique set of astrocytes and MTR in some GFAP positive cells. Thus, while the whole of folate metabolism is potentially possible in the CSF, it is separated across different cells in the cortex. As discussed in chapter 6, two immediate possibilities could explain these observations.

9.4.1. Model 1

FDH as the main transporter could deliver 5mTHF, 10 formyl THF, or THF throughout the cortex. 5mTHF must be converted to THF by MTR and MTRr as well as being used in the BH2-BH4 cycle for neurotransmitter and nitric oxide synthesis. Thus, MTR and MTRr in the neurones must be receiving 5mTHF to convert to THF and methylate homocysteine. However, Figure 6.6. shows that homocysteine may be separated in astrocytes and is not in neurones suggesting that all homocysteine is removed from neurones rapidly to avoid its toxic effects. Presence of other folate enzymes in neurones indicates the ability for limited conversion of folate metabolites for specific metabolic tasks in those cells.

9.4.2. Model 2

In this model the evidence indicates that folate metabolism occurs in the CSF and that FDH positive astrocytes then transport the metabolites throughout the cortex with specific metabolites used by different cells. In addition, it looks likely that some cells, particularly pyramidal cells in the cortex, may be able to take specific metabolites, perhaps with bound enzymes from the CSF at the pial surface where their apical processes originate.

In both models, the loss of FDH, observed in AD CSF, would have an effect on folate transported through the FDH-positive astrocyte network as this shows the need of FDH bound to folate to extract folate from the CSF. The alternative model would then allow

for folate to pass through other pathways and/or directly to neurones connected to the pial surface and/or via GFAP astrocytes.

9.5. Speculation on potential treatment

In neonatal hydrocephalus, it has been found that bypassing the FDH block to normal folate transport, through supplementation with alternative forms of folate, can reverse the effects of fluid drainage obstruction and also "repair" the drainage system to remove/prevent the hydrocephalus (Cains et al., 2009).

So, what would happen with a similar folate supplement for AD? Although AD occurs in ageing brains, it is possible that the supplement could "repair" the drainage faults and return the CSF pathway to something reflecting normal flow and drainage. In addition, or even in the absence of "fixing" the drains, alternative folates should enter the normal pathway for folate uptake, i.e., the FDH-positive astrocyte network, as well as the alternative pathways available. This would then potentially restore normal function, i.e., all functions dependent on folate supply including DNA synthesis, methylation, neurotransmitter synthesis, nitric oxide synthesis, etc., and perhaps arrest further pathology. Early treatment of mild cognitive impairment might, therefore, even prevent conversion to AD. This of course requires a larger study to determine if the observations reported in this study are generally the case in conditions of dementia and AD.

9.6. Conclusion

This study set out to determine whether a fault in cerebral folate exists in AD and whether this is similar to that reported for hydrocephalus. The study confirmed a similar loss of FDH in the CSF associated with a change in folate supply to the cortex and a change in metabolism towards hypermethylation. Thus, the study supports the hypothesis of a CSF drainage obstruction resulting in physiological changes in the brain CSF pathway for nutrient supply and consequential loss of function and neurodegeneration. Although the study is on a small number of patients, the results are sufficiently significant to support the conclusions and lay the foundation for a bigger investigation to confirm the general applicability of the findings.

9.7. Limitations

The study was funded by Commonwealth Scholarship Commission (CSC) due to which we were restricted to purchase limited number of brains. Moreover, due to the COVID-19 pandemic, it was very difficult to get antibodies and other consumables in time. The brains which were used in this study were formalin fixed so in some IHC sections it was difficult to see the antigen antibody interaction which was sorted out by positive and negative controls. Due to limited time (after COVID-19 pandemic) and funding constraints we couldn't conduct experiments to show specific CSF flow and clearance mechanisms. Other limitation is that Manchester brain bank don't have age at which AD is first time diagnosed.

9.8. Future perspectives

This study used post-mortem tissues exclusively. Thus, the study findings need to be confirmed, at least for CSF, in tissue taken from living patients with AD.

All folate enzymes were found to be present in CSF but only one or two were present in specific cells, not all, in the cortex. Thus, to determine which cells are generating the enzymes and which are simply taking them from the CSF, we need to carry out in situ PCR or hybridisation studies to identify which cells are making these enzymes. This is important to determine the details of folate metabolism in the brain. For example, does the CSF contain all of folate metabolism and simply supplies metabolites to cells as they need them? Where are the folate proteins coming from? Choroid plexus, FDH astrocytes, neurons, blood or other source?

Confocal microscopy and 3D reconstruction will allow a high-resolution determination of the interaction between cells containing folate, folate proteins and receivers. In addition, this will also allow visualisation of multiple proteins using different wavelengths of Alexa Fluor secondary antibodies. Thus, it may be possible to look at sub-cellular localisation of folate metabolism in astrocytes and neurons. Importantly, it will be possible to investigate the transport mechanisms at the ventricular ependymal barrier, the pial barrier and the blood-brain barrier.

With neurologists it may also be possible to test the utility of folate supplements in slowing/halting the progress of this debilitating condition. There are already reports of the beneficial effects of folate for dementia so this would be a promising direction into clinical work.

Chapter 10

References

Chapter 10

References

(2009). 'In this issue', *Proteomics,* 10(1).

Agnihotri, A. & Aruoma, O. I. (2020). 'Alzheimer's Disease and Parkinson's Disease: A Nutritional Toxicology Perspective of the Impact of Oxidative Stress, Mitochondrial Dysfunction, Nutrigenomics and Environmental Chemicals', *J Am Coll Nutr,* 39(1), pp. 16-27.

Aisen, P. S., Cummings, J., Jack, C. R., Jr., Morris, J. C., Sperling, R., Frolich, L., Jones, R. W., Dowsett, S. A., Matthews, B. R., Raskin, J., Scheltens, P. & Dubois, B. (2017). 'On the path to 2025: understanding the Alzheimer's disease continuum', *Alzheimers Res Ther,* 9(1), p. 60.

Al-Baradie, R. S. & Chaudhary, M. W. (2014). 'Diagnosis and management of cerebral folate deficiency. A form of folinic acid-responsive seizures', *Neurosciences (Riyadh),* 19(4), pp. 312-6.

Alam, C., Aufreiter, S., Georgiou, C. J., Hoque, M. T., Finnell, R. H., O'Connor, D. L., Goldman, I. D. & Bendayan, R. (2019). 'Upregulation of reduced folate carrier by vitamin D enhances brain folate uptake in mice lacking folate receptor alpha', *Proc Natl Acad Sci U S A,* 116(35), pp. 17531-17540.

An, Y., Feng, L., Zhang, X., Wang, Y., Wang, Y., Tao, L., Qin, Z. & Xiao, R. (2019). 'Dietary intakes and biomarker patterns of folate, vitamin B6, and vitamin B12 can be associated with cognitive impairment by hypermethylation of redox-related genes NUDT15 and TXNRD1', *Clin Epigenetics,* 11(1), p. 139.

Andreasen, N. C., Olsen, S. A., Dennert, J. W. & Smith, M. R. (1982). 'Ventricular enlargement in schizophrenia: relationship to positive and negative symptoms', *Am J Psychiatry,* 139(3), pp. 297-302.

Anguera, M. C., Field, M. S., Perry, C., Ghandour, H., Chiang, E. P., Selhub, J., Shane, B. & Stover, P. J. (2006). 'Regulation of folate-mediated one-carbon metabolism by 10-formyltetrahydrofolate dehydrogenase', *J Biol Chem,* 281(27), pp. 18335-42.

Apostolova, L. G., Green, A. E., Babakchanian, S., Hwang, K. S., Chou, Y. Y., Toga, A. W. & Thompson, P. M. (2012). 'Hippocampal atrophy and ventricular enlargement in normal aging, mild cognitive impairment (MCI), and Alzheimer Disease', *Alzheimer Dis Assoc Disord,* 26(1), pp. 17-27.

Babic Leko, M., Nikolac Perkovic, M., Klepac, N., Svob Strac, D., Borovecki, F., Pivac, N., Hof, P. R. & Simic, G. (2020). 'Relationships of Cerebrospinal Fluid Alzheimer's Disease Biomarkers and COMT, DBH, and MAOB Single Nucleotide Polymorphisms', *J Alzheimers Dis,* 73(1), pp. 135-145.

Bailey, S. W. & Ayling, J. E. (2009). 'The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake', *Proc Natl Acad Sci U S A,* 106(36), pp. 15424-9.

Benveniste, H., Lee, H. & Volkow, N. D. (2017). 'The Glymphatic Pathway: Waste Removal from the CNS via Cerebrospinal Fluid Transport', *Neuroscientist,* 23(5), pp. 454-465.

Berrios-Rivera, S. J., Bennett, G. N. & San, K. Y. (2002). 'Metabolic engineering of Escherichia coli: increase of NADH availability by overexpressing an NAD(+)-dependent formate dehydrogenase', *Metab Eng,* 4(3), pp. 217-29.

Bi, X. H., Zhao, H. L., Zhang, Z. X., Liu, Q. & Zhang, J. W. (2010). 'Association analysis of CbetaS 844ins68 and MTHFD1 G1958A polymorphisms with Alzheimer's disease in Chinese', *J Neural Transm (Vienna),* 117(4), pp. 499-503.

Bidla, G., Watkins, D., Chery, C., Froese, D. S., Ells, C., Kerachian, M., Saskin, A., Christensen, K. E., Gilfix, B. M., Gueant, J. L. & Rosenblatt, D. S. (2020). 'Biochemical analysis of patients with mutations in MTHFD1 and a diagnosis of methylenetetrahydrofolate dehydrogenase 1 deficiency', *Mol Genet Metab,* 130(3), pp. 179-182.

Bloom, G. S. (2014). 'Amyloid-beta and tau: the trigger and bullet in Alzheimer disease pathogenesis', *JAMA Neurol,* 71(4), pp. 505-8.

Bonkowsky, J. L., Ramaekers, V. T., Quadros, E. V. & Lloyd, M. (2008). 'Progressive encephalopathy in a child with cerebral folate deficiency syndrome', *J Child Neurol,* 23(12), pp. 1460-3.

Boston, P. F., McKirdy, S. J., Al-Turki, M. A., Barker, M. E. & Russell, J. M. (2020). 'Vitamin B12 and folate levels in progression of Alzheimer's disease - a short report', *Int J Psychiatry Clin Pract,* 24(1), pp. 68-70.

Bottiglieri, T. (2005). 'Homocysteine and folate metabolism in depression', *Prog Neuropsychopharmacol Biol Psychiatry,* 29(7), pp. 1103-12.

Braun, M. & Iliff, J. J. (2020). 'The impact of neurovascular, blood-brain barrier, and glymphatic dysfunction in neurodegenerative and metabolic diseases', *Int Rev Neurobiol,* 154, pp. 413-436.

Brennan, L. & de Roos, B. (2021). 'Nutrigenomics: lessons learned and future perspectives', *Am J Clin Nutr,* 113(3), pp. 503-516.

Bueno, D., Parvas, M., Nabiuni, M. & Miyan, J. (2020). 'Embryonic cerebrospinal fluid formation and regulation', *Semin Cell Dev Biol,* 102, pp. 3-12.

Cains, S., Shepherd, A., Nabiuni, M., Owen-Lynch, P. J. & Miyan, J. (2009). 'Addressing a folate imbalance in fetal cerebrospinal fluid can decrease the incidence of congenital hydrocephalus', *J Neuropathol Exp Neurol,* 68(4), pp. 404-16.

Campos-Escamilla, C. (2021). 'The role of transferrins and iron-related proteins in brain iron transport: applications to neurological diseases', *Adv Protein Chem Struct Biol,* 123, pp. 133-162.

Cao, X., Wolf, A., Kim, S. E., Cabrera, R. M., Wlodarczyk, B. J., Zhu, H., Parker, M., Lin, Y., Steele, J. W., Han, X., Ramaekers, V. T., Steinfeld, R., Finnell, R. H. & Lei, Y. (2020). 'CIC de novo loss of function variants contribute to cerebral folate deficiency by downregulating FOLR1 expression', *J Med Genet*.

Cario, H., Smith, D. E., Blom, H., Blau, N., Bode, H., Holzmann, K., Pannicke, U., Hopfner, K. P., Rump, E. M., Ayric, Z., Kohne, E., Debatin, K. M., Smulders, Y. & Schwarz, K. (2011). 'Dihydrofolate reductase deficiency due to a homozygous DHFR mutation causes megaloblastic anemia and cerebral folate deficiency leading to severe neurologic disease', *Am J Hum Genet,* 88(2), pp. 226-31.

Chance, S. A., Esiri, M. M. & Crow, T. J. (2003). 'Ventricular enlargement in schizophrenia: a primary change in the temporal lobe?', *Schizophr Res,* 62(1-2), pp. 123-31.

Chachaj, A., K. Gasiorowski, A. Szuba, A. Sieradzki and J. Leszek (2022). "Lymphatic system in the brain clearance mechanisms - new therapeutic perspectives for Alzheimer's disease." Curr Neuropharmacol.

Chanson, A., Rock, E., Martin, J. F., Liotard, A. & Brachet, P. (2007). 'Preferential response of glutathione-related enzymes to folate-dependent changes in the redox state of rat liver', *Eur J Nutr,* 46(4), pp. 204-12.

Chen, R., Liou, T. H., Miao, N. F., Chang, K. H., Yen, C. F., Liao, H. F., Chi, W. C. & Chou, K. R. (2020). 'Using World Health Organization Disability Assessment Schedule 2.0 in people with schizophrenia: a 4-year follow-up', *Eur Arch Psychiatry Clin Neurosci,* 270(3), pp. 301-310.

Child, D. F., Hudson, P. R., Jones, H., Davies, G. K., De, P., Mukherjee, S., Brain, A. M., Williams, C. P. & Harvey, J. N. (2004). 'The effect of oral folic acid on glutathione, glycaemia and lipids in Type 2 diabetes', *Diabetes Nutr Metab,* 17(2), pp. 95-102.

Chou, Y. Y., Lepore, N., Avedissian, C., Madsen, S. K., Parikshak, N., Hua, X., Shaw, L. M., Trojanowski, J. Q., Weiner, M. W., Toga, A. W., Thompson, P. M. & Alzheimer's Disease Neuroimaging, I. (2009). 'Mapping correlations between ventricular expansion and CSF amyloid and tau biomarkers in 240 subjects with Alzheimer's disease, mild cognitive impairment and elderly controls', *Neuroimage,* 46(2), pp. 394-410.

Collaborators, G. B. D. N. (2019). 'Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016', *Lancet Neurol,* 18(5), pp. 459-480.

Cushing, H. (1914). 'Studies on the cerebro-spinal fluid: I. Introduction', *The Journal of medical research,* 31(1), p. 1.

Dalaker, T. O., Zivadinov, R., Ramasamy, D. P., Beyer, M. K., Alves, G., Bronnick, K. S., Tysnes, O. B., Aarsland, D. & Larsen, J. P. (2011). 'Ventricular enlargement and mild cognitive impairment in early Parkinson's disease', *Mov Disord,* 26(2), pp. 297-301.

de Leon, M. J., Li, Y., Okamura, N., Tsui, W. H., Saint-Louis, L. A., Glodzik, L., Osorio, R. S., Fortea, J., Butler, T., Pirraglia, E., Fossati, S., Kim, H. J., Carare, R. O., Nedergaard, M., Benveniste, H. & Rusinek, H. (2017). 'Cerebrospinal Fluid Clearance in Alzheimer Disease Measured with Dynamic PET', *J Nucl Med,* 58(9), pp. 1471-1476.

Delmelle, F., Thony, B., Clapuyt, P., Blau, N. & Nassogne, M. C. (2016). 'Neurological improvement following intravenous high-dose folinic acid for cerebral folate transporter deficiency caused by FOLR-1 mutation', *Eur J Paediatr Neurol,* 20(5), pp. 709-13.

DeLong, G. R., Stanbury, J. B. & Fierro-Benitez, R. (1985). 'Neurological signs in congenital iodine-deficiency disorder (endemic cretinism)', *Dev Med Child Neurol,* 27(3), pp. 317-24.

DeMattos, R. B., Bales, K. R., Parsadanian, M., O'Dell, M. A., Foss, E. M., Paul, S. M. & Holtzman, D. M. (2002). 'Plaque-associated disruption of CSF and plasma amyloid-beta (Abeta) equilibrium in a mouse model of Alzheimer's disease', *J Neurochem,* 81(2), pp. 229-36.

Dorszewska, J., Florczak, J., Rozycka, A., Kempisty, B., Jaroszewska-Kolecka, J., Chojnacka, K., Trzeciak, W. H. & Kozubski, W. (2007). 'Oxidative DNA damage and level of thiols as related to polymorphisms of MTHFR, MTR, MTHFD1 in Alzheimer's and Parkinson's diseases', *Acta Neurobiol Exp (Wars),* 67(2), pp. 113-29.

Duarte, S., Cruz Martins, R., Rodrigues, M., Lourenco, E., Moreira, I., Alonso, I. & Magalhaes, M. (2020). 'Association of cerebral folate deficiency and hereditary spastic paraplegia', *Neurologia*.

Dubois, B., Feldman, H. H., Jacova, C., Dekosky, S. T., Barberger-Gateau, P., Cummings, J., Delacourte, A., Galasko, D., Gauthier, S., Jicha, G., Meguro, K., O'Brien, J., Pasquier, F., Robert, P., Rossor, M., Salloway, S., Stern, Y., Visser, P. J. & Scheltens, P. (2007). 'Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria', *Lancet Neurol,* 6(8), pp. 734-46.

Duong, S., Patel, T. & Chang, F. (2017). 'Dementia: What pharmacists need to know', *Can Pharm J (Ott),* 150(2), pp. 118-129.

Eells, J. T., Gonzalez-Quevedo, A., Santiesteban Freixas, R., McMartin, K. E. & Sadun, A. A. (2000). '[Folic acid deficiency and increased concentrations of formate in serum and cerebrospinal fluid of patients with epidemic optical neuropathy]', *Rev Cubana Med Trop,* 52(1), pp. 21-3.

Elkis, H., Friedman, L., Wise, A. & Meltzer, H. Y. (1995). 'Meta-analyses of studies of ventricular enlargement and cortical sulcal prominence in mood disorders. Comparisons with controls or patients with schizophrenia', *Arch Gen Psychiatry,* 52(9), pp. 735-46.

Erel, O., Cannon, T. D., Hollister, J. M., Mednick, S. A. & Parnas, J. (1991). 'Ventricular enlargement and premorbid deficits in school-occupational attainment in a high risk sample', *Schizophr Res,* 4(1), pp. 49-52.

Fame, R. M., Cortes-Campos, C. & Sive, H. L. (2020). 'Brain Ventricular System and Cerebrospinal Fluid Development and Function: Light at the End of the Tube: A Primer with Latest Insights', *Bioessays,* 42(3), p. e1900186.

Ferreira, P., Luco, S. M., Sawyer, S. L., Davila, J., Boycott, K. M. & Dyment, D. A. (2016). 'Late diagnosis of cerebral folate deficiency: Fewer seizures with folinic acid in adult siblings', *Neurol Genet,* 2(1), p. e38.

Ferri, C. P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., Hall, K., Hasegawa, K., Hendrie, H., Huang, Y., Jorm, A., Mathers, C., Menezes, P. R., Rimmer, E., Scazufca, M. & Alzheimer's Disease, I. (2005). 'Global prevalence of dementia: a Delphi consensus study', *Lancet,* 366(9503), pp. 2112-7.

Field, M. S., Shields, K. S., Abarinov, E. V., Malysheva, O. V., Allen, R. H., Stabler, S. P., Ash, J. A., Strupp, B. J., Stover, P. J. & Caudill, M. A. (2013). 'Reduced MTHFD1 activity in male mice perturbs folate- and choline-dependent one-carbon metabolism as well as transsulfuration', *J Nutr,* 143(1), pp. 41-5.

Fischi-Gomez, E., Bonnier, G., Ward, N., Granziera, C. & Hadjikhani, N. (2021). 'Ultra-high field in vivo characterization of microstructural abnormalities in the orbitofrontal cortex and amygdala in autism', *Eur J Neurosci*.

Fowler, B. (2001). 'The folate cycle and disease in humans', *Kidney Int Suppl,* 78, pp. S221-9.

Frye, R. E., Delhey, L., Slattery, J., Tippett, M., Wynne, R., Rose, S., Kahler, S. G., Bennuri, S. C., Melnyk, S., Sequeira, J. M. & Quadros, E. (2016). 'Blocking and Binding Folate Receptor Alpha Autoantibodies Identify Novel Autism Spectrum Disorder Subgroups', *Front Neurosci,* 10, p. 80.

Frye, R. E., Donner, E., Golja, A. & Rooney, C. M. (2003). 'Folinic acid-responsive seizures presenting as breakthrough seizures in a 3-month-old boy', *J Child Neurol,* 18(8), pp. 562-9.

Frye, R. E., Melnyk, S., Fuchs, G., Reid, T., Jernigan, S., Pavliv, O., Hubanks, A., Gaylor, D. W., Walters, L. & James, S. J. (2013). 'Effectiveness of methylcobalamin and folinic Acid treatment on adaptive behavior in children with autistic disorder is related to glutathione redox status', *Autism Res Treat,* 2013, p. 609705.

Frye, R. E., Rossignol, D. A., Scahill, L., McDougle, C. J., Huberman, H. & Quadros, E. V. (2020). 'Treatment of Folate Metabolism Abnormalities in Autism Spectrum Disorder', *Semin Pediatr Neurol,* 35, p. 100835.

Frye, R. E., Slattery, J., Delhey, L., Furgerson, B., Strickland, T., Tippett, M., Sailey, A., Wynne, R., Rose, S., Melnyk, S., Jill James, S., Sequeira, J. M. & Quadros, E. V. (2018). 'Folinic acid improves verbal communication in children with autism and language impairment: a randomized double-blind placebo-controlled trial', *Mol Psychiatry,* 23(2), pp. 247-256.

Frye, R. E., Slattery, J. C. & Quadros, E. V. (2017). 'Folate metabolism abnormalities in autism: potential biomarkers', *Biomark Med,* 11(8), pp. 687-699.

Garcia-Cazorla, A., Quadros, E. V., Nascimento, A., Garcia-Silva, M. T., Briones, P., Montoya, J., Ormazabal, A., Artuch, R., Sequeira, J. M., Blau, N., Arenas, J., Pineda, M. & Ramaekers, V. T. (2008). 'Mitochondrial diseases associated with cerebral folate deficiency', *Neurology,* 70(16), pp. 1360-2.

Gato, A., Alonso, M. I., Lamus, F. & Miyan, J. (2020). 'Neurogenesis: A process ontogenically linked to brain cavities and their content, CSF', *Semin Cell Dev Biol,* 102, pp. 21-27.

Giuffrida, M. L., Caraci, F., Pignataro, B., Cataldo, S., De Bona, P., Bruno, V., Molinaro, G., Pappalardo, G., Messina, A., Palmigiano, A., Garozzo, D., Nicoletti, F., Rizzarelli, E. & Copani, A. (2009). 'Beta-amyloid monomers are neuroprotective', *J Neurosci,* 29(34), pp. 10582-7.

Gonzalez, C., Armijo, E., Bravo-Alegria, J., Becerra-Calixto, A., Mays, C. E. & Soto, C. (2018). 'Modeling amyloid beta and tau pathology in human cerebral organoids', *Mol Psychiatry,* 23(12), pp. 2363-2374.

Gorelova, V., Ambach, L., Rebeille, F., Stove, C. & Van Der Straeten, D. (2017). 'Folates in Plants: Research Advances and Progress in Crop Biofortification', *Front Chem,* 5, p. 21.

Goukasian, N., Porat, S., Blanken, A., Avila, D., Zlatev, D., Hurtz, S., Hwang, K. S., Pierce, J., Joshi, S. H., Woo, E. & Apostolova, L. G. (2019). 'Cognitive Correlates of Hippocampal Atrophy and Ventricular Enlargement in Adults with or without Mild Cognitive Impairment', *Dement Geriatr Cogn Dis Extra,* 9(2), pp. 281-293.

Grapp, M., Just, I. A., Linnankivi, T., Wolf, P., Lucke, T., Hausler, M., Gartner, J. & Steinfeld, R. (2012). 'Molecular characterization of folate receptor 1 mutations delineates cerebral folate transport deficiency', *Brain,* 135(Pt 7), pp. 2022-31.

Grapp, M., Wrede, A., Schweizer, M., Huwel, S., Galla, H. J., Snaidero, N., Simons, M., Buckers, J., Low, P. S., Urlaub, H., Gartner, J. & Steinfeld, R. (2013). 'Choroid plexus transcytosis and exosome shuttling deliver folate into brain parenchyma', *Nat Commun,* 4, p. 2123.

Grzybowski, D. M., Holman, D. W., Katz, S. E. & Lubow, M. (2006). 'In vitro model of cerebrospinal fluid outflow through human arachnoid granulations', *Invest Ophthalmol Vis Sci,* 47(8), pp. 3664-72.

Guekht, A., Brodie, M., Secco, M., Li, S., Volkers, N. & Wiebe, S. (2021). 'The road to a World Health Organization global action plan on epilepsy and other neurological disorders', *Epilepsia,* 62(5), pp. 1057-1063.

Guptha, S. H., Holroyd, E. & Campbell, G. (2002). 'Progressive lateral ventricular enlargement as a clue to Alzheimer's disease', *Lancet,* 359(9322), p. 2040.

Hansen, F. J. & Blau, N. (2005). 'Cerebral folate deficiency: life-changing supplementation with folinic acid', *Mol Genet Metab,* 84(4), pp. 371-3.

Harrison, I. F., Ismail, O., Machhada, A., Colgan, N., Ohene, Y., Nahavandi, P., Ahmed, Z., Fisher, A., Meftah, S., Murray, T. K., Ottersen, O. P., Nagelhus, E. A., O'Neill, M. J., Wells, J. A. & Lythgoe, M. F. (2020). 'Impaired glymphatic function and clearance of tau in an Alzheimer's disease model', *Brain,* 143(8), pp. 2576-2593.

Harvey, I., McGuffin, P., Williams, M. & Toone, B. K. (1990). 'The ventricle-brain ratio (VBR) in functional psychoses: an admixture analysis', *Psychiatry Res,* 35(1), pp. 61-9.

Hasselmann, O., Blau, N., Ramaekers, V. T., Quadros, E. V., Sequeira, J. M. & Weissert, M. (2010). 'Cerebral folate deficiency and CNS inflammatory markers in Alpers disease', *Mol Genet Metab,* 99(1), pp. 58-61.

Ho, A., Michelson, D., Aaen, G. & Ashwal, S. (2010). 'Cerebral folate deficiency presenting as adolescent catatonic schizophrenia: a case report', *J Child Neurol,* 25(7), pp. 898-900.

Horga, G., Bernacer, J., Dusi, N., Entis, J., Chu, K., Hazlett, E. A., Haznedar, M. M., Kemether, E., Byne, W. & Buchsbaum, M. S. (2011). 'Correlations between ventricular enlargement and gray and white matter volumes of cortex, thalamus, striatum, and internal capsule in schizophrenia', *Eur Arch Psychiatry Clin Neurosci,* 261(7), pp. 467-76.

Hubbard, B. M. & Anderson, J. M. (1981). 'Age, senile dementia and ventricular enlargement', *J Neurol Neurosurg Psychiatry,* 44(7), pp. 631-5.

Hunsberger, H. C., Pinky, P. D., Smith, W., Suppiramaniam, V. & Reed, M. N. (2019). 'The role of *APOE4* in Alzheimer's disease: strategies for future therapeutic interventions', *Neuronal Signal,* 3(2), p. NS20180203.

Iliff, J. & Simon, M. (2019). 'CrossTalk proposal: The glymphatic system supports convective exchange of cerebrospinal fluid and brain interstitial fluid that is mediated by perivascular aquaporin-4', *J Physiol,* 597(17), pp. 4417-4419.

Iliff, J. J., Chen, M. J., Plog, B. A., Zeppenfeld, D. M., Soltero, M., Yang, L., Singh, I., Deane, R. & Nedergaard, M. (2014). 'Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury', *J Neurosci,* 34(49), pp. 16180-93.

Iliff, J. J., Wang, M., Liao, Y., Plogg, B. A., Peng, W., Gundersen, G. A., Benveniste, H., Vates, G. E., Deane, R., Goldman, S. A., Nagelhus, E. A. & Nedergaard, M. (2012). 'A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta', *Sci Transl Med,* 4(147), p. 147ra111.

Jackson, D. C., Irwin, W., Dabbs, K., Lin, J. J., Jones, J. E., Hsu, D. A., Stafstrom, C. E., Seidenberg, M. & Hermann, B. P. (2011). 'Ventricular enlargement in new-onset pediatric epilepsies', *Epilepsia,* 52(12), pp. 2225-32.

Jakobsen, J., Gyldensted, C., Brun, B., Bruhn, P., Helweg-Larsen, S. & Arlien-Soborg, P. (1989). 'Cerebral ventricular enlargement relates to neuropsychological measures in unselected AIDS patients', *Acta Neurol Scand,* 79(1), pp. 59-62.

Jann, M. W. (2014). 'Diagnosis and treatment of bipolar disorders in adults: a review of the evidence on pharmacologic treatments', *Am Health Drug Benefits,* 7(9), pp. 489-99.

Jara, C., Cerpa, W., Tapia-Rojas, C. & Quintanilla, R. A. (2020). 'Tau Deletion Prevents Cognitive Impairment and Mitochondrial Dysfunction Age Associated by a Mechanism Dependent on Cyclophilin-D', *Front Neurosci,* 14, p. 586710.

Jayaswal, S. K., Chawla, H. M., Goulatia, R. K. & Rao, G. S. (1988). 'Cerebral ventricular enlargement in chronic schizophrenia', *Br J Psychiatry,* 153, pp. 414-5.

Jerico, D., Luis, E. O., Cusso, L., Fernandez-Seara, M. A., Morales, X., Cordoba, K. M., Benito, M., Sampedro, A., Larriva, M., Ramirez, M. J., de Salamanca, R. E., Ortiz-de-Solorzano, C., Alegre, M., Prieto, J., Lanciego, J. L., D'Avola, D., Gonzalez-Aseguinolaza, G., Pastor, M. A., Desco, M. & Fontanellas, A. (2020). 'Brain ventricular enlargement in human and murine acute intermittent porphyria', *Hum Mol Genet*.

Jessen, N. A., Munk, A. S., Lundgaard, I. & Nedergaard, M. (2015). 'The Glymphatic System: A Beginner's Guide', *Neurochem Res,* 40(12), pp. 2583-99.

Jiang, Y., Xiao, X., Wen, Y., Wan, M., Zhou, L., Liu, X., Wang, X., Guo, L., Liu, H., Zhou, Y., Wang, J., Liao, X., Shen, L. & Jiao, B. (2021). 'Genetic effect of MTHFR C677T, A1298C, and A1793G polymorphisms on the age at onset, plasma homocysteine, and white matter lesions in Alzheimer's disease in the Chinese population', *Aging (Albany NY),* 13(8), pp. 11352-11362.

Jimenez, A. R., Naz, N. & Miyan, J. A. (2019). 'Altered folate binding protein expression and folate delivery are associated with congenital hydrocephalus in the hydrocephalic Texas rat', *J Cereb Blood Flow Metab,* 39(10), pp. 2061-2073.

Jones, P. B., Harvey, I., Lewis, S. W., Toone, B. K., Van Os, J., Williams, M. & Murray, R. M. (1994). 'Cerebral ventricle dimensions as risk factors for schizophrenia and affective psychosis: an epidemiological approach to analysis', *Psychol Med,* 24(4), pp. 995-1011.

Kametani, F. & Hasegawa, M. (2018). 'Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease', *Front Neurosci,* 12, p. 25.

Karin, I., Borggraefe, I., Catarino, C. B., Kuhm, C., Hoertnagel, K., Biskup, S., Opladen, T., Blau, N., Heinen, F. & Klopstock, T. (2017). 'Folinic acid therapy in cerebral folate deficiency: marked improvement in an adult patient', *J Neurol,* 264(3), pp. 578-582.

Kempton, M. J., Stahl, D., Williams, S. C. & DeLisi, L. E. (2010). 'Progressive lateral ventricular enlargement in schizophrenia: a meta-analysis of longitudinal MRI studies', *Schizophr Res,* 120(1-3), pp. 54-62.

Kokjohn, T. A. & Roher, A. E. (2009). 'Antibody responses, amyloid-beta peptide remnants and clinical effects of AN-1792 immunization in patients with AD in an interrupted trial', *CNS Neurol Disord Drug Targets,* 8(2), pp. 88-97.

Kronenberg, G., Harms, C., Sobol, R. W., Cardozo-Pelaez, F., Linhart, H., Winter, B., Balkaya, M., Gertz, K., Gay, S. B., Cox, D., Eckart, S., Ahmadi, M., Juckel, G., Kempermann, G., Hellweg, R., Sohr, R., Hortnagl, H., Wilson, S. H., Jaenisch, R. & Endres, M. (2008). 'Folate deficiency induces neurodegeneration and brain dysfunction in mice lacking uracil DNA glycosylase', *J Neurosci,* 28(28), pp. 7219-30.

Krsicka, D., Geryk, J., Vlckova, M., Havlovicova, M., Macek, M., Jr. & Pourova, R. (2017). 'Identification of likely associations between cerebral folate deficiency and complex

genetic- and metabolic pathogenesis of autism spectrum disorders by utilization of a pilot interaction modeling approach', *Autism Res,* 10(8), pp. 1424-1435.

Krupenko, S. A. & Krupenko, N. I. (2018). 'ALDH1L1 and ALDH1L2 Folate Regulatory Enzymes in Cancer', *Adv Exp Med Biol,* 1032, pp. 127-143.

Krupenko, S. A. & Krupenko, N. I. (2019). 'Loss of ALDH1L1 folate enzyme confers a selective metabolic advantage for tumor progression', *Chem Biol Interact,* 302, pp. 149-155.

Krupenko, S. A. & Oleinik, N. V. (2002). '10-formyltetrahydrofolate dehydrogenase, one of the major folate enzymes, is down-regulated in tumor tissues and possesses suppressor effects on cancer cells', *Cell Growth Differ,* 13(5), pp. 227-36.

Lee, Y., Choi, Y., Park, E. J., Kwon, S., Kim, H., Lee, J. Y. & Lee, D. S. (2020). 'Improvement of glymphatic-lymphatic drainage of beta-amyloid by focused ultrasound in Alzheimer's disease model', *Sci Rep,* 10(1), p. 16144.

Lemche, E. (2018). 'Early Life Stress and Epigenetics in Late-onset Alzheimer's Dementia: A Systematic Review', *Curr Genomics,* 19(7), pp. 522-602.

Leuzzi, V., Mastrangelo, M., Celato, A., Carducci, C. & Carducci, C. (2012). 'A new form of cerebral folate deficiency with severe self-injurious behaviour', *Acta Paediatr,* 101(11), pp. e482-3.

Liu, P. P., Xie, Y., Meng, X. Y. & Kang, J. S. (2019). 'History and progress of hypotheses and clinical trials for Alzheimer's disease', *Signal Transduct Target Ther,* 4, p. 29.

Liu, S., Wu, Y., Liu, X., Zhou, J., Wang, Z., He, Z. & Huang, Z. (2017). 'Lack of association between MTHFR A1298C variant and Alzheimer's disease: evidence from a systematic review and cumulative meta-analysis', *Neurol Res,* 39(5), pp. 426-434.

Liu, X., Wang, W., Chen, H. L., Zhang, H. Y. & Zhang, N. X. (2019). 'Interplay between Alzheimer's disease and global glucose metabolism revealed by the metabolic profile alterations of pancreatic tissue and serum in APP/PS1 transgenic mice', *Acta Pharmacol Sin,* 40(10), pp. 1259-1268.

Lou, Y., Carlock, C. & Wu, J. (2018). 'Glymphatic Efficiency Is a Critical Factor for Using Abnormal Tau in Peripheral Tissues as Biomarker for Alzheimer's Disease', *Biomark Appl,* 2018(3).

Lucock, M. (2000). 'Folic acid: nutritional biochemistry, molecular biology, and role in disease processes', *Mol Genet Metab,* 71(1-2), pp. 121-38.

Luxenberg, J. S., Haxby, J. V., Creasey, H., Sundaram, M. & Rapoport, S. I. (1987). 'Rate of ventricular enlargement in dementia of the Alzheimer type correlates with rate of neuropsychological deterioration', *Neurology,* 37(7), pp. 1135-40.

MacFarlane, A. J., Perry, C. A., McEntee, M. F., Lin, D. M. & Stover, P. J. (2011). 'Mthfd1 is a modifier of chemically induced intestinal carcinogenesis', *Carcinogenesis,* 32(3), pp. 427-33.

Madsen, S. K., Gutman, B. A., Joshi, S. H., Toga, A. W., Jack, C. R., Jr., Weiner, M. W. & Thompson, P. M. (2013). 'Mapping Dynamic Changes in Ventricular Volume onto Baseline Cortical Surfaces in Normal Aging, MCI, and Alzheimer's Disease', *Multimodal Brain Image Anal (2013),* 8159, pp. 84-94.

Mafi, S., Laroche-Raynaud, C., Chazelas, P., Lia, A. S., Derouault, P., Sturtz, F., Baaj, Y., Froget, R., Rio, M., Benoist, J. F., Poumeaud, F., Favreau, F. & Faye, P. A. (2020). 'Pharmacoresistant Epilepsy in Childhood: Think of the Cerebral Folate Deficiency, a Treatable Disease', *Brain Sci,* 10(11).

Mahajan, U. V., Varma, V. R., Griswold, M. E., Blackshear, C. T., An, Y., Oommen, A. M., Varma, S., Troncoso, J. C., Pletnikova, O., O'Brien, R., Hohman, T. J., Legido-Quigley, C. & Thambisetty, M. (2020). 'Dysregulation of multiple metabolic networks related to brain transmethylation and polyamine pathways in Alzheimer disease: A targeted metabolomic and transcriptomic study', *PLoS Med,* 17(1), p. e1003012.

Mak, E., Su, L., Williams, G. B., Firbank, M. J., Lawson, R. A., Yarnall, A. J., Duncan, G. W., Mollenhauer, B., Owen, A. M., Khoo, T. K., Brooks, D. J., Rowe, J. B., Barker, R. A., Burn, D. J. & O'Brien, J. T. (2017). 'Longitudinal whole-brain atrophy and ventricular enlargement in nondemented Parkinson's disease', *Neurobiol Aging,* 55, pp. 78-90.

Mangold, S., Blau, N., Opladen, T., Steinfeld, R., Wessling, B., Zerres, K. & Hausler, M. (2011). 'Cerebral folate deficiency: a neurometabolic syndrome?', *Mol Genet Metab,* 104(3), pp. 369-72.

Mantile, F. & Prisco, A. (2020). 'Vaccination against beta-Amyloid as a Strategy for the Prevention of Alzheimer's Disease', *Biology (Basel),* 9(12).

Martinez-Galan, J. R., Pedraza, P., Santacana, M., Escobar del Ray, F., Morreale de Escobar, G. & Ruiz-Marcos, A. (1997). 'Early effects of iodine deficiency on radial glial cells of the hippocampus of the rat fetus. A model of neurological cretinism', *J Clin Invest,* 99(11), pp. 2701-9.

Martola, J., Stawiarz, L., Fredrikson, S., Hillert, J., Bergstrom, J., Flodmark, O., Aspelin, P. & Kristoffersen Wiberg, M. (2008). 'Rate of ventricular enlargement in multiple sclerosis: a nine-year magnetic resonance imaging follow-up study', *Acta Radiol,* 49(5), pp. 570-9.

Maryam, R. S., Sahar, J., Hastono, S. P. & Harimurti, K. (2021). 'Common symptoms of Alzheimer's dementia that are easily recognizable by families', *Dement Neuropsychol,* 15(2), pp. 186-191.

May, C., Kaye, J. A., Atack, J. R., Schapiro, M. B., Friedland, R. P. & Rapoport, S. I. (1990). 'Cerebrospinal fluid production is reduced in healthy aging', *Neurology,* 40(3 Pt 1), pp. 500-3.

Melzer, L., Freiman, T. M. & Derouiche, A. (2021). 'Rab6A as a Pan-Astrocytic Marker in Mouse and Human Brain, and Comparison with Other Glial Markers (GFAP, GS, Aldh1L1, SOX9)', *Cells,* 10(1).

Mercimek-Mahmutoglu, S. & Stockler-Ipsiroglu, S. (2007). 'Cerebral folate deficiency and folinic acid treatment in hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) syndrome', *Tohoku J Exp Med,* 211(1), pp. 95-6; author reply 97.

Mielke, M. M., Przybelski, S. A., Lesnick, T. G., Kern, S., Zetterberg, H., Blennow, K., Knopman, D. S., Graff-Radford, J., Petersen, R. C., Jack, C. R., Jr. & Vemuri, P. (2021). 'Comparison of CSF neurofilament light chain, neurogranin, and tau to MRI markers', *Alzheimers Dement*.

Miller, A. L. (2003). 'The methionine-homocysteine cycle and its effects on cognitive diseases', *Altern Med Rev,* 8(1), pp. 7-19.

Miyan, J., Cains, S., Larcombe, S., Naz, N., Jimenez, A. R., Bueno, D. & Gato, A. (2020). 'Subarachnoid cerebrospinal fluid is essential for normal development of the cerebral cortex', *Semin Cell Dev Biol,* 102, pp. 28-39.

Miyan, J. A., Nabiyouni, M. & Zendah, M. (2003). 'Development of the brain: a vital role for cerebrospinal fluid', *Can J Physiol Pharmacol,* 81(4), pp. 317-28.

Miyan, J. A., Zendah, M., Mashayekhi, F. & Owen-Lynch, P. J. (2006). 'Cerebrospinal fluid supports viability and proliferation of cortical cells in vitro, mirroring in vivo development', *Cerebrospinal Fluid Res,* 3, p. 2.

Moretti, P., Sahoo, T., Hyland, K., Bottiglieri, T., Peters, S., del Gaudio, D., Roa, B., Curry, S., Zhu, H., Finnell, R. H., Neul, J. L., Ramaekers, V. T., Blau, N., Bacino, C. A., Miller, G. & Scaglia, F. (2005). 'Cerebral folate deficiency with developmental delay, autism, and response to folinic acid', *Neurology,* 64(6), pp. 1088-90.

Movsas, T. Z., Pinto-Martin, J. A., Whitaker, A. H., Feldman, J. F., Lorenz, J. M., Korzeniewski, S. J., Levy, S. E. & Paneth, N. (2013). 'Autism spectrum disorder is associated with ventricular enlargement in a low birth weight population', *J Pediatr,* 163(1), pp. 73-8.

Muller, M., Esser, R., Kotter, K., Voss, J., Muller, A. & Stellmes, P. (2013). 'Third ventricular enlargement in early stages of multiple sclerosis is a predictor of motor and neuropsychological deficits: a cross-sectional study', *BMJ Open,* 3(9), p. e003582.

Murdaca, G., Banchero, S., Tonacci, A., Nencioni, A., Monacelli, F. & Gangemi, S. (2021). 'Vitamin D and Folate as Predictors of MMSE in Alzheimer's Disease: A Machine Learning Analysis', *Diagnostics (Basel),* 11(6).

Murphy, M. P. & LeVine, H., 3rd (2010). 'Alzheimer's disease and the amyloid-beta peptide', *J Alzheimers Dis,* 19(1), pp. 311-23.

Naganawa, S. & Taoka, T. (2020). 'The Glymphatic System: A Review of the Challenges in Visualizing its Structure and Function with MR Imaging', *Magn Reson Med Sci*.

Nasrallah, H. A., McCalley-Whitters, M. & Jacoby, C. G. (1982). 'Cerebral ventricular enlargement in young manic males. A controlled CT study', *J Affect Disord,* 4(1), pp. 15-9.

Naz, N., Jimenez, A. R., Sanjuan-Vilaplana, A., Gurney, M. & Miyan, J. (2016). 'Neonatal hydrocephalus is a result of a block in folate handling and metabolism involving 10-formyltetrahydrofolate dehydrogenase', *J Neurochem,* 138(4), pp. 610-23.

Nestor, S. M., Rupsingh, R., Borrie, M., Smith, M., Accomazzi, V., Wells, J. L., Fogarty, J., Bartha, R. & Alzheimer's Disease Neuroimaging, I. (2008). 'Ventricular enlargement as a possible measure of Alzheimer's disease progression validated using the Alzheimer's disease neuroimaging initiative database', *Brain,* 131(Pt 9), pp. 2443-54.

Ng Kee Kwong, K. C., Mehta, A. R., Nedergaard, M. & Chandran, S. (2020). 'Defining novel functions for cerebrospinal fluid in ALS pathophysiology', *Acta Neuropathol Commun,* 8(1), p. 140.

Nikolac Perkovic, M. & Pivac, N. (2019). 'Genetic Markers of Alzheimer's Disease', *Adv Exp Med Biol,* 1192, pp. 27-52.

Nikolac Perkovic, M., Videtic Paska, A., Konjevod, M., Kouter, K., Svob Strac, D., Nedic Erjavec, G. & Pivac, N. (2021). 'Epigenetics of Alzheimer's Disease', *Biomolecules,* 11(2).

Norwitz, N. G., Saif, N., Ariza, I. E. & Isaacson, R. S. (2021). 'Precision Nutrition for Alzheimer's Prevention in *APOE4* Carriers', *Nutrients,* 13(4).

Novikova, G., Andrews, S. J., Renton, A. E. & Marcora, E. (2021). 'Beyond association: successes and challenges in linking non-coding genetic variation to functional consequences that modulate Alzheimer's disease risk', *Mol Neurodegener,* 16(1), p. 27.

Nuru, M., Muradashvili, N., Kalani, A., Lominadze, D. & Tyagi, N. (2018). 'High methionine, low folate and low vitamin B6/B12 (HM-LF-LV) diet causes neurodegeneration and subsequent short-term memory loss', *Metab Brain Dis,* 33(6), pp. 1923-1934.

Nzila, A., Ward, S. A., Marsh, K., Sims, P. F. & Hyde, J. E. (2005). 'Comparative folate metabolism in humans and malaria parasites (part I): pointers for malaria treatment from cancer chemotherapy', *Trends Parasitol,* 21(6), pp. 292-8.

O'Brien, R. J. & Wong, P. C. (2011). 'Amyloid precursor protein processing and Alzheimer's disease', *Annu Rev Neurosci,* 34, pp. 185-204.

Oberman, K., Gouweleeuw, L., Hoogerhout, P., Eisel, U. L. M., van Riet, E. & Schoemaker, R. G. (2020). 'Vaccination Prevented Short-Term Memory Loss, but Deteriorated Long-Term Spatial Memory in Alzheimer's Disease Mice, Independent of Amyloid-beta Pathology', *J Alzheimers Dis Rep,* 4(1), pp. 261-280.

Olaleye, O. A., Zaki, D. A. & Hamzat, T. K. (2021). 'Expectations of individuals with neurological conditions from rehabilitation: A mixed-method study of needs', *S Afr J Physiother,* 77(1), p. 1498.

Ott, B. R., Cohen, R. A., Gongvatana, A., Okonkwo, O. C., Johanson, C. E., Stopa, E. G., Donahue, J. E., Silverberg, G. D. & Alzheimer's Disease Neuroimaging, I. (2010). 'Brain ventricular volume and cerebrospinal fluid biomarkers of Alzheimer's disease', *J Alzheimers Dis,* 20(2), pp. 647-57.

Owen-Lynch, P. J., Draper, C. E., Mashayekhi, F., Bannister, C. M. & Miyan, J. A. (2003). 'Defective cell cycle control underlies abnormal cortical development in the hydrocephalic Texas rat', *Brain,* 126(Pt 3), pp. 623-31.

Papadopoulos, A., Seguin, D., Correa, S. & Duerden, E. G. (2021). 'Peer victimization and the association with hippocampal development and working memory in children with ADHD and typically-developing children', *Sci Rep,* 11(1), p. 16411.

Patel, C. N., Georrge, J. J., Modi, K. M., Narechania, M. B., Patel, D. P., Gonzalez, F. J. & Pandya, H. A. (2018). 'Pharmacophore-based virtual screening of catechol-o-methyltransferase (COMT) inhibitors to combat Alzheimer's disease', *J Biomol Struct Dyn,* 36(15), pp. 3938-3957.

Peng, Q., Lao, X., Huang, X., Qin, X., Li, S. & Zeng, Z. (2015). 'The MTHFR C677T polymorphism contributes to increased risk of Alzheimer's disease: evidence based on 40 case-control studies', *Neurosci Lett,* 586, pp. 36-42.

Peng, W., Achariyar, T. M., Li, B., Liao, Y., Mestre, H., Hitomi, E., Regan, S., Kasper, T., Peng, S., Ding, F., Benveniste, H., Nedergaard, M. & Deane, R. (2016). 'Suppression of glymphatic fluid transport in a mouse model of Alzheimer's disease', *Neurobiol Dis,* 93, pp. 215-25.

Ramaekers, V., Sequeira, J. M. & Quadros, E. V. (2013). 'Clinical recognition and aspects of the cerebral folate deficiency syndromes', *Clin Chem Lab Med,* 51(3), pp. 497-511.

Ramaekers, V. T. & Blau, N. (2004). 'Cerebral folate deficiency', *Dev Med Child Neurol,* 46(12), pp. 843-51.

Ramaekers, V. T., Blau, N., Sequeira, J. M., Nassogne, M. C. & Quadros, E. V. (2007a). 'Folate receptor autoimmunity and cerebral folate deficiency in low-functioning autism with neurological deficits', *Neuropediatrics,* 38(6), pp. 276-81.

Ramaekers, V. T., Quadros, E. V. & Sequeira, J. M. (2013). 'Role of folate receptor autoantibodies in infantile autism', *Mol Psychiatry,* 18(3), pp. 270-1.

Ramaekers, V. T., Rothenberg, S. P., Sequeira, J. M., Opladen, T., Blau, N., Quadros, E. V. & Selhub, J. (2005). 'Autoantibodies to folate receptors in the cerebral folate deficiency syndrome', *N Engl J Med,* 352(19), pp. 1985-91.

Ramaekers, V. T., Segers, K., Sequeira, J. M., Koenig, M., Van Maldergem, L., Bours, V., Kornak, U. & Quadros, E. V. (2018). 'Genetic assessment and folate receptor autoantibodies in infantile-onset cerebral folate deficiency (CFD) syndrome', *Mol Genet Metab,* 124(1), pp. 87-93.

Ramaekers, V. T., Sequeira, J. M., Artuch, R., Blau, N., Temudo, T., Ormazabal, A., Pineda, M., Aracil, A., Roelens, F., Laccone, F. & Quadros, E. V. (2007b). 'Folate receptor autoantibodies and spinal fluid 5-methyltetrahydrofolate deficiency in rett syndrome', *Neuropediatrics,* 38(4), pp. 179-83.

Ramaekers, V. T., Thony, B., Sequeira, J. M., Ansseau, M., Philippe, P., Boemer, F., Bours, V. & Quadros, E. V. (2014). 'Folinic acid treatment for schizophrenia associated with folate receptor autoantibodies', *Mol Genet Metab,* 113(4), pp. 307-14.

Rammling, M., Madan, M., Paul, L., Behnam, B. & Pattisapu, J. V. (2008). 'Evidence for reduced lymphatic CSF absorption in the H-Tx rat hydrocephalus model', *Cerebrospinal Fluid Res,* 5, p. 15.

Rasmussen, M. K., Mestre, H. & Nedergaard, M. (2018). 'The glymphatic pathway in neurological disorders', *Lancet Neurol,* 17(11), pp. 1016-1024.

Reddy, O. C. & van der Werf, Y. D. (2020). 'The Sleeping Brain: Harnessing the Power of the Glymphatic System through Lifestyle Choices', *Brain Sci,* 10(11).

Reeves, B. C., Karimy, J. K., Kundishora, A. J., Mestre, H., Cerci, H. M., Matouk, C., Alper, S. L., Lundgaard, I., Nedergaard, M. & Kahle, K. T. (2020). 'Glymphatic System Impairment in Alzheimer's Disease and Idiopathic Normal Pressure Hydrocephalus', *Trends Mol Med,* 26(3), pp. 285-295.

Requena-Jimenez, A., Nabiuni, M. & Miyan, J. A. (2021). 'Profound changes in cerebrospinal fluid proteome and metabolic profile are associated with congenital hydrocephalus', *J Cereb Blood Flow Metab,* 41(12), pp. 3400-3414.

Robinson, N., Grabowski, P. & Rehman, I. (2018). 'Alzheimer's disease pathogenesis: Is there a role for folate?', *Mech Ageing Dev,* 174, pp. 86-94.

Roman, G. C. (2015). 'MTHFR Gene Mutations: A Potential Marker of Late-Onset Alzheimer's Disease?', *J Alzheimers Dis,* 47(2), pp. 323-7.

Sachdev, P. S., Blacker, D., Blazer, D. G., Ganguli, M., Jeste, D. V., Paulsen, J. S. & Petersen, R. C. (2014). 'Classifying neurocognitive disorders: the DSM-5 approach', *Nat Rev Neurol,* 10(11), pp. 634-42.

Sadighi, Z., Butler, I. J. & Koenig, M. K. (2012). 'Adult-onset cerebral folate deficiency', *Arch Neurol,* 69(6), pp. 778-9.

Saijo, T., Abe, T., Someya, Y., Sassa, T., Sudo, Y., Suhara, T., Shuno, T., Asai, K. & Okubo, Y. (2001). 'Ten year progressive ventricular enlargement in schizophrenia: an MRI morphometrical study', *Psychiatry Clin Neurosci,* 55(1), pp. 41-7.

Sayo, A., Jennings, R. G. & Van Horn, J. D. (2012). 'Study factors influencing ventricular enlargement in schizophrenia: a 20 year follow-up meta-analysis', *Neuroimage,* 59(1), pp. 154-67.

Schenning, K. J., Murchison, C. F., Mattek, N. C., Silbert, L. C., Kaye, J. A. & Quinn, J. F. (2016). 'Surgery is associated with ventricular enlargement as well as cognitive and functional decline', *Alzheimers Dement,* 12(5), pp. 590-7.

Scott, M. L., Golden, C. J., Ruedrich, S. L. & Bishop, R. J. (1983). 'Ventricular enlargement in major depression', *Psychiatry Res,* 8(2), pp. 91-3.

Selhub, J. (2002). 'Folate, vitamin B12 and vitamin B6 and one carbon metabolism', *J Nutr Health Aging,* 6(1), pp. 39-42.

Serrano, M., Garcia-Silva, M. T., Martin-Hernandez, E., O'Callaghan Mdel, M., Quijada, P., Martinez-Aragon, A., Ormazabal, A., Blazquez, A., Martin, M. A., Briones, P., Lopez-Gallardo, E., Ruiz-Pesini, E., Montoya, J., Artuch, R. & Pineda, M. (2010). 'Kearns-Sayre syndrome: cerebral folate deficiency, MRI findings and new cerebrospinal fluid biochemical features', *Mitochondrion,* 10(5), pp. 429-32.

Serrano, M., Perez-Duenas, B., Montoya, J., Ormazabal, A. & Artuch, R. (2012). 'Genetic causes of cerebral folate deficiency: clinical, biochemical and therapeutic aspects', *Drug Discov Today,* 17(23-24), pp. 1299-306.

Shen, M. D. (2018). 'Cerebrospinal fluid and the early brain development of autism', *J Neurodev Disord,* 10(1), p. 39.

Shen, M. D., Kim, S. H., McKinstry, R. C., Gu, H., Hazlett, H. C., Nordahl, C. W., Emerson, R. W., Shaw, D., Elison, J. T., Swanson, M. R., Fonov, V. S., Gerig, G., Dager, S. R., Botteron, K. N., Paterson, S., Schultz, R. T., Evans, A. C., Estes, A. M., Zwaigenbaum, L., Styner, M. A., Amaral, D. G., Piven, J., Hazlett, H. C., Chappell, C., Dager, S., Estes, A., Shaw, D., Botteron, K., McKinstry, R., Constantino, J., Pruett, J., Schultz, R., Zwaigenbaum, L., Elison, J., Evans, A. C., Collins, D. L., Pike, G. B., Fonov, V., Kostopoulos, P., Das, S., Gerig, G., Styner, M., Gu, H., Piven, J. & Infant Brain Imaging Study, N. (2017). 'Increased Extra-axial Cerebrospinal Fluid in High-Risk Infants Who Later Develop Autism', *Biol Psychiatry,* 82(3), pp. 186-193.

Shen, M. D., Nordahl, C. W., Li, D. D., Lee, A., Angkustsiri, K., Emerson, R. W., Rogers, S. J., Ozonoff, S. & Amaral, D. G. (2018). 'Extra-axial cerebrospinal fluid in high-risk and normal-risk children with autism aged 2-4 years: a case-control study', *Lancet Psychiatry,* 5(11), pp. 895-904.

Shen, M. D., Nordahl, C. W., Young, G. S., Wootton-Gorges, S. L., Lee, A., Liston, S. E., Harrington, K. R., Ozonoff, S. & Amaral, D. G. (2013). 'Early brain enlargement and elevated extra-axial fluid in infants who develop autism spectrum disorder', *Brain,* 136(Pt 9), pp. 2825-35.

Silverberg, G., Mayo, M., Saul, T., Fellmann, J. & McGuire, D. (2006). 'Elevated cerebrospinal fluid pressure in patients with Alzheimer's disease', *Cerebrospinal Fluid Res,* 3, p. 7.

Silverberg, G. D. (2004). 'Normal pressure hydrocephalus (NPH): ischaemia, CSF stagnation or both', *Brain,* 127(Pt 5), pp. 947-8.

Silverberg, G. D., Heit, G., Huhn, S., Jaffe, R. A., Chang, S. D., Bronte-Stewart, H., Rubenstein, E., Possin, K. & Saul, T. A. (2001). 'The cerebrospinal fluid production rate is reduced in dementia of the Alzheimer's type', *Neurology,* 57(10), pp. 1763-6.

Silverberg, G. D., Huhn, S., Jaffe, R. A., Chang, S. D., Saul, T., Heit, G., Von Essen, A. & Rubenstein, E. (2002). 'Downregulation of cerebrospinal fluid production in patients with chronic hydrocephalus', *J Neurosurg,* 97(6), pp. 1271-5.

Silverberg, G. D., Mayo, M., Saul, T., Rubenstein, E. & McGuire, D. (2003). 'Alzheimer's disease, normal-pressure hydrocephalus, and senescent changes in CSF circulatory physiology: a hypothesis', *Lancet Neurol,* 2(8), pp. 506-11.

Steinfeld, R., Grapp, M., Kraetzner, R., Dreha-Kulaczewski, S., Helms, G., Dechent, P., Wevers, R., Grosso, S. & Gartner, J. (2009). 'Folate receptor alpha defect causes cerebral folate transport deficiency: a treatable neurodegenerative disorder associated with disturbed myelin metabolism', *Am J Hum Genet,* 85(3), pp. 354-63.

Stoccoro, A. & Coppede, F. (2018). 'Role of epigenetics in Alzheimer's disease pathogenesis', *Neurodegener Dis Manag,* 8(3), pp. 181-193.

Stoiljkovic, M., Horvath, T. L. & Hajos, M. (2021). 'Therapy for Alzheimer's disease: Missing Targets and Functional Markers?', *Ageing Res Rev*, p. 101318.

Strakowski, S. M., DelBello, M. P., Zimmerman, M. E., Getz, G. E., Mills, N. P., Ret, J., Shear, P. & Adler, C. M. (2002). 'Ventricular and periventricular structural volumes in first- versus multiple-episode bipolar disorder', *Am J Psychiatry,* 159(11), pp. 1841-7.

Strickland, K. C., Krupenko, N. I., Dubard, M. E., Hu, C. J., Tsybovsky, Y. & Krupenko, S. A. (2011). 'Enzymatic properties of ALDH1L2, a mitochondrial 10-formyltetrahydrofolate dehydrogenase', *Chem Biol Interact,* 191(1-3), pp. 129-36.

Suormala, T., Gamse, G. & Fowler, B. (2002). '5,10-Methylenetetrahydrofolate reductase (MTHFR) assay in the forward direction: residual activity in MTHFR deficiency', *Clin Chem,* 48(6 Pt 1), pp. 835-43.

Thome, U., Klima, P., Moosa, A. N., Gupta, A., Parikh, S. & Pestana Knight, E. M. (2016). 'Electrographic status epilepticus in sleep in an adult with cerebral folate deficiency', *Neurol Clin Pract,* 6(1), pp. e4-e7.

Tice, C., McDevitt, J. & Langford, D. (2020). 'Astrocytes, HIV and the Glymphatic System: A Disease of Disrupted Waste Management?', *Front Cell Infect Microbiol,* 10, p. 523379.

Torres, A., Newton, S. A., Crompton, B., Borzutzky, A., Neufeld, E. J., Notarangelo, L. & Berry, G. T. (2015). 'CSF 5-Methyltetrahydrofolate Serial Monitoring to Guide Treatment of Congenital Folate Malabsorption Due to Proton-Coupled Folate Transporter (PCFT) Deficiency', *JIMD Rep,* 24, pp. 91-6.

Troen, A. M. (2012). 'Folate and vitamin B12: function and importance in cognitive development', *Nestle Nutr Inst Workshop Ser,* 70, pp. 161-71.

Vellas, B., Aisen, P. S., Sampaio, C., Carrillo, M., Scheltens, P., Scherrer, B., Frisoni, G. B., Weiner, M., Schneider, L., Gauthier, S., Gispen-de Wied, C. C., Hendrix, S., Feldman, H., Cedarbaum, J., Petersen, R., Siemers, E., Andrieu, S., Prvulovic, D., Touchon, J. & Hampel, H. (2011). 'Prevention trials in Alzheimer's disease: an EU-US task force report', *Prog Neurobiol,* 95(4), pp. 594-600.

Vita, A., Dieci, M., Silenzi, C., Tenconi, F., Giobbio, G. M. & Invernizzi, G. (2000). 'Cerebral ventricular enlargement as a generalized feature of schizophrenia: a distribution analysis on 502 subjects', *Schizophr Res,* 44(1), pp. 25-34.

Wang, G. J., Volkow, N. D., Roque, C. T., Cestaro, V. L., Hitzemann, R. J., Cantos, E. L., Levy, A. V. & Dhawan, A. P. (1993). 'Functional importance of ventricular enlargement and cortical atrophy in healthy subjects and alcoholics as assessed with PET, MR imaging, and neuropsychologic testing', *Radiology,* 186(1), pp. 59-65.

Weed, L. H. (1914). 'Studies on Cerebro-Spinal Fluid. No. III : The pathways of escape from the Subarachnoid Spaces with particular reference to the Arachnoid Villi', *J Med Res,* 31(1), pp. 51-91.

Weggen, S. & Beher, D. (2012). 'Molecular consequences of amyloid precursor protein and presenilin mutations causing autosomal-dominant Alzheimer's disease', *Alzheimers Res Ther,* 4(2), p. 9.

Willemsen, M. A., Wevers, R. A. & Verbeek, M. M. (2005). 'Cerebral folate deficiency syndrome', *N Engl J Med,* 353(7), p. 740; author reply 740.

Wu, Y., Li, S., Wang, W. & Zhang, D. (2020). 'Associations of dietary vitamin B1, vitamin B2, niacin, vitamin B6, vitamin B12 and folate equivalent intakes with metabolic syndrome', *Int J Food Sci Nutr,* 71(6), pp. 738-749.

Ye, B. S., Lee, Y., Kwak, K., Park, Y. H., Ham, J. H., Lee, J. J., Shin, N. Y., Lee, J. M., Sohn, Y. H. & Lee, P. H. (2016). 'Posterior Ventricular Enlargement to Differentiate Dementia with Lewy Bodies from Alzheimer's Disease', *J Alzheimers Dis,* 52(4), pp. 1237-43.

Yuvaniyama, J., Chitnumsub, P., Kamchonwongpaisan, S., Vanichtanankul, J., Sirawaraporn, W., Taylor, P., Walkinshaw, M. D. & Yuthavong, Y. (2003). 'Insights into antifolate resistance from malarial DHFR-TS structures', *Nat Struct Biol,* 10(5), pp. 357-65.

Zalsman, G., Huang, Y. Y., Harkavy-Friedman, J. M., Oquendo, M. A., Ellis, S. P. & Mann, J. J. (2005). 'Relationship of MAO-A promoter (u-VNTR) and COMT (V158M) gene polymorphisms to CSF monoamine metabolites levels in a psychiatric sample of caucasians: A preliminary report', *Am J Med Genet B Neuropsychiatr Genet,* 132B(1), pp. 100-3.

Zalsman, G., Huang, Y. Y., Oquendo, M. A., Brent, D. A., Giner, L., Haghighi, F., Burke, A. K., Ellis, S. P., Currier, D. & Mann, J. J. (2008). 'No association of COMT Val158Met polymorphism with suicidal behavior or CSF monoamine metabolites in mood disorders', *Arch Suicide Res,* 12(4), pp. 327-35.

Zhang, C., Deng, X., Wen, Y., He, F., Yin, F. & Peng, J. (2020a). 'First case report of cerebral folate deficiency caused by a novel mutation of FOLR1 gene in a Chinese patient', *BMC Med Genet,* 21(1), p. 235.

Zhang, C., Luo, J., Yuan, C. & Ding, D. (2020b). 'Vitamin B12, B6, or Folate and Cognitive Function in Community-Dwelling Older Adults: A Systematic Review and Meta-Analysis', *J Alzheimers Dis,* 77(2), pp. 781-794.

Zhang, L., Chopp, M., Jiang, Q. & Zhang, Z. (2019). 'Role of the glymphatic system in ageing and diabetes mellitus impaired cognitive function', *Stroke Vasc Neurol,* 4(2), pp. 90-92.

Zhang, X., Bao, G., Liu, D., Yang, Y., Li, X., Cai, G., Liu, Y. & Wu, Y. (2021). 'The Association Between Folate and Alzheimer's Disease: A Systematic Review and Meta-Analysis', *Front Neurosci,* 15, p. 661198.

Zhao, H., Wei, T., Li, X. & Ba, T. (2018). 'Early life adversity induced third ventricular enlargement in young adult male patients suffered from major depressive disorder: a study of brain morphology', *Folia Morphol (Warsz),* 77(3), pp. 428-433.

Zhao, R., Diop-Bove, N., Visentin, M. & Goldman, I. D. (2011). 'Mechanisms of membrane transport of folates into cells and across epithelia', *Annu Rev Nutr,* 31, pp. 177-201.

Zou, Y., Kennedy, K. G., Grigorian, A., Fiksenbaum, L., Freeman, N., Zai, C. C., Kennedy, J. L., MacIntosh, B. J. & Goldstein, B. I. (2021). 'Antioxidative Defense Genes and Brain Structure in Youth Bipolar Disorder', *Int J Neuropsychopharmacol*.

Zhou, X. B., Y. X. Zhang, C. X. Zhou and J. J. Ma (2022). "Chinese Herbal Medicine Adjusting Brain Microenvironment via Mediating Central Nervous System Lymphatic Drainage in Alzheimer's Disease." Chin J Integr Med 28(2): 176-184.

Chapter 11

ASPM and microcephaly in a Pakistani family

Chapter 11

Preamble: Genetic analysis of neurological conditions in Pakistan

The PhD project at QAU originally aimed to analyse genetic defects associated with different neurological conditions, of which there are many in Pakistan. Identifying gene errors associated with neurological conditions is an important aspect of understanding their cause, aetiology and outcomes. This is particularly true in a country where consanguinity is very high and the likelihood of finding genetic causes increases. However, the consequences of abnormal genes needs additional research at the molecular and cellular, not to mention systems/physiological level.

With the author (SFN) successfully obtaining funding for a split-site PhD with Manchester University, the direction of the project was modified to include functional genomics, including nutrigenomics, as well as investigating the underlying physiological changes associated with the specific conditions of ageing, dementia and AD.

The paper presented here is a result of work carried out prior to the split-site PhD agreement and demonstrates the trajectory of the genetic studies at QAU. The body of the thesis demonstrates a multi-faceted approach to understanding a condition that includes genetics.

Original Manuscript Accepted for Publication in

Genetic Testing and Molecular Biomarker

A two-base pair deletion in IQ repeats in *ASPM* underlies microcephaly in a Pakistani family.

Authors: Syeda Farwa Naqvi[1], Rana Muhammad Kamran Shabbir[1], Aslıhan Tolun[2], Sulman Basit[3,] Sajid Malik[1]

[1]Human Genetics Program, Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan

[2]Department of Molecular Biology and Genetics, MOBGAM, Istanbul Technical University, Istanbul 34467, Turkey

[3]Taibah University Madinah, Kingdom of Saudi Arabia

Corresponding authors:

Sulman Basit, Taibah University Madinah, Kingdom of Saudi Arabia

E-mail: sbasit.phd@gmail.com ORCID 0000-0003-4294-6825

Sajid Malik, Human Genetics Program, Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. Tel: +92-51-90643158.
E-mail: malik@qau.edu.pk ORCID 0000-0001-5563-9053

Short running title: *ASPM* variant in primary microcephaly

Conflict of interest: None declared

ABSTRACT

Aims: Autosomal recessive primary microcephaly (MCPH) is a rare and clinically and genetically highly heterogeneous developmental disorder. Biallelic variants in *abnormal spindle-like microcephaly-associated* (*ASPM*) gene account for 40% to 68% of all MCPH cases. This study aimed to elucidate the genetic basis of MCPH in an extended family. In order to highlight recurrent mutations useful in implementing genetic testing programs, we further aimed to carry out a descriptive review of the reported *ASPM* mutations.

Materials and Methods: A large, inbred kindred with 7 affected members is investigated, and detailed clinical and behavioural assessments were carried out. SNP-based homozygosity mapping and exome sequencing were performed.

Results: Affected individuals have characteristic features including small head, receding forehead, mild to moderate intellectual disability, developmental delay, short stature, speech apraxia, and behavioural anomalies. We mapped the disease gene locus and detected rare frameshift deletion c.6854_6855del (p.(Leu2285GlnfsTer32)) in exon 18 of *ASPM*. A total of 215 mutations in *ASPM* have been reported in at least 453 families, nearly 50% of which are of Pakistani origin. Mutations are either recurrent, founder or private mutations in the Pakistani or some other populations.

Conclusion: SNP-based homozygosity mapping and exome sequencing are essential in delineating the genetically distinct microcephaly types. The highlighted recurrent mutations in *ASPM* could be useful in implementing genetic testing programs for MCPH.

Keywords: Intellectual disability; short stature; small head; developmental delay; speech apraxia

INTRODUCTION

Autosomal recessive primary microcephaly (MCPH; MIM-251200) is a clinically and genetically highly heterogeneous developmental anomaly that is characterized by prenatal onset of brain growth impairment resulting in reduced brain volume, which is measured as an occipitofrontal circumference equal to or >2 standard deviations (SDs) and 3 SDs below the age- and sex-matched means at birth and 6 months, respectively. This condition leads to intellectual disability (ID) without any significant neurological deficit (Von der Hagen *et al.,* 2014; Barbelanne and Tsang, 2014). At least 28 loci are known for MCPH, and the corresponding genes have been discovered (https://www.omim.org/phenotypicSeries/PS251200). MCPH1-17, MCPH19-25 and MCPH28 is inherited in an autosomal recessive fashion whereas MCPH18, MCPH26 and MCPH27 are autosomal dominant. Biallelic variants in *ASPM* gene (MIM-605481) are the most frequent cause of MCPH and account for 40-68% of primary microcephaly cases (Zaqout *et al.*, 2017; Letard *et al.*, 2018). Nearly half of all reported MCPH patients originate from Pakistan.

Genetically distinct microcephaly types share high phenotypic similarity such as congenital onset, short stature, mild to severe intellectual disability, receding forehead, decreased brain weight, disproportionately thin cerebral cortex, developmental and speech delay, and speech apraxia (OMIM; Faheem *et al.,* 2015; Shaheen *et al.,* 2019; Jean *et al.,* 2020). High throughput methods such as SNP-based genotyping and exome sequencing are essential to delineate genetically heterogeneous conditions like microcephaly.

We present Pakistani kindred with 7 microcephalic members. Through SNP-based genotyping and homozygosity mapping followed by exome sequencing we showed that a rare variant in *ASPM* segregates with the malformation in the family. We also carried out a descriptive review of the reported *ASPM* mutations and highlight recurrent mutations useful in implementing genetic testing programs.

MATERIALS AND METHODS

Sample Collection and Clinical Investigations

Peripheral blood samples were collected after obtaining informed consent according to the Helsinki II declaration. The study protocol was approved by the ethical review committee of Quaid-i-Azam University (DAS-1070) and the Istanbul Technical University Ethics Review Board (MBG.22/2014).

The family is from a remote area of Punjab, Pakistan. The five-generation pedigree strongly suggested an autosomal recessive pattern (Figure 1). Seven family members (3 males and 4 females) at ages 16 to 32 years were affected. In total 10 family members (5 affected and 5 unaffected) were physically examined with the help of local physicians. Photographs of were taken for all participants, and anthropometric measurements of affected subjects were obtained.

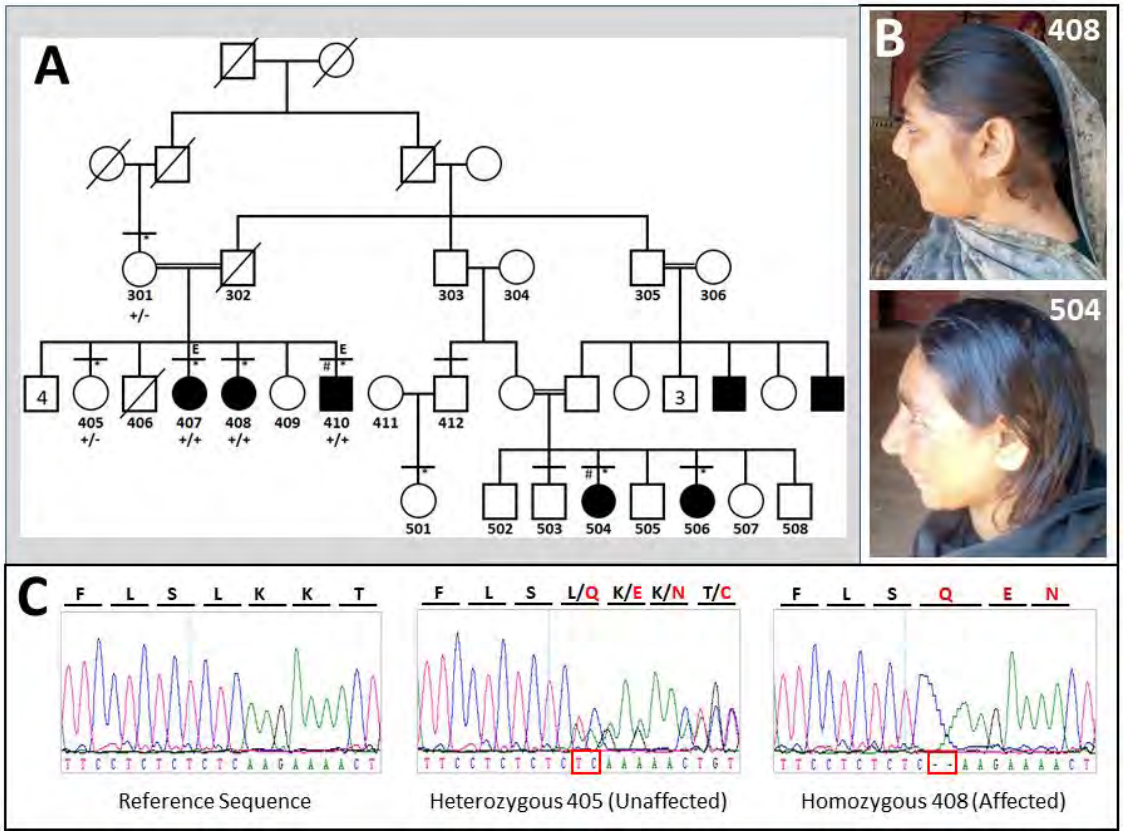


Figure 1: Family and patients. A. Pedigree of the family showing also genotypes for *ASPM* c.6854_6855del. +, variant; -, reference allele. DNA was available from individuals marked *. SNP genotype data were generated for individuals marked #. Exome data were generated for individuals marked E. Horizontal line above the symbol indicates that clinical examination was performed. B. Facial features of patients 408 and 504 showing small head, receding forehead, prominent nose and large ears. C. Electropherograms showing causative *ASPM* c.6854_6855del. Deleted nucleotides are in red box.

Genetic Analyses

Genotype data for 710K SNP markers for a mixture of DNA samples of two affected individuals (410 and 504) were generated using Illumina Human OmniExpress-24 BeadChip. Intervals >1Mb with shared homozygosity were detected through HomozygosityMapper and manual inspection on MS-Excel.

DNA sample of affected sibs 407 and 410 were subjected to exome sequencing with Illumina TruSeq Exome Capture kit followed by massively parallel paired-end sequencing with Illumina HiSeq 2000 (Illumina, USA). Variant calling and annotation of exome data were performed by using BWA, SAMTools and ANNOVAR (2019Oct24). The alignments were visualized with BamView. The threshold for sufficient coverage was assumed as four reads, and the minimum accepted threshold for quality score was 40. In candidate regions rare (frequency <0.01) and novel variants were selected according to the information in public databases gnomAD (v2.1.1) that contains thousands of Pakistani exomes, 1000 Genome and ESP6500 (SI-V2), and those possibly homozygous (alternate depths >0.60) and affecting protein structure were considered. Variants found in in-lab exome files were excluded. Sanger sequencing was carried out to assess the segregation of the variant with the disease. The genome assembly of hg19 was utilized.

RESULTS

Clinical findings

Patients have small heads, mild to moderate ID, short stature, narrow and oval shaped faces, receding foreheads, large ears and prominent noses (Table 1; Fig. 1B). They have attention deficit behaviour and speech apraxia. They never attended a school of any kind, because of low ID. Affected males spend time wandering in the streets and have no concept of money. They are not able to perform any conceptual work but recognize relatives and find their way home. They have sense of self-respect. They are comfortable with strangers and are friendly towards them. For instance, female individual 504 at age 34 always has a smiley face. According to family elders, affected relatives had delayed developmental landmarks including delayed crawling, walking and toilet training. They do not have hyperactive or aggressive behaviour,

and epilepsy and bipolar episodes were not observed. Anthropometric measurements showed marked developmental failures (Supp. Table 1).

Table 1. Physical and clinical features of affected family members

Pedigree IDs	**407**	**408**	**410**	**504**	**506**
Sex, age (years)	F, 22	F, 20	M, 17	F, 34	F, 16
Clinical features					
Intellectual disability*	Moderate	Moderate	Mild	Moderate	Moderate
Short stature	+	+	+	+	+
Small head	+	+	+	+	+
Receding forehead	+	+	+	+	+
Narrow oval face	+	+	+	+	+
Large ears	+	+	+	+	+
Prominent nose	+	+	+	+	+
Speech apraxia	+	+	–	+	+
Developmental features					
Developmental delay	+	+	+	+	+
Crawling and walking late	+	+	+	+	+
Speech delay	+	+	+	+	+
Behavioural features					
Attention deficit	+	+	+	+	+
Unable to attend school	+	+	+	+	+
Impulsivity	–	+	–	+	+
Aggression, short temper	–	–	–	–	–
Bipolar episodes	–	–	–	–	–
Epilepsy	–	–	–	–	–
Physical disability	–	–	–	–	–
Hyperactive	–	–	–	–	–

*criteria of the American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 5th Ed., Washington, DC. 2013.

+, feature present; −, feature absent

Genetic findings

SNP genotypes were used to detect 22 autosomal homozygous regions >1 Mb shared by the two affected siblings. Those regions were scrutinized for potential candidate genes through GeneDistiller. Exome filtration strategy revealed a total of eight variants common to the exome files of the two affected sibs (Supp. Table 2). Seven of those variants were functionally not relevant to the phenotype and did not fall in a homozygous interval. Frameshift variant c.6854_6855del (p.(Leu2285GlnfsTer32); NM_018136.4) in *ASPM* exon 18 was detected in a shared homozygosity region of 7.83 Mb at 1q31. It is extremely rare, with allele frequency 0.00003268 (1 allele in 30604 alleles from South Asian samples in gnomAD). It has been reported in compound heterozygous in an MCPH family (Rasool *et al.*, 2020). In the exome file no pathogenic variant was found in other MCPH-related genes.

Descriptive summary of *ASPM* mutations:

In order to investigate the recurrence and to understand the pattern of mutation, we compiled all *ASPM* variants assembled by Letard *et al.,* (2018) and Rasool *et al.,* (2020) and added more recently reported variants (Batool *et al.,* 2021; Khan *et al.,* 2021; Naseer *et al.,* 2021). In order to investigate recurrence and detect potential mutational hotspots, data were extracted from published reports employing large microcephaly cohorts (Tan *et al.*, 2014; Wang *et al.*, 2017; Ahmad *et al.*, 2017; Ahmad *et al.,* 2019; Shaheen *et al.,* 2019). Those data extend the number of different disease variants to 215 in at least 453 families.

Reported causative mutations are dispersed throughout the gene. A summary of the distribution is presented in Fig. 2. There is a direct relationship between exon length and the number of variants (Spearman correlation $R^2 = 0.9771$), indicating that there is no mutation hotspot. The majority (n=200) of the mutations fall across coding regions; only 15 are intronic. There are two complex re-arrangements and two large deletions that encompass several exons/introns. Majority of the mutations are nonsense (n=92), followed by small frameshift deletions (n=84), splicing (n=17), and small frameshift duplications (n=14) (Table 2). Missense variants are scarce (n=3), raising the question whether mild mutations are tolerated. Nonsense mutations are highest in exon 18 (n=36), followed by exon 13 (n=9). Small frameshift deletions are also more prevalent in exon 18 and 13. Highest number of splicing variants is in intron 10. Exon 7 is the smallest exon (68 bp) and has no known mutation.

Microcephaly families of Pakistani origin have the highest contribution to the mutational heterogeneity of *ASPM*. Tables 2 and 3 shows the recurrent mutations and the number of reported families for mutation types. The most common variant is c.3978G>A (p.(Trp1326Ter)) followed by c.7782_7783delGA (p.(Lys2595SerfsTer6)), occurring in at least 77 and 21 families, respectively (totally 22% of all families). At least seven mutations are likely founder mutations reported primarily or exclusively in the Pakistani population. Nearly half of the mutations (n=149) are private, with 30% contribution of novel mutations from Pakistani patients. Among at least 110 base substitutions, the C>T transition is the most frequent variant (n=51) followed by transversion G>A (n=15), both likely due to de-amination of a C. The small frameshift deletions and duplications comprising 98 of the variants could be due to DNA polymerase slippage.

Table 2. *ASPM* mutation types, number of affected families and the origin of the families

Mutation type	**No.**	**%age**	**Families**						
			Total	**Pakistani**	**European**	**Saudi/Yemen**	**Egyptian**	**Iranian**	**Turkish**
Nonsense	92	42.8	261	151	36	14	11	7	6
Small deletion (FS)	84	39.1	144	48	40	11	5	2	4
Splicing	17	7.9	20	4	3	2	1	3	0
Small duplication (FS)	14	6.5	16	4	1	0	1	1	2
Missense	3	1.4	7	1	0	1	0	5	0
Insertion/deletion (complex rearrangement)	2	0.9	2	0	1	0	0	0	0
Large deletion	2	0.9	2	0	2	0	0	0	0
Insertion	1	0.5	1	0	1	0	0	0	0
Total	**215**	**100.0**	**453**	**208**	**84**	**28**	**18**	**18**	**12**

FS, frameshift

Table 3. Recurrent mutations and number of families with the variant

Exon	Mutation	Effect on protein	Consequence	Total families	Pakistani families	*Other common origin*
17	c.3978G>A	p.Trp1326*	Nonsense	77	75	Turkish (2)
18	c.7782_7783delGA	p.Lys2595Serfs*6	Small deletion (FS)	21	4	European (7); Algerian (4)
24	c.9730C>T	p.Arg3244*	Nonsense	10	9	African
18	c.8508_8509delGA	p.Lys2837Metfs*34	Small deletion (FS)	9	9	-
3	c.1138C>T	p.Gln380*	Nonsense	7	0	Saudi (6)
23	c.9557C>G	p.Ser3186*	Nonsense	7	7	-
11	c.3055C>T	p.Arg1019*	Nonsense	6	2	European (3)
3	c.1260_1266del	p.Gln421Hisfs*32	Small deletion (FS)	5	5	-
4	c.1959_1962del	p.Asn653Lysfs*14	Small deletion (FS)	5	0	Saudi (3)
16	c.3796G>T	p.Glu1266*	Nonsense	5	0	African (2)
18	c.4795C>T	p.Arg1599*	Nonsense	5	3	European
18	c.5584A>C	p.Lys1862Gln	Missense	5	0	Iranian (5)
18	c.8017C>T	p.Gln2673*	Nonsense	5	0	Saudi (3)
21	c.9190C>T	p.Arg3064*	Nonsense	5	2	Egyptian (2)
23	c.9492T>G	p.Tyr3164*	Nonsense	5	5	-
24	c.9697C>T	p.Arg3233*	Nonsense	5	1	Saudi/Egyptian (2)
	Mutations reported in 4 families			4 x 4	4	European (5)
	Mutations reported in 3 families			3 x 10	13	European (5)
	Mutations reported in 2 families			2 x 36	26	European (9)
	Private			149	44	European (45)

FS, frameshift

DISCUSSION

Here we demonstrate that homozygous frameshift deletion c.6854_6855del (p.(Leu2285GlnfsTer32)) underlies MCPH microcephaly in a large Pakistani kindred. Due to high genetic heterogeneity in MCPH, we initially launched SNP-based homozygosity mapping and detected homozygous intervals shared between two affected relatives in different branches of the kindred. SNP genotyping coupled with exome sequencing ruled out linkage to any other known recessive MCPH locus. The 2-base pair deletion detected in the present family falls in the IQ repeats of *ASPM* and is deduced to cause a shift in the translational frame and incorporation of 32 non-native amino acids before termination, or NMD could occur prior to translation. The resulting truncation causes the deletion of approximately 33% of the native protein. Functional studies on *ASPM* have revealed that calmodulin binding to IQ motifs induces a conformational change in proteins that regulate the binding of actin to the amino terminal CH domains. It has been proposed that changes in *ASPM* alter the orientation of the mitotic spindle of neuroblasts, which induces symmetric mitosis that results in two progenitor cells (Kouprina *et al.,* 2004). IQ motifs have been suggested to play an essential role in determining brain size (Zhang *et al.,* 2003).

ASPM is a large gene with 28 exons and codes for a 3477-amino acid protein. Majority of the reported mutations are nonsense, small deletions, splicing or small duplications. Those mutations are spread throughout the protein, and the majority are predicted to generate either a premature truncated protein or unstable RNA that is degraded by nonsense-mediated RNA decay (NMD) (Abdel-Hamid *et al.,* 2016; Letard *et al.,* 2018). In summary, the majority of *ASPM* mutations likely cause loss-of-function of the encoded protein (Letard *et al.,* 2018). Based on the accumulated data on recurrent and founder mutations, it should be possible to implement genetic testing and molecular diagnosis of MCPH. Furthermore, as remarked by Letard *et al.* (2018), functional studies are warranted to prove the pathogenicity of the reported variants.

Previously, it was observed that there was no straightforward genotype–phenotype correlation between mutation type or site and the head size and other clinical features associated with MCPH (Nicholas *et al.,* 2009). It is, however, pertinent to mention that the genotype–phenotype studies are limited due to unavailability of detailed clinical description, quantitative evaluations of cognitive, neurodevelopmental and behavioural variables, and neuro-imaging studies. It also remains to be elucidated whether the functional and phenotypic impact of

frameshift and nonsense mutations occurring in the initial exons is milder than such mutations occurring at the end of gene, or vice versa. Furthermore, the consequences of the mutations falling in four distinguishable regions of *ASPM* protein remains unknown.

ASPM is the human ortholog of the *Drosophila melanogaster* 'abnormal spindle' gene (*asp*), which has a pivotal role in normal mitotic spindle function in embryonic neuroblasts. In mouse, it has been shown that Aspm protein has a role in the regulation of mitotic spindle and neurogenesis (Fish *et al.,* 2006). Mutations in *ASPM* are also known to cause reduction in the size of brain in mice (Pulvers *et al.,* 2010). These evidences are suggestive that mutations in *ASPM* impair the development of brain by perturbing the orientation of the mitotic spindle and can decrease the number of neuronal cells by affecting the asymmetrical to symmetrical cell division ratios (Thornton *et al.,* 2009). Further molecular studies are required in order to understand the detailed cellular mechanisms involved in the pathogenicity of *ASPM* defects in microcephaly.

Conclusions: SNP-based homozygosity mapping and exome sequencing are essential in delineating the genetically distinct microcephaly types. The mutation spectrum of *ASPM* comprises recurrent, founder and private mutations some of which have been reported primarily or exclusively in the Pakistani population. The highlighted recurrent mutations in *ASPM* could be useful in implementing genetic testing programs for MCPH.

Acknowledgments: We thank family members for their cooperation.

Authorship confirmation statement: All persons who meet **authorship** criteria are listed as **authors**

Author(s') disclosure statement(s): None declared

Funding: This study was supported by URF-QAU, Pakistan [grant number DFBS/2014/3230, 2013-2014] and the Scientific and Technological Research Council of Turkey [TÜBITAK, grant number 114Z829].

REFERENCES

Abdel-Hamid MS, Ismail MF, Darwish HA, *et al.* (2016) Molecular and phenotypic spectrum of ASPM-related primary microcephaly: identification of eight novel mutations. Am J Med Genet A 170A:2133–2140.

Ahmad I, Baig SM, Abdulkareem AR, *et al.* (2017). Genetic heterogeneity in Pakistani microcephaly families revisited. Clin Genet 92:62–68. doi.org/10.1111/cge.12955

Ahmed J, Windpassinger C, Salim M, *et al.* (2019). Genetic study of Khyber-Pukhtunkhwa resident Pakistani families presenting primary microcephaly with intellectual disability. J Pak Med Assoc 69:1812–1816. https://doi.org/10.5455/JPMA.300681

APA. American Psychiatric Association, Diagnostic and statistical manual of mental disorders. 5th Ed, Washington, DC. 2013.

Barbelanne M, Tsang WT (2014) Molecular and Cellular Basis of Autosomal Recessive Primary Microcephaly. Biomed Res Int 547986. doi:10.1155/2014/547986.

Batool T, Irshad S, Mahmood K (2021) Novel Pathogenic Mutation Mapping of ASPM Gene in Consanguineous Pakistani Families with Primary Microcephaly. Braz J Biol 83:e246040. doi:10.1590/1519-6984.246040.

Faheem M, Naseer MI, Rasool M, *et al.* (2015) Molecular genetics of human primary microcephaly: an overview. BMC Med Genomics 8:S4. https://doi.org/10.1186/1755-8794-8-S1-S4

Fish JL, Kosodo Y, Enard W, *et al.* (2006) Aspm specifically maintains symmetric proliferative divisions of neuroepithelial cells. Proc Natl Acad Sci USA 103:10438–10443.

Jean F, Stuart A, Tarailo-Graovac M (2020) Dissecting the Genetic and Etiological Causes of Primary Microcephaly. Front Neurol 15;11:570830. doi:10.3389/fneur.2020.570830.

Khan NM, Hussain B, Zheng C, *et al.* (2021) Updates on Clinical and Genetic Heterogeneity of ASPM in 12 Autosomal Recessive Primary Microcephaly Families in Pakistani Population. Frontiers in Pediatrics 9:695133. https://doi.org/10.3389/fped.2021.695133

Kouprina N, Pavlicek A, Mochida GH, *et al.* (2004) Accelerated evolution of the ASPM gene controlling brain size begins prior to human brain expansion. PLoS Biol 2:e126.

Letard P, Drunat S, Vial Y, *et al.* (2018) Autosomal recessive primary microcephaly due to ASPM mutations: An update. Hum Mut 39:319–332. Doi:10.1002/humu.23381.

Naseer MI, Abdulkareem AA, Muthaffar OY, *et al.* (2021) Whole Exome Sequencing Identifies Three Novel Mutations in the ASPM Gene From Saudi Families Leading to Primary Microcephaly. Frontiers in Pediatrics 8:627122. https://doi.org/10.3389/fped.2020.627122

Nicholas AK, Swanson EA, Cox JJ, *et al.* (2009) The molecular landscape of ASPM mutations in primary microcephaly. J Med Genet 46:249–253

Passemard S, Titomanlio L, Elmaleh E, *et al.* (2009). Expanding the clinical and neuroradiologic phenotype of primary microcephaly due to ASPM mutations. Neurology 73:962–969. doi.org/10.1212/WNL.0b013e3181b8799a

Pulvers JN, Bryk J, Fish JL, *et al.* (2010) Mutations in mouse Aspm (abnormal spindle-like microcephaly associated) cause not only microcephaly but also major defects in the germline. Proc Nat Acad Sci USA 107:16595-16600. Doi:10.1073/pnas.1010494107.

Rasool S, Baig JM, Moawia A, *et al.* (2020) An update of pathogenic variants in ASPM, WDR62, CDK5RAP2, STIL, CENPJ, and CEP135 underlying autosomal recessive primary microcephaly in 32 consanguineous families from Pakistan. Mol Genet Genomic Med 8:e1408. doi: 10.1002/mgg3.1408.

Shaheen R, Maddirevula S, Ewida N, *et al.* (2019) Genomic and phenotypic delineation of congenital microcephaly. Genet Med 21:545-552. doi: 10.1038/s41436-018-0140-3.

Tan CA, del Gaudio D, Dempsey MA, *et al.* (2014) Analysis of ASPM in an ethnically diverse cohort of 400 patient samples: perspectives of the molecular diagnostic laboratory. Clin Genet 85(4):353-8.

Thornton GK, Woods CG. (2009) Primary microcephaly: do all roads lead to Rome? Trends Genet 25:501-510. Doi:10.1016/j.tig.2009.09.011.

Von der Hagen M, Pivarcsi M, Liebe J, *et al.* (2014) Diagnostic approach to microcephaly in childhood: A two-center study and review of the literature. Dev Med Child Neurol 56:732. doi:10.1111/dmcn.12425.

Wang R, Khan A, Han S, *et al.* (2017) Molecular analysis of 23 Pakistani families with autosomal recessive primary microcephaly using targeted next-generation sequencing. J Hum Genet 62:299–304.

Zaqout S, Morris-Rosendahl D, Kaindl AM. (2017) Autosomal Recessive Primary Microcephaly (MCPH): An update. Neuropediatrics 48:135–142. Doi:10.1055/s-0037-1601448.

Zhang J (2003) Evolution of the human ASPM gene, a major determinant of brain size. Genetics 165:2063–70.

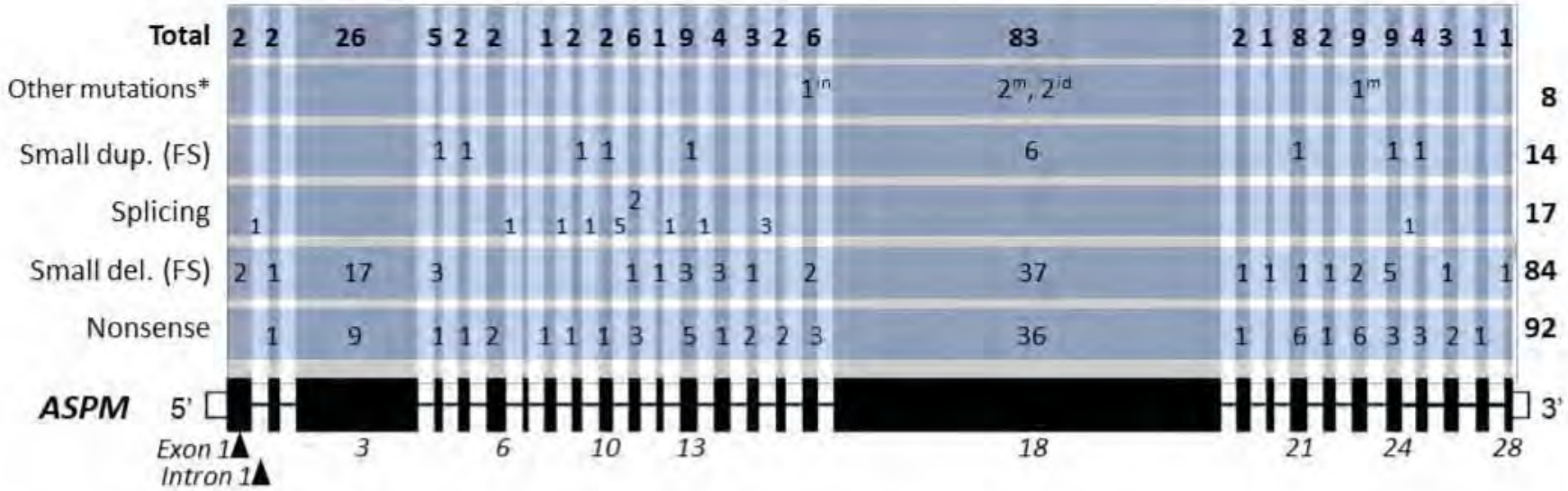
Supplementary data:

Supp. Figure 2. Characteristics of genetic defects identified.

Supp. Table 1: Anthropometric measurements of affected family members.

Supp. Table 2. Homozygous exonic variants shared by the two affected siblings.

Supp. Figure 2 Characteristics of genetic defects identified.



FS, frameshift; id, insertion/deletion (complex rearrangement); in, insertion; m, missense; *two cases of large deletions

Supp. Table 1: Anthropometric measurements of affected family members. Percentiles are given in parentheses.

Pedigree ID	407	408	410	504	506
Sex, age (years)	F, 22	F, 20	M, 17	F, 34	F, 16
Standing height*	138 (<1)	152 (<5)	143 (<1)	135 (<1)	145 (<1)
Sitting height†	66 (<1)	70 (<1)	69 (<1)	66 (<1)	75 (<1)
Arm span§	126 (<1)	142 (<5)	141 (<1)	120 (<1)	133 (<1)
Head circumference‡	42 (<1)	39.5 (<1)	41 (<1)	39 (<1)	37 (<1)
Neck circumference	28.5	30	27	29	28

All measurements are in cm. Head circumference is with respect to age and sex.

*Percentiles are from WHO Growth Reference: http://www.who.int/growthref/who2007_height_for_age/en/

†Kelly AM, Shaw NJ, Thomas AM, Pynsent PB, Baker DJ. Growth of Pakistani children in relation to the 1990 growth standards. Arch Dis Child. 1997;77:401-5.

§With reference to height. Chen WY, Lin YT, Chen Y, Chen KC, Kuo BI, Tsao PC, Lee YS, Soong WJ, Jeng MJ. Reference equations for predicting standing height of children by using arm span or forearm length as an index. J Chin Med Assoc. 2018;81:649-56.

‡ James HE, Perszyk AA, MacGregor TL, Aldana PR. The value of head circumference measurements after 36 months of age: a clinical report and review of practice patterns. J Neurosurg Pediatr. 2015;16(2):186-94.

Supp. Table 2. Homozygous exonic variants shared by the two affected siblings.

S.No	Chr	Start	End	Ref	Alt	Func.refG	Gene.refG	ExonicFunc.refGene	AAChange.refGene	1000G_SA	ExAC_SAS	gnomAD_e	SIFT_pr	Polyphe	Polyphe	LRT_pre	Mutatid	PROVEA	Otherinfo	Otherinfo	Otherinfo	Otherinfo	Otherinfo	Otherinfo	Otherinfo	Otherinfo	Otherinfo
1	chr01	33430102	33430102	T	G	exonic	RNF19B	nonsynonymous SNV	RNF19B:NM_00112				T	B	B	.	P	N	hom	39.74	2	rs1138403	PASS	AC=2;SAM	GT:AD:DP	1/1:0,2:2:6:67,6,0
2	chr01	197071526	197071527	GA	-	exonic	ASPM	frameshift deletion	ASPM:NM_018136:e			3.25E-05	D	D	D	.	D	D	hom	2626.77	64	.	PASS	AC=2;SAM	GT:AD:DP	1/1:0,64:64:99:2655,193,0
3	chr01	207643169	207643169	T	A	exonic	CR2	nonsynonymous SNV	CR2:NM_001006658				T	B	B	.	N	N	hom	1911.77	58	.	PASS	AC=2;SAM	GT:AD:DP	1/1:1,57:58:99:1940,163,0
4	chr02	44049988	44049988	C	T	exonic	ABCG5	nonsynonymous SNV	ABCG5:NM_022436	0.0072	0.0045	0.004	T	B	B	N	N	N	hom	4666.77	147	rs1434027	PASS	AC=2;SAM	GT:AD:DP	1/1:0,147:147:99:4695,439,0
5	chr02	74718268	74718268	G	A	exonic	TTC31	nonsynonymous SNV	TTC31:NM_022492:e	0.0031	0.001	0.0011	D	P	B	N	N	D	hom	2929.77	85	rs5591699	PASS	AC=2;SAM	GT:AD:DP	1/1:0,85:85:99:2958,255,0
6	chr02	84846883	84846883	A	T	exonic	DNAH6	nonsynonymous SNV	DNAH6:NM_001370	0.0031	0.0064	0.0061	D	D	D	.	D	D	hom	1654.77	48	rs3675512	PASS	AC=2;SAM	GT:AD:DP	1/1:0,48:48:99:1683,144,0
7	chr02	97166488	97166488	C	T	exonic	NEURL3	nonsynonymous SNV	NEURL3:NM_00128		0.0011	0.0006	.	B	B	.	.	.	hom	898.77	27	.	PASS	AC=2;SAM	GT:AD:DP	1/1:0,27:27:81:927,81,0
8	chr02	98429152	98429152	T	C	exonic	TMEM131	nonsynonymous SNV	TMEM131:NM_0153	0.018	0.0165	0.0155	T	B	B	D	D	N	hom	783.77	21	rs5322092	PASS	AC=2;SAM	GT:AD:DP	1/1:0,21:21:63:812,63,0
9	chr02	105687920	105687920	C	G	exonic	MRPS9	nonsynonymous SNV	MRPS9:NM_182640		0.001	0.0011	D	D	D	D	D	D	hom	710.77	20	.	PASS	AC=2;SAM	GT:AD:DP	1/1:0,20:20:60:1|1:105687920_C_G:739,60,0
10	chr03	16926544	16926544	T	G	exonic	PLCL2	unknown	UNKNOWN	.	.	.	.	.	.	.	.	.	hom	241.84	6	rs3682845	PASS	AC=2;SAM	GT:AD:DP	1/1:0,6:6:18:1|1:16926521_CT_C:270,18,0
11	chr03	16926607	16926607	T	C	exonic	PLCL2	unknown	UNKNOWN	.	.	1	.	.	.	.	.	.	hom	663.77	16	rs7626448	PASS	AC=2;SAM	GT:AD:DP	1/1:0,16:16:48:1|1:16926599_T_G:692,48,0
12	chr03	124646682	124646682	G	A	exonic	MUC13	nonsynonymous SNV	MUC13:NM_033049	.	.	3.28E-05	.	B	B	N	N	.	hom	3374.77	88	.	PASS	AC=2;SAM	GT:AD:DP	1/1:5,83:88:99:1|1:124646682_G_A:3403,243,
13	chr04	22404353	22404353	T	C	exonic	ADGRA3	nonsynonymous SNV	ADGRA3:NM_14529	0.01	0.0091	0.0084	T	B	B	N	N	N	hom	2479.77	71	rs2001517	PASS	AC=2;SAM	GT:AD:DP	1/1:0,71:71:99:2508,213,0
14	chr04	144617804	144617804	A	C	exonic	FREM3	nonsynonymous SNV	FREM3:NM_001168	0.0031	0.0015	0.0015	D	.	.	D	D	D	hom	3093.77	89	rs5333749	PASS	AC=2;SAM	GT:AD:DP	1/1:0,89:89:99:3122,267,0
15	chr09	102677532	102677532	A	G	exonic	STX17	nonsynonymous SNV	STX17:NM_017919:e	0.0051	0.0033	0.0034	D	B	B	D	D	N	hom	1041.77	33	rs5764902	PASS	AC=2;SAM	GT:AD:DP	1/1:1,32:33:59:1070,59,0
16	chr10	47000004	47000004	T	C	exonic	GPRIN2	nonsynonymous SNV	GPRIN2:NM_01469	.	.	.	.	.	.	.	.	.	hom	9525.77	287	rs3127822	PASS	AC=2;SAM	GT:AD:DP	1/1:1,286:287:99:9554,823,0
17	chr11	96117596	96117596	C	T	exonic	CCDC82	nonsynonymous SNV	CCDC82:NM_00131	.	0.0002	0.0002	T	B	B	N	N	N	hom	2162.77	57	.	PASS	AC=2;SAM	GT:AD:DP	1/1:0,57:57:99:2191,172,0
18	chr11	117280468	117280468	G	A	exonic	CEP164	nonsynonymous SNV	CEP164:NM_001271	.	0.0003	0.0004	T	B	B	N	N	N	hom	1045.77	31	rs3707938	PASS	AC=2;SAM	GT:AD:DP	1/1:0,31:31:93:1074,93,0
19	chr11	117321348	117321348	C	G	exonic	DSCAML1	nonsynonymous SNV	DSCAML1:NM_0206	.	0.0004	0.0005	T	B	B	.	D	N	hom	4254.77	133	rs1481856	PASS	AC=2;SAM	GT:AD:DP	1/1:0,133:133:99:4283,398,0
20	chr12	53207583	53207583	-	CACCAAAGCCACCAG	exonic	KRT4	nonframeshift insertion	KRT4:NM_002272:e	.	.	.	.	.	.	.	.	.	hom	1977.77	26	rs1126739	PASS	AC=2;SAM	GT:AD:DP	1/1:0,26:26:99:2006,129,0
21	chr13	78272267	78272267	-	GG	exonic	SLAIN1	frameshift insertion	SLAIN1:NM_001242	.	.	.	.	.	.	.	.	.	hom	499.77	11	rs2013804	PASS	AC=2;SAM	GT:AD:DP	1/1:0,11:11:36:1|1:78272267_T_TGG:528,36,0
22	chr16	21848694	21848694	T	A	exonic	NPIPB4	nonsynonymous SNV	NPIPB4:NM_00131	.	.	0.0002	T	B	B	.	.	N	hom	189.78	9	rs7609223	MG_SNP_	AC=2;SAM	GT:AD:DP	1/1:0,9:9:26:218,26,0
23	chr16	88502598	88502598	G	C	exonic	ZNF469	nonsynonymous SNV	ZNF469:NM_001127	.	0.0006	0.0006	D	B	B	.	N	N	hom	785.77	27	.	PASS	AC=2;SAM	GT:AD:DP	1/1:0,27:27:81:814,81,0
24	chr17	72889676	72889676	-	GGCTCCGTAGGTTCCA	exonic	FADS6	nonframeshift insertion	FADS6:NM_178128:exon1:c.17_18insGATGGAACCTACGGAGCCCATGGAACCTACGGAGCCCATGGAACCT										hom	6388.77	96	.	PASS	AC=2;SAM	GT:AD:DP	1/1:0,96:96:99:6417,481,0
25	chr19	55350963	55350963	-	CCCGGAGCTCCTATGA	exonic	KIR2DS4	frameshift insertion	KIR2DS4:NM_00128	.	.	.	.	.	.	.	.	.	hom	1451.77	11	rs5514567	PASS	AC=2;SAM	GT:AD:DP	1/1:0,11:11:97:1480,97,0
26	chr20	47841503	47841503	G	C	exonic	DDX27	nonsynonymous SNV	DDX27:NM_001348	0.002	0.005	0.005	D	B	B	N	N	N	hom	1663.77	50	rs1423992	PASS	AC=2;SAM	GT:AD:DP	1/1:0,50:50:99:1692,150,0
27	chr20	49576353	49576353	G	C	exonic	MOCS3	nonsynonymous SNV	MOCS3:NM_014484	0.01	0.0141	0.0145	T	B	B	N	N	N	hom	4531.77	134	rs2020566	PASS	AC=2;SAM	GT:AD:DP	1/1:0,134:134:99:4560,402,0
28	chr21	10920098	10920098	T	C	exonic	TPTE	nonsynonymous SNV	TPTE:NM_00129022	.	.	.	.	.	.	.	.	.	hom	2731.77	83	rs212146	PASS	AC=2;SAM	GT:AD:DP	1/1:0,83:83:99:2760,249,0

Yellow highlighted: Causative mutation

Gray highlighted: filtered out due to presence in in-house exomes (n=45)

Blue highlighted: filtered out due to high frequency in allele frequncy databases

Pink highlighted: predicted benign or neutral effect on the protein by more than one online prediction tools

No highlight: Gene not relevant to the trait

Supplementary materials

Sample LifecodeGX® Nutrigenomics Report

These reports were generated from the pathway SNP analyses carried out by LGC Genomics Ltd on DNA supplied from frozen post-mortem human normal ageing and AD brains. The data were extracted from the 50 reports and input into excel to generate heat maps as well as carry out statistical analyses.

Report Contents are separate reports with pages:

Nervous System

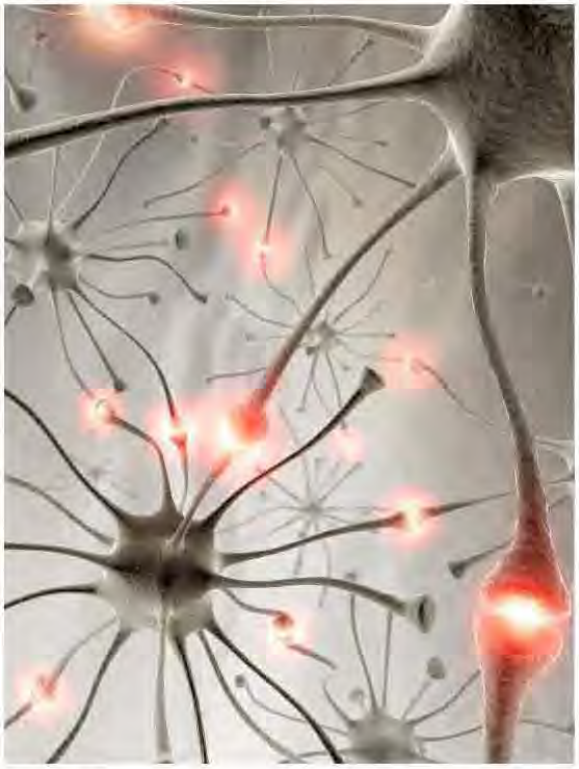
Nervous System

The nervous system is a complex system which enables the transmission of messages around the mind and body, enabling an individual to respond to their environment.

The messages are communicated via neurons which are supported and nourished by glial cells. A neurotransmitter is a molecule that carries signals between neurons and across nerve junctions (synapses).

In order for us to interact effectively with our environment the excitatory and inhibitory neurotransmitters must remain in balance.

This report examines the genes, nutrients, pharmaceutical drugs and lifestyle and environmental factors that can impact the nervous system. It provides a personalised analysis based on genetic results.



Nervous System

Neurotransmitters

The nervous system includes excitatory and inhibitory neurotransmitters.

The key inhibitory (calming) neurotransmitters are: gamma amino butyric acid (GABA) and serotonin.

The main excitatory (stimulating) neurotransmitters are dopamine, adrenaline, noradrenaline, histamine, acetylcholine, glutamate and phenethylamine (PEA).

Lifecycle

The neurotransmitter lifecycle involves:

- Synthesis - dependent on availability of substrates, cofactors, and environmental stimuli
- Signalling - by receptors, which can have a direct effect on a nerve cell or activate a secondary message cascade
- Transport - removal of neurotransmitters from the synapse back into to the cell for storage
- Metabolism - the breakdown of neurotransmitters and removal of metabolites

What can go wrong?

Neurotransmitter imbalances can have serious physical and mental health effects.

Symptoms of neurotransmitter imbalance include: mood disorders and depression, attention deficit and obsessive compulsive disorders, addictive behaviours, motor control disruption, anger, aggression and restlessness.

What can be done?

As with all imbalances, it is important to establish the root cause. There are many candidates - stress, inflammation, obesity, toxicity, gut disfunction, age, diet, lifestyle and genetic predisposition.

Commonly prescribed medications such as SSRIs (selective serotonin re-uptake inhibitors) can cause undesirable side effects and do not work for everyone.

The use of genetic testing and analysis enables a more personalised approach.

Nutritional support including precursors (for example tryptophan), cofactors (B vitamins) and inhibitors (such as curcumin) may be beneficial.

Other lifestyle adjustments to sleep, exposure to sunlight, exercise, stress management and meditation, can also have a significant effect.

Serotonin and Melatonin

Serotonin

Serotonin, also known as 5-hydroxy-tryptamine or 5-HT, is associated with well-being and is popularly referred to as the 'happiness neurotransmitter'.

The majority (about 90%) is made in the gut where it regulates gastrointestinal movements. The remainder is synthesised in the central nervous system (CNS) where, with melatonin, it affects mood, appetite and sleep. It also affects cognitive functions including memory and learning.

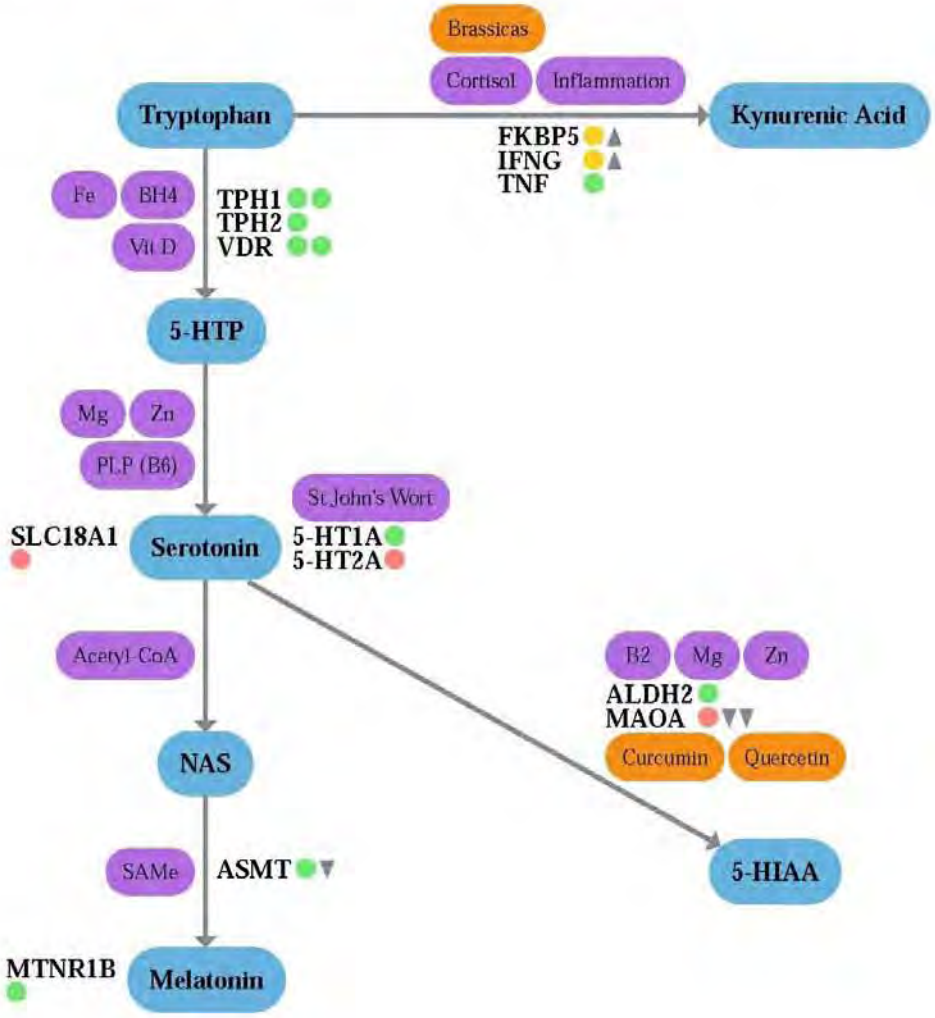
Serotonin was initially thought to increase vascular **tone** by virtue of its presence in **serum** – hence the name **serotonin**.

Melatonin

Melatonin, also known as N-acetyl-5-methoxy-tryptamine, is a sleep hormone naturally produced in the pineal gland of the brain. It regulates sleep and plays a role in maintaining circadian rhythm, the body's natural time clock. It is also an antioxidant. It suppresses insulin which is not needed during sleep.

Serotonin and Melatonin

Your Results in Context



Key

- the effect of the variant is negative
- the effect of the variant is neutral (no effect) or is subjective
- the effect of the variant is positive
- ▲ up-regulates or increases the activity and effect of the gene
- ▼ down-regulates or decreases the activity and effect of the gene
- No arrow - no effect on the activity of the gene

Serotonin and Melatonin

Imbalance

Serotonin and melatonin levels can be impacted by nutrition and lifestyle factors such as protein intake and exercise. There are many different triggers of imbalance, the most common being stress, inflammation, light exposure and genetics.

Tryptophan Availability

Reduced availability of tryptophan to make serotonin can be a major factor in depression. In addition, reduced melatonin synthesis can cause circadian dysrhythmia and insomnia.

Conversely, too much tryptophan can have an inhibitory effect on TPH activity, thereby reducing serotonin production.

Inflammation and Stress

Raised cortisol levels due to stress, or inflammation resulting from infection or injury, may cause tryptophan to be redirected to the kynurenic pathway. This 'tryptophan steal' can slow the rate of serotonin synthesis.

The extent of inflammation can be modulated by genetics, in particular variants that up-regulate pro-inflammatory molecules. These include IFN-gamma, TNF-alpha, and the TDO and IDO enzymes that catalyse kynurenine synthesis.

Serotonin - Dopamine Competition

The synthesis, re-uptake and metabolic pathways of serotonin and dopamine are intertwined. This means that imbalances in one often affect the other. Both serotonin and dopamine are synthesised using the enzyme DDC and metabolised (broken down) by MAOs.

Methylation

Hypo-methylation is associated with lower serotonin and melatonin levels. Neurotransmitter synthesis is slowed due to insufficiency of the (methyl-folate dependent) cofactor BH4 (tetrahydrobiopterin). De-methylation also increases transporter expression and more efficient re-uptake (removal) of neurotransmitters.

Hyper-methylation is associated with higher serotonin and melatonin levels. Methyl donors support neurotransmitter synthesis and suppress transporter activity, thereby slowing the rate of neurotransmitter re-uptake.

Serotonin and Melatonin

Serotonin Symptoms

An imbalance in serotonin levels can lead to an array of problems. Whilst most people are aware of the connection between low serotonin and depression, high levels of serotonin can also be problematic.

Low Serotonin

- Anxiety or worry
- Depression or low mood
- Appetite, hunger or cravings
- Increased pain sensitivity
- Migraines
- Obsessive compulsive disorder (OCD)
- Insomnia

High Serotonin

- Anxiety, irritability or restlessness
- Bone loss
- High blood pressure
- Gut sensitivity or diarrhoea
- Carcinoid syndrome
- Headache
- Fatigue
- Weight gain

Anti-depressants SSRIs do not work for everyone. In particular people who experience depressive symptoms due to high serotonin levels.

Serotonin syndrome is a consequence of excess serotonin which can be fatal. Symptoms include autonomic, cognitive and somatic effects. It usually occurs as a result of (antidepressant) drug overdose or drug interaction.

Serotonin and Melatonin

Melatonin Symptoms

An imbalance in melatonin levels can lead to various problems. However, excess of melatonin does not seem to be a problem.

Low Melatonin

- Mood disorders (seasonal affective disorder, bipolar disorder and major depressive disorder)
- Sleep disturbances

Studies show that the blue light emitted by screens (such as TV, computer and phone) suppresses melatonin levels making it more difficult to fall asleep.

Serotonin and Melatonin

Lifecycle

Synthesis

Serotonin synthesis is a two step process starting with the essential amino acid tryptophan. Tryptophan is converted to 5-HTP by the enzyme tryptophan-5-hydroxylase which exists in two different forms - TPH2 in the brain and TPH1 in the digestive system. 5-HTP is then converted to serotonin by dopa decarboxylase (DDC).

In the evening, stimulated by darkness, noradrenaline activates the cAMP dependent protein kinase A (PKA). This increases arylalkylamine N-acetyl-transferase (AANAT) activity by up to 100x creating melatonin and initiating sleep.

Melatonin is synthesised from N-acetyl-serotonin (NAS). NAS is produced from serotonin by the AANAT enzyme and is converted to melatonin by the acetylserotonin O-methyltransferase (ASMT) enzyme.

Kynurenine

The kynurenine pathway is catalysed by tryptophan 2, 3-dioxygenase (TDO) in the brain and indoleamine 2, 3-dioxygenase (IDO) in the liver. It can be up-regulated by cortisol activation of FK506 binding protein 5 (FKBP5) or immune activation of tumour necrosis factor (TNF) or interferon gamma (INFG), depleting the tryptophan available for serotonin synthesis.

Transport

The serotonin transporter SERT, moves serotonin out of the synapse terminating its action. Various agents can inhibit 5-HT re-uptake including cocaine, tricyclic antidepressants and selective serotonin re-uptake inhibitors.

Unlike other transporters, the vesicular monoamine transporter VMAT1 (also called SLC18A1), moves serotonin and other neurotransmitters into the vesicles, ready to be released into the synapse. Thus an increase in VMAT1 activity results in higher levels of neurotransmitters.

Receptors

The serotonin receptors are activated by serotonin and control the release of a number of excitatory and inhibitory neurotransmitters including glutamate, dopamine, adrenaline, noradrenalin and acetylcholine as well as the hormones cortisol, corticotropin, vasopressin and prolactin. They are the target of many drugs including antidepressants, antipsychotics and anti-migraine agents.

Metabolism

Serotonin is broken down to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase A (MAOA) and aldehyde dehydrogenase 2 (ALDH2).

Serotonin and Melatonin

Follow Up and Testing

Speak to a health professional about clinical testing such as:

Organic acids

- 5-hydroxyindoleacetate (5-HIAA) (serotonin)

Inflammatory markers

- Kynurenate (KYN)
- Quinolinate (QUIN)
- Picolinate

Methylation markers

- Methylmalonate (B12)
- Formiminoglutamate (FIGLU)
- Xanthurenate (B6)
- SAH: SAMe
- Homocysteine

Nutrition and Lifestyle

Nutrition

If low serotonin

- Cofactors (methylation support) - B6, Zn, Mg, methionine and SAMe
- Substrates - tryptophan and 5-HTP
- Anti-inflammatories - brassica foods
- Turmeric and quercetin (decrease MAOA activity - metabolism)

If high serotonin

- Cofactors: Cu (balance Zn) and acetyl CoA (conversion to melatonin)
- B2 - increases metabolism (MAOA activity)

If low melatonin

Consider supplements. Melatonin can be taken as a pill over the counter in some countries.

Manage Stress

Meditation - let go of your thoughts, stay in the present, to help you wind down.

Exercise

Running, walking, yoga, swimming.

Sleep

Ensure a dark environment, since the less sunlight the eye receives, the more melatonin is released by the pineal gland, thereby enhancing and regulating sleep.

Serotonin and Melatonin

Genetic Results

5-HT1A 5-Hydroxytryptamine Receptor 1A

5-HT1A is a subtype of the 5-HT receptor. This receptor has a major role since it binds to the neurotransmitter serotonin.

Variants on this gene have been associated with increased anxiety and stress response.

5-HT1A Variant		Result	Description
rs6295	1019CG	GC	Neutral genotype - no impact on 5-HT1A expression. Normal sensitivity to serotonin.

5-HT2A 5-Hydroxytryptamine Receptor 2A

The serotonin receptor gene 5-HT2A is activated by serotonin. It has an excitatory effect including stimulating smooth muscle contraction in the GI tract (increasing motility).

Variants on the 5-HT2A gene are associated with higher expression, however this can sometimes lead to serotonin resistance and symptoms associated with low serotonin including anxiety, depression and insomnia. Higher 5-HT2A expression is also reported to have anti-inflammatory effects and reduce symptoms of rheumatoid arthritis.

5-HT2A Variant		Result	Description
rs6311	-1438G>A	TT	The T allele is associated with increased receptor expression, however in the long-term this can lead to serotonin resistance so more serotonin is needed to have the same effect. This has been linked with higher risk of anxiety and depressive disorders. St John's Wort increases 5-HT2A expression and increases serotonin sensitivity, and may be useful to offset the effects of the T allele.

ALDH2 Aldehyde Dehydrogenase 2 Family (mitochondrial)

Aldehyde dehydrogenase (ALDH2) belongs to the aldehyde dehydrogenase gene family. There are two major forms of ALDH in the liver: cytosolic ALDH1 and mitochondrial ALDH2. Most Caucasians have both forms, while approximately 50% of East Asians have the cytosolic but not the mitochondrial form. ALDH2 is the second enzyme of the major oxidative pathway of alcohol metabolism and is also needed to breakdown the the amine neurotransmitters.

A higher frequency of acute alcohol intoxication among Asians could be related to the absence of an active form of mitochondrial ALDH2.

ALDH2 Variant		Result	Description
rs671	Glu487Lys	GC	Normal (good) ability to break down the metabolites of catecholamine neurotransmitters, including serotonin. Serotonin metabolism may also be affected by other genetic variants, particularly on the MAOA gene. Support this pathway by limiting alcohol consumption and increasing co-factors - vitamins B2 and B3, magnesium, molybdenum and zinc.

ASMT Acetylserotonin O-Methyltransferase

The ASMT gene is located in the pseudoautosomal region of the short arms of X and Y chromosomes. N-Acetylserotonin O-Methyltransferase catalyses the final step in melatonin synthesis from N-Acetyl serotonin with SAMe as cofactor.

ASMT Variant	Result	Description
rs4446909	GA	Normal (good) ASMT expression and conversion of N-acetyl-serotonin (NAS) to melatonin. The G allele is the risk allele, but is recessive, so the A allele overrides its effect.

FKBP5 FK506 Binding Protein 5

FKBP5 is an important stress regulating gene responsible for controlling the body's response to cortisol by signalling to the body to lower the levels after they have been raised in response to stress.

Variants in this gene are associated with prolonged stress response and increased reactivity due to impaired lowering of cortisol levels after a stressful event. It is also linked to stress-related disorders such as depression, anxiety and post traumatic stress disorder (PTSD) in adulthood particularly as a result of childhood trauma.

FKBP5 Variant	Result	Description
rs1360780	CT	Increased FKBP5 expression and cortisol can stimulate the kynurenine pathway via interferon gamma (INFG) and tumour necrosis factor (TNF), and depletion of tryptophan for serotonin synthesis. Limit lifestyle behaviours that raise cortisol. Stress reduction techniques such as mediation, yoga and massage may be helpful.

IFN-gamma Interferon Gamma

Interferon-gamma (IFNG), or type II interferon, is a critical part of the body's immune response to viral and intracellular bacterial infections and for tumour control. It is produced predominantly by NK cells as part of the innate immune response, and by CD4 & CD8 once antigen-specific immunity develops.

IFNG over expression is associated with a number of inflammatory and autoimmune diseases such as rheumatoid arthritis and SLE (Lupus). It also stimulates IDO which can up-regulate the kynurenine pathway and reduce tryptophan availability for serotonin synthesis.

IFN-gamma Variant		Result	Description
rs2430561	+874AT	AT	The T allele is associated with increased IFNG expression and stimulation of the kynurenine pathway which can 'steal' the tryptophan needed for serotonin synthesis and result in lower serotonin levels. Follow an anti-inflammatory diet including omega 3 (found in oily fish) and brassica foods which inhibit the kynurenine pathway.

MAOA Monoamine Oxidase A

MAOA is a member of the monoamine oxidase gene family whose enzymes catalyse the deactivation of monoaminergic neurotransmitters serotonin, melatonin, noradrenaline and adrenaline. It also metabolises dopamine, tyramine and tryptamine, equally with MAOB. MAOA is located on the X chromosome, so males only carry one allele inherited from their mother. We report results for males as homozygous as they will not inherit a 'balancing' allele.

MAOA is nicknamed the 'warrior gene' because variants are associated with anger and aggression due to slower neurotransmitter breakdown - effects which may be amplified if COMT variants are also present. Conversely, a combination of wild alleles has been labelled the 'worrier' genotype, associated with low mood due to rapid breakdown of neurotransmitters.

MAOA Variant		Result	Description
rs6323	R297R	TT	Low MAOA enzyme activity and slower breakdown of monoamine neurotransmitters which can contribute to higher levels. This is sometimes known as the 'warrior' genotype. If symptoms such as anxiety and outward anger are experienced vitamin B2, magnesium and zinc may increase MAOA activity.

MTNR1B Melatonin Receptor 1B

The MTNR1B gene is found mainly in the eyes and brain and is involved in melatonin response to the onset of darkness or light. Melatonin is involved in several processes in the body including circadian rhythms, mood regulation, anxiety, sleep, appetite, immune responses and heart function.

Variants in MTNR1B are associated with disturbed sleeping patterns (particularly early waking) and increased risk of impaired blood glucose metabolism linked to type 2 diabetes.

MTNR1B Variant	Result	Description
rs10830963	CC	Normal melatonin receptor activity. No impact on sleep patterns or blood sugar metabolism.

QDPR Quinoid Dihydropteridine Reductase

QDPR, also known as DHPR, catalyses the regeneration of tetrahydrobiopterin (BH4) from quinonoid dihydrobiopterin (BH2), a reaction requiring active folate (5-MTHF). BH4 is an important cofactor for the synthesis of the neurotransmitters serotonin and dopamine, and for nitric oxide production.

Variants may result in BH4 deficiency. Excess ammonia may also deplete BH4.

QDPR Variant		Result	Description
rs1031326	690A>G	CC	Wild genotype. Normal (good) recycling of BH4 from BH2, to support serotonin synthesis. Low 5-MTHF (methyl-folate) will reduce the recycling of BH4 regardless of genotype. Ensure good methylation and methyl-folate status.

SLC18A1 Solute Carrier Family 18 Member A1

Also known as VMAT1 (Vesicular monoamine transporter 1), SLC18A1 is an integral membrane protein, which is embedded in synaptic vesicles. It serves to transfer monoamines, such as noradrenaline, adrenaline, dopamine, and serotonin, into the vesicles, ready to release the neurotransmitters into synapses as chemical messages to postsynaptic neurons. Therefore, unlike other transporters VMAT1 activity supports, or raises, neurotransmitter levels. VMAT1 is expressed in neuroendocrine cells.

SLC18A1 function is essential to the correct activity of the monoaminergic systems that have been implicated in several human neuropsychiatric disorders including bipolar disorder and schizophrenia. Variants on SLC18A1 may increase its activity thereby raising levels of monoamine neurotransmitters.

SLC18A1 Variant		Result	Description
rs1390938	Thr136Ile	GG	This is reported as negative because the variant A allele has a positive effect, increasing the transporter activity.

TNF Tumor Necrosis Factor

Tumor necrosis factor (TNF) helps regulate the immune response involved in inflammation, fever and the inhibition of tumour growth.

Variants on TNF are associated with an overactive immune response and susceptibility to a range of inflammatory health conditions including arthritis, asthma, migraine and Alzheimer's. It can up-regulate catabolic pathways and suppress protein synthesis in skeletal muscle, impacting physical performance.

TNF Variant		Result	Description
rs1800629	-308GA	GG	Normal TNF activity, and immune response. Not associated with excessive inflammation or kynurenine pathway disruption to serotonin synthesis.

TPH1 Tryptophan Hydroxylase 1

TPH1 encodes tryptophan hydroxylase 1, an isoenzyme found in peripheral serotonin biosynthesis. This enzyme catalyses the formation of 5-hydroxytryptophan (5-HTP) from tryptophan. A subsequent reaction produces serotonin.

TPH1 Variant		Result	Description
rs1799913	A779C	GG	Neutral genotype - no impact on serotonin synthesis. Ensure sufficient intake of tryptophan, found in turkey, chicken, bananas, avocados and many other foods, and cofactors - BH4 and iron.
rs1800532	A218C	GG	Neutral genotype - no impact on serotonin synthesis. Ensure sufficient intake of tryptophan, found in turkey, chicken, bananas, avocados and many other foods, and cofactors - BH4 and iron.

TPH2 Tryptophan Hydroxylase 2

TPH2 encodes tryptophan hydroxylase 2, an isoenzyme found in neural serotonin biosynthesis. This enzyme catalyses the formation of 5-hydroxytryptophan (5-HTP) from tryptophan. A subsequent reaction produces serotonin.

TPH2 Variant		Result	Description
rs4570625	844G>T	GG	Neutral genotype - no impact on serotonin synthesis. Ensure sufficient intake of tryptophan (in turkey, chicken, bananas, avocados and many other foods), carbohydrate - to support transport across the blood brain barrier, and cofactors BH4 and iron.

VDR Vitamin D (1,25- dihydroxyvitamin D3) Receptor

VDR encodes the nuclear hormone receptor for vitamin D3 (the active form of vitamin D in the body). This receptor shows sequence similarity to the steroid and thyroid hormone receptors and mediates an increase in dopamine production in response to Vitamin D, requiring less methyl groups. Vitamin D3 is also crucial for bone formation, modulation of the immune system, and cell proliferation and differentiation.

Variants in VDR are linked to lower vitamin D sensitivity, and reduced dopamine and serotonin synthesis. This can balance COMT variants (which slow metabolism) as there will be less circulating dopamine to break down. Individuals with variants on COMT but normal VDR activity may have higher dopamine levels, as less need for, and tolerance of, methyl donors and dopamine precursors, and susceptibility to mood swings.

VDR Variant		Result	Description
rs1544410	BsmI	CC	Normal (good) response to Vitamin D which will support serotonin synthesis. However, if you have variants on the MAOA gene, which slow down the breakdown of serotonin, this can result in high levels of serotonin. This has been associated with anxiety, restlessness and fatigue, and lower tolerance to methylated supplements.
rs731236	TaqI	AA	Normal (good) response to Vitamin D which will support serotonin synthesis. However, if you have variants on the MAOA gene, which slow down the breakdown of serotonin, this can result in high levels of serotonin. This has been associated with anxiety, restlessness and fatigue, and lower tolerance to methylated supplements.

Dopamine and Adrenergic

Dopamine

Dopamine is a powerful neurotransmitter sometimes called the 'feel good' neurotransmitter. It is not only involved in pleasure but also in reward (motivation) and in motion.

Dopamine is produced in different areas of the brain including in the substantia nigra and the ventral tegmental area.

Noradrenaline

Noradrenaline is an organic chemical responsible for mobilising the brain and body for action. It is responsible for our response to stressful situations.

An increase in noradrenaline raises blood pressure and heart rate, triggers glucose release, stimulates wakefulness, and provokes sweating.

Adrenaline

Adrenaline is the hormone and neurotransmitter resposible for increasing blood flow. This plays a particularly important role in the 'fight or flight' response.

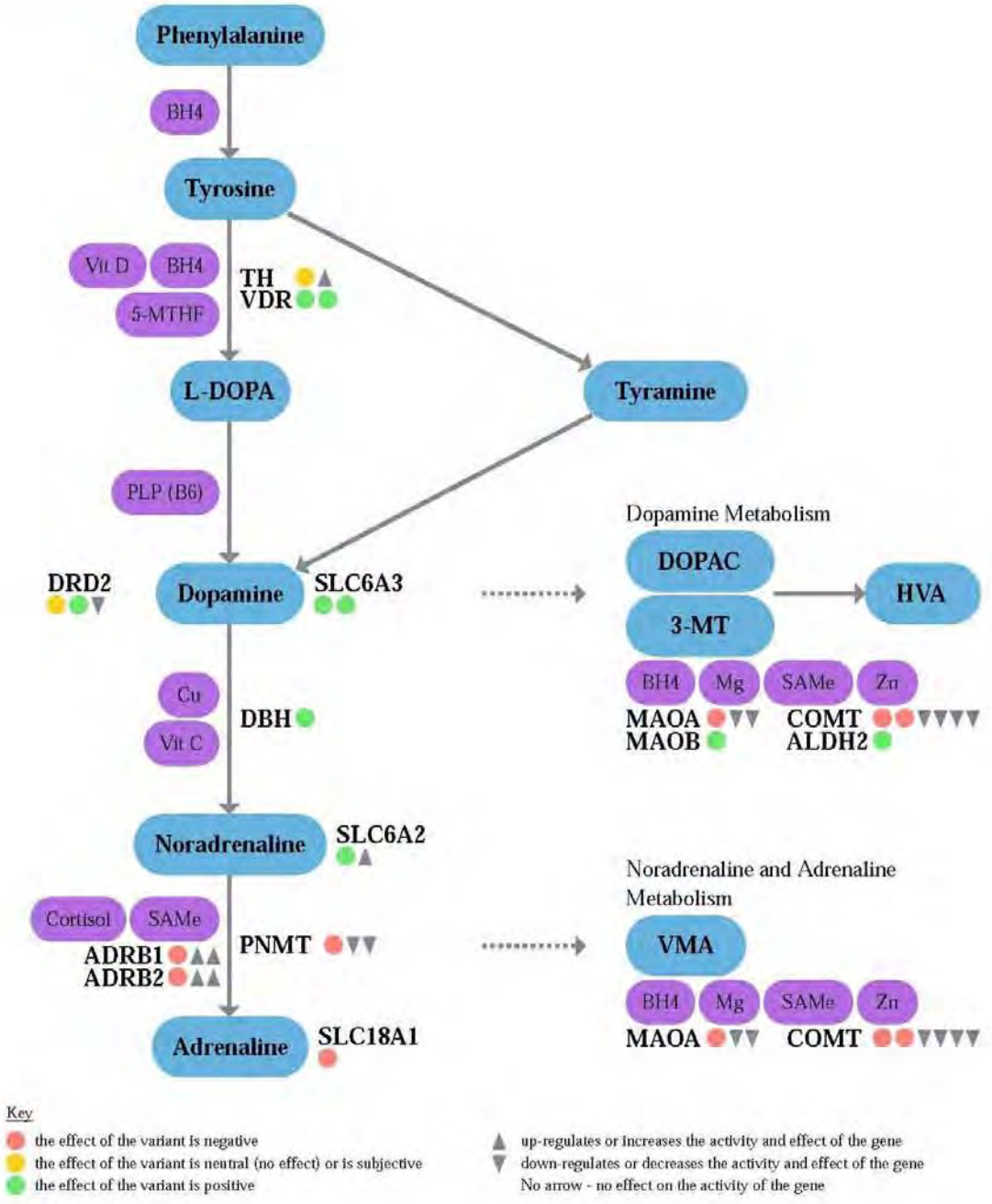
It is often used as medication in extreme situations such as cardiac arrest, superficial bleeding and anaphylaxis. An excess of adrenaline can cause tachycardia, cardiac arrhythmia, hypertension, anxiety and panic attacks.

Adrenaline is a hormone created and secreted by the kidneys of humans and animals to help cope with distress.

Both **adrenaline and noradrenaline** play an important role in the fight or flight response.

Dopamine and Adrenergic

Your Results in Context



Key

- the effect of the variant is negative
- the effect of the variant is neutral (no effect) or is subjective
- the effect of the variant is positive
- ▲ up-regulates or increases the activity and effect of the gene
- ▼ down-regulates or decreases the activity and effect of the gene
- No arrow - no effect on the activity of the gene

Dopamine and Adrenergic

Imbalance

Dopamine, noradrenaline and adrenaline levels can all be impacted by nutrition and lifestyle aspects such as exercise and sleep. Both too high and too low levels can be problematic.

Lack of Motivation

Dopamine is mostly produced in two brain regions: the substantia nigra, involved in movement and speech, and the ventral tegmental region, involved in reward. If dopamine is too low, both these areas will be under-stimulated and the individual will lack energy and motivation.

Addictions and Cravings

Low dopamine makes someone feel like they need more dopamine (which is true). Therefore, the individual is more likely to consume addictive substances such as foods and drugs.

Serotonin - Dopamine Competition

The metabolism, synthesis, and re-uptake pathways of serotonin and dopamine are intertwined. This means that imbalances in one often affect the other. Both serotonin and dopamine are synthesised using the enzyme DDC and metabolised (broken down) by MAO enzymes.

High Dopamine

High dopamine over-stimulates the substantia nigra and ventral tegmental regions, which leads to hyper-activity and hyper-movement (inability to focus).

Dopamine and Adrenergic

Dopamine Symptoms

An imbalance in dopamine levels can lead to a multitude of issues. Both low and high dopamine levels can be problematic.

Low Dopamine

- Lack of motivation
- Fatigue or insomnia
- Addictions and cravings
- Mood issues
- Depression
- Parkinson's disease (resting tremor)
- Decreased libido
- Anxiety

High Dopamine

- Hyperactivity
- Inability to focus
- Anxiety

Drugs such as marijuana and cocaine trigger dopamine release but then deplete its reserves. The individual then consumes more drugs in order to have the same effect, leading to addiction.

A study shows that rats with more dopamine climbed a fence to a larger pile of food, while rats with lower amounts opted for the safer small pile.

Dopamine and Adrenergic

Adrenergic Symptoms

An imbalance in adrenaline and noradrenaline levels can lead to an array of problems.

Low Adrenaline and Noradrenaline

- Depression or low mood
- Poor attention and lack of focus
- Addictions and cravings
- Alzheimer's disease
- Anorexia nervosa
- Fatigue
- Obsessive behaviour (adrenaline)
- Hypotension

High Adrenaline and Noradrenaline

- ADHD
- Anxiety and depression
- Bipolar disorder
- Hyperglycaemia and hyperinsulinaemia
- Obstructive sleep apnea
- PTSD
- Anger or violent behaviour
- Migraine
- Orthostatic intolerance

Did you know?

Adrenaline increases your concentration so much that you forget about pain.

Dopamine and Adrenergic

Lifecycle

Synthesis

Dopamine synthesis is composed of three steps. First, phenylalanine is converted into another amino acid, tyrosine. This reaction is catalysed by the phenylalanine hydroxylase enzyme with tetrahydrobiopterin (BH4) as a cofactor. Then, tyrosine hydroxylase catalyses the conversion of tyrosine to L-DOPA. Finally, L-DOPA is converted into dopamine via the DDC enzyme.

Noradrenaline is formed from dopamine using the dopamine beta-hydroxylase (DBH) enzyme and copper, oxygen and vitamin C as cofactors. However, copper overload can up-regulate the pathway, depleting dopamine and creating an excess of noradrenaline.

Noradrenaline is converted to adrenaline via the phenylethanolamine N-methyltransferase (PNMT) enzyme, with cortisol and SAMe as cofactors.

Tyramine

When the minor tyramine pathway is activated, tyrosine is converted into tyramine instead of L-DOPA. Tyramine can then directly form dopamine, increasing noradrenaline, leading to blood vessel constriction and increased blood pressure.

Transport

The dopamine transporter DAT (also called SLC6A3), is a membrane-spanning protein that pumps the neurotransmitter dopamine out of the synaptic cleft back into the cytosol. This system permits the active re-uptake of dopamine from the synapse and therefore regulates dopaminergic neurotransmission.

The noradrenaline transporter NET (also called SLC6A2), is a monoamine transporter and is responsible for the sodium-chloride dependent re-uptake of extracellular noradrenaline.

Receptors

Dopamine receptors (DRDs) are a class of G protein-coupled receptors that are activated by dopamine. When activated, they inhibit the enzyme adenylate cyclase, thus reducing the intracellular concentration of the second messenger cyclic AMP (cAMP).

The adrenergic receptors (ADRs) are a class of G protein-coupled receptors that are activated by noradrenaline and adrenaline.

Metabolism

Dopamine is broken down into inactive metabolites by several enzymes: MAOA, MAOB, COMT and ALDH2. Dopamine's main metabolic pathway leads to the end-product homovanillic acid (HVA). From the bloodstream, HVA is the filtered out by the kidneys and then excreted in the urine.

Both noradrenaline and adrenaline are degraded by MAOA and COMT.

Dopamine and Adrenergic

Follow Up and Testing

Speak to a health professional about clinical testing such as:

Organic acids

- Homovanillate (HVA) (dopamine)
- Vanilmandelate (VMA) (adrenaline and noradrenaline)

Cofactors

- Vitamin D

Methylation markers

- Methylmalonate (B12)
- Formiminoglutamate (FIGLU)
- Xanthurenate (B6)
- SAH: SAMe
- Homocysteine

Nutrition and Lifestyle

Nutrition

Foods that contain the amino acids tyrosine and phenylalanine boost dopamine levels the most, as they are dopamine precursors.

Increase cofactors for synthesis and transport inhibition: vitamins D, B6 and& B5, methionine, SAMe - found in fish, chicken, beef, pork, turkey, whole grains, seeds, nuts, eggs, beans, cottage cheese and spinach.

Limit intake of caffeine, alcohol, drugs and sugar.

Manage Stress

Studies have shown that stress over-activates dopamine. Relaxation and meditation can help reduce dopamine levels.

Exercise

Exercise helps to support dopamine levels.

Sleep

Studies have shown that sleep deprived people have less dopamine binding and lower response to dopamine.

Recipe for Ricotta Cream (high in tyrosine)

- 1 cup of ricotta
- 1 tbsp of honey
- Juice and zest of 1/2 lemon

Mix all ingredients until well combined. Enjoy on toast or apple slices.

Dopamine and Adrenergic

Genetic Results

ADRB1 Adrenoceptor Beta 1

The ADRB1 gene encodes a subtype of adrenergic receptors, the beta-1 adrenergic receptor. These adrenergic receptors are G protein-coupled receptors that bind to noradrenaline and adrenaline.

The adrenergic receptors (subtypes alpha 1, alpha 2, beta 1, and beta 2) mediate the physiological effects of the hormone adrenaline and the neurotransmitter noradrenaline. Specific polymorphisms in this gene have been shown to affect the resting heart rate and can be involved in heart failure. Variants may also affect clinical response to beta blockers.

ADRB1 Variant		Result	Description
rs1801253	Arg389Gly	CC	Relatively high sensitivity to adrenaline and noradrenaline (due to the C allele). Greater stimulation of noradrenaline release has been associated with heart failure. Carriers of the C allele are reported to respond well to beta blocker drugs to lower blood pressure. Adrenaline and noradrenaline levels can be reduced by limiting consumption of stimulants such as caffeine.

ADRB2 Beta-2-Adrenergic Receptor

The beta-2 adrenergic receptor, ADRB2, controls the physiological response to adrenaline, priming the body for action or 'fight or flight'. It stimulates heart rate, blood flow, and availability of glucose for immediate energy.

The G (Gly) allele is the ancestral, more sensitive version, and is associated with greater adrenergic response to exercise or stress.

ADRB2 Variant		Result	Description
rs1042713	Arg16Gly	GG	The G allele is associated with a greater fight or flight response to adrenaline including increases in heart rate, vasodilation, and energy release (glycolysis). This genotype may be more vulnerable to physiological effects, such as hypertension and metabolic disfunction, in response to chronic stress.

ALDH2 Aldehyde Dehydrogenase 2 Family (mitochondrial)

Aldehyde dehydrogenase (ALDH2) belongs to the aldehyde dehydrogenase gene family. There are two major forms of ALDH in the liver: cytosolic ALDH1 and mitochondrial ALDH2. Most Caucasians have both forms, while approximately 50% of East Asians have the cytosolic but not the mitochondrial form. ALDH2 is the second enzyme of the major oxidative pathway of alcohol metabolism and is also needed to breakdown the the amine neurotransmitters.

A higher frequency of acute alcohol intoxication among Asians could be related to the absence of an active

form of mitochondrial ALDH2.

ALDH2 Variant		Result	Description
rs671	Glu487Lys	GG	Normal (good) ability to break down the metabolites of catecholamine neurotransmitters - serotonin, dopamine, noradrenaline, adrenaline and histamine. Support this pathway by limiting alcohol consumption and increasing cofactors - vitamins B2 and B3, magnesium, molybdenum and zinc.

COMT Catechol-O-Methyltransferase

COMT breaks down the neurotransmitters: dopamine, adrenaline, and noradrenaline by using a methyl group from SAMe to methylate the catechol molecule, preparing it for excretion. COMT is also involved in oestrogen metabolism, converting active oestrogen to less active oestrogen. SAMe and SAH compete for the binding site on the COMT molecule, therefore a build up of SAH will reduce COMT activity.

Variants on COMT may reduce its activity and result in excess methyl groups which may cause irritability, heightened stress response, hyperactivity, heightened pain sensitivity and slower detoxification of oestrogen.

COMT Variant		Result	Description
rs4633	H62H	TT	Reduced COMT activity and slower breakdown of dopamine. Whilst this is often viewed as negative and associated with high dopamine, it can balance out variants on VDR, which slow dopamine synthesis, and normalise dopamine levels. However, low COMT activity and wild (no variance) on VDR can result in high dopamine and susceptibility to mood swings. To support COMT (and reduce dopamine) and ensure adequate intake B vitamins, zinc and magnesium (to provide methyl cofactors).
rs4680	V158M	AA	Reduced COMT activity and slower breakdown of dopamine. Whilst this is often viewed as negative and associated with high dopamine, it can balance out variants on VDR, which slow dopamine synthesis, and normalise dopamine levels. However, low COMT activity and wild (no variance) on VDR can result in high dopamine and susceptibility to mood swings. To support COMT (and reduce dopamine) and ensure adequate intake B vitamins, zinc and magnesium (to provide methyl cofactors).

DBH Dopamine Beta-Hydroxylase

The DBH gene encodes the dopamine beta-hydroxylase enzyme, which catalyses the oxidative hydroxylation of dopamine to noradrenaline.

Variants on DBH are associated with reduced activity and symptoms such as depression, poor attention, fatigue and hypotension.

DBH Variant		Result	Description
rs1611115	C-970T	CC	Neutral genotype - no impact on noradrenaline levels.

DRD2 Dopamine Receptor D2

The DRD2 gene is a G-protein coupled receptor located on postsynaptic dopaminergic neurons that is centrally involved in reward-mediating pathways that control dopamine synthesis and release.

Signaling through dopamine D2 receptors governs physiologic functions related to locomotion, hormone

production, and substance misuse. D2 receptors are also known targets of antipsychotic drugs that are used to treat neuropsychiatric disorders such as schizophrenia.

DRD2 Variant		Result	Description
rs1076560	811-83G>T	CC	Wild type, no variance. No impact on DRD2 dopamine receptor activity or dopamine response. This genotype is associated balanced dopamine levels and lower risk of opioid, cocaine and alcohol dependence.
rs6277	957C>T	AG	The G allele is associated with higher DRD2 expression which can inhibit dopamine. AG is a balanced dopamine type associated with better executive function, cognitive ability and working memory and less risk of addiction and abnormal reward seeking (impulsive) behaviours.

MAOA Monoamine Oxidase A

MAOA is a member of the monoamine oxidase gene family whose enzymes catalyse the deactivation of monoaminergic neurotransmitters serotonin, melatonin, noradrenaline and adrenaline. It also metabolises dopamine, tyramine and tryptamine, equally with MAOB. MAOA is located on the X chromosome, so males only carry one allele inherited from their mother. We report results for males as homozygous as they will not inherit a 'balancing' allele.

MAOA is nicknamed the 'warrior gene' because variants are associated with anger and aggression due to slower neurotransmitter breakdown - effects which may be amplified if COMT variants are also present. Conversely, a combination of wild alleles has been labelled the 'worrier' genotype, associated with low mood due to rapid breakdown of neurotransmitters.

MAOA Variant		Result	Description
rs6323	R297R	TT	Low MAOA enzyme activity and slower breakdown of monoamine neurotransmitters which can contribute to higher levels. This is sometimes known as the 'warrior' genotype. If symptoms such as anxiety and outward anger are experienced vitamin B2, magnesium and zinc may increase MAOA activity.

MAOB Monoamine Oxidase B

MAOB is a member of the monoamine oxidase gene family whose enzymes catalyse the deactivation of monoaminergic neurotransmitters. It is the main catalyst for the breakdown of phenethylamine (PEA), benzylamine and histamine. It also metabolises dopamine, tyramine and tryptamine, equally with MAOA. MAOB is located on the X chromosome, so males only carry one allele, inherited from their mother. We report results for males as homozygous as they will not inherit a 'balancing' allele.

Variants on the MAOB gene are associated with reduced enzyme activity and slower breakdown of neurotransmitters. MAOB is a target for MAO inhibitor drugs used to raise dopamine levels and to improve motor function in Parkinson's disease patients.

MAOB Variant		Result	Description
rs1799836	A644G	TT	No variance. Normal (efficient) MAOB activity and efficient breakdown of neurotransmitters, which can contribute to lower dopamine levels. If symptoms of low dopamine are present curcumin and quercetin can help inhibit MAOB activity.

PNMT Phenylethanolamine N-Methyltransferase

The PNMT enzyme catalyses the last step of the catecholamine biosynthesis pathway, which methylates noradrenaline to form adrenaline and therefore plays a key role in regulating adrenaline production. During environmental or physiological stress such as exercise, pituitary corticotrophin (ACTH) release promotes the secretion of glucocorticoids that induce PNMT.

Variants on PNMT are reported to reduce its activity and slow down the conversion of noradrenaline to adrenaline.

PNMT Variant		Result	Description
rs876493	G-161A	AA	Reduced PNMT activity and slower conversion of noradrenaline to adrenaline, which has been linked to hypertension. As SAMe is a cofactor for PNMT ensure sufficient B vitamins, zinc and magnesium to support SAMe synthesis.

QDPR Quinoid Dihydropteridine Reductase

QDPR, also known as DHPR, catalyses the regeneration of tetrahydrobiopterin (BH4) from quinonoid dihydrobiopterin (BH2), a reaction requiring active folate (5-MTHF). BH4 is an important cofactor for the synthesis of the neurotransmitters serotonin and dopamine, and for nitric oxide production.

Variants may result in BH4 deficiency. Excess ammonia may also deplete BH4.

QDPR Variant		Result	Description
rs1031326	690A>G	CC	Wild genotype. Normal (good) recycling of BH4 from BH2, to support dopamine synthesis. Low 5-MTHF (methyl-folate) will reduce the recycling of BH4 regardless of genotype. Ensure good methylation and methyl-folate status.

SLC18A1 Solute Carrier Family 18 Member A1

Also known as VMAT1 (Vesicular monoamine transporter 1), SLC18A1 is an integral membrane protein, which is embedded in synaptic vesicles. It serves to transfer monoamines, such as noradrenaline, adrenaline, dopamine, and serotonin, into the vesicles, ready to release the neurotransmitters into synapses as chemical messages to postsynaptic neurons. Therefore, unlike other transporters VMAT1 activity supports, or raises, neurotransmitter levels. VMAT1 is expressed in neuroendocrine cells.

SLC18A1 function is essential to the correct activity of the monoaminergic systems that have been implicated in several human neuropsychiatric disorders including bipolar disorder and schizophrenia. Variants on SLC18A1 may increase its activity thereby raising levels of monoamine neurotransmitters.

SLC18A1 Variant		Result	Description
rs1390938	Thr136Ile	GG	Wild genotype. No impact (increase) on the transporter activity. This is reported as negative relative to carriers of an A allele which is associated with increased adrenaline release. This (wild) genotype is associated with lower resilience to affective anxiety, depressiveness and alcohol use disorders.

SLC6A2 Solute Carrier Family 6 Member 2

Also known as NET or NET1, SLC6A2 encodes a noradrenaline transporter. It is responsible for the re-uptake of noradrenaline into presynaptic nerve terminals and is a regulator of noradrenaline homeostasis.

Variants on this gene have been associated with major depressive disorder, and separately, orthostatic intolerance, a syndrome characterized by lightheadedness, fatigue, altered mental activity and syncope (fall in blood pressure).

SLC6A2 Variant		Result	Description
rs5569	G1287A	AG	No impact on the transport or removal of noradrenaline (not up-regulated) or negative impact on noradrenaline levels. Although the G allele is linked to increased transporter activity it is overridden by the dominant A allele.

SLC6A3 Solute Carrier Family 6 Member 3

Also known as DAT or DAT1, SLC6A3 codes for a dopamine transporter which is a member of the sodium and chloride dependent neurotransmitter transporter family. It pumps dopamine out of the synaptic cleft back into the cytosol. It is responsible for the active re-uptake (removal) of dopamine from the synapse and thus regulates dopaminergic neurotransmission.

Variants on this gene have been associated with idiopathic epilepsy, attention-deficit hyperactivity disorder, dependence on alcohol and cocaine, susceptibility to Parkinson's disease and protection against nicotine dependence.

SLC6A3 Variant		Result	Description
rs27072	328G>A	CC	No impact on transporter activity (not decreased) nor on dopamine levels.
rs6347		TT	Neutral genotype. No impact (increase) on dopamine transporter or decrease in dopamine levels.

TH Tyrosine Hydroxylase

Tyrosine hydroxylase catalyses the conversion of tyrosine to L-DOPA (di-hydroxyphenylalanine), which is then converted into dopamine, noradrenaline and adrenaline. As the rate limiting enzyme, TH is also known as the master catecholamine controller. It is found mainly in the central nervous system and adrenal medulla.

Genetic variants on TH have been associated with various nervous system diseases, including bipolar disorders, schizophrenia, and Parkinson's disease, and with hypertension.

TH Variant		Result	Description
rs10770141	C-824T	CA	The A allele is associated with higher activity, faster synthesis and higher levels of dopamine and noradrenaline. This may be positive or negative depending on the context. Increased risk of hypertension (high blood pressure) in response to stress; however dopamine and noradrenaline support cognitive functioning so can reduce the risk of developing neurodegenerative conditions such as Alzheimer's and Parkinson's disease.

VDR Vitamin D (1,25- dihydroxyvitamin D3) Receptor

VDR encodes the nuclear hormone receptor for vitamin D3 (the active form of vitamin D in the body). This receptor shows sequence similarity to the steroid and thyroid hormone receptors and mediates an increase in dopamine production in response to Vitamin D, requiring less methyl groups. Vitamin D3 is also crucial for bone formation, modulation of the immune system, and cell proliferation and differentiation.

Variants in VDR are linked to lower vitamin D sensitivity, and reduced dopamine and serotonin synthesis. This can balance COMT variants (which slow metabolism) as there will be less circulating dopamine to break down. Individuals with variants on COMT but normal VDR activity may have higher dopamine levels, as less need for, and tolerance of, methyl donors and dopamine precursors, and susceptibility to mood swings.

VDR Variant		Result	Description
rs1544410	BsmI	CC	Normal (good) response to Vitamin D which will support normal (to high) dopamine levels. However, variants on the COMT gene, which slow down the breakdown of dopamine, can result in high levels of dopamine, noradrenaline and adrenaline. This has been associated with mood swings and intolerance to methylated supplements.
rs731236	TaqI	AA	Normal (good) response to Vitamin D which will support normal (to high) dopamine levels. However, variants on the COMT gene, which slow down the breakdown of dopamine, can result in high levels of dopamine, noradrenaline and adrenaline. This has been associated with mood swings and intolerance to methylated supplements.

GABA

GABA

GABA, gamma-aminobutyric acid, is not only a neurotransmitter but also an amino acid. It sometimes knows as the 'off' switch. Indeed, it is the major inhibitory neurotransmitter in the brain: at a synapse level, GABA decreases a neuron's action potential, or excitability. It is critical for relaxation, improves memory and mood, relieves anxiety, promotes sleep, moderates blood pressure, and influences catecholamine release and cytokine and hormone production.

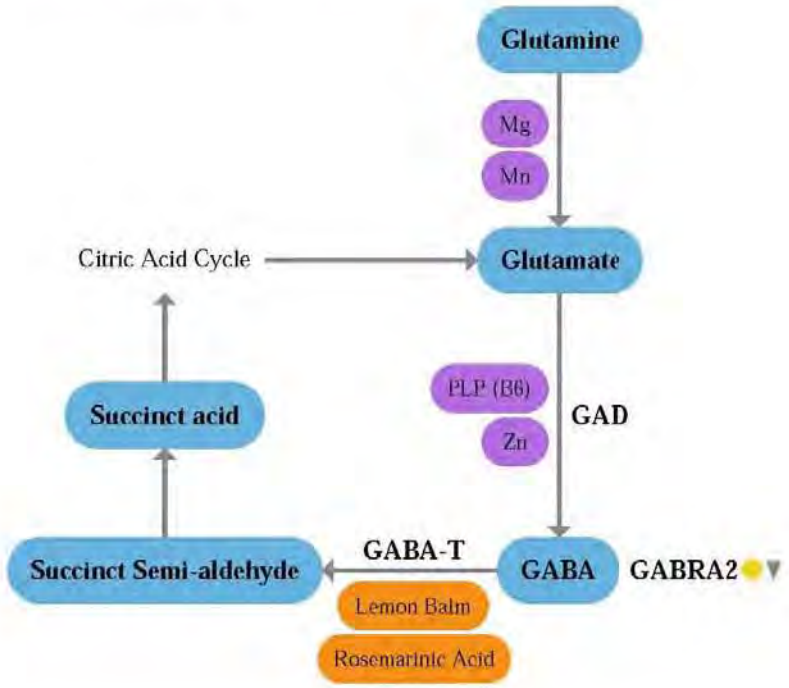
Because GABA can reduce neural transmission, increased GABA activity can have sedative effects. Accordingly, drugs such as alcohol and benzodiazepines, create similar sedative effects by interacting with GABA receptors.

Glutamate

Glutamate is the major excitatory neurotransmitter, sometimes known as the 'on' switch. At a synapse level, glutamate increases a neuron's action potential. It optimises memory and learning, inhibits sleep, improves libido, regulates appetite and increases gut motility.

GABA

Your Results in Context



Key

- the effect of the variant is negative
- the effect of the variant is neutral (no effect) or is subjective
- the effect of the variant is positive

- up-regulates or increases the activity and effect of the gene
- down-regulates or decreases the activity and effect of the gene
- No arrow - no effect on the activity of the gene

GABA

Imbalance

The most significant impacts on the effect of GABA and glutamate are due to variations in the amount of transmitter and number of receptors.

Anxiety

The amygdala is an almond-shaped group of neurons located within the medial temporal lobe. It acts as a kind of alarm bell. It is activated when someone experiences danger, fear or aggression. When GABA levels are low, every single action potential fires, causing the amygdala to be over-activated. Thus someone feels constantly in danger, and experiences fear or aggression, leading to anxiety.

OCD

Obsessional compulsive disorder (OCD) affects both cognition and motor behaviour. It is characterised by obsessions - unwanted but insistent, repetitive thoughts; and by compulsions to repeat an action over and over until the anxiety and negative thoughts are neutralised.

High glutamate levels are typical in OCD patients, and can over-stimulate the orbitofrontal cortex (OFC) and the anterior cingulate gyrus (ACG) regions of the brain. If these regions are stimulated beyond normal range (hyper-excited), they may fire inappropriately and send erroneous messages to the basal ganglia, resulting in the feeling that something is wrong.

The basal ganglia is where sequences of habitual and reflexive movement are stored. Hence, over stimulation of the basal ganglia is associated with development of habitual routines.

GABA

GABA Symptoms

An imbalance in GABA levels can lead to an array of problems. Both low and high levels can be a problem due to their effect on action potential firing.

Low GABA

- Anxiety
- Inability to focus or ADHD
- Low energy
- Panic attacks or disorders
- General or social anxiety disorders or phobias
- Seizures or convulsions
- Muscle tremors or spasms

High GABA

- Anxiety
- Excessive need for sleep
- Lethargy
- Decreased drive and motivation

Understanding GABA's effect in the brain

Without GABA's inhibitory signal, every single action potential reaches its target.

However, if there is too much GABA, a neuron's threshold potential is never reached, and it will not excite any nearby neurons.

Glutamate Symptoms

An imbalance in glutamate levels can lead to an array of problems. Both low and high levels can be a problem due to their effect on action potential firing.

Low Glutamate

- Agitation
- Insomnia
- Chronic fatigue
- Depression
- Lethargy
- Migraines

High Glutamate

- Anxiety
- Insomnia
- Panic
- Biopolar disorder or OCD
- Depression
- Hyperthyroidism

GABA

GABA Lifecycle

Synthesis

GABA is synthesised from glutamate by the glutamate decarboxylase (GAD) enzyme. GAD uses PLP (B5) and zinc as cofactors.

Transport

GABA's main transporters in the brain are GAT1 and GAT3 encoded by SLC6A1 and SLC6A11 respectively. They enable the re-uptake of GABA by removing it from the synaptic cleft into neurons or glial cells where it is degraded by mitochondrial enzymes.

Variants on GABA transporters are linked to anxiety disorders with panic symptoms.

Receptors

There are two types of GABA receptors: GABA-A and GABA-B.

GABA-A receptors are ligand-gated chloride channels (also known as inotropic receptors). When activated, Cl^- ions pass through the neuron's membrane, it causes its hyper-polarisation, leading to inhibitory actions.

GABA-B receptors are G protein-coupled receptors (also known as metabotropic receptors). When activated, they cause the opening of potassium channels. Therefore, K^+ ions flow out of the neuron, hyper-polarising its membrane and again, leading to inhibitory actions.

Metabolism

GABA is broken down by the GABA transaminase (GABA-T) enzyme. It catalyses the conversion of GABA and 2-oxoglutarate into succinate semialdehyde and glutamate.

GABA

Glutamate Lifecycle

Synthesis

Glutamate is synthesised from glutamine by the glutaminase (GLS) enzyme.

Transport

Glutamate transporters move glutamate across the neurons' membranes. The excitatory amino acid transporters (EAATs) re-uptake 90% of glutamate from the synaptic cleft and extra-synaptic sites into glial cells and neurons. Without these transporters, glutamate would build up and kill cells due to excitotoxicity, due to over activation of glutamate receptors.

Receptors

Glutamate's main receptors are NMDA receptors. These are ion channel proteins found in nerve cells. When activated by the binding of glutamate and glycine, positively charged ions flow though the cell membrane and start the metabolic cascade.

Metabolism

Glutamate is broken down into glutamine by the glutamine synthetase (GS) enzyme. This is essential and protective since the enzyme catalyses the condensation of glutamate and ammonia (which is toxic to the brain) into glutamine. Magnesium and manganese are cofactors.

GABA

Follow Up and Testing

Speak to a health professional about clinical testing such as:

- Urine tests for GABA and glutamate levels

Glutamate cannot pass the blood-brain barrier, therefore it must be synthesised in the brain and glutamine food must be consumed.

Nutrition and Lifestyle

Nutrition

If low GABA

- Glutamine - foods include bone broth, cabbage, meats (beef, chicken), spirulina, fermented foods, eggs and dairy
- Cofactors - B6 and zinc
- Valerian
- L-theanine
- Kava and skullcap (herbs)
- Rosemarinic acid - found in lemon balm, rosemary, basil, sage, thyme and peppermint
- Limit intake of coffee, alcohol and tobacco

If high glutamate

- N-acetyl cysteine
- Reduce glutamine foods

Manage stress

Meditation can boost GABA levels.

Exercise

Intense exercise increases levels of glutamate and GABA.

Sleep

Activation of GABA-A receptors by GABA helps to support sleep.

GABA

Genetic Results

GABRA2 Gamma-Aminobutyric Acid Type A Receptor Alpha 2 Subunit

GABA is the major inhibitory neurotransmitter in the mammalian brain where it acts at GABA-A receptors, which are ligand gated chloride channels. Chloride conductance of these channels can be modulated by agents such as benzodiazepines (valium) that bind to the GABA-A receptor.

GABRA2 Variant	Result	Description
rs279858	CT	Decreased GABRA2 receptor activity. The variant C allele decreases the levels of GABRA2 expression, reducing sensitivity to GABA. This genotype has been associated with increased risk of alcohol dependence as alcohol activates GABA receptors promoting relaxation and reducing anxiety. The medicinal herb valerian activates GABA receptors and has similar sedative effects as alcohol, without the negative side effects. L-theanine and rosemarinic acid (found in rosemary, lemon balm, sage, thyme and peppermint) can help support GABA levels by inhibiting its break down.

Pharmacology

Drug Metabolism

Pharmacology is the science concerned with the study of drugs and how they affect living organisms. It focuses on the biological action of drugs and chemicals, and the way they work at the molecular, cellular and systems levels. Systems pharmacology focuses on agents affecting specific physiological systems, such as the nervous system (neuropharmacology), which is the focus of this report.

The liver is the main site of drug metabolism. Other sites include the small intestine, kidneys and lungs. Drugs administered orally are absorbed into the portal circulation and go directly to the liver. Thus drug concentration is often drastically reduced before it reaches general circulation - known as the first pass effect (this reduces bioavailability of the drug) whereas injected, inhaled, topical and sublingual drugs bypass the first pass effect. However all circulating drugs are metabolised by the liver eventually. This biotransformation happening in the liver acts as a detoxifier of the drugs.

Drug metabolism is divided into three phases.

In phase I substances are primed for further metabolism by the addition or exposure of a binding site via oxidation, reduction or hydrolysis. Substances are often made more reactive, and toxic, during phase I.

During phase II (conjugation) substrates are 'deactivated' and made more water-soluble, a process that prepares them for excretion via the bile to the small intestine or via the kidneys to urine.

Phase III (anti-porter activity) involves the transport of substances across cellular barriers such as in the liver, gastrointestinal tract, kidneys and blood-brain barrier.

"The dose makes the poison" – Paracelsus

Any substance can produce a harmful effect and be toxic for the organ or body if it is in too high concentration.

Pharmacology

Genetic Results

ADRB2 Beta-2-Adrenergic Receptor

The beta-2 adrenergic receptor, ADRB2, controls the physiological response to adrenaline, priming the body for action or 'fight or flight'. It stimulates heart rate, blood flow, and availability of glucose for immediate energy.

The G (Gly) allele is the ancestral, more sensitive version, and is associated with greater adrenergic response to exercise or stress.

ADRB2 Variant		Result	Description
rs1042713	Arg16Gly	GG	The G allele is associated with increased ADRB2 sensitivity to adrenaline. As this genotype is more resistant to agonist induced desensitisation an individual is less likely to develop adverse effects to inhaler use (albuterol or salmeterol).

CYP2C19 Cytochrome P450, Family 2, Subfamily C, Polypeptide 19

CYP2C19 is a member of the cytochrome P450 superfamily of enzymes. It metabolises a wide variety of pharmaceutical drugs including some anticonvulsants, proton pump inhibitors, antidepressants, sedatives and antimalarials.

Polymorphisms of this gene are known to have different effects. The rs4244285 (A) allele is associated with decreased enzyme activity (poor metaboliser) whereas the rs12248560 (T) allele is associated with significantly increased enzyme activity (extensive metaboliser). Dosage adjustment may be needed to achieve optimal therapeutic benefit.

CYP2C19 Variant		Result	Description
rs12248560	-806C>T	CC	Normal (not increased or decreased) metabolism of substrates including anticoagulants and other pharmaceutical drugs
rs4244285	681G>A	GG	Normal (not increased or decreased) metabolism of anticoagulants such as clopidogrel and other pharmaceutical drugs.

CYP2D6 Cytochrome P450, Family 2, Subfamily D, Polypeptide 6

CYP2D6 is a member of the cytochrome P450 superfamily of enzymes. It is known to metabolise as many as twenty five percent of commonly prescribed drugs as well as lipids, hormones and toxins. Its substrates include antidepressants, antipsychotics, analgesics and anti-tussives, beta adrenergic blocking agents, antiarrythmics and antiemetics.

Polymorphisms of this gene have different effects ranging from ultra-rapid metabolisers – with 3 or more functional alleles – to poor metabolisers – where certain alleles, or even the whole gene, may be absent. Poor

metabolisers do not metabolise codeine to morphine and thus experience no analgesic effect, whereas ultra rapid metabolisers can experience morphine toxicity. Dosage adjustment may be needed to achieve optimal therapeutic benefit. Always refer to your GP or specialist before adjusting dosage of any prescribed medication.

CYP2D6 Variant		Result	Description
rs1135840	S486T	GG	Normal (not increased or decreased) enzyme activity and metabolism of commonly prescribed drugs such as tramadol (a pain relief drug), and conversion of codeine to morphine, and of lipids and hormones.
rs16947	R296C	AA	Slower metaboliser of substrates including commonly prescribed drugs - such as tramadol, a pain relief drug, and conversion of codeine to morphine (which may reduce or delay efficacy).
rs35742686	2549delA	II	Normal (not increased or decreased) enzyme activity and metabolism of commonly prescribed drugs - such as conversion of codeine to morphine, fluoxetine and fluvoxamine (antidepressants used to treat depression, panic disorder and OCD), and of lipids and hormones.
rs3892097	1846G>A	CC	Normal (not increased or decreased) enzyme activity and metabolism of commonly prescribed drugs - including fluoxetine and fluvoxamine (antidepressants used to treat depression, panic disorder and OCD) tamoxifen (used to treat breast cancer), conversion of codeine to morphine, and of lipids and hormones.

CYP3A4 Cytochrome P450, Family 3, Subfamily A, Polypeptide 4

The CYP3A4 enzyme is involved in the metabolism of approximately half the drugs in use today, including acetaminophen, codeine, cyclosporin A, diazepam and erythromycin. It also metabolises some steroids and hormones, particularly the biosynthesis of 16aOH-E1 and the conversion of oestradiol (E2) to oestriol (E3). CYP3A4 expression is induced by glucocorticoids and some pharmacological agents and is strongly inhibited by grapefruit, antifungals and antibiotics.

Up-regulated enzyme activity can result in high amounts of circulating pro-carcinogens if phase II detoxification pathways (such as methylation, sulphonation/ sulphoconjugation, glucuronidation or glutathione conjugation etc.) are not working optimally.

CYP3A4 Variant		Result	Description
rs2740574	-392G>A	TT	Normal CYP3A4 enzyme activity and metabolism of pharmaceutical drugs. Lower risk of oxidative damage.

Bonus SNPs

Genetic Results

BDNF Brain Derived Neurotrophic Factor

The BDNF gene encodes the brain-derived neurotrophic factor. This protein is a nerve growth factor, meaning it promotes the growth, differentiation and survival of neurons and synapses in the central and peripheral nervous systems. Expression of this gene is reduced in Alzheimer's, Parkinson's, and Huntington's disease patients.

BDNF may play a role in the regulation of the stress response and in the biology of mood disorders.

BDNF Variant		Result	Description
rs6265	Val66Met	TC	Slightly decreased BDNF expression. Low concentrations of BDNF are associated with increased risk of neuronal damage linked with Alzheimer's and Parkinson's. Some research has linked met66 (T allele) carriers to eating disorders. It has been suggested that BDNF can be increased by intense exercise, vitamin D, curcumin, green tea, DHA (a component of omega-3 fatty acid) and resveratrol.

DIO1 Iodothyronine Deiodinase 1

The DIO1 gene encodes the enzyme type I iodothyronine deiodinase, a selenoprotein, requiring selenium for its synthesis. DIO1 catalyzes the activation, as well as the inactivation of thyroid hormone. The activation reaction involves the conversion of the prohormone thyroxine (T4), secreted by the thyroid gland, to the bioactive thyroid hormone (T3). This gene is expressed predominantly in the liver and kidney and provides most of the circulating T3, which is essential for growth, differentiation and basal metabolism.

We report results for SNP rs223554 [C/A] which is in linkage disequilibrium (LD), or correlation, with SNP rs11206244 [C/T].

DIO1 Variant		Result	Description
rs2235544	34C>A	AC	Reduced conversion of T4 to T3. The A allele of this SNP is associated with lower deiodinase 1 (D1) function, lower free T3 and free T3/T4 ratio and higher serum free T4 and rT3. It has also been associated with depression in white female subjects.

DIO2 Iodothyronine Deiodinase 2

The DIO2 gene encodes the enzyme type II iodothyronine deiodinase, a selenoprotein, requiring selenium for its synthesis. DIO2 catalyzes the conversion of the prohormone thyroxine (T4) to the bioactive thyroid hormone (T3). This gene is widely expressed, including in thyroid, placenta, pituitary and brain. It is thought to be responsible for the 'local' production of T3, and thus important in influencing thyroid hormone action in these tissues.

It has also been reported to be highly expressed in thyroids of patients with Grave's disease, and in follicular

adenomas. The intrathyroidal T4 to T3 conversion by this enzyme may contribute significantly to the relative increase in thyroidal T3 production in these patients.

DIO2 Variant		Result	Description
rs12885300	Gly3Asp	CC	The wild C allele is associatec with increased risk of bipolar disorder, particularly in combination with the higher risk DIO2 rs225014 C allele. Both DIO2 SNPs should be examined together, and rs225014 has a stronger impact.
rs225014	Thr92Ala	TT	No variance. No impact on DIO2 expression or activity. No association with hypothyroidism or depression or osteoarthritis.

FKBP5 FK506 Binding Protein 5

FKBP5 is an important stress regulating gene responsible for controlling the body's response to cortisol by signalling to the body to lower the levels after they have been raised in response to stress.

Variants in this gene are associated with prolonged stress response and increased reactivity due to impaired lowering of cortisol levels after a stressful event. It is also linked to stress-related disorders such as depression, anxiety and post traumatic stress disorder (PTSD) in adulthood particularly as a result of childhood trauma.

FKBP5 Variant	Result	Description
rs1360780	CT	Increased FKBP5 levels and impaired regulation of cortisol. This is associated with low stress resilience and increased risk of depressive disorders. Increasing physical activity has been shown to improve stress resilience and sleep regulation.

HNMT Histamine N-Methyltransferase

HNMT controls the neurotransmitter activity of histamine in the brain and plays an important role in regulating the airway response to histamine. Variants have been reported to increase susceptibility to asthma. HNMT inactivates histamine via methylation - using SAMe as the methyl donor - therefore genetic variants that impact methylation (such as MTHFR) may also affect HNMT activity. The resultant N-Methylhistamine is then oxidatively deaminated to N-methyl-imidazole acetaldehyde by MAOB or by DAO.

HNMT Variant		Result	Description
i3000469	314CT	CC	No variance. No reported impact on HNMT activity or effect on histamine metabolism.

OPRM1 Opioid Receptor Mu 1

The OPRM1 gene encodes the mu opioid receptor (MOR). Opioids such as morphine, heroin, fentanyl, and methadone bind to this receptor. It is also the primary receptor for endogenous opioid peptides and opioid analgesic agents such as beta-endorphin and enkephalins. The mu opioid receptor also has an important role in dependence to other drugs of abuse, such as nicotine, cocaine, and alcohol via its modulation of the dopamine system.

OPRM1 Variant		Result	Description
rs1799971	A118G	GA	Decreased OPRM1 activity and less sensitivity to opioids. Patients with the G allele are may be less sensitive to certain drugs including morphine (for pain management) and L-asparaginase (a chemotherapeutic drug). Studies have sought associations with alcohol dependence but findings are inconsistent.

SLCO1C1 Solute Carrier Family 21, Member 1C1

Also known as OATP1C1, this gene encodes a member of the organic anion transporter family, a transmembrane receptor that mediates the sodium-independent uptake of thyroid hormones in brain tissues. This protein has particularly high affinity for the thyroid hormones thyroxine, tri-iodothyronine and reverse tri-iodothyronine. Polymorphisms in the gene encoding this protein may be associated with fatigue and depression in patients suffering from hypothyroidism.

SLCO1C1 Variant		Result	Description
rs10770704	Intron3C>T	CT	Variants are associated with reduced transport of T4 across the blood brain barrier, and with fatigue and depression.

Nervous System

References

Precision Nutrition, Genetics: The Universe Within - Can knowing more about your genes help you eat, move, and live better? By Krista Scott-Dixon, PhD With John Berardi, Phd, Alaina Hardie, and Helen Kollias, PhD

U.S Food & Drug Administration (https://www.fda.gov/Drugs/ScienceResearch/ucm572698.htm)

5-HT1A 5-Hydroxytryptamine Receptor 1A

Donalson et al. (2016). The functional serotonin 1a receptor promoter polymorphism, rs6295, is associated with psychiatric illness and differences in transcription. Translational Psychiatry, 2016 Mar; 6(3): e746. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4872437/)

Kantzky et al. (2017). The influence of the rs6295 gene polymorphism on serotonin-1A receptor distribution investigated with PET in patients with major depression applying machine learning. Translational Psychiatry, 2017 Jun; 7(6): e1150. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5537636/)

5-HT2A 5-Hydroxytryptamine Receptor 2A

Chang et al. (2017). Serotonin 2A receptor (5-HT2A) gene promoter variant interacts with chronic perceived stress to modulate resting parasympathetic activity in humans. Psychoneuroendocrinology, 2017 Feb;76:119-126. (https://www.ncbi.nlm.nih.gov/pubmed/27912162)

Parsons et al. (2004). The -1438A/G polymorphism in the 5-hydroxytryptamine type 2A receptor gene affects promoter activity. Biological Psychiatry, 2004 Sep 15;56(6):406-10. (https://www.ncbi.nlm.nih.gov/pubmed/15364038)

ADRB1 Adrenoceptor Beta 1

Bruck et al. (2005). The Arg389Gly Beta1-Adrenoceptor Polymorphism and Catecholamine Effects on Plasma Renin Activity. Journal of the American College of Cardiology Volume 46, Issue 11, 6 December 2005, Pages 2111-2115. (http://www.sciencedirect.com/science/article/pii/S0735109705021959)

Johnson AD, Newton-Cheh C, Chasman DI, et al. ASSOCIATION OF HYPERTENSION DRUG TARGET GENES WITH BLOOD PRESSURE AND HYPERTENSION IN 86,588 INDIVIDUALS. Hypertension. 2011;57(5):903-910. doi:10.1161/HYPERTENSIONAHA.110.158667. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3099407/)

Kersten M. Small et al. (2003). Pharmacology and Physiology of Human Adrenergic Receptor polymorphisms. Annual review of pharmacology and toxicology, 2003. 43:381–411. (https://pdfs.semanticscholar.org/bac3/2e49afb21ccf0183e6b1795b94207a401e52.pdf)

ADRB2 Beta-2-Adrenergic Receptor

Adrenergic beta(2) receptor polymorphism and athletic performance. Vishnu Sarpeshkar and David J Bentley. J Hum Genet. 2010 Aug;55(8):479-85. doi: 10.1038/jhg.2010.42. Epub 2010 Apr 30. (https://pdfs.semanticscholar.org/735d/bd52384920347e78f9e188593b957887e2f6.pdf)

Hussein et al. (2017). Beta2-adrenergic receptor gene haplotypes and bronchodilator response in Egyptian patients with chronic obstructive pulmonary disease. Advances in Medical Sciences, 2017 Mar;62(1):193-201. (https://www.ncbi.nlm.nih.gov/pubmed/28327457)

Kim et al. (2009). Genetic association analysis of COPD candidate genes with bronchodilator responsiveness. Respiratory Medicine 2009 Apr;103(4):552-7. (https://www.ncbi.nlm.nih.gov/pubmed/19114454?dopt=Abstract)

Turner et al. (2016). Childhood asthma exacerbations and the Arg-16 beta2 receptor polymorphism: a meta-analysis stratified by treatment. Journal of Allergy and Clinical Immunology, 2016 Jul; 138(1): 107–113.e5. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4931969/)

ALDH2 Aldehyde Dehydrogenase 2 Family (mitochondrial)

Cai Q , Wu J , Cai Q , Chen EZ , Jiang ZY, (2015), Association between Glu504Lys polymorphism of ALDH2 gene and cancer risk: a meta-analysis, PloS one; 10(2): e0117173. (http://europepmc.org/abstract/MED/25680116)

Li D, Zhao H, Gelernter J, (2012), Strong protective effect of the aldehyde dehydrogenase gene (ALDH2) 504lys (*2) allele against alcoholism and alcohol-induced medical diseases in Asians, Human Genetics; 131 (5), pp. 725–737. (https://link.springer.com/article/10.1007/s00439-011-1116-4)

Nervous System

ASMT Acetylserotonin O-Methyltransferase

Galecki et al. (2010). Single nucleotide polymorphisms and mRNA expression for melatonin synthesis rate-limiting enzyme in recurrent depressive disorder. Journal of Pineal Research, 2010 May;48(4):311-7. (https://www.ncbi.nlm.nih.gov/pubmed/20433639)

Geoffroy et al. (2014). An ASMT variant associated with bipolar disorder influences sleep and circadian rhythms: a pilot study. Genes, Brain and Behavior, 2014 Mar;13(3):299-304. (https://www.ncbi.nlm.nih.gov/pubmed/24308489)

Kripke et al. (2011). Polymorphisms in melatonin synthesis pathways: possible influences on depression. Journal of Circadian Rhythms, 2011; 9: 8. (https://www.ncbi.nlm.nih.gov/pubmed/21827647?dopt=Abstract)

BDNF Brain Derived Neurotrophic Factor

Park et al. (2017). The BDNF Val66Met Polymorphism Affects the Vulnerability of the Brain Structural Network. Frontiers in Human Neuroscience, 2017; 11: 400. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5541016/)

COMT Catechol-O-Methyltransferase

Stein DJ, Newman TK, Savitz J, Ramesar R. (2006). Warriors versus worriers: the role of COMT gene variants. CNS Spectr;11(10): pp. 745-8. (http://www.ncbi.nlm.nih.gov/pubmed/17008817?dopt=Abstract)

Xu K1, Ernst M, Goldman D. (2006). Imaging genomics applied to anxiety, stress response, and resiliency. Neuroinformatics; 4(1):51-64. (http://www.ncbi.nlm.nih.gov/pubmed/16595858)

CYP2C19 Cytochrome P450, Family 2, Subfamily C, Polypeptide 19

De Vos et al. (2011). Association between CYP2C19*17 and metabolism of amitriptyline, citalopram and clomipramine in Dutch hospitalized patients. The Pharmacogenomics Journal (2011) 11, 359–367. (https://www.ncbi.nlm.nih.gov/pubmed/20531370)

Kim JY, Cheong HS, Park TJ, Shin HJ, Seo DW, Na HS, Chung MW and Shin HD, (2014). Screening for 392 polymorphisms in 141 pharmacogenes, Biomed Rep; 2 (4): 463–476. (http://europepmc.org/articles/PMC4051470)

CYP2D6 Cytochrome P450, Family 2, Subfamily D, Polypeptide 6

Bijl MJ, Visser LE, Hofman A, Vulto AG, van Gelder T, Stricker BH and van Schaik RH, (2008). Influence of the CYP2D6*4 polymorphism on dose, switching and discontinuation of antidepressants, Br J Clin Pharmacol; 65(4): pp. 558-64. (http://www.ncbi.nlm.nih.gov/pubmed/18070221)

Johansson I, Lundqvist E, Bertilsson L, Dahl ML, Sjöqvist F, Ingelman-Sundberg M. Inherited amplification of an active gene in the cytochrome P450 CYP2D locus as a cause of ultrarapid metabolism of debrisoquine. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(24):11825-11829. (http://www.ncbi.nlm.nih.gov/pubmed/7903454)

Zhou SF, (2009), Polymorphism of human cytochrome P450 2D6 and its clinical significance. Part I. Clin Pharmacokinet. 2009; 48(11):pp. 689-723. (http://www.ncbi.nlm.nih.gov/pubmed/19817501)

CYP3A4 Cytochrome P450, Family 3, Subfamily A, Polypeptide 4

Amirimani B, Ning B, Deitz AC, Weber BL, Kadlubar FF, Rebbeck TR. (2003), Increased transcriptional activity of the CYP3A4*1B promoter variant, Environ Mol Mutagen; 42(4): 299-305. (http://www.ncbi.nlm.nih.gov/pubmed/14673875)

DBH Dopamine Beta-Hydroxylase

Barrie et al. (2014). Regulatory Polymorphisms in Human DBH Affect Peripheral Gene Expression and Sympathetic Activity. Circulation Research, 2014 Dec 5; 115(12): 1017–1025. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4258174/)

Shao et al. (2016). Association of Dopamine Beta-Hydroxylase (DBH) Polymorphisms with Susceptibility to Parkinson's Disease. Medical Science Monitor, 2016; 22: 1617–1622. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4915320/)

DIO1 Iodothyronine Deiodinase 1

Marco Medici, W. Edward Visser, Theo J. Visser, Robin P. Peeters; Genetic Determination of the Hypothalamic-Pituitary-Thyroid Axis: Where Do We Stand?, Endocrine Reviews, Volume 36, Issue 2, 1 April 2015, Pages 214–244, https://doi.org/10.1210/er.2014-1081 (https://academic.oup.com/edrv/article/36/2/214/2354676)

Panicker V, Cluett C, Shields B, et al. A Common Variation in Deiodinase 1 Gene DIO1 Is Associated with the Relative Levels of Free Thyroxine and Triiodothyronine. The Journal of Clinical Endocrinology and Metabolism. 2008;93(8):3075-3081. doi:10.1210/jc.2008-0397. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2515080/)

Philibert RA, Beach SRH, Gunter TD, et al. The Relationship of Deiodinase 2 Genotype and Thyroid Function to Lifetime History of Major Depression in Three Independent Populations. American journal of medical genetics Part B, Neuropsychiatric genetics: the official publication of the International Society of Psychiatric Genetics. 2011;156(5):593-599. doi:10.1002/ajmg.b.31200. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3236034/)

Nervous System

DIO2 Iodothyronine Deiodinase 2

Galecka et al. (2014). Association of the DIO2 gene single nucleotide polymorphisms with recurrent depressive disorder. Acta Biochim Pol. 2015;62(2):297-302. (https://www.ncbi.nlm.nih.gov/pubmed/26098717)

He B, Li J, Wang G, Ju W, Lu Y, Shi Y, et al. Association of genetic polymorphisms in the type II deiodinase gene with bipolar disorder in a subset of Chinese population. Prog Neuropsychopharmacol Biol Psychiatry. 2009;33:986–90 (https://www.ncbi.nlm.nih.gov/pubmed/19427350)

Vijay Panicker, Ponnusamy Saravanan, Bijay Vaidya, Jonathan Evans, Andrew T. Hattersley, Timothy M. Frayling, Colin M. Dayan: Common Variation in the DIO2 Gene Predicts Baseline Psychological Well-Being and Response to Combination Thyroxine Plus Triiodothyronine Therapy in Hypothyroid Patients, The Journal of Clinical Endocrinology & Metabolism, Volume 94, Issue 5, 1 May 2009, Pages 1623–1629, https://doi.org/10.1210/jc.2008-1301 (https://www.ncbi.nlm.nih.gov/pubmed/19190113)

Yalakanti et al. (2015). Association of Type II 5′ Monodeiodinase Thr92Ala Single Nucleotide Gene Polymorphism and Circulating Thyroid Hormones Among Type 2 Diabetes Mellitus Patients. Indian J Clin Biochem. 2016 Apr; 31(2): 152–161. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4820430/)

DRD2 Dopamine Receptor D2

Betcheva et al. (2009). Case-control association study of 59 candidate genes reveals the DRD2 SNP rs6277 (C957T) as the only susceptibility factor for schizophrenia in the Bulgarian population. Journal of Human Genetics. 2009 Feb;54(2):98-107. (https://www.ncbi.nlm.nih.gov/pubmed/19158809)

Clarke et al. (2014). The dopamine receptor D2 (DRD2) SNP rs1076560 is associated with opioid addiction. Annals of Human Genetics. 2014 Jan; 78(1): 33–39. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4013426/)

He et al. (2016). Associations between dopamine D2 receptor gene polymorphisms and schizophrenia risk: a PRISMA compliant meta-analysis. Neuropsychiatric Disease and Treatment, 2016; 12: 3129–3144. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5158172/)

Sasabe et al. (2007). Association analysis of the dopamine receptor D2 (DRD2) SNP rs1076560 in alcoholic patients. Neuroscience Letters, 2007 Jan 29;412(2):139-42. (https://www.ncbi.nlm.nih.gov/pubmed/17196743)

Zheng et al. (2012). Rs1076560, a functional variant of the dopamine D2 receptor gene, confers risk of schizophrenia in Han Chinese. Neuroscience Letters. 2012 Jun 14;518(1):41-4. (https://www.ncbi.nlm.nih.gov/pubmed/22569179)

FKBP5 FK506 Binding Protein 5

Brooks AK, Lawson MA, Smith RA, Janda TM, Kelley KW, McCusker RH. Interactions between inflammatory mediators and corticosteroids regulate transcription of genes within the Kynurenine Pathway in the mouse hippocampus. Journal of Neuroinflammation. 2016;13:98. doi:10.1186/s12974-016-0563-1. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC485547/)

Fujii et al. (2014). The common functional FKBP5 variant rs1360780 is associated with altered cognitive function in aged individuals. Scientific Reports, 2014; 4: 6696. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4204028/)

Han et al. (2017). Influence of FKBP5 polymorphism and DNA methylation on structural changes of the brain in major depressive disorder. Scientific Reports, 2017; 7: 42621. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5309810/)

Rao et al. (2016). Common variants in FKBP5 gene and major depressive disorder (MDD) susceptibility: a comprehensive meta-analysis. Scientific Reports, 2016; 6: 32687. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5013409/)

GABRA2 Gamma-Aminobutyric Acid Type A Receptor Alpha 2 Subunit

Lieberman et al. (2015). GABRA2 alcohol dependence risk allele is associated with reduced expression of chromosome 4p12 GABAA subunit genes in human neural cultures. Alcoholism, Clinical and Experimental Research. 2015 Sep; 39(9): 1654–1664. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4558268/)

HNMT Histamine N-Methyltransferase

Preuss, C. V., Wood, T. C., Szumlanski, C. L., Raftogianis, R. B., Otterness, D. M., Girard, B., Scott, M. C., Weinshilboum, R. M. Human histamine N-methyltransferase pharmacogenetics: common genetic polymorphisms that alter activity. Molec. Pharm. 53: 705-717, 1998. [PubMed: 9547362]. (http://www.ncbi.nlm.nih.gov/pubmed/9547362)

Szczepankiewicz A, Breborowicz A, Sobkowiak P, Popiel A (2010). "Polymorphisms of two histamine-metabolizing enzymes genes and childhood allergic asthma: a case control study". Clin Mol Allergy. 8: 14. doi:10.1186/1476-7961-8-14. (http://europepmc.org/abstract/MED/21040557)

Yan, L., Galinsky, R. E., Bernstein, J. A., Liggett, S. B., Weinshilboum, R. M. (2000). Histamine N-methyltransferase pharmacogenetics: association of a common functional polymorphism with asthma. Pharmacogenetics; 10: pp. 261-266. (http://www.ncbi.nlm.nih.gov/pubmed/10803682)

Nervous System

IFN gamma Interferon Gamma

Tang et al. (2014). Associations of IFN- rs2430561 T/A, IL28B rs12979860 C/T and ER rs2077647 T/C polymorphisms with outcomes of hepatitis B virus infection: a meta-analysis. Journal of Biomedical Research. 2014 Nov; 28(6): 484–493. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4250527/)

Wei et al. (2017). A single nucleotide polymorphism in the interferon- gene (IFNG +874 T/A) is associated with susceptibility to tuberculosis. Oncotarget, 2017 Aug 1; 8(31): 50415–50429. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5584145/)

Wu et al. (2016). Association of Interferon Gamma +874T/A Polymorphism and Leukemia Risk. Medicine (Baltimore), 2016 Mar; 95(12). (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4998384/)

MAOA Monoamine Oxidase A

Antypa N, Giegling I, Calati R, Schneider B, Hartmann AM, Friedl M, Konte B, Lia L, De Ronchi D, Serretti A, Rujescu D. (2013). MAOA and MAOB polymorphisms and anger-related traits in suicidal participants and controls. European Archives of Psychiatry and Clinical Neuroscience, 263(5):393-403. (http://europepmc.org/abstract/MED/23111930)

Zhang J, Chen Y, Zhang K, Yang H, Sun Y, Fang Y, Shen Y, Xu Q. (2010). A cis-phase interaction study of genetic variants within the MAOA gene in major depressive disorder. Biological Psychiatry, 68(9):795-800. (http://europepmc.org/abstract/MED/20691428)

MAOB Monoamine Oxidase B

Netter P, Montag C, Reuter M, Baars M and Gallhofer B, (2015), Genetic Variation of the MAO B Gene is Related to Shorter Reaction Times in Alcohol Dependent Patients, Journal of Addiction Medicine and Therapy, 3 (1): pp. 1014. (https://www.jscimedcentral.com/Addiction/addiction-3-1014.pdf)

MTNR1B Melatonin Receptor 1B

Comai S. PhD and Gobbi G. MD, PhD. (2014). Unveiling the role of melatonin MT2 receptors in sleep, anxiety and other neuropsychiatric diseases: a novel target in psychopharmacology, J Psychiatry Neurosci, 39(1): pp. 6–21. (http://europepmc.org/articles/PMC3868666)

Garaulet M, Gómez-Abellán P, Rubio-Sastre P, Madrid JA, Saxena R, Scheer FA. Common type 2 diabetes-risk variant in MTNR1B worsens the deleterious effect of melatonin on glucose tolerance in humans. Metabolism: clinical and experimental. 2015;64(12):1650-1657. doi:10.1016/j.metabol.2015.08.003. (https://www.ncbi.nlm.nih.gov/pubmed/26440713)

OPRM1 Opioid Receptor Mu 1

Hajj A, Halepian L, Osta NE, Chalhine G, Kattan J, Rabbaa Khabbaz L. OPRM1 c.118A>G Polymorphism and Duration of Morphine Treatment Associated with Morphine Doses and Quality-of-Life in Palliative Cancer Pain Settings. Angelini S, Raveguini G, Tegeder I, eds. International Journal of Molecular Sciences. 2017;18(4):669. doi:10.3390/ijms18040669. (https://www.ncbi.nlm.nih.gov/pubmed/28346387/)

Kang SM, Rosales JL, Meier-Stephenson V, Kim S, Lee KY, Narendran A. Genome-wide loss-of-function genetic screening identifies opioid receptor 1 as a key regulator of L-asparaginase resistance in pediatric acute lymphoblastic leukemia. Oncogene. 2017;36(42):5910-5913. doi:10.1038/onc.2017.211. (https://www.ncbi.nlm.nih.gov/pubmed/28650467/)

Kong X, Deng H, Gong S, Alston T, Kong Y, Wang J. Lack of associations of the opioid receptor mu 1 (OPRM1) A118G polymorphism (rs1799971) with alcohol dependence: review and meta-analysis of retrospective controlled studies. BMC Medical Genetics. 2017;18:120. doi:10.1186/s12881-017-0478-4. (https://www.ncbi.nlm.nih.gov/pubmed/29070014/)

PNMT Phenylethanolamine N-Methyltransferase

Rodríguez-Flores JL, Zhang K, Kang SW, et al. Conserved regulatory motifs at phenylethanolamine N-methyltransferase (PNMT) are disrupted by common functional genetic variation: an integrated computational/experimental approach. Mammalian Genome. 2010;21(3-4):195-204. doi:10.1007/s00335-010-9253-y. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2844968/)

QDPR Quinoid Dihydropteridine Reductase

Shi J, Badner JA, Hattori E, Potash JB, Willour VL, McMahon FJ, Gershon ES, and Liu C. (2008). Neurotransmission and Bipolar Disorder: A Systematic Family-based Association Study, Am J Med Genet B Neuropsychiatr Genet; 147B(7): pp. 1270–1277 (http://europepmc.org/articles/PMC2574701)

Nervous System

SLC18A1 Solute Carrier Family 18 Member A1

Vaht et al. (2016). A Functional Vesicular Monoamine Transporter 1 (VMAT1) Gene Variant Is Associated with Affect and the Prevalence of Anxiety, Affective, and Alcohol Use Disorders in a Longitudinal Population-Representative Birth Cohort Study. Search Results International Journal of Neuropsychopharmacology, 2016 Jul; 19(7) (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4966275/)

SLC6A2 Solute Carrier Family 6 Member 2

Li et al. (2012). The norepinephrine transporter gene is associated with the retardation symptoms of major depressive disorder in the Han Chinese population. Neural Regeneration Research, 2012 Sep 5; 7(25): 1985–1991. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4298894/)

Ueda et al. (2016). Relationship between G1287A of the NET Gene Polymorphisms and Brain Volume in Major Depressive Disorder: A Voxel-Based MRI Study. PLoS One, 2016; 11(3). (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4784887/)

SLC6A3 Solute Carrier Family 6 Member 3

Dumontheil et al. (2014). Preliminary investigation of the influence of dopamine regulating genes on social working memory. Society for Neuroscience, 2014 Oct; 9(5): 437–451. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4131246/)

Pinsonneault et al. (2011). Dopamine Transporter Gene Variant Affecting Expression in Human Brain is Associated with Bipolar Disorder. Neuropsychopharmacology, 2011 Jul; 36(8): 1644–1655. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3138671/)

Roussotte et al. (2014). Carriers of a common variant in the dopamine transporter gene have greater dementia risk, cognitive decline, and faster ventricular expansion. Alzheimer's & Dementia, 2015 Oct; 11(10): 1153–1162. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4465053/)

SLCO1C1 Solute Carrier Family 21, Member 1C1

van der Deure, W., Peeters, R., & Visser, T. (2010). Molecular aspects of thyroid hormone transporters, including MCT8, MCT10, and OATPs, and the effects of genetic variation in these transporters. Journal of Molecular Endocrinology, 44(1), 1–11. (https://jme.bioscientifica.com/view/journals/jme/44/1/1.xml)

TH Tyrosine Hydroxylase

Lee et al. (2016). Genetic Variations of Tyrosine Hydroxylase in the Pathogenesis of Hypertension. Electrolyte & Blood Pressure, 2016 Dec; 14(2): 21–26. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5337429/)

Nielsen et al. (2010). Tyrosine hydroxylase polymorphism (C-824T) and hypertension: a population-based study. American Journal of Hypertension, 2010 Dec;23(12):1306-11. (https://www.ncbi.nlm.nih.gov/pubmed/20706199)

Sanada H, Jones JE, Jose PA. Genetics of Salt-Sensitive Hypertension. Current hypertension reports. 2011;13(1):55-66. doi:10.1007/s11906-010-0167-6. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4019234/)

TNF Tumor Necrosis Factor

Abraham et al. (1999). Impact of the -308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease. Journal of Leukocyte Biology, 1999 Oct;66(4):562-6. (https://www.ncbi.nlm.nih.gov/pubmed/?term=Impact%20of%20the%20-308%20TNF%20promoter%20polymorphism%20on%20the%20transcriptional%20regulation%20of%20the%20TNF%20gene:%20relevance%20to%20disease)

Oxenkrug GF. Tryptophan-Kynurenine Metabolism as a Common Mediator of Genetic and Environmental Impacts in Major Depressive Disorder: The Serotonin Hypothesis Revisited 40 Years Later. The Israel journal of psychiatry and related sciences. 2010;47(1):56-63. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3021918/)

TPH1 Tryptophan Hydroxylase 1

Chen et al. (2012). Association between the TPH1 A218C polymorphism and risk of mood disorders and alcohol dependence: evidence from the current studies. Journal of Affective Disorders, 138(1-2):27-33. (https://www.ncbi.nlm.nih.gov/pubmed/21601290?dopt=Abstract)

Gonzalez-Castro et al. (2014). Association of TPH-1 and TPH-2 gene polymorphisms with suicidal behavior: a systematic review and meta-analysis. BMC Psychiatry, 14: 196. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4099217/)

Janusz K. Rybakowski and Alessandro Serretti. Genetic Influences on Response to Drug Treatment for Major Psychiatric Disorders, 2016. (http://urlz.fr/67L4)

Nielsen et al. (1994). Suicidality and 5-Hydroxyindoleacetic Acid Concentration Associated With a Tryptophan Hydroxylase Polymorphism. Archives of General Psychiatry, 51(1):34-38. (http://jamanetwork.com/journals/jamapsychiatry/article-abstract/496468)

The Neurobiology and Genetics of Nicotine and Tobacco, p. 68.
(https://books.google.co.uk/books?id=00KMBgAAQBAJ&pg=PA68&lpg=PA68&dq=rs1799913+C+allele&source=bl&ots=TdPsFhg2K&sig=FXXyfdmfnzBUZKwHD6FQQk5YgM4&hl=en&sa=X&ved=0ahUKEwj95ljwyqDWAhVEblAKHQGhDmSQ6AEIPTAD#v=onepage&q=rs1799913%20C%20allele&f=false)

TPH2 Tryptophan Hydroxylase 2

Latsko et al. (2016). A Novel Interaction between Tryptophan Hydroxylase 2 (TPH2) Gene Polymorphism (rs4570625) and BDNF Val66Met Predicts a High-Risk Emotional Phenotype in Healthy Subjects. PLoS One, 2016; 11(10).
(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5047464/)

Villalba et al. (2016). Serotonin Related Gene Polymorphisms and Asymptomatic Neurocognitive Impairment in HIV-Infected Alcohol Abusers. Genetics Research International, 2016; 7169172. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4812279/)

VDR Vitamin D (1,25 - dihydroxyvitamin D3) Receptor

Cui X1, Pelekanos M, Liu PY, Burne TH, McGrath JJ, Eyles DW. (2013). The vitamin D receptor in dopamine neurons; its presence in human substantia nigra and its ontogenesis in rat midbrain. J. Neuroscience (16), 236:77-87.
(http://www.ncbi.nlm.nih.gov/pubmed/23352937)

Wang L, Ma J, Manson JE, Buring JE, Gaziano JM, Sesso HD. (2013). A prospective study of plasma vitamin D metabolites, vitamin D receptor gene polymorphisms, and risk of hypertension in men. Eur J Nutr, 52, (7):1771-9.
(http://www.ncbi.nlm.nih.gov/pubmed/23262750)

Nervous System

Lifecode GX ®

— Professional Genotype Analysis —

Disclaimer

The information provided should not be used for diagnostic or treatment purposes and is not a substitute for personal medical advice. Use the information provided by Lifecode Gx® solely at your own risk.

Lifecode Gx® makes no warranties or representations as to the accuracy of information provided herein. If you have any concerns about your health, please consult a qualified health professional.

Report for Jaleel Miyan 09_31 (CP00000931)

Lifecode GX ®
— Professional Genotype Analysis —

Methylation

Methylation, also referred to as one carbon metabolism, is a process by which methyl groups are added to molecules. It is involved in almost every biochemical reaction in the body, occurring billions of times every second in our cells and contributing to numerous crucial bodily functions, including:

- Detoxification
- DNA integrity
- Energy production
- Inflammation control
- Immune function
- Gene expression / suppression
- Neurotransmitter balance
- Telomere protection (ageing)

Environmental factors such as diet, chemical or drug exposure and stress are known to play a role in supporting or hampering methylation. Important dietary co-factors include vitamin B6, B9, B12, methionine, betaine(TMG), choline and S-adenosylmethionine (SAMe). Insufficiency or deficiency of any of these co-factors may also hinder methylation.

Impaired methylation may contribute to major chronic conditions, including:

- Cardiovascular disease
- Unexplained miscarriages
- Problems during pregnancy
- Mood and psychiatric disorders
- Cancer
- Free radical damage (premature ageing)
- Diabetes
- Infertility
- Neural tube defects
- Adult neurological conditions
- Chronic fatigue syndrome

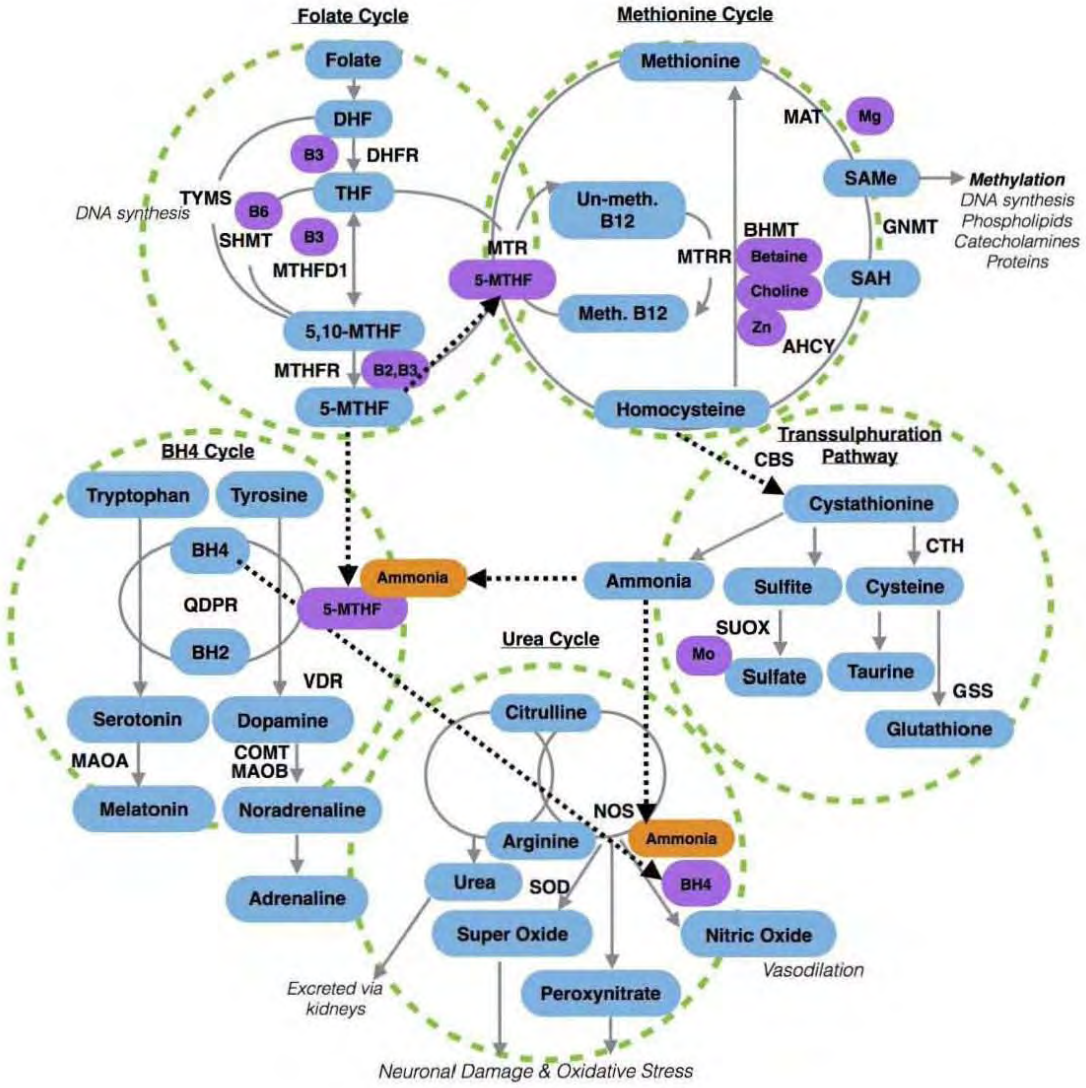
The Role of Genes in Methylation

The purpose of analysing genetic variants (or single nucleotide variants (SNVs)) in the context of the methylation pathway is to understand the likely effect, such as up or down regulation and subsequent impact on gene function, in order to provide guidance on how to support or bypass weaknesses or bottlenecks. Although an individual's genes cannot be changed, the rate and manner of gene expression, and therefore protein synthesis, can be regulated.

This report provides a personalised genotype analysis organised by the following methylation sub-cycles:

- The Folate Cycle
- The Methionine Cycle
- The Transsulphuration Pathway
- The BH4 Cycle / Neurotransmitter Metabolism
- The Urea Cycle

The Methylation Cycle Summary



Key:

- Co-factor
- Inhibitor

Folate Cycle

Folate, or vitamin B9, is the generic term for naturally occurring dietary folate and folic acid (the monoglutamate form of the vitamin found in supplements and fortified foods).

Folate is converted into dihydrofolate (DHF) in the presence of Vitamin B3. DHF is then converted to THF, also with the aid of B3.

The cyclical part of the process involves the conversion of tetrahydrofolate (THF) into 5,10-methylenetetrahydrafolate which in turn gets converted to 5-methyltetrahydrofolate (5-MTHF). 5-MTHF is then converted back into THF.

5-MTHF is an important product of the folate cycle as it is required by the methionine cycle for the conversion of homocysteine to methionine and to drive the conversion of BH2 to BH4 to support the neurotransmitter cycle. Another folate-dependent reaction, the methylation of deoxyuridylate (dUMP) to thymidylate (dTMP) in the formation of DNA, is required for proper cell division. An impairment of this reaction initiates a process that can lead to megaloblastic anemia, one of the indicators of folate deficiency.

Genetics

Absorption of folate may be impacted by variants on the FOLH1 gene (food form) and on the RFC1 or DHFR genes (either form).

The MTHFR A1298C variant impacts the conversion of dihydrobiopterin (BH2) to tetrahydrobiopterin (BH4) leading to low levels of neurotransmitters. In addition to the strain on the BH4 cycle, the amount of BH4 will also affect the functioning of the urea cycle.

The MTHFR C677T variant slows down the production of 5-MTHF which not only affects the regeneration of THF in the folate cycle but also the transfer of methyl groups to regenerate methionine in the methionine cycle. A homozygous genotype (AA) has more impact than a heterozygous (AG).

Variants on the MTHFD1 and SHMT1 genes can also slow the conversion of THF to 5,10 Methylene and subsequently impact 5-MTHF levels. Variants on the SHMT1 gene can also affect the conversion of serine to glycine.

Variants on MTHFD1 can impact synthesis of purines and on TYMS can affect thymidine synthesis, both of which are important for cell proliferation and growth.

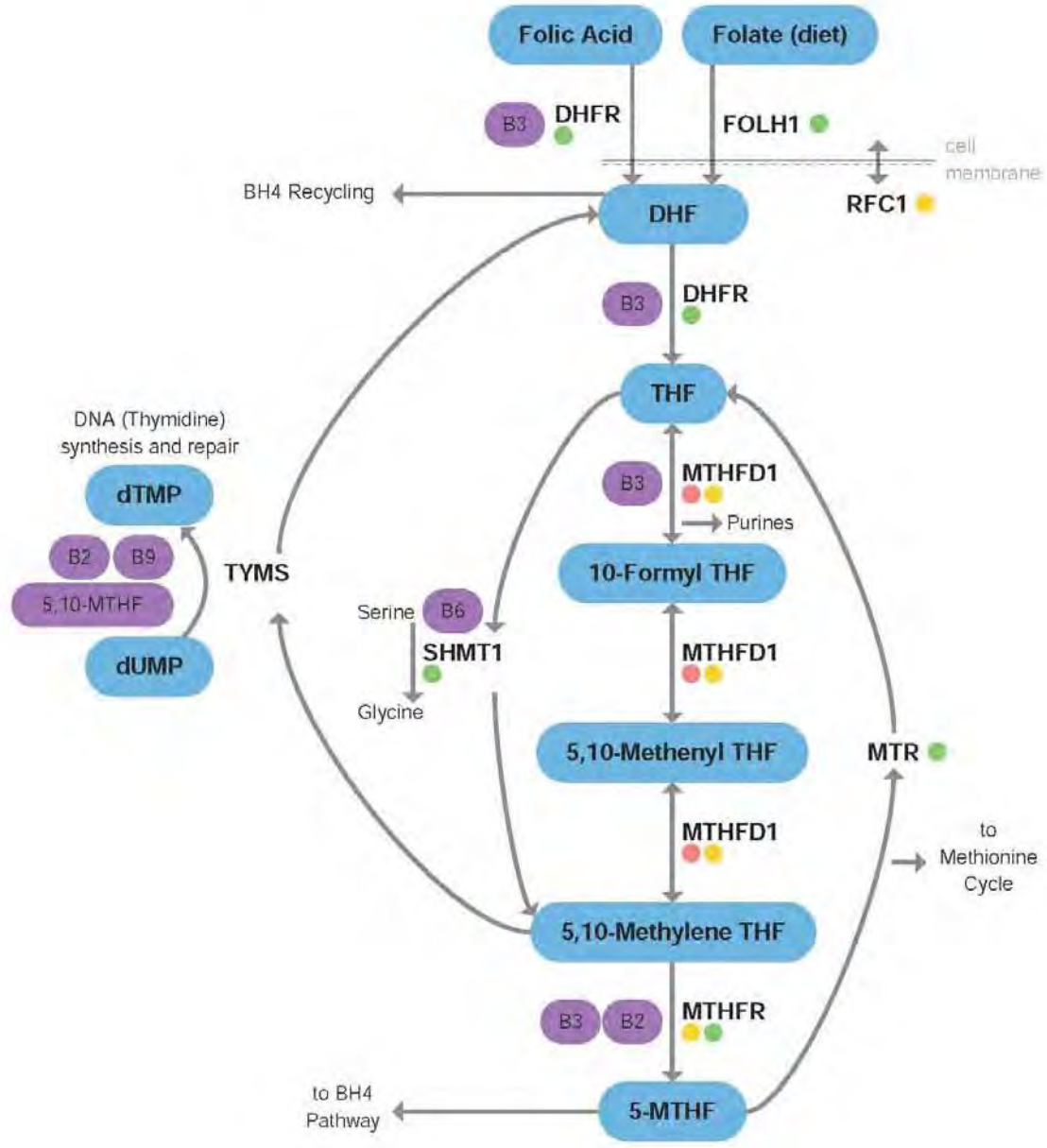
Further Investigation

Functional testing of serum and erythrocyte folate levels may be considered. As serum folate levels are sensitive to recent dietary intake, erythrocyte levels are more indicative of tissue folate stores.

Management and Lifestyle

Ensure adequate intakes of B vitamins - particularly B9 (folates) B2, B3 and B6. Methylated forms of B vitamins may be appropriate depending on variants.

Your Folate Cycle Results:



Key:

- Co-factor
- Inhibitor
- Protective - neutral
- Neutral - negative
- Negative

DHFR Dihydrofolate Reductase

SNV		Result	Description
rs70991108	19bp DEL	II	Neutral genotype - both copies of the DHFR gene are intact. No impact on DHFR gene expression or assimilation of folate.

The enzyme dihydrofolate reductase catalyses the conversion of dihydrofolate (DHF) into tetrahydrofolate (THF), a methyl group shuttle required for the synthesis of purines, thymidine and nucleic acids - precursors to DNA and RNA. The action of DHFR on folic acid (synthetic folate) absorbed in the liver is slower than on dietary folate absorbed in the intestine.

Anti-folate drugs such as methotrexate target DHFR to deplete cells of reduced folate resulting in the suppression of purine and pyrimidine precursor synthesis.

Variants on the DHFR gene may down regulate or up regulate activity. Lower activity may protect against certain cancers (colorectal cancer and childhood leukaemia), similar to the action of methotrexate, however, the consequent deficiency of folate can increase susceptibility to megaloblastic anaemia, neural tube defects and spina bifida. Higher enzyme activity can deplete 5,10 Methylene-THF and 5-MTHF required for synthesis of SAMe (the master methyl donor) and may tilt the balance in favour of DNA synthesis at the expense of methyl supply which can lead to aberrant DNA methylation and instability. High intake of folic acid (synthetic folate) has been linked to higher DHFR activity and increased risk of breast cancer in DHFR 19-bp deletion carriers.

FOLH1 Folate Hydrolase

SNV		Result	Description
rs202700	C1561T	CC	Normal intestinal absorption of dietary folate

FOLH1, also known as GPC 2 (Glutamate Carboxypeptidase II), is anchored to the intestinal brush border and facilitates the transfer of dietary folate into the body by converting polyglutamylated folates to monoglutamyl folates. Folic acid (a synthetic form of folate) is a monoglutamate, so does not require this conversion.

Variants are associated with down regulation of the gene resulting in impaired intestinal absorption of dietary folate, resulting in lower blood folate levels and consequent hyperhomocysteinemia.

MTHFD1 Methylenetetrahydrofolate Dehydrogenase 1

SNV		Result	Description
rs1076991	C105T	TT	Reduced gene activity which may reduce the supply of methyl-folate to recycle homocysteine to methionine (via the 'long route'). Folate insufficiency has been linked to risk of neural tube defects. Dependency on the short route (via BHMT) and betaine (as co-factor) and its substrate choline (found in eggs). Depletion of choline may increase risk of endometriosis and infertility.
rs2236225	G1958A	AG	Possible reduction in gene activity which may reduce the supply of methyl-folate to recycle homocysteine to methionine (via the 'long route'). Folate insufficiency has been linked risk of neural tube defects. Possible increased dependency on the short route (via BHMT) and betaine (as co-factor) and its substrate choline (found in eggs). Depletion of choline may increase risk of endometriosis, and related infertility

MTHFD1 possesses three distinct activities which catalyse the sequential reactions in the interconversion of the carbon-1 derivatives of THF, which are substrates for methionine, thymidylate, and de novo purine synthesis. These are reversible reactions that can be directed towards 5-MTHF - and homocysteine re-methylation - or away from it and can, therefore, impact the methionine cycle.

Variants in MTHFD1 are associated with down regulation of the gene activity and can impact availability of the various THF substrates required for nucleotide biosynthesis, DNA synthesis and repair and increase the demand for choline as a methyl-group donor (in the BHMT 'short cut' pathway of the methionine cycle). Variants have been linked to increased risk of folate sensitive neural tube defects and endometriosis related infertility due to choline depletion.

MTHFR Methylenetetrahydrofolate Reductase (NAD(P)H)

SNV		Result	Description
rs1801131	A1298C	GT	Reduced gene function which may result in lower 5-MTHF (methyl-folate) and slower conversion of BH2 to BH4 - needed for neurotransmitter synthesis. This genotype should be examined in the context of the BH4/ Neurotransmitter cycle. Methylation can be supported by adequate consumption of folate containing foods (such as green leafy vegetables, citrus fruits, beans and liver) and cofactors (vitamins B2 and B3).
rs1801133	C677T	GG	Neutral genotype. No impact on 5-MTHF and homocysteine levels.

The MTHFR gene is responsible for making the protein methylenetetrahydrofolate reductase (MTHFR), the rate-limiting enzyme in the methylation cycle which catalyses the conversion of folate to 'active' folate (5-MTHF) needed to support the re-methylation of homocysteine to methionine, DNA synthesis and repair (vital for healthy cell division), and the metabolism of neurotransmitters, phospholipids and proteins such as myelin.

Variants on the MTHFR gene usually result in lower enzyme activity. The C677T variant, which occurs in about 30% of people, can result in significantly reduced 5-MTHF levels - up to 40% reduction for heterozygotes and 70% for homozygotes (AA). MTHFR activity can be supported by increasing the intake of folate (B9) and the cofactors riboflavin (vitamin B2), niacin (vitamin B3), cobalamin (vitamin B12) and zinc. The A1298C variant has less direct impact on 5-MTHF levels but is associated with depletion of BH4 - vital for neurotransmitter synthesis.

MTR 5-Methyltetrahydrofolate-Homocysteine Methyltransferase

SNV		Result	Description
rs1805087	A2756G	AA	Neutral genotype. No impact on recycling of methyl-folate (5-MTHF) to THF

Also known as cobalamin-dependent methionine synthase (MS), MTR catalyses the final step in methionine synthesis from homocysteine. It also supplies folate to the cycles that produce purines and pyrimidines for DNA synthesis. MTR eventually becomes inactive due to the oxidation of its cobalamin co-factor.

Variants in MTR can increase the activity of this gene product so that it leads to a greater need for B12 as the enzyme is using up B12 at a faster rate. MTR activity can be supported by ensuring adequate B12. The MTR and MTRR composite status is also important as MTRR helps to recycle B12 for use by MTR.

RFC1 Reduced Folate Carrier 1

SNV		Result	Description
rs1051266	A80G	CT	Reduced ability to take up, retain, and metabolise folate which could result in raised homocysteine levels. Associated with reduced transport of and poorer response to methotrexate treatment

RFC1, also known as SLC19A1 (Solute Carrier Family 19), is a transporter of folate and is involved in the regulation of intracellular concentrations of folate. It has a higher affinity for reduced folate than folic acid.

Variants on this gene are associated with reduced ability to take up, retain, and metabolise folate resulting in reduced bioavailable folate (5-MTHF) which impacts DNA methylation, and impacts the methionine cycle - contributing to increased homocysteine levels, and the BH4/ neurotransmitter cycle - decreased BH4 levels.

SHMT1 Serine hydroxymethyltransferase 1 (Soluble)

SNV		Result	Description
rs1979277	C1420T	GG	Normal conversion of THF to 5,10-Methylene THF needed for synthesis of methyl-folate, purines, thymidine, needed for DNA synthesis and repair, and for conversion of serine to glycine.

SHMT is a vitamin B6 dependent enzyme which catalyzes the reversible conversion of serine to glycine and of tetrahydrofolate to 5,10-methylene tetrahydrofolate needed for the synthesis of purine, thymidine and methionine. Variances causing disturbances in SHMT1 expression and activity lower the concentration of available 5,10-MTHF, leading to lower synthesis of purines and DNA and lower availability of 5-MTHF for methylation processes.

TYMS Thymidylate Synthetase

SNV	Result	Description
rs2790	?	No result

Thymidylate synthase catalyses the methylation of deoxyuridylate to deoxythymidylate using 5,10-methylenetetrahydrofolate as a co-factor. This function maintains the dTMP (thymidine-5-prime monophosphate) pool critical for DNA replication and repair.

Functional genetic variants in TYMS may impact DNA stability and increase the risk of certain cancers.

Methionine Cycle

The methionine cycle is also known as the SAMe or methylation cycle. It is the cycle that is responsible for the process of methylation - adding or removing methyl groups from one chemical to another – by SAMe. SAMe is called the universal methyl donor as it is the primary source of methyl groups for most other biochemical reactions including methylation of DNA, RNA, proteins, creatine etc.

The major intermediates involved in this cycle are methionine, S-adenosylmethionine (SAM or SAMe), S-adenosylhomocysteine (SAH) and homocysteine. It involves the regeneration of methionine from homocysteine with the help of methylated vitamin B12 (methylcobalamin) and 5-MTHF, which is an important intermediate in the folate cycle. There is also an alternative 'short cut' conversion pathway that is catalysed by BHMT. Methionine is converted into the various intermediates such as SAMe, SAH and (back) to homocysteine.

Homocysteine may also be removed from the methionine cycle by conversion into cystathionine (see transsulphuration cycle).

Genetics

Methionine is converted to SAMe in the presence of magnesium (Mg) and ATP (universal energy donor) by the enzyme MAT. Variants in MAT may down regulate its activity and impact the rate of SAMe synthesis.

SAMe, once it donates its methyl group to the various reactions, gets converted to SAH. A high ratio of SAH to SAMe may inhibit the conversion of SAMe to SAH and therefore the rate of methylation. This may occur if the rate of SAH conversion to homocysteine is slowed either due to down-regulation of the AHCY gene or if homocysteine levels are high.

The 'long route' reaction that converts homocysteine back to methionine involves the MTR mediated transfer of a methyl group from 5-MTHF (from the folate cycle) to form methylated B12. The B12 methyl group is then used to re-methylate homocysteine to methionine. Some of the un-methylated B12 is re-methylated by the enzyme MTRR using SAMe as the methyl donor. This reaction can be impacted by variants in MTR, MTRR genes or in the folate cycle (particularly MTHFR) or by vitamin B12 or SAMe deficiency.

The 'short cut' pathway for conversion of homocysteine to methionine does not involve B12 or the folate cycle. The BHMT enzyme catalyses the conversion of betaine (TMG) to DMG by transferring a methyl group to homocysteine for it to become methionine. This pathway can be impacted by variants in the BHMT gene or betaine or choline deficiency.

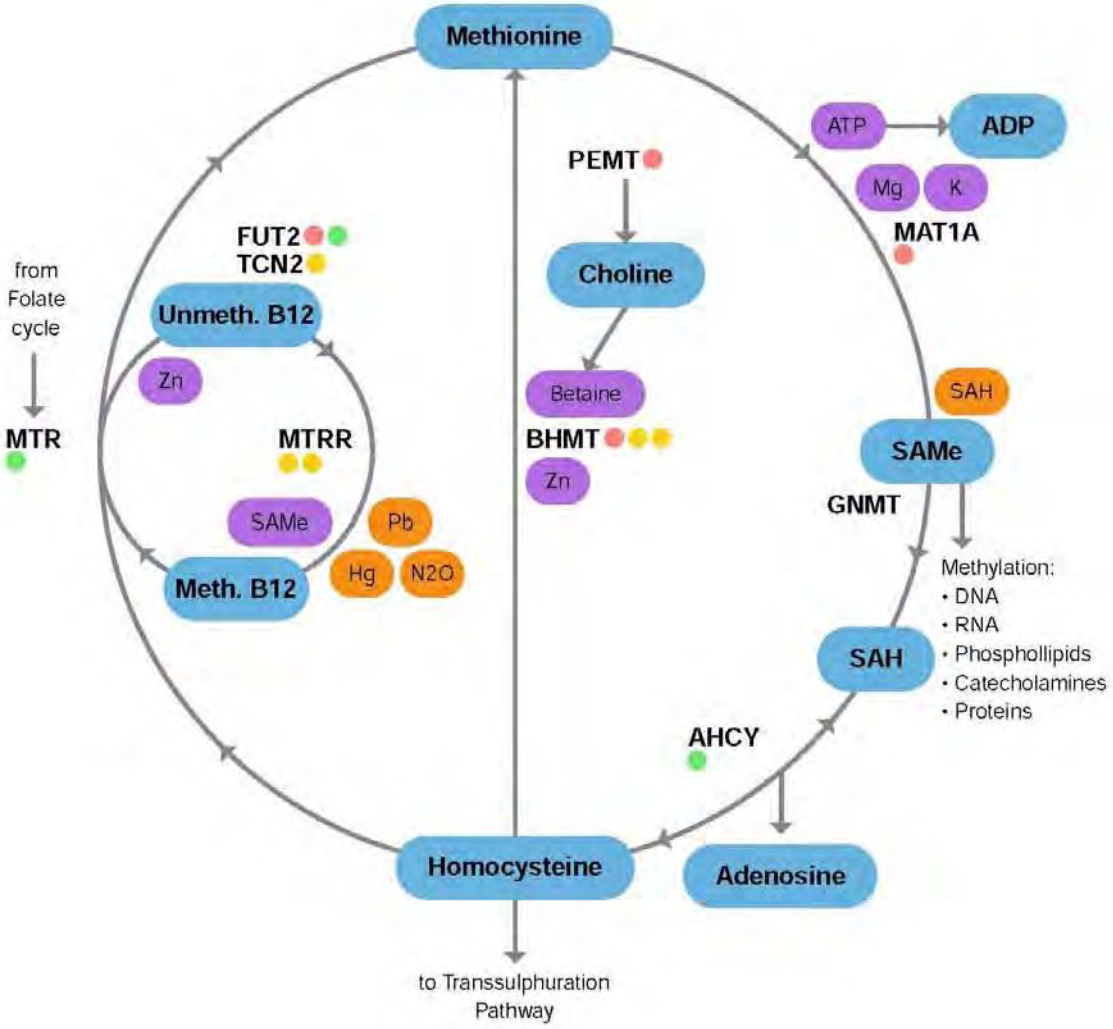
Further Investigation

Functional testing of homocysteine, methionine, B12 and SAMe levels may be considered. The ratio of SAH: SAMe is also a useful indicator of SAMe conversion.

Management and Lifestyle

Ensure adequate intakes of vitamin B9 (see Folate cycle), B12, betaine (found in beetroot) and choline. SAMe supplementation may be considered.

Your Methionine Cycle Results:



Key:

- Co-factor
- Inhibitor
- Protective - neutral
- Neutral - negative
- Negative

AHCY S-Adenosylhomocysteine Hydrolase

SNV		Result	Description
i5000928	Tyr143Cys	TT	Neutral genotype - no impact on conversion of SAH to homocysteine.

AHCY, which is also known as SAHH, catalyses the reversible hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and homocysteine.

Although genetic deficiency of AHCY activity in humans has been reported in only a few cases, metabolic effects of AHCY deficiency include elevated plasma SAH, SAMe, and methionine. The same effects may more likely result from high homocysteine levels triggering the reverse reaction metabolising and increasing levels of SAH. A high SAH to SAMe ratio can inhibit SAMe conversion and cause build up of methionine.

BHMT Betaine-homocysteine S-methyltransferase

SNV		Result	Description
rs3733890	R239Q	AG	This genotype is associated with down-regulated BHMT activity resulting in a less effective 'short cut' pathway for the conversion of homocysteine to methionine and risk of high homocysteine. It is also reported to increase the risk of NTDs (neural tube defects). BHMT can be supported by increasing intake of co-factors including foods containing zinc - such as beef, lamb, chicken, chickpeas, pumpkin seeds, cashews, betaine - from quinoa, spinach and beetroot, and choline (substrate of betaine) - found in eggs.
rs567754	BHMT/2	TC	This genotype is associated with down-regulated BHMT activity resulting in a less effective 'short cut' pathway for the conversion of homocysteine to methionine. BHMT can be supported by increasing intake of co-factors including foods containing zinc - such as beef, lamb, chicken, chickpeas, pumpkin seeds, cashews, betaine - from quinoa, spinach and beetroot, and choline (substrate of betaine) - found in eggs.
rs651852	BHMT/8	TT	This genotype is associated with down-regulated BHMT activity resulting in a less effective 'short cut' pathway for the conversion of homocysteine to methionine. BHMT can be supported by increasing intake of co-factors including foods containing zinc - such as beef, lamb, chicken, chickpeas, pumpkin seeds, cashews, betaine - from quinoa, spinach and beetroot, and choline (substrate of betaine) - found in eggs.

BHMT catalyses the transfer of a methyl group from betaine to homocysteine to form methionine. It uses a 'short cut' mechanism rather than the B12-dependent 'long route'. The BHMT pathway is zinc-dependent and requires adequate levels of TMG - trimethylglycine (betaine) to function properly. This reaction is also required for the irreversible oxidation of choline. BHMT activity can also be affected by cortisol levels (stress) and may play a role in ADD/ADHD by affecting noradrenaline levels.

Variants in BHMT may contribute to increased homocysteine levels particularly if there are also variants on the MTR or MTRR genes affecting the 'long route' re-methylation of homocysteine.

FUT2 Fucosyltransferase 2

SNV		Result	Description
rs1047781	A385T	AA	Secretor genotype (Asian populations) - susceptibility to H. pylori infection and gastritis linked to reduced B12 absorption

SNV		Result	Description
rs601338	W143X	AA	Non-secretor (non-Asian populations) - protective against H. pylori and gastritis, linked to improved B12 absorption. Individuals homozygous for the FUT2 nonsecretor genotype appear to be resistant to infection with Norovirus.

The classic human secretor locus (Se), FUT2 gene, encodes alpha-(1,2)fucosyltransferase which regulates the expression of the H antigen, a precursor of the blood group A and B antigens, on the gastrointestinal mucosa. Absorption of B12 requires the secretion of the glycoprotein intrinsic factor (IF) from the gastric cells, binding of IF to vitamin B12 and a functional gastrointestinal absorption system.

The FUT2 secretor status has been associated with both H. pylori infection and gastritis; patients with vitamin B12 malabsorption and low levels of serum vitamin B12 have higher prevalence of H. pylori infection. Secretor status is also associated with increased Bifido bacterium in the host. In addition the milk sugar 2'FL found in maternal breast milk stimulates the growth of Bifido bacteria in the microbiome of the breast fed infant.

The FUT non-secretor status is associated with resistance to Norwalk/ Norovirus and resilience to H. Pylori resulting in better B12 status. It is however linked to lower Bifido bacteria and less diverse and populated 'friendly' bacteria and increased risk of Celiac and other autoimmune diseases.

The homozygous genotypes W143X (AA) in non Asian populations and A385T (TT) in Asian populations have been reported as reliable indicators of an inactive FUT2 gene and non secretor status. About 20% of people are non secretors.

MAT1A Methionine Adenosyltransferase I, Alpha

SNV		Result	Description
rs1985908	T1297C	GG	Down regulation of MAT activity linked to hypermethioninemia and low SAMe. MAT activity can be supported by ensuring adequate intake of co-factors potassium and magnesium

MAT catalyses a two-step reaction that involves the transfer of the adenosyl from ATP to methionine to form S-adenosylmethionine (SAMe) and tripolyphosphate. SAMe is the main source of methyl groups for most biological methylations and is known as the master methyl donor.

Variants on the MAT genes partially inactivate MAT activity and may lead to hypermethioninemia, low SAMe and therefore slow methylation.

MTR 5-Methyltetrahydrofolate-Homocysteine Methyltransferase

SNV		Result	Description
rs1805087	A2756G	AA	Neutral genotype - no impact on MTR activity or B12 levels

Also known as cobalamin-dependent methionine synthase (MS), MTR catalyses the final step in methionine synthesis from homocysteine. It also supplies folate to the cycles that produce purines and pyrimidines for DNA synthesis. MTR eventually becomes inactive due to the oxidation of its cobalamin co-factor.

Variants in MTR can increase the activity of this gene product so that it leads to a greater need for B12 as the enzyme is using up B12 at a faster rate. MTR activity can be supported by ensuring adequate B12. The MTR and MTRR composite status is also important as MTRR helps to recycle B12 for use by MTR.

MTRR 5-Metyltetrahydrofolate-homocysteine S-Methyltransferase Reductase

SNV		Result	Description
rs162036	K350A	GA	Reduced ability to re-methylate vitamin B12 which is needed for MTR conversion of homocysteine and can contribute to hyperhomocysteinemia. Supplementing methylated B12 may be beneficial to support methylation.
rs1801394	A66G	GA	Reduced ability to re-methylate vitamin B12 which is needed for MTR conversion of homocysteine and can contribute to hyperhomocysteinemia. Supplementing methylated B12 may be beneficial to support methylation.

MTRR (methionine synthase reductase) regenerates MTR via a methylation reaction that uses SAMe as donor. MTRR also supports MTR activity by recycling and converting vitamin B12 into its methylated form.

Variants in MTRR can result in down-regulation of the gene activity and reduce its effectiveness in supporting MTR and contribute to high homocysteine levels.

PEMT Phosphatidylethanolamine N-methyltransferase

SNV		Result	Description
rs7946	V175M	TT	Potential for reduced choline synthesis, which can impact betaine levels needed to support the BHMT 'short cut' conversion of homocysteine to methionine. As PEMT activity is stimulated by oestrogen, this variant may have more impact on males and post-menopausal females. Dependency on PEMT activity can be reduced by ensuring adequate dietary intake of choline (found in eggs, beef, chicken and fish).

PEMT encodes an enzyme which converts phosphatidylethanolamine to phosphatidylcholine by sequential methylation in the liver, a significant source of choline relative to dietary intake. Choline is a major source of methyl groups via its metabolite betaine - which catalyzes the methylation of homocysteine to form methionine. Oestrogen induces expression of the PEMT gene and allows premenopausal women to make more of their required choline endogenously compared to postmenopausal women, and men.

Polymorphisms in the PEMT gene alter the endogenous synthesis of choline which can impact the 'short cut' re-methylation of homocysteine to methionine by BHMT and may therefore increase susceptibility to high homocysteine levels particularly in combination with variants on MTHFR, MTR or MTRR genes.

TCN2 Transcobalamin II

SNV		Result	Description
rs1801198	C776G	GC	The G allele decreases the activity of the TCN2 gene and the cellular and plasma concentration of transcobalamin, the carrier protein which delivers vitamin B12 to cells. The variant has been associated with developmental disorders and pregnancy loss. It does not appear to impact homocysteine levels.

This gene encodes transcobalamin II (TCII), a member of the vitamin B12-binding protein family. This plasma protein binds cobalamin and mediates its transport from the intestine into blood cells

Variants on the gene may reduce ability to absorb cobalamin (vitamin B12).

Transsulphuration Pathway

The transsulphuration pathway is a metabolic pathway involving the interconversion of cysteine and homocysteine, through the intermediate, cystathionine. This pathway generates the antioxidant glutathione, as well as the amino acids taurine and cysteine. The negative by-products: ammonia - which depletes BH4 leading to low dopamine and serotonin (see BH4 cycle); sulphites - which stimulate cortisol and produce brain fog; and glutamate - which leads to excitotoxicity, are also generated in this process.

Genetics

CBS regulates the enzyme that converts homocysteine to cystathionine and its downstream metabolites. The majority of variants on this gene cause up-regulation, making the enzyme work too fast, pulling homocysteine at a high rate from the methionine cycle, preventing it from being recycled via MTR and BHMT and compromising our ability to recycle homocysteine back to SAMe, the universal methyl donor. Homocysteine is then rapidly converted into taurine, cysteine and ammonia leading to high levels of sulphites and low levels of glutathione. Excess ammonia floods the urea cycle, weakening NOS activity (see urea cycle) and decreases BH4 which disrupts neurotransmitter metabolism (see BH4 cycle). The CBS C699T variant has the strongest effect, thought to increase CBS activity by up to 10 times.

Variants in BHMT aggravate and frequently co-exist with CBS variants.

CTH and GSS mediate the conversion of cysteine and glutathione respectively. Variants on either gene will lead to low glutathione synthesis.

Variants on SUOX will exacerbate high sulphite levels caused by up-regulated CBS due to slow degradation and detoxification of sulphites. This can result in sulphite sensitivity and neurological abnormalities.

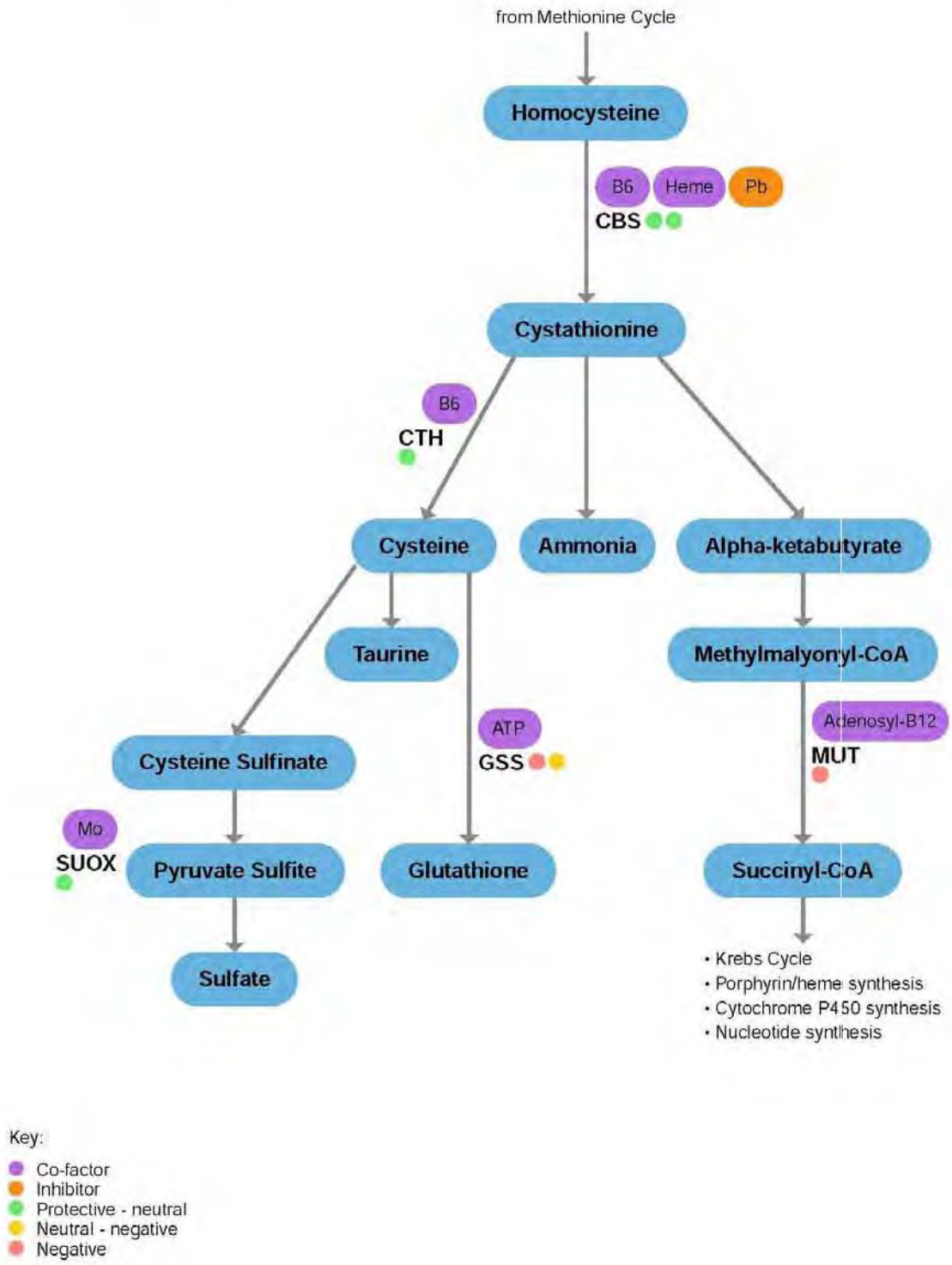
Further Investigation

A urine or plasma amino acid profile will identify homocysteine, taurine, glutathione, ammonia and sulphur-containing amino acids: cysteine and methionine. A urine dipstick test will identify sulphur in the urine.

Management and Lifestyle

Support BHMT to balance CBS up-regulation (TMG, phosphatidylserine, phosphatidylcholine and zinc). Neutralise ammonia (charcoal, probiotics to stop bacterial production of ammonia, limit animal protein). Limit sulphur-containing foods such as eggs, garlic, onions and broccoli, and supplements, e.g. cysteine, since sulphur sensitivity may occur (avoid completely if homozygous for SUOX). Supplementing B6 (P5P) will ensure proper functioning of the pathway and molybdenum will support SUOX activity.

Your Transsulphuration Pathway Results:



Key:

- Co-factor
- Inhibitor
- Protective - neutral
- Neutral - negative
- Negative

CBS Cystathionine Beta-Synthase

SNV		Result	Description
rs1801181	C1080T	GG	Wild genotype - typically exhibits normal CBS enzyme activity
rs234706	C699T	GG	Wild genotype - typically exhibits normal CBS enzyme activity

The CBS gene converts homocysteine (generated from methionine from the methionine cycle) to cystathionine, the first step in the transsulphuration pathway requiring vitamin B6 and heme as co-factors. The CBS enzyme acts as an "open gate" between homocysteine and the transsulphuration pathway, draining homocysteine, preventing it from being recycled into methionine, depleting B6 and B12, and preventing the synthesis of SAMe. Instead, homocysteine is diverted and converted into cysteine and taurine, a process which generates ammonia. High ammonia puts pressure on the urea cycle and causes low BH4, disrupting neurotransmitter metabolism. High cysteine creates toxic sulphites putting pressure on the SUOX gene. Glutathione synthesis is also negatively affected by the flooding of this pathway. CBS enzyme deficiency is less common but can occur and causes high homocysteine levels due to the blockage of the transsulphuration pathway.

The C699T (A) variant is thought to have the strongest up-regulating effect on the CBS enzyme. CBS should be assessed together with variants on MTHFR, MTR , BHMT and MUT.

CTH Cystathionine Gamma-Lyase

SNV		Result	Description
rs1021737	G1112T	GG	Wild genotype - typically exhibits normal CTH activity (conversion of cystathionine to cysteine). Insufficiency of vitamin B6 will compromise this enzyme regardless of genotype

CTH encodes an enzyme that converts cystathionine into cysteine. This is the second step in the transsulphuration pathway requiring vitamin B6 as a co-factor. Glutathione synthesis in the liver is dependent upon the availability of cysteine and is important for healthy detoxification.

Variants on this gene cause compromised conversion of cystathionine to cysteine

GSS Glutathione Synthetase

SNV		Result	Description
rs1801310	59270A>G	AA	Low GSS enzyme activity - may lead to slow glutathione synthesis. This enzyme requires ATP as a co-enzyme to function optimally.
rs6088659	A5997G	CT	Reduced GSS enzyme activity - may lead to low glutathione synthesis. This enzyme requires ATP as a co-enzyme to function optimally

GSS controls the second step of glutathione biosynthesis, the ATP-dependent conversion of gamma-L-glutamyl-Lcysteine to glutathione. Glutathione is important for a variety of biological functions including protection of cells from oxidative damage by free radicals, detoxification of xenobiotics, and membrane transport.

Variants on this gene may cause low synthesis of glutathione leading to possible deficiency.

MUT Methylmalonyl-CoA Mutase

SNV		Result	Description
I6060254	G1595A	TT	Associated with low circulating B12 levels and elevated homocysteine. Possible reduced ability to convert methylmalyonyl-CoA to succinyl-CoA which may affect the Krebs cycle. Ensure adequate adenosyl-B12 to support enzyme function

MUT is a mitcochondrial enzyme that converts methylmalonyl Co-enzyme A to succinyl-Co-enzyme A requiring adenosylcobalamin (adenosyl-B12) as co-factor. Succinyl-CoA is an important enzyme in the Krebs cycle and is crucial for the synthesis of heme, cytochrome P450s and nucleotides.

Mutations in this gene may lead to various types of methylmalonic aciduria.

SUOX Sulfite Oxidase

SNV		Result	Description
rs705703	C5444T	CC	Wild genotype - typically indicates normal SUOX enzyme activity leading to normal conversion of sulphites to sulphates. Molybdenum insufficiency will lead to reduced enzyme function regardless of genotype

SUOX catalyses the oxidation of sulphite to sulphate, the final molybdenum-dependent reaction in the oxidative degradation of the sulphur amino acids cysteine and methionine. This gene product helps to detoxify sulphites in the body.

Variants on SUOX may result in sulphite sensitivity and neurological abnormalities, and should be regarded in combination with up-regulated CBS. Sulphites are generated as a natural byproduct of the methylation cycle as well as ingested from foods we eat and give off the gas sulphur dioxide, which can cause irritation in the lungs, severe asthma attack in those who suffer from asthma, nausea, hives and, in rare cases, more severe allergic reactions.

BH4 Cycle / Neurotransmitter Metabolism

Tetrahydrobiopterin, or BH4, is a naturally occurring chemical compound requiring active folate (5-MTHF) and S-adenosylmethionine (SAMe) to help convert several amino acids such as phenylalanine, tyrosine and tryptophan into the neurotransmitters noradrenaline, dopamine, serotonin, melatonin and thyroid hormones. Without the participation of 5-MTHF in this process, SAMe and neurotransmitter levels decrease in the cerebrospinal fluid, contributing to depression.

BH4 is crucial for neutralising ammonia and for generating nitric oxide from arginine in the urea cycle (without BH4, the free radical superoxide, is created instead). BH4 also protects nerve cells from heavy metal toxicity and glutathione depletion.

Low levels of crucial neurotransmitters can cause mood imbalances, poor memory and concentration, sleep disturbances and aggressive behaviour.

Genetics

BH4 deficiency can occur as a result of variants on QDPR, the gene responsible for converting BH2 to BH4 with the help of active folate from the folate cycle. Variants on CBS, BHMT and MTHFR A1298C can also cause BH4 deficiency due to high ammonia and low active folate.

Variants on COMT, MAOA & MAOB result in poor breakdown of neurotransmitters and may lead to imbalances causing mood disorders. SAMe and SAH compete for the SAMe binding site on the COMT molecule (think of the SAMe binding site as the 'on-off' switch for COMT). A build up of SAH will thus reduce COMT activity.

Variants on VDR TaqI, BsmI and ApaI lead to lower vitamin D levels causing low dopamine production. COMT variants can be beneficial as there will be less circulating dopamine in need of being broken down. Those with VDR variants but without COMT variants will have low dopamine levels and increased need for methyl donors and dopamine precursors. Conversely those with COMT variants but without VDR variants will have the highest levels of dopamine and low need for and tolerance of methyl groups and dopamine precursors.

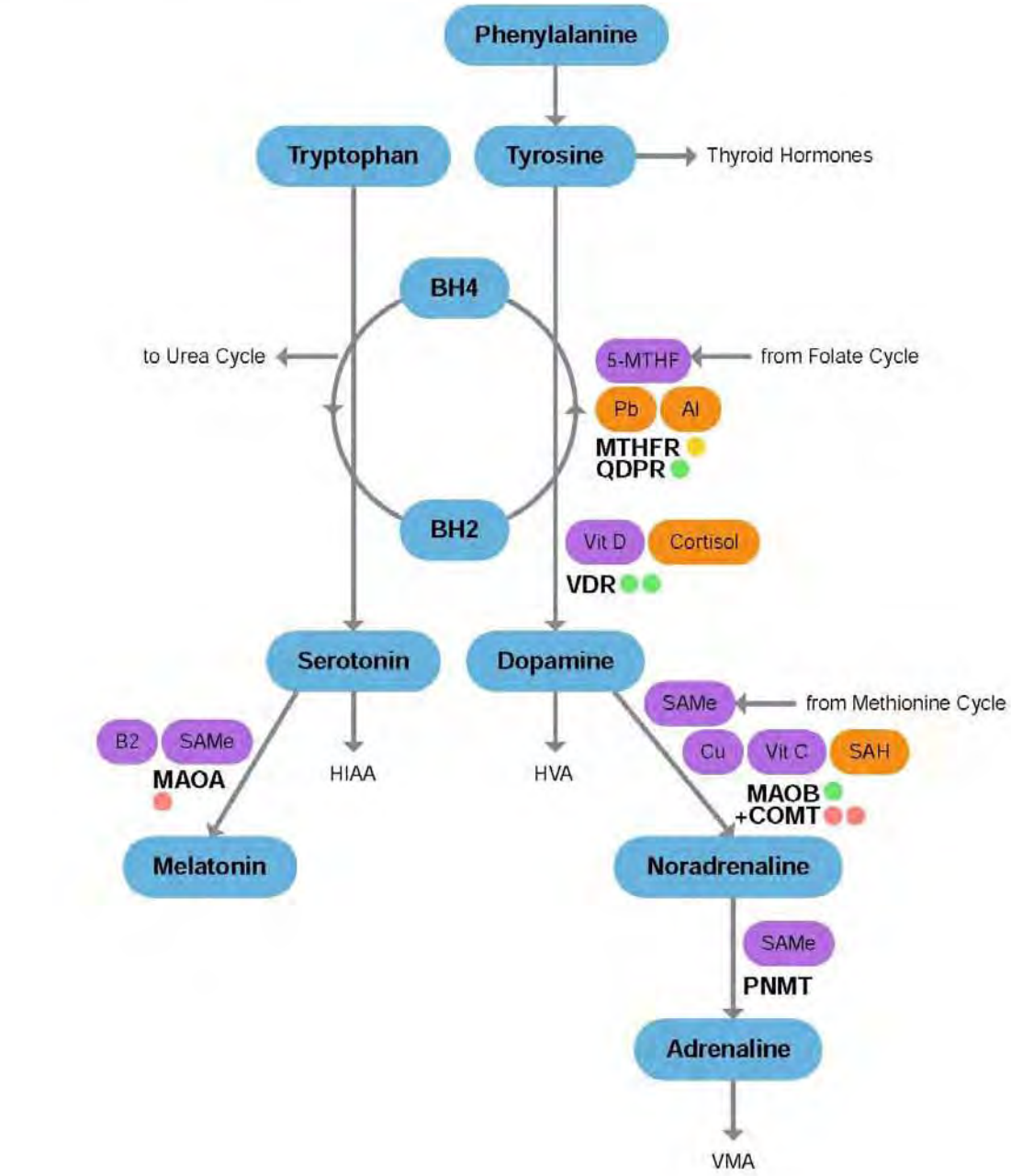
Further Investigation

Investigate neurotransmitter balance and SAH: SAMe ratio (since SAH inhibits COMT).

Management and Lifestyle

Focus on removing any heavy metals (especially mercury, lead and aluminium). Consider supplementing BH4 and 5-MTHF, however, avoid supplementing methyl donors if there are variants on VDR and COMT as these will not be well tolerated and may lead to irritability and mood disorders. Avoid foods rich in tyrosine (dopamine precursor) as it competes with tryptophan (serotonin precursor) for uptake and may cause a high dopamine / low serotonin imbalance. Instead, emphasise foods rich in tryptophan. Individuals with COMT variants should avoid coffee/caffeine as it releases catecholamines, leading to adrenalin overload.

Your BH4 Cycle / Neurotransmitter Metabolism Results:



Key:

- Co-factor
- Inhibitor
- Protective - neutral
- Neutral - negative
- Negative

COMT Catechol-O-Methyltransferase

SNV		Result	Description
rs4633	H62H	TT	Reduced COMT activity causing slower breakdown of catecholamines. This is mostly a negative trait, however, in combination with variants in VDR (low activity) this can be positive since dopamine synthesis and break down is slow leading to normal circulating levels. Those with normal (higher) VDR activity will have higher dopamine levels and low need for and tolerance of methyl donors and dopamine precursors, and the greatest susceptibility to mood swings. Low SAMe/ high SAH will further reduce COMT activity.
rs4680	V158M	AA	Reduced COMT activity causing slower breakdown of catecholamines. This is mostly a negative trait, however, in combination with variants in VDR (low activity) this can be positive since dopamine synthesis and break down is slow leading to normal circulating levels. Those with normal (higher) VDR activity will have higher dopamine levels and low need for and tolerance of methyl donors and dopamine precursors, and the greatest susceptibility to mood swings. Low SAMe/ high SAH will further reduce COMT activity

COMT breaks down the neurotransmitters: dopamine, adrenaline, and noradrenaline by using a methyl group from SAMe to methylate the catechol molecule, preparing it for excretion. COMT is also involved in oestrogen metabolism, converting active oestrogen to less active oestrogen. SAMe and SAH compete for the binding site on the COMT molecule, therefore a build up of SAH will reduce COMT activity.

Variants on COMT may reduce its activity and result in excess methyl groups which may cause irritability, heightened stress response, hyperactivity, heightened pain sensitivity and slower detoxification of oestrogen.

MAOA Monoamine Oxidase A

SNV		Result	Description
rs6323	R297R	TT	Low MAOA enzyme activity and slower breakdown of monoamine neurotransmitters which can contribute to higher levels. This is sometimes known as the 'warrior' genotype. If symptoms such as anxiety and outward anger are experienced vitamin B2, magnesium and zinc may increase MAOA activity.

MAOA is a member of the monoamine oxidase gene family whose enzymes catalyse the deactivation of monoaminergic neurotransmitters serotonin, melatonin, noradrenaline and adrenaline. It also metabolises dopamine, tyramine and tryptamine, equally with MAOB. MAOA is located on the X chromosome, so males only carry one allele inherited from their mother. We report results for males as homozygous as they will not inherit a 'balancing' allele.

MAOA is nicknamed the 'warrior gene' because variants are associated with anger and aggression due to slower neurotransmitter breakdown - effects which may be amplified if COMT variants are also present. Conversely, a combination of wild alleles has been labelled the 'worrier' genotype, associated with low mood due to rapid breakdown of neurotransmitters.

MAOB Monoamine Oxidase B

SNV		Result	Description
rs1799836	A644G	TT	Wild genotype - typically exhibits normal MAOB enzyme activity, efficient breakdown of substrates including neurotransmitters and reduced susceptibility to negative moods

MAOB is a member of the monoamine oxidase gene family whose enzymes catalyse the deactivation of monoaminergic neurotransmitters. It is the main catalyst for the breakdown of phenethylamine (PEA), benzylamine and histamine. It also metabolises dopamine, tyramine and tryptamine, equally with MAOA. MAOB is located on the X chromosome, so males only carry one allele, inherited from their mother. We report results for males as homozygous as they will not inherit a 'balancing' allele.

Variants on the MAOB gene are associated with reduced enzyme activity and slower breakdown of neurotransmitters. MAOB is a target for MAO inhibitor drugs used to raise dopamine levels and to improve motor function in Parkinson's disease patients.

MTHFR Methylenetetrahydrofolate Reductase (NAD(P)H)

SNV		Result	Description
rs1801131	A1298C	GT	Reduced gene function which may result in lower 5 MTHF (methyl folate) and slower conversion of BH2 to BH4 - needed for neurotransmitter synthesis. This genotype should be examined in the context of the BH4/ Neurotransmitter cycle. Methylation can be supported by adequate consumption of folate containing foods (such as green leafy vegetables, citrus fruits, beans and liver) and cofactors (vitamins B2 and B3).

The MTHFR gene is responsible for making the protein methylenetetrahydrofolate reductase (MTHFR), the rate-limiting enzyme in the methylation cycle which catalyses the conversion of folate to 'active' folate (5-MTHF) needed to support the re-methylation of homocysteine to methionine, DNA synthesis and repair (vital for healthy cell division), and the metabolism of neurotransmitters, phospholipids and proteins such as myelin.

Variants on the MTHFR gene usually result in lower enzyme activity. The C677T variant, which occurs in about 30% of people, can result in significantly reduced 5-MTHF levels – up to 40% reduction for heterozygotes and 70% for homozygotes (AA). MTHFR activity can be supported by increasing the intake of folate (B9) and the cofactors riboflavin (vitamin B2), niacin (vitamin B3), cobalamin (vitamin B12) and zinc. The A1298C variant has less direct impact on 5-MTHF levels but is associated with depletion of BH4 - vital for neurotransmitter synthesis.

QDPR Quinoid Dihydropteridine Reductase

SNV		Result	Description
rs1031326	690A>G	CC	Wild genotype - typically exhibits normal recycling of BH4 from BH2. Low 5-MTHF will reduce the recycling of BH4 regardless of genotype

QDPR, also known as DHPR, catalyses the regeneration of tetrahydrobiopterin (BH4) from quinonoid dihydrobiopterin (BH2), a reaction requiring active folate (5-MTHF). BH4 is an important cofactor for the synthesis of the neurotransmitters serotonin and dopamine, and for nitric oxide production.

Variants may result in BH4 deficiency. Excess ammonia may also deplete BH4.

VDR Vitamin D (1,25- dihydroxyvitamin D3) Receptor

SNV		Result	Description
rs1544410	BsmI	CC	Wild genotype - typically exhibits normal VDR enzyme activity and normal/high dopamine synthesis. Low vitamin D and stress (cortisol) will reduce enzyme activity regardless of genotype. Variants on COMT may interact with this genotype and cause mood swings and intolerance to methyl donors due to high production and slow breakdown of dopamine
rs731236	TaqI	AA	Wild genotype - typically exhibits normal VDR enzyme activity and normal/high dopamine synthesis. Low vitamin D and stress (cortisol) will reduce enzyme activity regardless of genotype. Variants on COMT may interact with this genotype and cause mood swings and intolerance to methyl donors due to high production and slow breakdown of dopamine

VDR encodes the nuclear hormone receptor for vitamin D3 (the active form of vitamin D in the body). This receptor shows sequence similarity to the steroid and thyroid hormone receptors and mediates an increase in dopamine production in response to Vitamin D, requiring less methyl groups. Vitamin D3 is also crucial for bone formation, modulation of the immune system, and cell proliferation and differentiation.

Variants in VDR are linked to lower vitamin D sensitivity, and reduced dopamine and serotonin synthesis. This can balance COMT variants (which slow metabolism) as there will be less circulating dopamine to break down. Individuals with variants on COMT but normal VDR activity may have higher dopamine levels, as less need for, and tolerance of, methyl donors and dopamine precursors, and susceptibility to mood swings.

Urea Cycle

The urea cycle (also known as the ornithine cycle) is a cycle of biochemical reactions occurring primarily in the liver, and to a lesser extent in the kidney whereby ammonia is converted to less toxic urea.

In the presence of BH4, Nitric Oxide Synthase (NOS) converts arginine to nitric oxide, a reactive free radical which acts as a biological mediator of the cardio vascular system by helping to resist plaque formation, vasospasm and abnormal clotting. In the brain and peripheral nervous system nitric oxide displays many properties of a neurotransmitter, and has been implicated in neurotoxicity associated with stroke and neurodegenerative diseases and neural regulation of smooth muscle, including peristalsis and penile erection. Nitric oxide also has antimicrobial and anti-tumoral properties.

NOS is also important for the detoxification of ammonia (from the transsulphuration pathway) - a process that uses up BH4 which may compromise serotonin and dopamine production. If there is insufficient BH4 arginine is converted into the damaging free radicals superoxide or peroxynitrate instead of being converted to nitric oxide.

Genetics

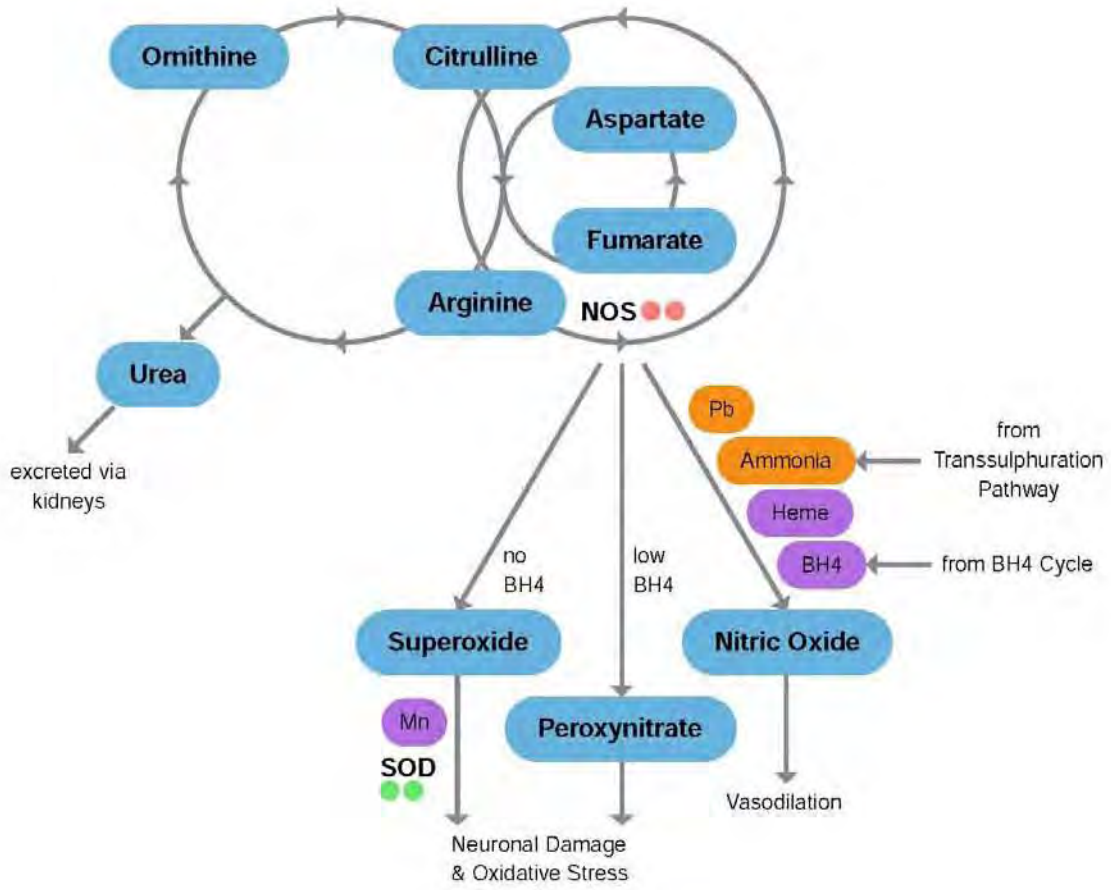
The NOS D298E and C19635A variants result in dysfunctional NOS enzymes which are less effective in breaking down ammonia and generating nitric oxide. This creates higher levels of the free radicals, superoxide and peroxynitrate.

Variants on SOD2 result in low superoxide dismutase activity (neutralisation of mitochondrial superoxide to hydrogen peroxide) and therefore susceptibility to oxidative stress.

Management and Lifestyle

Focus on decreasing the ammonia burden and increasing antioxidant intake to counteract free radical damage. BH4 supplementation may improve the generation of nitric oxide. Consider supplementing vitamin C or SOD to support break down of superoxide and 5-MTHF to address peroxynitrate.

Your Urea Cycle Results:



Key:

- Co-factor
- Inhibitor
- Protective - neutral
- Neutral - negative
- Negative

NOS3 Endothelial Nitric Oxide Synthase

SNV		Result	Description
i6015641	-786TC	CC	Reduced eNOS activity and nitric oxide linked to slower ammonia detoxification and higher free radical levels. Increased risk of hypertension and coronary artery disease. Ensure adequate levels of methyl-folate to support BH4 production. Increase antioxidants to reduce free radical damage. Moderate intake of ammonia generating foods (protein).
rs1799983	G894T	TT	Reduced eNOS activity and nitric oxide linked to slower ammonia detoxification and higher free radical levels. Increased risk of hypertension and coronary artery disease. Ensure adequate levels of methyl-folate to support BH4 production. Increase antioxidants to reduce free radical damage. Moderate intake of ammonia generating foods (protein)

eNOS is responsible for synthesising nitric oxide (NO) from l-arginine with the help of BH4 (tetrahydrobiopterin). Nitric oxide is involved in growth of new blood vessels and also controls endothelium-dependent vasodilatation reducing blood pressure and supporting transport of oxygen and other nutrients around the body.

Low eNOS activity due to genetic variants or inadequate BH4 can generate the free radicals peroxynitrate and superoxide instead of NO, compromising cardiovascular function. eNOS also assists in the detoxification of ammonia by converting it into less toxic urea.

SOD2 Superoxide Dismutase 2, Mitochondrial

SNV		Result	Description
rs2758331	G816T	CC	Wild genotype - associated with normal SOD enzyme activity and ability to neutralise superoxide. Low manganese levels will reduce SOD activity regardless of genotype, ensure adequate levels to support SOD activity
rs4880	A16V	AA	Wild genotype - associated with normal SOD enzyme activity and ability to neutralise superoxide. Low manganese levels will reduce SOD activity regardless of genotype, ensure adequate levels to support SOD activity

SOD2, also known as MnSOD, is a member of the superoxide dismutase family and encodes a mitochondrial protein that is one of the body's major antioxidant defense system against oxidative damage.

Variants decrease superoxide dismutase activity and therefore the ability to break down the free radical, superoxide, resulting in higher risk of oxidative stress. Ensure adequate levels of the co-factor manganese and increase antioxidant support.

References

AHCY S-Adenosylhomocysteine Hydrolase

Baric, I., Fumic, K., Glenn, B., Cuk, M., Schulze, A., Finkelstein, J. D., James, S. J., Mejaski-Bosnjak, V., Pazanin, L., Pogribny, I. P., Rados, M., Sarnavka, V., Scukanec-Spoljar, M., Allen, R. H., Stabler, S., Uzelac, L., Vugrek, O., Wagner, C., Zeisel, S., Mudd, S. H. (2004). S-adenosylhomocysteine hydrolase deficiency in a human: a genetic disorder of methionine metabolism. Proc. Nat. Acad. Sci. 101: 4234-4239. (http://www.ncbi.nlm.nih.gov/pubmed/15024124)

BHMT Betaine-homocysteine S-methyltransferase

Boyles AL, Billups AV, Deak KL, Siegel DG, Mehltretter L, Slifer SH, Bassuk AG, Kessler JA, Reed MC, Nijhout HF, George TM, Enterline DS, Gilbert JR, Speer MC, NTD Collaborative Group. Neural tube defects and folate pathway genes: family-based association tests of gene-gene and gene-environment interactions. Environ Health Perspect. 2006 Oct;114(10) 1547-1552. doi:10.1289/ehp.9166. PMID. 17035141; PMCID: PMC1626421. (http://europepmc.org/abstract/MED/17035141)

Clifford AJ, Chen K, McWade L, Rincon G, Kim SE, Holstege DM, Owens JE, Liu B, Müller HG, Medrano JF, Fadel JG, Moshfegh AJ, Baer DJ, Novotny JA. (2012). Gender and single nucleotide polymorphisms in MTHFR, BHMT, SPTLC1, CRBP2, CETP, and SCARB1 are significant predictors of plasma homocysteine normalized by RBC folate in healthy adults. J Nutr. 2012 Sep;142(9):1764-71. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3417835/)

Tanaka T, Scheet P, Giusti B, (2009), Genome-wide Association Study of Vitamin B6, Vitamin B12, Folate, and Homocysteine Blood Concentrations. American Journal of Human Genetics, 84(4):477-482. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2667971/)

CBS Cystathionine Beta-Synthase

Aras O, Hanson NQ, Yang F, Tsai MY. (2000). Influence of 699C->T and 1080C->T polymorphisms of the cystathionine beta-synthase gene on plasma homocysteine levels. Clinical Genetics. Dec;58(6):455-9 (http://www.ncbi.nlm.nih.gov/pubmed/11149614)

COMT Catechol-O-Methyltransferase

Stein DJ, Newman TK, Savitz J, Ramesar R. (2006). Warriors versus worriers: the role of COMT gene variants. CNS Spectr;11(10): pp. 745-8 (http://www.ncbi.nlm.nih.gov/pubmed/17008817?dopt=Abstract)

Xu K1, Ernst M, Goldman D. (2006). Imaging genomics applied to anxiety, stress response, and resiliency. Neuroinformatics; 4(1):51-64 (http://www.ncbi.nlm.nih.gov/pubmed/16595858)

CTH Cystathionine Gamma-Lyase

Kraus JP, Hasek J, Kozich V, Collard R, Venezia S, Janošíková B, Wang J, Stabler SP, Allen RH, Jakobs C, Finn CT, Chien YH, Hwu WL, Hegele RA, Mudd SH. (2009). Cystathionine gamma-lyase: Clinical, metabolic, genetic, and structural studies. Molecular Genetics and Metabolism. 97(4): 250-259 (http://europepmc.org/abstract/MED/19428278)

Rajendran S, Shen X, Glawe J, Kolluru GK, Kevil CG. Nitric Oxide and Hydrogen Sulfide Regulation of Ischemic Vascular Growth and Remodeling. Compr Physiol. 2019;9(3):1213-1247. Published 2019 Jun 12. doi:10.1002/cphy.c180026 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6938731/)

DHFR Dihydrofolate Reductase

Kalmbach RD, Choumenkovitch SF, Troen AP, Jacques PF, D'Agostino R, Selhub J. A 19-Base Pair Deletion Polymorphism in Dihydrofolate Reductase Is Associated with Increased Unmetabolized Folic Acid in Plasma and Decreased Red Blood Cell Folate. The Journal of Nutrition. 2008;138(12):2323-2327. doi:10.3945/jn.108.096404. (http://www.ncbi.nlm.nih.gov/pubmed/19022952)

Xu X, Gammon MD, Wetmur JG, Rao M, Gaudet MM, Teitelbaum SL, Britton JA, Neugut AI, Santella RM, et al. A functional 19-base pair deletion polymorphism of dihydrofolate reductase (DHFR) and risk of breast cancer in multivitamin users. Am J Clin Nutr. 2007;85:1098-102. (http://ajcn.nutrition.org/content/85/4/1098.long)

FOLH1 Folate Hydrolase

Devlin, A. M., Ling, E., Peerson, J. M., Fernando, S., Clarke, R., Smith, A. D., Halsted, C. H. (2000). Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia. Hum. Molec. Genet. 9: 2837-2844. (http://www.ncbi.nlm.nih.gov/pubmed/11092759)

Divyya S, Naushad SM, Addlagatta A, Murthy PV, Reddy ChR, Digumarti RR, Gottumukkala SR, Kumar A, Rammurti S, Kutala VK. (2012) Paradoxical role of C1561T glutamate carboxypeptidase II (GCPII) genetic polymorphism in altering disease susceptibility. Gene. Apr 15;497(2):273-9. (http://www.ncbi.nlm.nih.gov/pubmed/22310383)

FUT2 Fucosyltransferase 2

Hazra A, Kraft P, Lazarus R, et al. Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway. Human Molecular Genetics. 2009;18(23):4677-4687. doi:10.1093/hmg/ddp428 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2773275/)

Hazra, A., Kraft, P., Selhub, J., Giovannucci, E. L., Thomas, G., Hoover, R. N., Chanock, S. J., Hunter, D. J. (2008). Common variants of FUT2 are associated with plasma vitamin B12 levels. Nature Genet. 40: 1160-1162. (http://www.ncbi.nlm.nih.gov/pubmed/18776911)

Kelly, R. J., Rouquier, S., Giorgi, D., Lennon, G. G., Lowe, J. B. (1995). Sequence and expression of a candidate for the human secretor blood group alpha (1,2)fucosyltransferase gene (FUT2): Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. J. Biol. Chem. 270: 4640-4649. (http://www.ncbi.nlm.nih.gov/pubmed/7876235)

Kudo, T., Iwasaki, H., Nishihara, S., Shinya, N., Ando, T., Narimatsu, I., Narimatsu, H. (1996). Molecular genetic analysis of the human Lewis histo-blood group system. II. Secretor gene inactivation by a novel single missense mutation A385T in Japanese nonsecretor individuals. J. Biol. Chem. 271: 9830-9837. (http://www.ncbi.nlm.nih.gov/pubmed/8621666)

Rouquier, S., Lowe, J. B., Kelly, R. J., Fertitta, A. L., Lennon, G. G., Giorgi, D. (1995) Molecular cloning of a human genomic region containing the H blood group alpha-(1,2)fucosyltransferase gene and two H locus-related DNA restriction fragments: isolation of a candidate for the human

secretor blood group locus. J. Biol. Chem. 270: 4632-4639. (http://www.ncbi.nlm.nih.gov/pubmed/7876234)

Tanaka, T., Scheet, P., Giusti, B., Bandinelli, S., Piras, M. G., Usala, G., Lai, S., Mulas, A., Corsi, A. M., Vestrini, A., Sofi, F., Gori, A. M., Abbate, R., Guralnik, J., Singleton, A., Abecasis, G. R., Schlessinger, D., Uda, M., Ferrucci, L. Genome-wide association study of vitamin B6, vitamin B12, folate, and homocysteine blood concentrations. Am. J. Hum. Genet. 84: 477-482, 2009. Note: Erratum: Am. J. Hum. Genet. 84: 712 only, 2009. (http://www.cell.com/ajhg/fulltext/S0002-9297(09)00097-4)

GSS Glutathione Synthetase

de Andrade M, Li Y, Marks RS, Deschamps C, Scanlon P, Olswold CL, Jiang R, Swensen SJ, Sun Z, Cunningham J, Wampfler JA, Limper AH, Midthun DE & Yang P. (2011). Genetic Variants Associated with the Risk of Chronic Obstructive Pulmonary Disease with and without Lung Cancer. Cancer Prev Res (Phila); 5(3): pp. 365-373 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3414259/)

MAOA Monoamine Oxidase A

Antypa N, Giegling I, Calati R, Schneider B, Hartmann AM, Friedl M, Konte B, Lin L, De Ronchi D, Serretti A, Rujescu D. (2013). MAOA and MAOB polymorphisms and anger related traits in suicidal participants and controls. European Archives of Psychiatry and Clinical Neuroscience. 263(5):393-403 (http://europepmc.org/abstract/MED/23111930)

Zhang J, Chen Y, Zhang K, Yang H, Sun Y, Fang Y, Shen Y, Xu Q. (2010). A cis-phase interaction study of genetic variants within the MAOA gene in major depressive disorder. Biological Psychiatry, 68(9):795-800 (http://europepmc.org/abstract/MED/20691428)

MAOB Monoamine Oxidase B

Dlugos AM, Palmer AA, de Wit H. (2009). Negative emotionality: monoamine oxidase B gene variants modulate personality traits in healthy humans. J Neural Transm (Vienna); 116(10): pp. 1323-34 (http://www.ncbi.nlm.nih.gov/pubmed/19657847?dopt=Abstract)

MTHFD1 Methylenetetrahydrofolate Dehydrogenase 1

Brody LC, Conley M, Cox C, Kirke PN, McKeever MP, Mills JL, Molloy AM, O'Leary VB, Parle-McDermott A, Scott JM, Swanson DA. (2002). A polymorphism, R653Q, in the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase is a maternal genetic risk factor for neural tube defects: report of the Birth Defects Research Group. Am J Hum Genet. 2002 Nov;71(5):1207-15. (http://www.ncbi.nlm.nih.gov/pubmed/12384833/)

Hol FA, van der Put NMJ, Geurds MPA, Heil SG, Trijbels FJM, Hamel BCJ, Mariman ECM, Blom HJ (1998) Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase) in patients with neural tube defects. Clin Genet 53:119–125 (http://www.ncbi.nlm.nih.gov/pubmed/9611072)

Imbard A, Benoist J F, Blom HJ. (2013). Neural Tube Defects, Folic Acid and Methylation. International Journal of Environmental Research and Public Health. 10(9):4352-4389. doi:10.3390/ijerph10094352. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3799525/)

Zeisel SH. (2008). Genetic polymorphisms in methyl group metabolism and epigenetics: lessons from humans and mouse models. Brain Res. Oct 27;1237:5-11. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2587491/)

MTHFR Methylenetetrahydrofolate Reductase (NAD(P)H)

Bhatia, P. and Singh, N. (2015), Homocysteine excess: delineating the possible mechanism of neurotoxicity and depression. Fundam Clin Pharmacol, 29: 522–528. doi:10.1111/fcp.12145 (https://www.ncbi.nlm.nih.gov/pubmed/26376956)

Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci (2001) 22:195-201.10.1016/S0165-6147(00)01675-8 (https://www.ncbi.nlm.nih.gov/pubmed/11282420)

van der Put NM, van Straaten HW, Trijbels FJ, Blom HJ. Folate, homocysteine and neural tube defects: an overview. Exp Biol Med (Maywood). 2001 Apr;226(4):243-70. Review. PubMed PMID: 11368417. (http://www.ncbi.nlm.nih.gov/pubmed/11368417)

MTRR 5-Metyltetrahydrofolate-homocysteine S-Methyltransferase Reductase

Wang Y, Liu Y, Ji W, Qin H, Wu H, Xu D, Tukebai T, Wang Z. Analysis of MTR and MTRR Polymorphisms for Neural Tube Defects Risk Association. Medicine (Baltimore). 2015 Sep;94(35) e1367. doi:10.1097/md.0000000000001367 PMID: 26334892; PMCID: PMC4616500 (http://europepmc.org/abstract/MED/26334892)

MTR 5-Methyltetrahydrofolate-Homocysteine Methyltransferase

Imbard A, Benoist JF, Blom HJ. (2013). Neural tube defects, folic acid and methylation. Int J Environ Res Public Health. Sep 17;10(9):4352-89 (http://www.ncbi.nlm.nih.gov/pubmed/24048206)

Ma J, Stampfer MJ, Christensen B, et al. .A polymorphism of the methionine synthase gene: association with plasma folate, vitamin B12, homocyst(e)ine, and colorectal cancer risk. Cancer Epidemiol Biomarkers Prev. 1999;8:835–9. (http://cebp.aacrjournals.org/content/8/9/835)

MUT Methylmalonyl-CoA Mutase

Collin S.M., Metcalfe C., Palmer T.M., Refsum H., Lewis S.J., Davey-Smith G., Cox A., Davis M., Marsden G., Johnston C., Lane A., Donovan J., Neal D.E., Hamdy F.C., Smith D.A., and Martin R.M. (2001). The causal roles of vitamin B(12) and transcobalamin in prostate cancer: can Mendelian randomization analysis provide definitive answers? International Journal of Molecular Epidemiology and Genetics; 2(4): 316-327. (http://europepmc.org/abstract/MED/22199995)

Kinoshita M, Numata S, Tajima A, Nishi A, Muraki S, Tsuchiya A, Umehara H, Watanabe S, Imoto S, Ohmori T. (2016). Cumulative effect of the plasma total homocysteine related genetic variants on schizophrenia risk, Psychiatry Research; 10 (17) (https://www.researchgate.net/publication/309298235_Cumulative_effect_of_the_plasma_total_homocysteine-related_genetic_variants_on_schizophrenia_risk)

PEMT Phosphatidylethanolamine N-methyltransferase

Ivanov A, Nash-Barboza S, Hinkis S, Caudill MA. (2009). Genetic variants in phosphatidylethanolamine N-methyltransferase and methylenetetrahydrofolate dehydrogenase influence biomarkers of choline metabolism when folate intake is restricted. J Am Diet Assoc. Feb;109(2):313-8. (http://www.ncbi.nlm.nih.gov/pubmed/19167960)

QDPR Quinoid Dihydropteridine Reductase

Shi J, Badner JA, Hattori E, Potash JB, Willour VL, McMahon FJ, Gershon ES, and Liu C. (2008). Neurotransmission and Bipolar Disorder: A Systematic Family-based Association Study. Am J Med Genet B Neuropsychiatr Genet; 147B(7): pp. 1270–1277 (http://europepmc.org/articles/PMC2574701)

RFC1 Reduced Folate Carrier 1

Imbard A, Benoist JF, Blom HJ. (2013). Neural tube defects, folic acid and methylation. Int J Environ Res Public Health. Sep 17;10(9):4352-89 (http://www.ncbi.nlm.nih.gov/pubmed/24045206)

SHMT1 Serine hydroxymethyltransferase 1 (Soluble)

Guerreiro CS, Carmona B, Gonçalves S, Carolino E, Hidalgo P, Brito M, Leitão CN, and Cravo M. (2008). Risk of colorectal cancer associated with the C677T polymorphism in 5,10-methylenetetrahydrofolate reductase in Portuguese patients depends on the intake of methyl-donor nutrients. Am J Clin Nutr November 2008 vol. 88 no. 5 1413-1418 (http://ajcn.nutrition.org/content/88/5/1413.full)

Ilan J. N. Koppen, Frederik J. R. Hermans and Gertjan J. L. Kaspers. (2010). Folate related gene polymorphisms and susceptibility to develop childhood acute lymphoblastic leukaemia. British Journal of Haematology Volume 148, Issue 1, pages 3–14, January 2010. (http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2141.2009.07898.x/full)

Locasale JW. Serine, glycine and the one-carbon cycle: cancer metabolism in full circle. Nature reviews Cancer. 2013;13(8):572-583. doi:10.1038/nrc3557. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3806315/)

SOD2 Superoxide Dismutase 2, Mitochondrial

Bastaki M, et al. Genotype-activity relationship for Mn-superoxide dismutase, glutathione peroxidase 1 and catalase in humans. Pharmacogenet Genomics. 2006;16(4):279–86. (https://www.ncbi.nlm.nih.gov/pubmed/16538174)

Gallagher CJ, Ahn K, Knipe AL, Dyer AM, Richie JP Jr, Lazarus P, Muscat JE. (2009) Association between haplotypes of manganese superoxide dismutase (SOD2), smoking, and lung cancer risk. Free Radic Biol Med. 2009 Jan 1;46(1):20-4. doi: 10.1016/j.freeradbiomed.2008.09.018. (http://www.ncbi.nlm.nih.gov/pubmed/18930810)

Holley AK, Bakthavatchalu V, Velez-Roman JM, and St. Clair DK. (2011). Manganese superoxide dismutase: guardian of the powerhouse. Int J Mol Sci; 12(10): pp. 7114–7162 (http://europepmc.org/articles/PMC3211030)

Wang P, Zhu Y, Xi S, Li S, Zhang Y. Association between MnSOD Val16Ala Polymorphism and Cancer Risk: Evidence from 33,098 Cases and 37,831 Controls. Dis Markers. 2018;2018:3061974. Published 2018 Sep 2. doi:10.1155/2018/3061974 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6139213/)

SUOX Sulfite Oxidase

Garrett RM, Johnson JL, Graf TN, Feigenbaum A, Rajagopalan KV. (1998). Human sulfite oxidase R160Q: identification of the mutation in a sulfite oxidase-deficient patient and expression and characterization of the mutant enzyme. Proc Natl Acad Sci USA; 95(11): pp. 6394-8 (https://www.ncbi.nlm.nih.gov/pubmed/9600976?dopt=Abstract)

TCN2 Transcobalamin II

Guéant, J., Chabi, N. W., Guéant-Rodriguez, R., Mutchinick, O. M., Debard, R., Payet, C. Namour, F. (2007). Environmental influence on the worldwide prevalence of a 776C→G variant in the transcobalamin gene (TCN2). Journal of Medical Genetics, 44(6), 363–367. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2740879/)

Namour F, Olivier J, Abdelmouttaleb I, Adjalla C, Debard R, Salvat C, Guéant J L. (2001). Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: relation to transcobalamin and homocysteine concentration in blood. Blood. 97(4), 1092–1098. (http://www.ncbi.nlm.nih.gov/pubmed/11159542)

TYMS Thymidylate Synthetase

Shen R, Liu E, Wen J, Liu Z, Wang LE, Wang Q, Tan D, Ajani JA, Wei Q. (2015). Genetic polymorphisms in the microRNA binding sites of the thymidylate synthase gene predict risk and survival in gastric cancer. Mol Carcinog. Sep;54(9):880-8. (http://www.ncbi.nlm.nih.gov/pubmed/24756984)

Simeon V, Todoerti K, La Rocca F, et al. Molecular Classification and Pharmacogenetics of Primary Plasma Cell Leukemia: An Initial Approach toward Precision Medicine. Angelini S, ed. International Journal of Molecular Sciences. 2015;16(8):17514-17534. doi:10.3390/ijms160817514. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4581206/)

Xu J, Tian S, Yin Z, Wu S, Liu L, Qian Y, Pei D, Gao W, Xu J, Yin Y, Liu P, Shu Y. (2014) MicroRNA-binding site SNPs in deregulated genes are associated with clinical outcome of non-small cell lung cancer. Lung Cancer. Sep;85(3):442-8. (http://www.ncbi.nlm.nih.gov/pubmed/24997136)

VDR Vitamin D (1,25- dihydroxyvitamin D3) Receptor

Cui X1, Pelekanos M, Liu PY, Burne TH, McGrath JJ, Eyles DW. (2013). The vitamin D receptor in dopamine neurons; its presence in human substantia nigra and its ontogenesis in rat midbrain. J. Neuroscience (16), 236:77-87 (http://www.ncbi.nlm.nih.gov/pubmed/23352937)

Wang L, Ma J, Manson JE, Buring JE, Gaziano JM, Sesso HD. (2013). A prospective study of plasma vitamin D metabolites, vitamin D receptor gene polymorphisms, and risk of hypertension in men. Eur J Nutr, 52, (7):1771-9 (http://www.ncbi.nlm.nih.gov/pubmed/23262750)

APOE Report

Introduction

What is APOE?

Apolipoprotein E (APOE) is a protein that is best known for its role in lipid metabolism by helping to remove cholesterol from the blood stream.

It can exist in three main forms known as E2, E3 and E4. Variances on the APOE gene determine which forms of the APOE protein are present. The different forms of APOE work in different ways.

What Can Go Wrong?

The E4 (epsilon 4) form of the APOE gene has been associated with disorders of lipid metabolism (increased plasma cholesterol and triglyceride levels), susceptibility to cardiovascular disease (heart attacks or strokes due to atherosclerosis), insulin resistance and Alzheimer's disease.

Having the genetic variant that creates the APOE E4 version of the protein is one of many risk factors and does not mean you will get any disease associated with it.

Supporting APOE

If you have a higher risk version of APOE you can reduce your risk of developing cardiovascular disease or Alzheimer's disease by making changes to your diet and lifestyle.

APOE Report

APOE and Alzheimer's disease

Whilst **APOE E4** alleles represent the strongest single genetic risk factor for Alzheimer's disease, examination of additional genetic variances can be helpful in considering the different sub-types.

According to Dale Bredesen MD, an expert in the mechanisms of neurodegenerative diseases, Alzheimer's disease is not a single disease but has three major subtypes: (i) hot (inflammatory); (ii) cold (atrophic); and (iii) vile (toxic).

(i) Hot (Inflammatory)

An up-regulated immune system can increase inflammation and the risk of cardiovascular disease, insulin resistance, arthritis and the 'hot' sub-type of Alzheimer's disease.

Variants on **APOE**, **TNF** (Tumour Necrosis Factor) and **IFN-gamma** (Interferon Gamma) genes can up-regulate carriers' immune and inflammatory response.

(ii) Cold (Atrophic)

The 'cold' sub-type of Alzheimer's is associated with reduced support from hormones - thyroid (T3), adrenal (cortisol), sex hormones (oestrogen, progesterone, testosterone) - and Vitamin D, and is often accompanied by increased homocysteine (see Methylation) and insulin resistance.

APOE E4 alleles are a risk factor for this sub-type. In addition, variants on **BDNF** (Brain Derived Neurotrophic Factor) can reduce support for neuronal and synaptic growth, survival and protection.

(iii) Vile (Toxic)

The 'toxic' sub-type of Alzheimer's disease is atypical, partly in that it occurs at a younger age, with no family history, and more often in **APOE E3 carriers (risk is not elevated by APOE A4)**. Symptom onset usually follows a period of stress, sleep loss, anaesthesia or menopause.

It is characterised by hypothalamic-pituitary adrenal axis (HPA) disfunction, metal toxicity (mercury, lead or iron), high homocysteine and low zinc (and elevated copper) and/ or chronic inflammatory response syndrome (CIRS) - a reaction to mycotoxins (found in mould). The **GST** (Glutathione S-Transferase) family of genes play an important part in detoxification and individual responses to these toxins.

Methylation

Methylation is a process by which methyl-groups (CH3) are added to molecules, contributing to numerous biological functions including cell division and repair, inflammation control, neurotransmitter synthesis and detoxification.

MTHFR (Methylenetetrahydrofolate Reductase) gene variants can result in reduced availability of methyl-folate (B9) needed for homocysteine metabolism - a factor in all three sub-types of Alzheimer's disease, and synthesis of SAMe - the master methyl-donor.

BDNF has been described as 'nutrition for brain cells'.

APOE Report

Your Results

APOE Genotypes

The APOE gene has three common versions which are determined by examining the DNA present at two specific locations in the genome. The locations are identified by the references rs429358 (Cys130Arg) and rs7412 (Arg158Cys).

Risk Assessment

The risk assessment below shows the relative susceptibility of different APOE types to Alzheimer's disease. As described elsewhere in this report, many other factors can influence this risk, including other genetic variances and lifestyle.

Your APOE Type

APOE type is determined by the examining the alleles inherited from each parent - the left hand and then the right hand alleles for each SNP. A 'TT' result codes for E2, 'TC' for E3 and 'CC' for E4.

According to the methodology described, your APOE type is: **E3/E3**

APOE Type	rs429358 Cys130Arg	rs7412 Arg158Cys	Alzheimer's Disease Risk Assessment*
E2/E2	TT	TT	least risk x 0.6
E2/E3	TT	TC	low risk x 0.6
E3/E3	TT	CC	most common and neutral odds ratio 1
E2/E4	TC	TC	above average risk x 2.6
E3/E4	TC	CC	elevated risk x 3.2
E4/E4	CC	CC	highest risk x 14.9

The combinations rs429358 CC and rs7412 TC or TT are very rare and are not reported.

* Global average risk odds - Farrer LA et al, 1997

APOE Report

Additional SNPs

The results below may indicate your genetic predisposition to inflammation (TNF and IFNG) and neuronal damage (BDNF).

BDNF Brain Derived Neurotrophic Factor

The BDNF gene encodes the brain-derived neurotrophic factor. This protein is a nerve growth factor, meaning it promotes the growth, differentiation and survival of neurons and synapses in the central and peripheral nervous systems. Expression of this gene is reduced in Alzheimer's, Parkinson's, and Huntington's disease patients.

BDNF may play a role in the regulation of the stress response and in the biology of mood disorders.

BDNF Variant		Result	Description
rs6265	Val66Met	TC	Reduced BDNF expression. Low concentrations of BDNF are associated with increased risk of neuronal damage linked with Alzheimer's and Parkinson's. Research suggests that BDNF can be increased by intense exercise, vitamin D, vitamin B3 (niacin), curcumin, green tea, DHA (a component of omega-3 fatty acid) and resveratrol.

GSTM1 Glutathione S-Transferase Mu 1

The Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification by catalysing the conjugation of many compounds with reduced glutathione, making them easier to excrete. GSTM1 is vital for the detoxification of compounds including carcinogens, pharmaceutical drugs, environmental toxins and products of oxidative stress.

Variations in GSTM1 can change an individual's susceptibility to carcinogens and toxins as well as affect the toxicity and efficacy of certain drugs. This gene is known to be highly polymorphic with over 50% of Caucasians having a null genotype (both copies absent) which is linked to little or no enzyme activity.

GSTM1 Variant		Result	Description
GSTM1	Insert/Delete	DD	The GSTM1 gene is absent (null). Loss of function of the GSTM1 gene, poor glutathione transferase activity and inability to neutralise damaging 3,4 semi-quinones. Address causes of oxidative stress and inflammation and increase intake of antioxidants including glutathione, vitamin C and vitamin E.

GSTP1 Glutathione S-Transferase Pi 1

The Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification by catalysing the conjugation of many compounds with reduced glutathione making them easier to excrete. GSTP1 is a polymorphic gene encoding active, functionally different GSTP1 variant proteins that are thought to function in xenobiotic metabolism.

Variants in GSTP1 are associated with reduced glutathione transferase activity and play a role in susceptibility to cancer, and other diseases.

GSTP1 Variant		Result	Description
rs1695	I105V	AA	No impact on glutathione transferase activity. High levels of oxidative stress and low glutathione levels will slow GST activity regardless of genotype. Address causes of oxidative stress and inflammation and increase intake of antioxidants including glutathione, vitamin C and vitamin E.

GSTT1 Glutathione S-Transferase (GST) Theta 1

The Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification by catalysing the conjugation of many compounds with reduced glutathione making them easier to excrete. GSTT1 is highly polymorphic and is often entirely absent - the GSTT1 null form has been associated with various conditions including cancer.

The frequency of the GSTT1 null genotype varies widely in different populations: approximately 50-60% in Asians, 15% in Caucasians, 15-20% in Africans, and less than 10% in Hispanic populations.

GSTT1 Variant		Result	Description
GSTT1	Insert/Delete	II	The GSTT1 gene is present. Normal GSTT1 glutathione transferase activity. High levels of oxidative stress and low glutathione levels will slow GSTM1 activity regardless of genotype. Address causes of oxidative stress and inflammation and increase intake of antioxidants including glutathione, vitamin C and vitamin E.

IFN-gamma Interferon Gamma

Interferon-gamma (IFNG), or type II interferon, is a critical part of the body's immune response to viral and intracellular bacterial infections and for tumour control. It is produced predominantly by NK cells as part of the innate immune response, and by CD4 & CD8 once antigen specific immunity develops.

IFNG over expression is associated with a number of inflammatory and autoimmune diseases such as rheumatoid arthritis and SLE (Lupus). It also stimulates IDO which can up-regulate the kynurenine pathway and reduce tryptophan availability for serotonin synthesis.

IFN-gamma Variant		Result	Description
rs2430561	+874AT	AT	The T allele is associated with increased IFNG expression which helps defend against viral infection. However, over-expression of IFNG has been associated with increased inflammation and a slight increase in Alzheimer's disease risk.

MTHFR Methylenetetrahydrofolate Reductase (NAD(P)H)

The MTHFR gene is responsible for making the protein methylenetetrahydrofolate reductase (MTHFR), the rate-limiting enzyme in the methylation cycle which catalyses the conversion of folate to 'active' folate (5-MTHF) needed to support the re-methylation of homocysteine to methionine, DNA synthesis and repair (vital for healthy cell division), and the metabolism of neurotransmitters, phospholipids and proteins such as myelin.

Variants on the MTHFR gene usually result in lower enzyme activity. The C677T variant, which occurs in about 30% of people, can result in significantly reduced 5-MTHF levels - up to 40% reduction for heterozygotes and 70% for homozygotes (AA). MTHFR activity can be supported by increasing the intake of folate (B9) and the cofactors riboflavin (vitamin B2), niacin (vitamin B3), cobalamin (vitamin B12) and zinc. The A1298C variant has less direct impact on 5-MTHF levels but is associated with depletion of BH4 - vital for neurotransmitter synthesis.

MTHFR Variant		Result	Description
rs1801131	A1298C	GT	Reduced gene function which may result in lower 5-MTHF (methyl-folate) and slower conversion of BH2 to BH4 - needed for neurotransmitter synthesis. Methylation can be supported by adequate consumption of folate containing foods (such as green leafy vegetables, citrus fruits, beans and liver) and cofactors (vitamins B2 and B3).
rs1801133	C677T	GG	Neutral genotype. No impact on 5-MTHF (methyl-folate) synthesis or on homocysteine levels

TNF Tumor Necrosis Factor

Tumor necrosis factor (TNF) helps regulate the immune response involved in inflammation, fever and the inhibition of tumour growth.

Variants on TNF are associated with an overactive immune response and susceptibility to a range of inflammatory health conditions including arthritis, asthma, migraine and Alzheimer's. It can up-regulate catabolic pathways and suppress protein synthesis in skeletal muscle, impacting physical performance.

TNF Variant		Result	Description
rs1800629	-308GA	GG	No variance. Normal TNF levels and normal inflammatory response. Not associated with increased risk of Alzheimer's disease.

APOE Report

Nutrition and Lifestyle

The risk of developing Alzheimer's disease (AD) can be reduced by adopting healthy lifestyle behaviours.

Reduce Inflammation

Inflammation is a significant risk factor for the 'hot' subtype of AD. Anti-inflammatory supplements such as turmeric, fish oil, quercetin and resveratrol can be useful, particularly for those who are genetically predisposed.

It is also vital to identify and remove the root cause(s) such as dietary sugars or damaging trans-fats, leaky gut, insulin resistance, viral or bacterial infections (including oral bacteria and Lyme disease) and psychological or physical stress.

Avoid Insulin Resistance

Insulin resistance is perhaps the single greatest metabolic contributor to AD risk. To reduce risk experts recommend:

- Minimising intake of simple carbohydrates (sugar) found in processed foods, starchy foods (such as potatoes and white rice) and alcohol.
- Consuming unsaturated fats sourced from fatty fish, avocados, nuts and olive oil (preferably extra-virgin, cold pressed).
- Fasting for at least 12 hours between your last meal of the day and the first the next morning.
- Maintaining a healthy body weight.

Individuals with APOE E4 alleles should avoid or minimise consumption of saturated fats, found in butter, meats, egg yolks and palm oil.

Balance Hormones

Hormone optimisation can help prevent or reverse cognitive decline associated with the 'cold' and 'toxic' subtypes of AD.

Thyroid hormones can get out of balance (hyper or hypo) due to genetic variances, insufficient or excess of cofactors (iodine or selenium), inhibitors (cortisol), or damage or disruption to the thyroid gland.

Oestrogen and Progesterone have protective effects on the brain and in many cases onset of cognitive changes can be linked with menopause.

Testosterone (males and females) is critical for maintenance of synapses.

Whilst **cortisol** is protective against pathogens, high levels (due to stress) can damage neurons and can also deplete the pregnelolone needed to make oestrogen and testosterone.

As this is a complex topic, you are advised to work with a heath professional.

Sleep enables your body and mind to rest and repair. Melatonin - the sleep hormone - has powerful antioxidant, anti-inflammatory and immune properties. For maximum benefit, aim for 7-9 hours of sleep each night.

APOE Report

Nutrition and Lifestyle

Detoxify

Exposure to toxic substances can contribute to cognitive decline associated with 'toxic' AD. Toxins such as heavy metals - lead, iron and mercury (found in predatory fish, paint and amalgam fillings), medications (including proton pump inhibitors), pesticides, alcohol, general anaesthetic, Lyme disease (tick bites), (endogenous) homocysteine, and mould (found in water damaged buildings) can contribute to toxic load.

Risk can be reduced by:

Limiting toxic exposure - exclude foods that are likely to contain toxins or allergens, limit alcohol intake, minimise pharmaceutical drug use, use air and water filters and understand vulnerabilities to specific toxins.

Improving detoxification and elimination through diet, supplementation (such as glutathione or N-acetyl cysteine), sweating (using a sauna and taking regular exercise) and drinking plenty of filtered water.

Detoxifying foods include cruciferous vegetables - broccoli, cabbage, Brussels sprouts, bok choy (also good for methylation) - coriander (or cilantro), garlic, ginger and lemon.

Regular exercise - at least 4 or 5 days a week for 45-60 minutes each day will help avoid insulin resistance, reduce stress, improve sleep, vascular function and mood!

Optimise Methylation

Homocysteine can be a factor in all three sub-types of Alzheimer's. Homocysteine is a toxin that can damage blood vessels and increase inflammation.

High homocysteine levels can be indicative of impaired methylation. To optimise methylation ensure sufficient supply of B6, B9, B12 (bioactive, or 'methylated' forms), magnesium and zinc.

APOE Report

Further Testing

The following functional and genetic tests may be useful:

Insulin Resistance

- Fasting glucose measures the level of glucose in the blood after fasting
- Glucose tolerance measures the level of glucose in the blood after fasting and again, 2 hours after taking a glucose drink
- HbA1C test - shows the average level of blood sugar (glucose) over the previous 3 months
- BMI (Body Mass Index)

Inflammation

- C-reactive protein (CRP) - a higher concentration is a sign of inflammation
- Erythrocyte sedimentation rate (ESR) - used to diagnose conditions associated with inflammation, or to confirm infection

Detoxification

- Detoxification DNA test
- Heavy metal testing - mercury, arsenic, cadmium, lead and chromium. Aluminium (a light metal) may also be tested.
- 8-Hydroxy-2-deoxyguanosine (8-OHdG) test - a biomarker of oxidative stress and to estimate DNA damage
- Copper, zinc and iron tests
- Mycotoxin (mould) testing
- Microbiome (stool) testing

Cardiovascular

- Blood cholesterol tests - total cholesterol, HDL, Non-HDL, LDL, triglycerides, lipid profile
- Blood pressure monitoring
- Heart rate variability (HRV)

Hormone Balance

- Oestrogen Balance DNA test
- Thyroid DNA tests
- Sex hormones testing - oestrogen, progesterone and testosterone
- Melatonin testing
- Cortisone and free cortisol
- Thyroid hormone tests

Methylation

- Methylation DNA test
- Homocysteine
- B12 and MMA (methylmalonic acid)
- B9 (folate)
- SAMe, SAH and SAME:SAH ratio

Food Intolerance

- Celiac, and lactose intolerance DNA tests
- Gluten intolerance tests

APOE Report

References

Bredesen DE. Inhalational Alzheimer's disease: an unrecognized—and treatable—epidemic. Aging (Albany NY). 2016;8(2):304-313. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4789584/

Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM (1997). "Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium". JAMA. 278 (16): 1349–56. doi:10.1001/jama.1997.03550160069041. PMID 9343467. https://www.ncbi.nlm.nih.gov/pubmed/9343467

APOE Apolipoprotein E

Bu G. Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. Nature reviews Neuroscience. 2009;10(5):333-344. doi:10.1038/nrn2620. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2908393/)

Fallaize R, Carvalho-Wells AL, Tierney AC, et al. APOE genotype influences insulin resistance, apolipoprotein CII and CIII according to plasma fatty acid profile in the Metabolic Syndrome. Scientific Reports. 2017;7:6274. doi:10.1038/s41598-017-05802-2. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5524844/)

Giri M, Shah A, Upreti B, Rai JC. Unraveling the genes implicated in Alzheimer's disease. Biomedical Reports. 2017;7(2):105-114. doi:10.3892/br.2017.927. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5526178/#b56-br-0-0-927)

Kim J, Basak JM, Holtzman DM. The Role of Apolipoprotein E in Alzheimer's Disease. Neuron. 2009;63(3):287-303. doi:10.1016/j.neuron.2009.06.026. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3044446/)

BDNF Brain Derived Neurotrophic Factor

Coelho F. G., Vital T. M., Stein A. M., et al. Acute aerobic exercise increases brain-derived neurotrophic factor levels in elderly with Alzheimer's disease. Journal of Alzheimer's Disease. 2014;39(2):401–408. doi: 10.3233/JAD-131073. (https://www.ncbi.nlm.nih.gov/pubmed/24164734)

Park et al. (2017). The BDNF Val66Met Polymorphism Affects the Vulnerability of the Brain Structural Network. Frontiers in Human Neuroscience, 2017; 11: 400. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5541016/)

Wu A, Ying Z, Gomez-Pinilla F. DHA dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. Neuroscience. 2008;155(3):751-759. doi:10.1016/j.neuroscience.2008.05.061. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3208643/#__abstractidm140630145270736title)

GSTM1 Glutathione S-Transferase Mu 1

Wang, M., Li, Y., Lin, L. et al. Mol Neurobiol (2016) 53: 1355. https://doi.org/10.1007/s12035-015-9092-7 (https://www.ncbi.nlm.nih.gov/pubmed/25633095)

GSTP1 Glutathione S-Transferase Pi 1

Bernardini S, Bellincampi L, Ballerini S, Federici G, Iori R, Trequattrini A, et al. (2005) Glutathione S-transferase P1 *C allelic variant increases susceptibility for late-onset Alzheimer disease: association study and relationship with apolipoprotein E epsilon4 allele. Clin Chem 51: 944–951. (https://www.ncbi.nlm.nih.gov/pubmed/15805147)

Pocernich CB, Butterfield DA. Elevation of Glutathione as a Therapeutic Strategy in Alzheimer Disease. Biochimica et Biophysica Acta. 2012;1822(5):625-630. doi:10.1016/j.bbadis.2011.10.003. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3277671/)

GSTT1 Glutathione S-Transferase (GST) Theta 1

Zahra Jafarian, Kioomars Salimi nejad, Koorosh Kamali, Mina Ohadi, Ali Kowsari, Leila Naeshi & Hamid Reza Khorram Khorshed. Association of glutathione S-transferases M1, P1 and T1 variations and risk of late-onset Alzheimer's disease. Neurological Research Vol. 40, Iss. 1, 2018 (https://www.ncbi.nlm.nih.gov/pubmed/29072550)

IFN gamma Interferon Gamma

TNFalpha plus IFNgamma induce the production of Alzheimer beta-amyloid peptides and decrease the secretion of APPs. Blasko I, Marx F, Steiner E, Hartmann T, Grubeck-Loebenstein B FASEB J. 1999 Jan; 13(1):63-8. (https://www.ncbi.nlm.nih.gov/pubmed/9872930/)

a M., Rafa H., Medjeber O., et al. IFN- and TNF- are involved during Alzheimer disease progression and correlate with nitric oxide production: a study in Algerian patients. Journal of Interferon and Cytokine Research. 2014;34(11):839–847. doi: 10.1089/jir.2013.0085 (https://www.ncbi.nlm.nih.gov/pubmed/24831467)

APOE Report

MTHFR Methylenetetrahydrofolate Reductase (NAD(P)H)

Rai, V. (2017), Methylenetetrahydrofolate Reductase (MTHFR) C677T Polymorphism and Alzheimer Disease Risk: a Meta-Analysis. Mol Neurobiol (2017) 54: 1173. https://doi.org/10.1007/s12035-016-9722-8 (https://www.ncbi.nlm.nih.gov/pubmed/26820674)

Bhatia, P. and Singh, N. (2015), Homocysteine excess: delineating the possible mechanism of neurotoxicity and depression. Fundam Clin Pharmacol, 29: 522–528. doi:10.1111/fcp.12145 (https://www.ncbi.nlm.nih.gov/pubmed/26376956)

Mansoori, Nasim et al. MTHFR (677 and 1298) and IL-6-174 G/C genes in pathogenesis of Alzheimer's and vascular dementia and their epistatic interaction. Neurobiology of Aging , Volume 33 , Issue 5 , 1003.e1 - 1003.e8 (https://www.ncbi.nlm.nih.gov/pubmed/22015309)

TNF Tumor Necrosis Factor

Chang R, Yee K-L, Sumbria RK. Tumor necrosis factor α Inhibition for Alzheimer's Disease. Journal of Central Nervous System Disease. 2017;9:1179573517709278. doi:10.1177/1179573517709278. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5436834/)

Tan ZS, Beiser AS, Vasan RS, et al. Inflammatory markers and the risk of Alzheimer disease: the Framingham study. Neurology. 2007;68(22):1902–1908. (https://www.ncbi.nlm.nih.gov/pubmed/17536046)

Tobinick E, Gross H, Weinberger A, Cohen H. TNF-alpha Modulation for Treatment of Alzheimer's Disease: A 6-Month Pilot Study Medscape General Medicine. 2006;8(2):25. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1785182/)

APOE Report

Lifecode GX ®

— Professional Genotype Analysis —

Disclaimer

The information provided should not be used for diagnostic or treatment purposes and is not a substitute for personal medical advice. Use the information provided by Lifecode Gx® solely at your own risk.

Lifecode Gx® makes no warranties or representations as to the accuracy of information provided herein. If you have any concerns about your health, please consult a qualified health professional.

Supplementary materials

Published paper from Chapter 11

A Two-Base Pair Deletion in IQ Repeats in ASPM Underlies Microcephaly in a Pakistani Family

Syeda Farwa Naqvi, Rana Muhammad Kamran Shabbir, Aslıhan Tolun, Sulman Basit, and Sajid Malik

in

GENETIC TESTING AND MOLECULAR BIOMARKERS

Volume 26, Number 1, 2022, Pp. 37–42

DOI: 10.1089/gtmb.2021.0231

Turnitin Originality Report

Molecular and Genetic Studies of the Cerebral Folate System in Human Aging & Alzheimer's Brain by Syeda Farwa Naqvi .

From CL QAU (DRSML)

- Processed on 02-Feb-2022 08:48 PKT
- ID: 1753206535
- Word Count: 43647

Similarity Index
11%
Similarity by Source

Internet Sources:
8%
Publications:
7%
Student Papers:
5%

Focal Person (Turnitin)
Quaid-i-Azam University
Islamabad

sources:

1 1% match (Internet from 29-Aug-2019)
https://journals.sagepub.com/doi/10.1177/1715163517690745

2 1% match (student papers from 29-Aug-2019)
Submitted to The University of Manchester on 2019-08-29

3 < 1% match (student papers from 02-May-2019)
Submitted to The University of Manchester on 2019-05-02

4 < 1% match (student papers from 12-Aug-2021)
Submitted to The University of Manchester on 2021-08-12

5 < 1% match (student papers from 27-May-2021)
Submitted to The University of Manchester on 2021-05-27

6 < 1% match (student papers from 08-Jul-2015)
Submitted to The University of Manchester on 2015-07-08

7 < 1% match (student papers from 18-Sep-2012)
Submitted to King's College on 2012-09-18

8 < 1% match (student papers from 30-Aug-2017)
Submitted to King's College on 2017-08-30

9 < 1% match (student papers from 12-Aug-2021)
Submitted to King's College on 2021-08-12

10 < 1% match (Internet from 29-Oct-2020)
https://link.springer.com/chapter/10.1007%2F978-981-32-9721-0_3

11 < 1% match (Internet from 16-Jul-2020)
https://link.springer.com/article/10.1186/s13195-017-0283-5?code=9ea528e1-cbb2-4002-9976-d04c11235a94&error=cookies_not_supported

12 < 1% match (Internet from 03-Jan-2022)
https://link.springer.com/article/10.1007/s10311-019-00944-3?code=852a8cc9-abbb-4782-aa94-e7ffdbeade58&error=cookies_not_supported

13 < 1% match (Internet from 11-May-2019)
https://link.springer.com/article/10.1007%2Fs00438-016-1277-x

14 < 1% match (student papers from 19-Dec-2016)
Submitted to Higher Education Commission Pakistan on 2016-12-19

15 < 1% match (student papers from 24-May-2013)
Submitted to Higher Education Commission Pakistan on 2013-05-24

10-2-2022

< 1% match (student papers from 18-Dec-2017)