The role of inflammation, telomere length and hippocampal neurogenesis in the aetiology and treatment of psychiatric disorders

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THE ROLE OF INFLAMMATION, TELOMERE LENGTH AND HIPPOCAMPAL NEUROGENESIS IN THE AETIOLOGY AND TREATMENT OF PSYCHIATRIC DISORDERS

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ABSTRACT

Psychiatric disorders such as major depressive disorder, bipolar disorder and schizophrenia are a tremendous burden on society, globally accounting for more than 30% of years lived with a disability and more than 10% of disability-adjusted life-years. Although there is a global effort to prevent the aetiology and progression of these disorders, we still know very little about the biological mechanisms that are responsible for their onset. This is partly due to the complexity of these disorders, with genetic factors and environmental factors both conferring a degree of influence. Nevertheless, due to studies showing strong associations between these disorders and certain environmental factors, as well as advancements in large-scale collaborative genetic studies and molecular biology tools, we are now better able to understand the aetiology of these disorders and find better ways of treating them.

This PhD seeks to explore the role of three biological mechanisms in relation to psychiatric disorder aetiology and/or drug treatment: inflammation, telomere length and hippocampal neurogenesis.

Inflammatory markers are reported to be higher amongst major depressive disorder patients (e.g. C-reactive protein and interleukin-6). The first two data chapters within this PhD aim to tease apart the causes of heightened inflammation in major depressive disorder, using childhood maltreatment data and polygenic epidemiology. This work reveals the potential importance of body mass index in driving heighted inflammation amongst major depressive disorder patients.

Telomere length is marker of cell age and is generally shorter (indicative of faster ageing) amongst those at risk for age-related disease and amongst psychiatric disorder patients. In the third and fourth data chapters, we study the genetic architecture of telomere length and the repositioning potential of lithium as an anti-ageing medication. Our work revealed that polygenic risk for psychiatric disorders does not predict shorter telomere length in a UK population sample, implicating the importance of environmental factors in driving shorter telomere length amongst patients. Nevertheless, using linkage disequilibrium score regression our work confirmed that telomere length is a polygenic trait, and we identify the first polygenic risk score capable of explaining over 4% of the variance in telomere length. We confirm that chronic lithium use is associated with longer telomere length amongst bipolar disorder patients, suggesting that lithium may have anti-ageing properties, but we found this effect to be moderated by inter-individual genetic variation related to telomere length.

Adult hippocampal neurogenesis describes the birth of new neurons in the adult hippocampus. It is a cellular mechanism that reduces with age and is hypothesized to be inhibited amongst psychiatric disorder patients and reversed with treatment. In this final chapter, we attempt to better understand lithium's telomere-lengthening and neurogenic mechanism of action by studying its effects using an *in vitro* human hippocampal ageing model. We show that hippocampal cell ageing is associated with cellular senescence, although long-term lithium exposure can promote hippocampal cell differentiation.

ACKNOWLEDGMENTS

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I must thank all my friends who listened to hours and hours of me talking about my research in the pub and did well at pretending to sound interested. Thank you for nodding and asking me obscure questions, I feel like I learn something new every time we talk.

And finally, I must thank Erin. Thank you for putting up with me, for being you and for making me smile every day. Thank you for listening to my issues and quite often solving them for me. You have helped me in more ways than you know, and I am very lucky to have you.

The reason why I decided to thank so many people is that although not all of them have had a direct impact on my academic work, they have all been there for me. When you are doing something difficult in life, just knowing that you have such a vast network of family and friends that support you, help you and guide you, is enough to provide you with that little bit of confidence needed to persevere and accomplish whatever task you have at hand.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

LIST OF ABBREVIATIONS

DAPI	4′,6-diamidino-2-phenylindole
4-OHT	4-hydroxytamoxifen
ACTH	Adrenocorticotropic Hormone
AD	Alzheimer's Disease
ANOVA	Analysis of Variance
ADD	AntiDepressants in Depression
bFGF	Basic Fibroblast Growth Factor
BD	Bipolar Disorder
BMI	Body Mass Index
BrdU	Bromodeoxyuridine
CNS	Central Nervous System
CTQ	Childhood Trauma Questionnaire
CNV	Copy Number Variants
CAD	Coronary Artery Disease
CRP	C-Reactive Protein
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorder V
DMSO	Dimethyl Sulfoxide
DZ	Dizygotic
DCX	Doublecortin
EGF	Epidermal Growth Factor
EDTA	Ethylenediaminetetraacetic Acid
GxE	Gene and Environment

GIANT	Genetic Investigation of Anthropometric Traits
GWAS	Genome-Wide Association Study
GSK3β	Glycogen Synthase Kinase 3 β
GAB1	RB2-associated-binding-protein 1
HAM-D	Hamilton Rating Scale for Depression
HPCs	Hippocampal Progenitor Cells
ICC	Immunocytochemistry
IGF	Insulin-like Growth Factor
IFN	Interferon
IL	Interleukin
ICD-10	International Classification of Diseases 10
LRRC34	Leucine-rich-repeat-containing protein 34
LTL	Leukocyte Telomere Length
LD	Linkage Disequilibrium
LiCl	Lithium Chloride
MRI	Magnetic Resonance Imaging
MDD	Major Depressive Disorder
mRNA	Messenger Ribonucleic Acid
MAP2	Microtubule-Associated Protein 2
MZ	Monozygotic
NCDN	Neurochondrin
NEK6	NIMA Related Kinase 6
NAF1	Nuclear Assembly Factor 1
PFA	Paraformaldehyde
PD	Parkinson's Disorder

PTSD	Post-Traumatic Stress Disorder
PGC	Psychiatric Genomics Consortium
qPCR	Quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RTL	Relative Telomere Length
RNA	Ribonucleic Acid
S100β	S100 calcium-binding protein B
SCZ	Schizophrenia
SSRIs	Selective Serotonin Reuptake Inhibitors
SNP	Single Nucleotide Polymorphisms
SELCoH	South East London Community Health Study
SBNO2	Strawberry Notch Homolog 2
SGZ	Subgranular Zone
SVZ	Subventricular zone
TERT	Telomerase Reverse Transcriptase
TERC	Telomerase RNA Component
TNF	Tumor Necrosis Factor
VIM	Vimentin
WHO	World Health Organization
ZNF257	Zinc Finger Protein 257

1 – INTRODUCTION

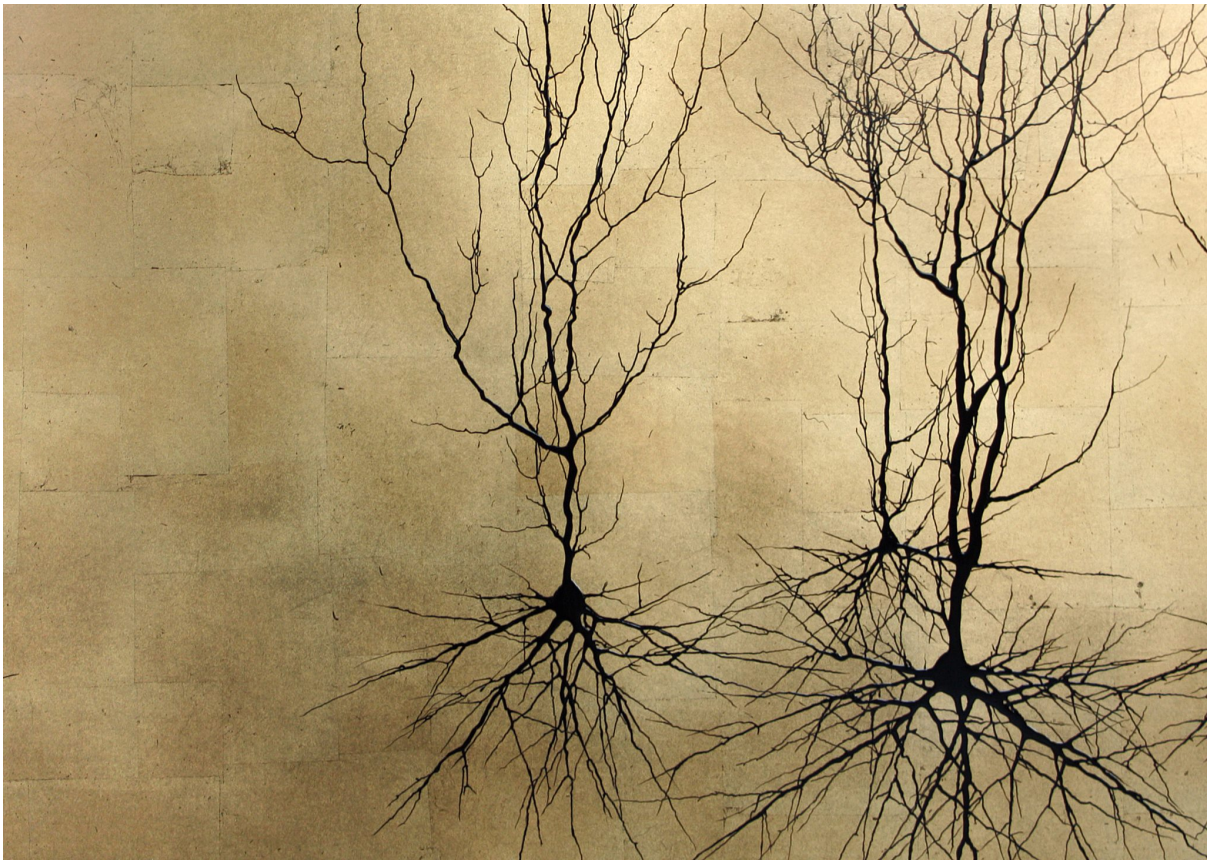

Figure 1.1.1 – Pyramidal neurons.
This image was created by Greg Dunn who is a neuroscientist with a passion for art. His work reflects the beauty of molecular neuroscience and present science in an artistic framework. Taken from: www.gregdunn.com

1.1 PSYCHIATRIC DISORDERS: CLINICAL PRESENTATION AND EPIDEMIOLOGY

1.1.1 MAJOR DEPRESSIVE DISORDER

Major depressive disorder (MDD) is a complex and heterogeneous psychiatric disorder, often considered as the predominant mental health disorder worldwide (1). Individuals are diagnosed with MDD if they meet a set number of diagnostic criteria in the Diagnostic and Statistical Manual of Mental Disorder V (DSM-V) and International Classification of Diseases 10 (ICD-10). The primary diagnoses centre around a persistently low mood, a loss of interest or pleasure for at least a two-week period and at least another four accompanying symptoms which may include changes in appetite, sleep, loss of energy, feelings of worthlessness, diminished concentration and recurrent suicidal thoughts (2). A number of clinical scales have been developed to measure depressive symptoms and their severity over time, including the Hamilton Rating Scale for Depression (HAM-D; (3)) and the Massachusetts General Hospital Treatment Resistant Depression staging score (4). In addition, there are several reliable interviews such as the Clinical Interview Schedule (CIS-R), often conducted in primary health clinics (5).

1.1.2 EPIDEMIOLOGY OF MDD

The World Health Organisation (WHO) estimates that more than 300 million people suffer from MDD worldwide and studies have suggested that MDD accounts for 8.2% of years lived with a disability, making it the second leading cause of global disability across the globe (6, 7).

In 2014, 19.7% of people in the UK aged 16 and over showed symptoms of anxiety or depression, which was a 1.5% increase from the previous year, suggesting that the incidence of MDD is increasing (8). This prevalence of MDD is higher in females (9) compared to males

(10), which has been attributed to differences in hormones, coping strategies, and help-seeking behaviours (1, 7, 11, 12). A number of social and behavioural factors have been associated with depressive symptoms, including unhealthy food consumption, negative self-perception, general health status, limited physical activity, adverse childhood experiences and psychological abuse (13, 14). Childhood adversity in particular has been strongly linked with MDD, showing associations with the severity of MDD, length of time that people suffer from MDD and the recurrence of MDD (15, 16). A number of biological mechanisms have been suggested as the mediating factors between childhood maltreatment and MDD, with increased circulating cortisol levels and systemic inflammation being two of the most studied to-date (17-20).

Although depressive episodes can be characterized as mild, moderate or severe, MDD involves repeated and chronic depressive episodes. In fact, around 80% of those diagnosed with MDD will experience two or more depressive episodes during their lifetime, with some factors socioeconomic (such as low-income and fewer years of education) increasing the duration and rate of episode recurrence (4, 7, 21-24). MDD is also prevalent in people suffering from physical disorders such as cancer, stroke and acute coronary syndrome, having a negative impact on the course of these disorders and reducing the quality of life of the patient (25). Conversely, patients with severe health disorders are also more likely to suffer from MDD (26).

1.1.3 BIPOLAR DISORDER

Bipolar disorder (BD) and MDD are both considered “mood disorders” and share many common features, aetiologies and clinical manifestations (27). As with MDD, BD patients experience episodes of depression (characterised by low mood, loss of interest, and low energy); however, BD patients also experience episodes of mania, which are characterised by symptoms such as talkativeness, increased distractibility and expanded self-esteem (28).

Episodes of mania and depression often last for several weeks or months, with people usually experiencing periods of "normal" mood (euthymia) between episodes (29). However, patterns of BD are not the same in all individuals, with some people experiencing rapid cycling (repeatedly swinging from mania to depression without having a "normal" period) and a mixed state (experiencing symptoms of mania and depression together) (29).

BD is further subdivided into Bipolar I disorder, which involves one or more manic episodes and may include a depressive episode; and Bipolar II disorder, which involves one or more severe depressive episodes and at least one hypomanic episode. Bipolar II does not disrupt daily functioning as much as Bipolar I, although the symptoms still cause severe distress at home, at work, in school and with relationships (30).

1.1.4 EPIDEMIOLOGY OF BIPOLAR DISORDER

Epidemiological studies have suggested BD to have a lifetime prevalence of around 2.4%, with a prevalence of 0.6% for Bipolar I and 0.4% for Bipolar II disorder (27, 31, 32). The mean age for the onset of BD appears to be in the early twenties, although findings from various studies vary between 20 and 30 years at onset (31).

Many studies have attempted to investigate sociodemographic variables associated with BD, although the findings appear to be inconsistent. Some studies suggest that higher rates of BD are present in low income, unemployed and unmarried groups, whereas other studies report BD to be associated with higher socioeconomic status, higher occupational level and increased creativity (32).

As with MDD, one of the biggest environmental risk factors for BD is childhood maltreatment (33). A recent meta-analysis of childhood trauma in bipolar disorder patients found a significant association between BD and physical, sexual and emotional abuse, with emotional

abuse showing the strongest association, being four times more likely to occur in bipolar disorder patients compared to healthy controls (33, 34). Moreover, higher rates of childhood maltreatment were reported in BD patients compared to MDD patients, and patients with a history of childhood maltreatment were more likely to transition to BD following a depressive episode (31). As with MDD, childhood adversity in BD has been associated with changes in hypothalamic-pituitary-adrenal (HPA) axis functioning and an increase in circulating pro-inflammatory cytokines, although the exact mechanisms remain unclear (35, 36).

BD is known to have high comorbidity with a number of medical and physical conditions, with increasing evidence showing an association between BD and irritable bowel syndrome, asthma, obesity, cardiovascular disease and diabetes (37, 38). Interestingly, many of these medical conditions are also associated with ageing, probing the notion that BD may be associated with faster biological ageing (39).

1.1.5 SCHIZOPHRENIA

Schizophrenia (SCZ) is a severe mental disorder that can affect the way a person thinks, feels and behaves. The DSM-V classifies an individual as having SCZ if they suffer from at least two positive symptoms which include delusions, hallucinations, disorganised speech and catatonic behaviour, and/or negative symptoms which include emotional flatness, anhedonia (the inability to experience pleasure), avolition (a lack of drive), or alogia (speech impairment), which occur for a significant portion of one month alongside a general disturbance in thought and behaviour for six months (40).

Symptoms vary in type, severity and length, with some people experiencing varying degrees of worsening, and remission, and some people experiencing permanent symptoms (41, 42). Overall, studies suggest that 25% of SCZ show good recovery, 50% show moderate recovery and 25% live with residual symptoms (43, 44). Although treatments are available, SCZ is

associated with increased mortality, with the lifespan of people with SCZ being shorter by around 19 years compared to the general population (45). Given the high rates of suicide in people with SCZ, it is unsurprising that it is one of the most studied psychiatric disorders to-date (46).

1.1.6 EPIDEMIOLOGY OF SCHIZOPHRENIA

Schizophrenia is in the top 25 leading causes of disability worldwide and is ranked as one of the top 10 illnesses contributing to the global burden of disease (47). The prevalence of SCZ (the number of cases in the population at any timepoint) is estimated to be around 1% internationally, and the incidence (the number of new cases annually) is estimated to be 1.5 per 10,000 people (48, 49). SCZ is a big social, health and economic burden on the patient, the caregivers and the wider society. The WHO estimates that the cost of SCZ in Western countries ranges from 1.6% to 2.6% of total health expenditures, with the economic burden of SCZ in the USA likely to be more than $60 billion per year (50, 51).

The age of onset of SCZ is typically during adolescence, with some reports of early-childhood and late-life cases (52). More men are diagnosed with SCZ compared to women, although women tend to be diagnosed in later life (53) compared to men (18 – 25 years) (54).

The pathophysiological events appear to be active before clinical manifestations of SCZ, suggesting that neural mechanisms associated with SCZ may be well established by the time the disorder is diagnosed (55, 56). The modern theory for the development of SCZ has been built upon the neurodevelopmental theory, which suggests that a prenatal event may disrupt the normal maturation of the brain, leading to biological changes which later result in clinical phenotypes (57, 58). More recent studies have suggested that this may not be the sole risk factor, as the model is unable to explain longitudinal changes in brain volume which occur after birth (59, 60). Thus, it is hypothesized that a “two hit” model may exist whereby prenatal

and genetic factors serve as the "first hits" and subsequent environmental factors such as childhood trauma, viral infections, drug misuse and social defeat serve as "second hits", tipping the balance towards mental ill health (61, 62). It is becoming clear that multiple "hits", some conferring greater risk than others, impact a genetically primed individual in different ways across periods of neurodevelopment and eventually culminate in the clinical symptoms of schizophrenia (61). Many of these risk factors have small effect sizes and complex interactions between these environmental factors and genetic risk are likely to play a role in the pathogenesis of SCZ (63, 64).

1.1.7 SUMMARY

Psychiatric disorders such as MDD, BD and SCZ are highly debilitating, resulting in a tremendous burden on the patients, the carers and the economy. As science is beginning to uncover reliable treatments for common medical conditions such as cardiovascular disease, psychiatric disorders are quickly becoming the leading causes of disability across the world, prompting a significant push to understand the risks, causes and treatments for these disorders.

Due to the complexity of psychiatric disorders and the difficulty in studying living brain tissue, it has been difficult to identify the biological mechanisms that might be driving symptoms in patients and to develop novel pharmacological treatments for these disorders. However, recent advancements in the fields of human genetics and molecular biology have helped researchers identify risk factors, aberrant biological mechanisms and biological targets that may be present in patients compared to controls. In addition, a better understand of the human body and breakthroughs in the field of stem cell technology have allowed scientists to model disease mechanisms *in vitro* using human or patient-derived tissue, leading to a better understanding of the aetiology of psychiatric disorders and how these pathologies may be reversed.

This PhD thesis has utilised advancements in the field of genetics to better understand the aetiology of psychiatric disorders, to model the pathology seen in some of these disorders, and to try and rescue this pathology using currently prescribed medication.

1.2 GENETIC RISK FACTORS FOR PSYCHIATRIC DISORDERS

1.2.1 TWIN STUDIES – HERITABILITY OF PSYCHIATRIC DISORDERS

More than 2,700 twin studies have been published in the last fifty years, helping researchers understand the aetiology of more than 17,800 traits, based on more than 14.5 million twin pairs (65). One of the main reasons for their popularity is that twin studies enable scientists to study the unobserved, by the logic of study design, investigating both genetic and environmental effects on human traits without directly observing genes and environments (66). A classic twin study design involves monozygotic (MZ) and dizygotic (DZ) twins raised together and the within-pair similarities are used as the cornerstones of subsequent analyses. In other words, the twin-cotwin similarities among MZ pairs are compared against the DZ pairs and the observed correlations are used to estimate the contributions of genetic and environmental factors (67).

Twin studies have been particularly effective in establishing the heritability of psychiatric disorders. MDD is known to be highly familial, with twin studies estimating heritability to be around 40%. The heritability of BD has been estimated to be more than 70%, and the heritability of SCZ has been estimated to be as high as 79% (68, 69). However, twin heritability estimates are subject to some shortfalls. Firstly, heritability estimates only give researchers an estimation of the additive genetic effects and cannot give insight into specific genetic loci or their associated biological mechanisms, which could be used as targets for treatment. Secondly, heritability is not constant in and between populations, with changes in the method of measurement, the environment, migration, selection and inbreeding all having an effect on

heritability estimates. Finally, heritability estimates are significantly larger compared to the findings from genome-wide association studies (GWAS), which can explain around 5% of the variation in traits such as height, compared twin heritability estimates of around 80%. This has become known as the "missing heritability problem", and may relate to pleiotropy, gene-environment interactions, rare genetic variants and epigenetic factors inflating twin heritability estimates.

1.2.2 GENOME-WIDE ASSOCIATION STUDIES

By sequencing the first entire human genome one base at a time, an effort called 'The human genome project', scientists have been able to find locations of genes, promoters, enhancers and many more genomic regions (70, 71). In addition, scientists have begun understanding how and which bases differ between individuals (72), giving us insight into phenotypic differences and disease aetiology (73).

Single nucleotide polymorphisms (SNPs) are variations in single nucleotides in the genome, occurring on average every 1000 bases see **Figure 1.2.1**. For example, a 'T' nucleotide may be replaced by an 'A' nucleotide in a minority of individuals, meaning the possible nucleotide variations at this locus – T or A – are alleles for this locus (74). SNPs are responsible for the diversity amongst individuals and susceptibility to common and complex diseases as well as psychiatric disorders; they may change the conformation of an amino acid or occur in non-coding regions of the genome, influence gene expression as well as mRNA regulation and localisation (75). The identification of SNPs in genes has been and will be a key factor in identifying genetic risk factors as well as functional biological pathways that can explain disease aetiology and inform us on potential treatments, with genome wide association studies already being key in providing these insights over the last few decades (76).

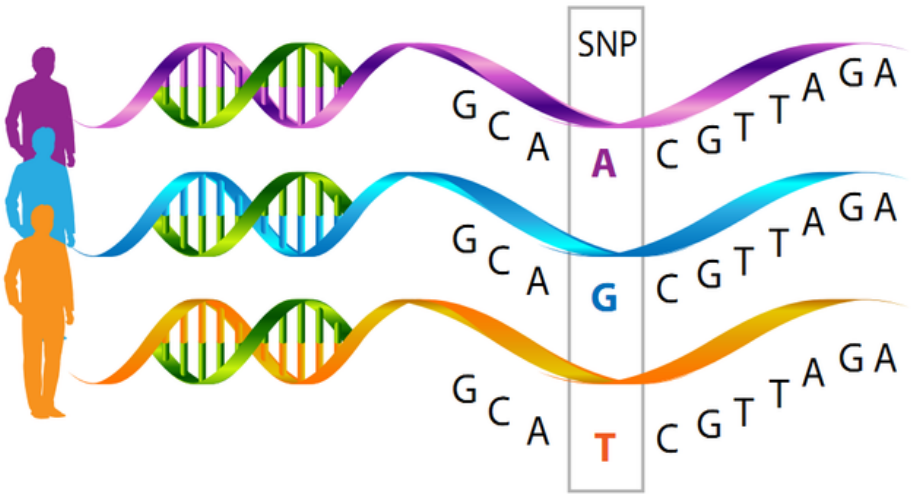

Figure 1.2.1 – Figure illustrating a single nucleotide polymorphism in a population.
This figure clearly illustrates how one nucleotide can differ in an individual, without affecting the rest of the nucleotide sequence. This is called a single nucleotide polymorphism. Taken from: https://steemit.com/biology/@alv/single-nucleotide-polymorphisms-single-letter-changes-in-dna-provide-road-signs-to-map-human-traits.

GWAS involve comparing hundreds of thousands of SNPs in disease cases and controls, in order to determine whether an association exists between any given SNP and disease risk (77). Sequencing an entire human genome is expensive and not feasible on a population level. However, due a biological phenomenon called linkage disequilibrium (LD), only a relatively small number of SNPs need to be sequenced to get an estimate of the entire genome.

LD describes non-random association of alleles at different loci (78), see **Figure 1.2.2**. Patterns of LD in the human genome can be divided into haplotype blocks – regions of high LD that can be separated from other haplotype blocks due to many historical and evolutionary recombination events (78-80).

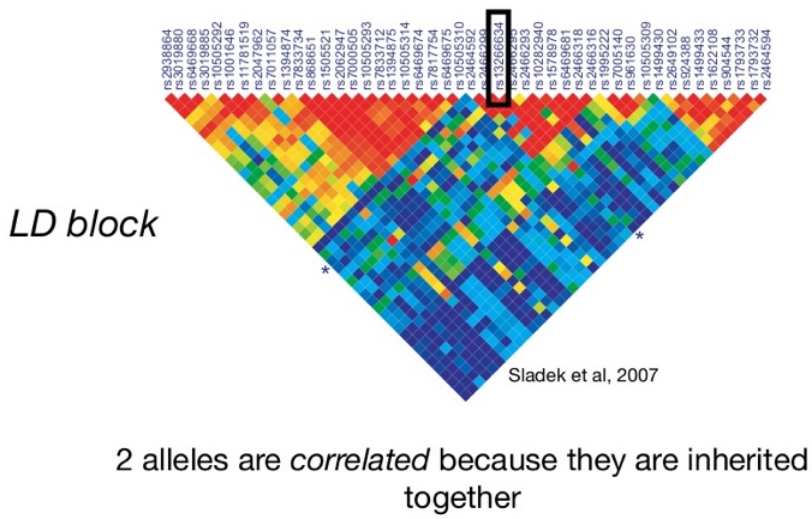

Figure 1.2.2 – Figure demonstrating linkage disequilibrium.
This figure represents an LD block, with the tag SNP (highlighted by the black rectangular box) being in high LD (shown in red) with surrounding SNPs. Taken from Sladek et al (2007).

When SNPs are in strong LD, the alleles of a few SNPs (the tag SNPs) on a haplotype can infer the alleles of other SNPs on the same haplotype. This means that fewer common SNPs need to be assessed in order to figure out the relevant haplotypes in any population (80). This was initially made possible via the HAPMAP project, which helped develop a map of common haplotype patterns throughout the genome, subdividing SNPs into haplotype blocks and tagging a subset of SNPs to each haplotype (81).

In practical term this means that the cost of SNP genotyping has been greatly reduced, allowing for the cheap and quick assessment of genetic differences in large cohorts. It is these large-scale studies that have provided valuable insights into the genetic architecture of many traits and disorders.

1.2.3 SCHIZOPHRENIA GWAS

One of the first big breakthroughs in GWAS came in the field of schizophrenia. The pathophysiology of schizophrenia is largely unknown, making treatment very difficult.

Previous twin studies have estimated the heritability of SCZ to be around 80%, suggesting a major role for inherited genetic variants, although SNP heritability from early GWAS was found to be much lower (82). It was therefore hypothesized that many SNPs conferring small effect sizes could be contributing to twin heritability estimates, and that current GWAS were simply underpowered. The SCZ Working Group of the Psychiatric Genomics Consortium (83) combined all available SCZ GWAS data into one mega-analysis consisting of 36,989 cases and 113,075 controls and identified 108 independent associated loci (84), see **Figure 1.2.3**. The SCZ mega-GWAS was the first to show that very large GWAS datasets can identify robust risk loci implicated in SCZ. Subsequent analyses, such as gene-level and gene-set enrichment analyses have further provided important insights into which genes, cell types and tissues genetic risk likely exerts its effects (84).

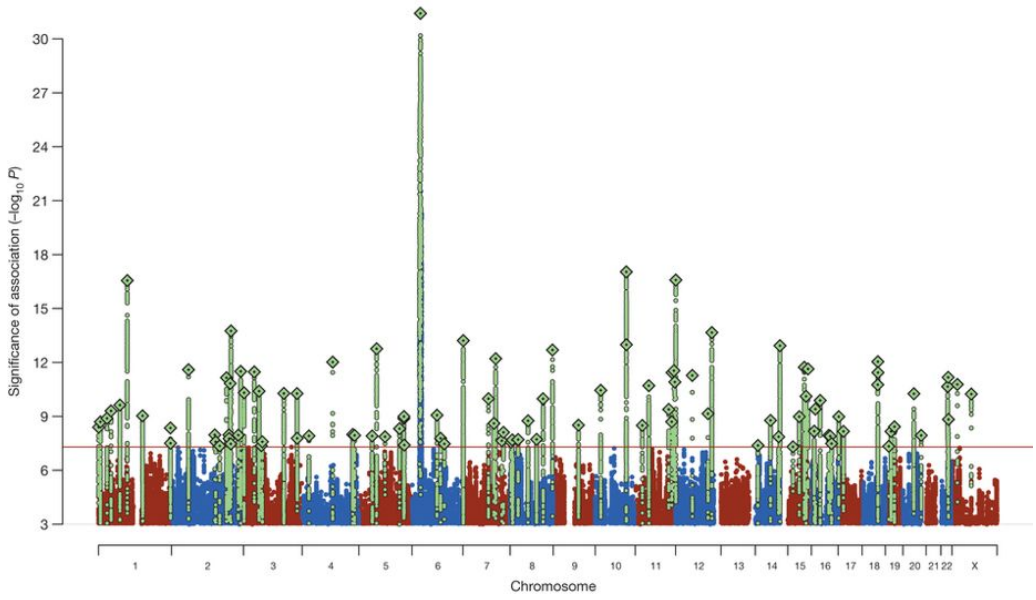

Figure 1.2.3 – Manhattan plot showing schizophrenia associations.
This plot is from the largest schizophrenia GWAS and demonstrates the genome-wide associations between SNPs and schizophrenia. The x-axis shows the chromosome number and the y-axis shows the significance of associations derived by logistic regression. The red line indicated the genome-wide significance threshold. The points in green indicate SNPs which were above the genome-wide significance threshold. Taken from Ripke et al (2014).

1.2.4 MAJOR DEPRESSIVE DISORDER GWAS

A more recent study utilised the same mega-GWAS approach in the field of MDD, analysing 135,458 cases and 344,901 controls and revealing 44 independent significant loci (85), see **Figure 1.2.4**. The results also showed that significant SNPs converge on genes most likely to be expressed highly in the brain, including in the cortex and hippocampus. Furthermore, there were significant genetic correlations between MDD and other psychiatric disorders, and body mass index (BMI), amongst others (85). This shows how large GWAS do not only reveal novel significant variants, but also aid in our understanding of disease aetiology.

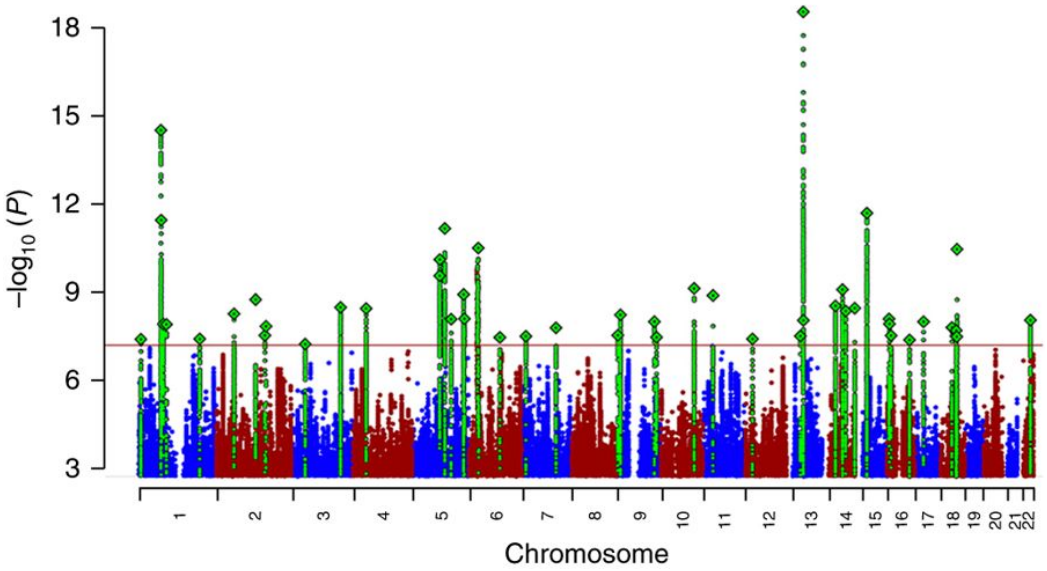

Figure 1.2.4 – Manhattan plot showing major depressive disorder associations.
A Manhattan plot from the largest GWAS carried out for MDD. The x-axis shows the chromosome number and the y-axis shows the significance of associations derived by logistic regression. The red line indicates genome-wide significance. The points in green represent SNPs which were above the genome-wide significance threshold. Taken from Wray et al (2018).

1.2.5 BIPOLAR DISORDER GWAS

Similarly, the Bipolar Disorder Working Group of the PGC performed the largest GWAS on BD consisting of 20,352 cases and 31,358 controls and found that 30 loci reach genome-wide significance, addressing key clinical questions and providing new insights into the biological aetiology of this disorder (86), see **Figure 1.2.5**.

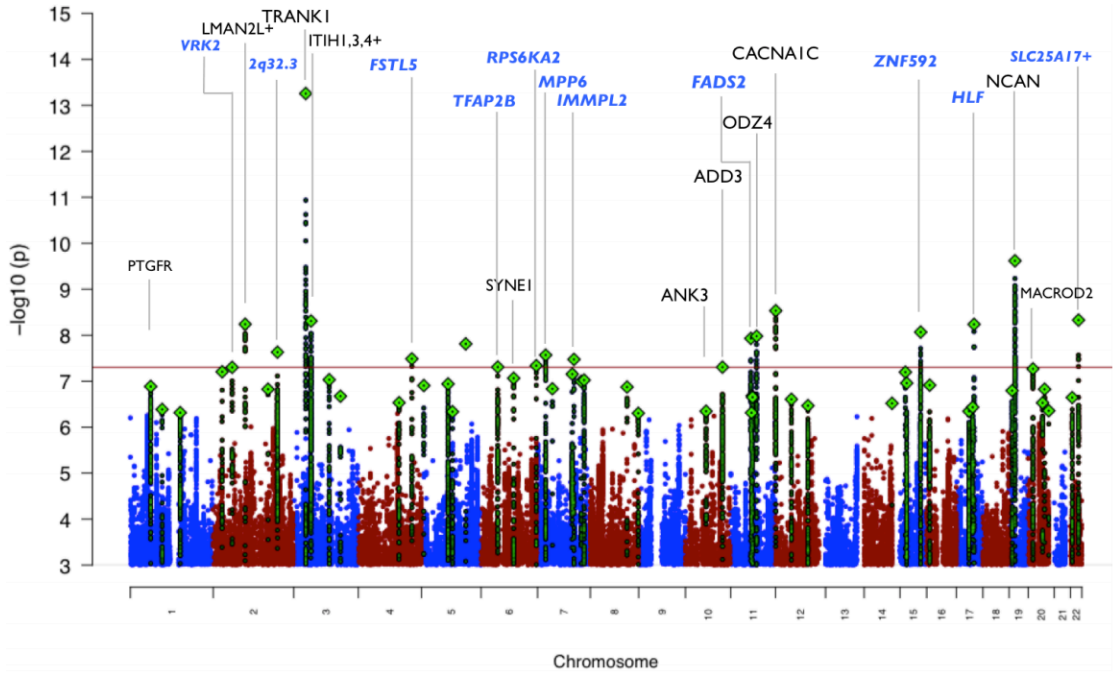

Figure 1.2.5 – Manhattan plot showing bipolar disorder associations.
This plot is taken from the largest GWAS carried out for BD. The x-axis shows the chromosome number and the y-axis shows the significance of associations derived by logistic regression. The red line indicates the genome-wide significance threshold. The genes in blue indicate SNPs which were above the genome-wide significance threshold. Taken from Stahl et al (2018).

1.2.6 GWAS FOR OTHER TRAITS

Large scale GWAS have also been applied to other traits such as BMI. The Genetic Investigation of Anthropometric Traits (GIANT) consortium is an international collaboration that aims to identify genetic loci that modulate human body shape, size, height and obesity.

The most recent genome-wide association study revealed 941 SNPs associated with BMI, explaining 6% of the variance in BMI in an independent sample (87), see

Figure 1.2.6. The sample size in this analysis was around 700,000 individuals, confirming the notion that increasing GWAS sample sizes can indeed aid the discovery of new loci and increase prediction accuracy for genetic biomarkers.

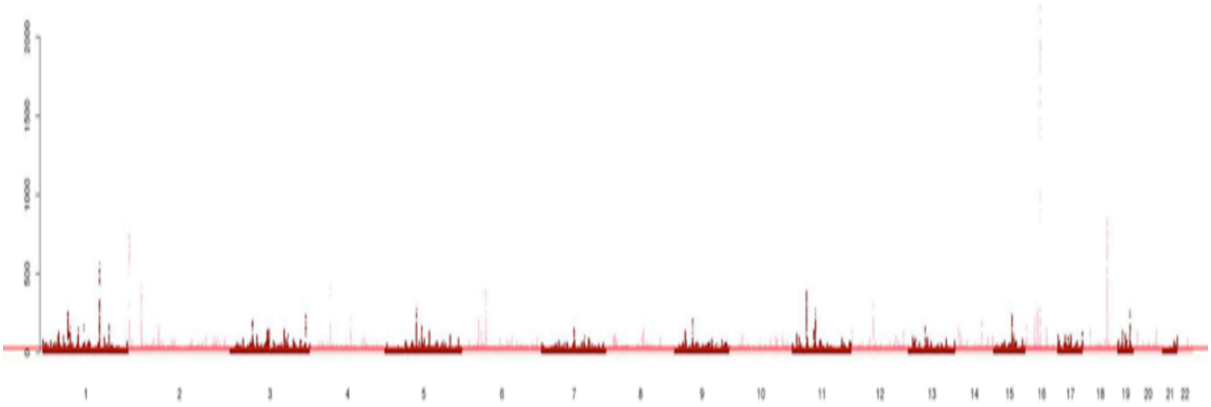

Figure 1.2.6 – Manhattan plot showing BMI associations.
This Manhattan plot is taken from a large-scale meta-analysis of GWAS for BMI. The x-axis shows the chromosome number and the y-axis shows the significance of associations derived by logistic regression. A total of 941 genome-wide significant SNPs were identified. Taken from Yengo et al, (2018)

1.3 POLYGENIC RISK SCORES AND THEIR USE

1.3.1 POLYGENIC DISORDERS

GWAS carried out at the beginning of the 21st century detected significant associations between hundreds of SNPs and a wide range of diseases (88). However, the associated SNPs only explained a limited amount of heritability and there was a struggle to find significant associations for complex diseases and psychiatric disorders such as schizophrenia (89, 90).

It was hypothesized that complex diseases and psychiatric disorders are polygenic, whereby a large number of SNPs with weaker effects and a small number of SNPs with stronger effects have a combined influence on the heritability of a given disease (91), see **Figure 1.3.1**. This suggests that the 'missing' heritability from initial GWAS studies is present but is likely to be 'hidden' due to small sample sizes.

Indeed, older GWAS on height could only explain around 5% of the genetic variability, whereas a polygenic model (which also includes a subset of non-significantly associated SNPs) explains up to 45% (92).

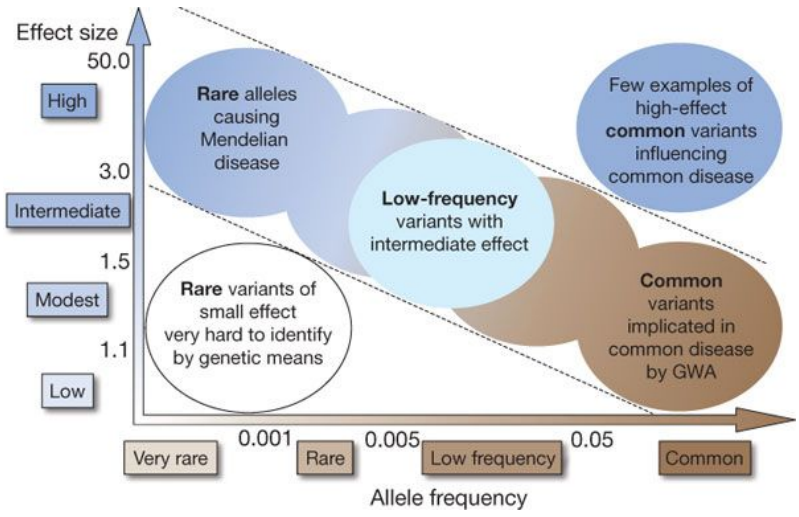

Figure 1.3.1 – The frequency of variants and disease susceptibility.
This figure illustrates how rare variants with a high effect size are primarily responsible for rare and Mendelian diseases, but confer a lot of risk, whereas common variants with a low effect size are implicated in a wide range of diseases but individually confer a small amount of risk. The allele frequency (ranging from very rare to very common) is displayed on the x-axis and the effect size (ranging from low to high) is displayed on the y-axis. Taken from Manolio et al 2009.

Polygenic risk scores (PRS) show great promise as they can be used to identify those at a higher risk of developing certain disorders and allow clinicians to focus more on these individuals, minimising their exposure to environmental risk factors that may increase the risk even further.

The field of psychiatric genetics has seen significant achievements in PRS modelling in recent years, mainly due to technological advancements, computational advancements and ultimately the growth of genome-wide association studies (93).

1.3.2 CALCULATING POLYGENIC RISK SCORES

Provided there are GWAS summary statistics available for a trait, PRS can be calculated using software such as PRSice (94). It calculates a PRS by utilising information from GWAS summary statistics pertaining to a trait of interest (base dataset) and applying it to an independent genetic dataset (target dataset). For each individual in the target dataset, it calculates the number of risk alleles at each SNP, and then multiplies this by the effect size defined in the GWAS summary statistics. This result is then summed across all SNPs (defined by the user) to determine individualised risk scores. The optimal number of SNPs to include in the PRS (the best signal-to-noise ratio) is often tested systematically, whereby the researcher will test a different combination of SNPs under varying p-value cut-offs (base dataset) to determine the polygenic predictor explaining the most variance within their target dataset, see **Figure 1.3.2**.

The utility of PRS may include detecting shared genetic aetiology between traits, establishing the presence of genetic signal in underpowered studies, working out the genetic architecture of a trait, screening for clinical trials and acting as a biomarker for a given trait.

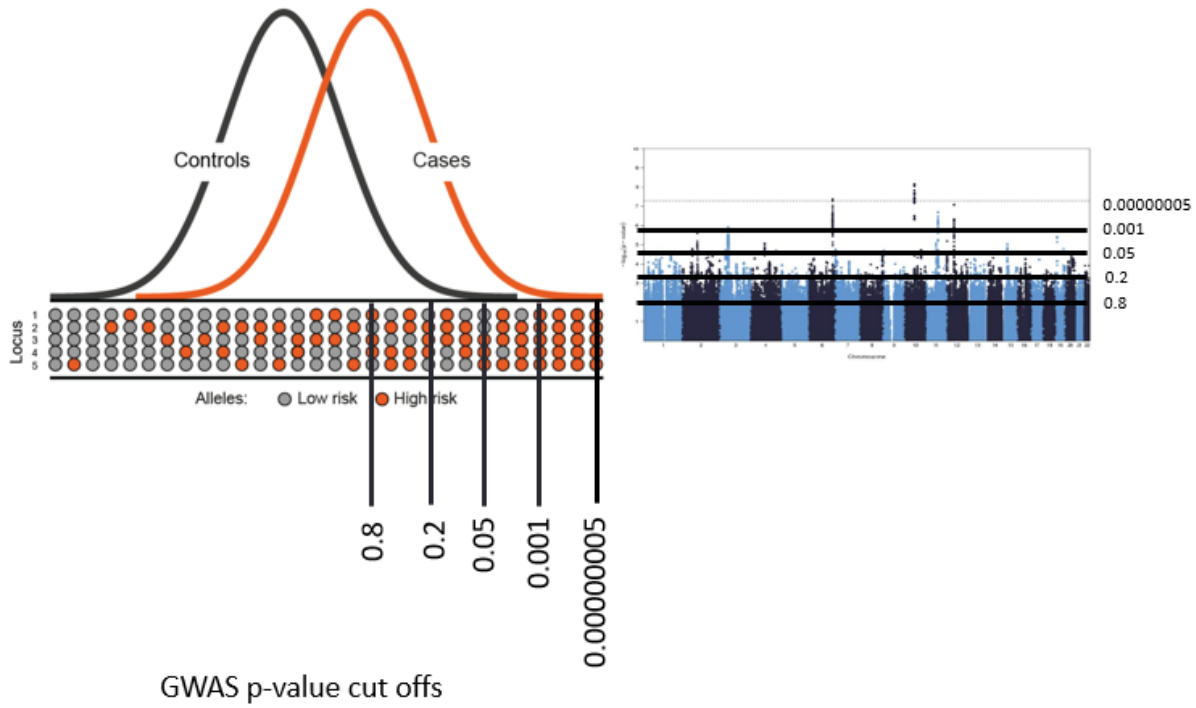

Figure 1.3.2 – Figure showing the basic principles of calculating polygenic risk scores.

The genome-wide significant P-value threshold (P < 5 X 10-8) is relaxed in order to encompass more risk alleles and thus a greater amount of the genetic contribution to the trait. The resulting threshold might be 0.05, which would see the inclusion of more SNPs, as shown by the black lines. Taken from Wiffin & Houlston (2014)

By using PRS it is possible to predict those who are at a three, four or fivefold increased risk for developing certain disorders compared to the rest of the population. Studies have routinely verified that cases show significantly higher PRS compared to controls and that the prevalence of disorders such as cardiovascular disease is positively associated with an increased PRS (95), see **Figure 1.3.3**.

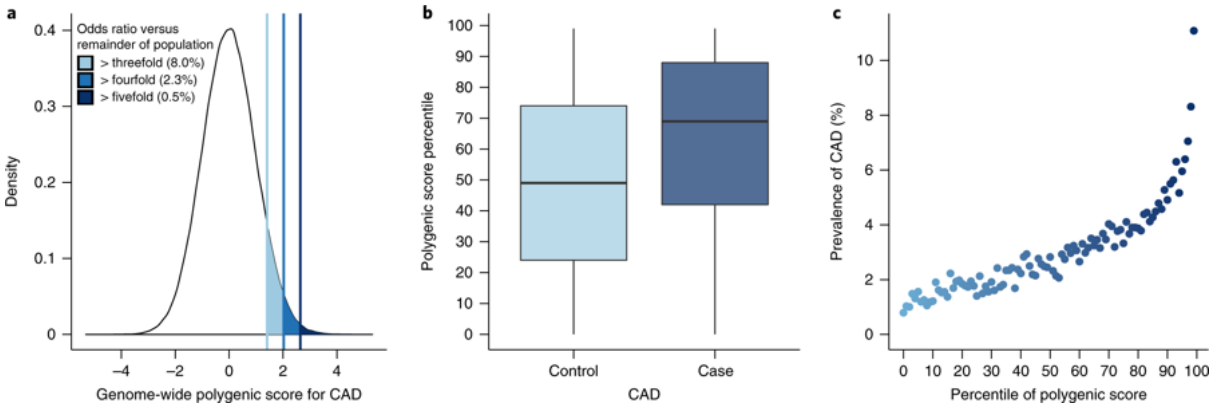

Figure 1.3.3 – A figure illustrating the risk for coronary artery disease according to polygenic risk scores.

(A) A distribution of PRSs for coronary artery disease (CAD) in the UK Biobank. The x-axis represents PRSs with values scaled to a mean of 0 and a standard deviation of 1. Shading represents the -fold increase in risk relative to the general population. (B) PRS for CAD percentiles in CAD cases versus controls. Each boxplot covers the interquartile range and the horizontal line represents the median. (C) Prevalence of CAD in 100 groups binned according to the percentile of PRS for CAD. Taken from Khera et al (2018)

1.3.3 THE UTILITY AND LIMITATIONS OF POLYGENIC RISK SCORES

Estimating the susceptibility of an individual to a disease is central to clinical decision making and can be a powerful tool for advancing human health. It is the earliest measurable contributor to disease risk and although a single measure of PRS still cannot provide enough clinical utility, it can be a useful prediction tool when combined with other factors such as environmental influence and high risk copy number variants (CNVs) (96).

For example, using PRS it is possible to identify those with the greatest risk for a disorder, adapt their lifestyle to offset the disorder as much as possible, and provide treatment at the right time should the disorder arise. In the field of cancer, studies have shown that a PRS would lead to a recommendation to initiate a colonoscopy screening at 42 years for individuals with the higher PRS, compared to a screening at 52 years for individuals with the lowest PRS (97).

One of the biggest factors holding PRS back from clinical utility is that they are currently only able to explain between 1% and 15% of the variation between cases and controls, which is deemed as insufficient to allow robust translation into a clinical context (96, 98, 99). This once again brings up the issue of "missing heritability", which has several possible explanations. One of the explanations is that many common risk variants with very small effect sizes can explain this "missing heritability", but only in extremely large sample sizes (100). Another is that risk is also conferred by rare (de novo) variants and that unknown non-additive genetic variation could be part of the genetic risk (101, 102).

On the other hand, people have argued that the ability of PRS to explain 15% of risk for disorders such as coronary artery disease (CAD) means that individuals at the top of the 15% may actually be at three to five times higher risk than the general population, making current PRS already more useful for identifying people with the highest risk for common disorders than some clinical monogenic tests (95).

1.3.4 POLYGENIC RISK SCORES IN PSYCHIATRY AND RESEARCH

As opposed to CAD where a number of biological screening measures can be taken from a patient, the field of psychiatry relies mainly on diagnoses based on self-reports and observations. PRS therefore hold great promise for being able to detect the risk for psychiatric disorders before symptoms arise and tailor preventions and treatments based on genetic predictors. For example, a recent study showed that an individualised PRS can accurately predict conversion from a prodromal risk syndrome to psychosis (103). By identifying those at risk of psychosis early on, clinicians may be able to offer these patients preventions or treatments before they experience a psychotic episode.

Advances in the field of psychiatric genetics also point towards a future where PRS can be used in precision medicine to tailor clinical decisions according to a patient's individual

biology in order to maximise treatment and minimise adverse side-effects (104). Seeing as DNA is stable from birth, as sample sizes in genetic studies increase, the accuracy of PRS will also increase (104).

Polygenic risk scores have greatly advanced the field of psychiatric research. Patients suffering from psychiatric disorders such as MDD and SCZ are much more likely to engage in detrimental behaviours such as smoking, alcohol and drug misuse (102, 105). They are also more likely to have experienced traumatic life events and turbulent upbringings (e.g. via evocative gene-environment correlation (106-108). These experiences have been shown to have negative consequences on the body and brain, making research into the biological causes of these disorders very difficult to discern from confounding factors. Polygenic risk scores allow researchers to calculate the genetic risk for disorders such as MDD, BD and SCZ in a healthy population and use these as continuous predictor variables, instead of case or control status. Although there is still a need to control for various factors (109), modelling risk for psychiatric disorders in unaffected populations allows researchers to separate out genetic risk mechanisms from confounders.

Additionally, PRS can be used to study gene by environment interactions such as the effect of environmental stress on those with the highest and lowest genomic liability for a given disorder (110, 111). For example, it may be possible to determine whether someone who has a higher PRS for a given disorder is more likely to show psychiatric disorder symptoms if they have suffered childhood trauma compared to someone who has not. The identification of gene by environment interactions can improve the accuracy and precision of genetic and environmental influences and allow for the discovery of new mechanisms related to disease aetiology (112).

Furthermore, it has become apparent that psychiatric disorders share genetic risk with other psychiatric disorders as well as non-disease traits such as educational attainment (113). This

has resulted in progress regarding shared biological pathways and causal relationships. For example, one study has shown that a higher genetic risk for schizophrenia is associated with increased cannabis use, which may in turn exacerbate the SCZ risk (114). This knowledge is valuable in developing treatments and preventative measures.

1.3.5 SUMMARY

The field of psychiatric genetics has come a long way in recent years. With the help of collaborative consortia and technological and computational advancements, GWAS have been able to identify a greater number of significant SNPs, paving the way for a large number of downstream analyses to elucidate disease aetiology, drug effect and environmental risk factors. In addition, scientists are beginning to understand the role that non-coding regions of the genome play in regulating gene expression and gene regulation, and how this affects biological processes in the human body.

A great promise for the future is to be able to predict risk for a number of disorders via genetic risk factors, and to tailor the environment accordingly. In addition, there is promise that genetic predictors can be used to tailor drug and treatment response for a number of psychiatric disorders.

In this thesis, we use the predictive power of PRS to model disease risk in a healthy population. By doing so, we have been able to better avoid confounding factors associated with psychiatric disorders (e.g. medication use) and better understand the biological mechanisms involved in the aetiology of these disorders. These are novel tools for studying psychiatric disorders and our findings provide unique insights into possible biological mechanisms associated with disease progression. In addition, we have used publicly available GWAS to estimate heritability for a given trait, perform genetic correlation analyses with other phenotypes and identify genes that are associated with SNPs from GWAS in order to elucidate aberrant

biological mechanisms and inform translational experiments as to which genes, pathways and compounds should be studied further.

1.4 ENVIRONMENTAL RISK FACTORS FOR PSYCHIATRIC DISORDERS

1.4.1 STRESS AND THE IMMUNE RESPONSE

Stress-induced immune system dysregulation has been the focus of much research in recent years, describing the interaction between the central nervous system (CNS), the endocrine system and the immune system, and how this interaction has an effect on physical and mental health (115-117). A wide range of stressors (such as daily hassles, exams, or a death of a loved one) can dysregulate the immune response by affecting this interaction, and studies have shown that this can in turn have a negative effect on health (117, 118).

The CNS is a key regulator of the immune response, involving a highly complex network of signals, receptors and bi-directional communication pathways between the nervous, endocrine and immune systems (119, 120). The HPA axis is one of the major pathways through which the immune system can be altered during exposure to threatening stress (121, 122). Activation of the HPA axis is the body's response to a stressor, which can be both physical (a virus or an infection) or psychological (death in the family or victimisation) (123).

This thesis will focus primarily on psychological stressors, which are strongly associated with an increased risk of psychiatric disorders such as MDD, BD and SCZ (124-126).

1.4.2 ENVIRONMENTAL STRESS AND INFLAMMATION

A wide range of daily occurrences are classified as stressors of varying degrees of severity, requiring the body to respond with physiological adaptations in order to restore homeostasis (127). Broadly speaking, psychological stress often occurs when certain events or

environmental demands exceed an individual's ability to cope (128). Stressors are often categorized by their duration and their course (129), although both have been shown to have negative consequences on physical and mental health (130, 131). Childhood maltreatment is one of the most common stressors to occur to children under 18 years of age, leading to disruption in early brain development and changes to nervous and immune systems (132, 133). Adults who have been maltreated are at an increased risk of physical, behavioural and mental health problems such as depression, obesity, smoking, high-risk behaviours, unintended pregnancy, and alcohol and drug misuse (134).

The WHO states that worldwide, 1 in 4 people report suffering childhood physical abuse and 12% of children report being sexually abused each year (135). Some studies have suggested that in European countries, more than 10% of people report moderate emotional neglect and 20% of people report moderate physical neglect in childhood (136). The WHO European report on preventing child maltreatment states that over 55 million children are affected by childhood maltreatment (137), with a 22.9% prevalence of physical abuse (138). Childhood maltreatment is often reported as a single measure, although it includes categories such as emotional abuse, emotional neglect, physical abuse, physical neglect, and sexual abuse.

Adults who have experienced childhood maltreatment show a reduction in hippocampal volume, suggesting that childhood maltreatment experiences could lead to long-lasting changes in a brain region implicated in disorders such as MDD, BD and SCZ (139, 140). In fact, some studies have shown that the association between depression and a smaller hippocampal volume is lost once maltreatment is taken into account (141). In addition, maltreatment seems to alter the development of sensory systems that process stressful experiences (such as the amygdala), leading to augmented responses to threatening stimuli later in life (140). Interestingly, there is a subset of individuals who have experienced childhood maltreatment but do not show any

over psychopathology, suggesting that they possess some biological or psychological resilience qualities (140). This subset of individuals is of great interest to the field of psychiatry, as they may shed light on useful treatments for those who are not resilient to psychopathologies resulting from experiences of childhood maltreatment.

A number of studies have examined the association between environmental stressors such as childhood maltreatment and inflammation, strongly supporting the notion that childhood maltreatment is associated with increased inflammatory activity, measured by levels of circulating pro-inflammatory cytokines (17, 18, 142). Although a transient increase in circulating levels of pro-inflammatory cytokines is part of a natural response to environmental stressors, prolonged exposure (such as repeated experiences of childhood trauma) can damage this natural response and result in aberrant functioning of both the nervous and immune systems in adulthood.

One reason for sustained pro-inflammatory cytokine release in adulthood has been attributed to HPA axis-related inflammation via glucocorticoid resistance (143). Glucocorticoid feedback inhibition of immune response gene transcription is the fundamental mechanism to protect the organism against excessive inflammation, and a malfunction in this mechanism due to prolonged exposure to environmental stress can lead to physical and psychological problems (144, 145). Repeated exposure to acute stress has been shown to alter glucocorticoid sensitivity and social cues indicating physical danger have been associated with glucocorticoid resistance in both humans and animals (146, 147). Given that pro-inflammatory cytokines and cortisol are part of the same response, glucocorticoid resistance in an individual can lead to permanently elevated levels of both, due to the inability to slow down or shut down the inflammatory response (144). These increased levels of pro-inflammatory cytokines as a consequence of childhood maltreatment, glucocorticoid sensitivity and aberrant stress response regulation,

have been associated with a number of psychiatric disorders such as depression, bipolar and anxiety disorder. (148, 149).

As a result, this thesis will focus on the difference in levels of circulating inflammatory markers between maltreated individuals with MDD, non-maltreated individuals with MDD, maltreated controls and non-maltreated controls. The aim is to try and understand whether those who have experienced maltreatment and have MDD are more likely to have higher levels of circulating pro-inflammatory markers compared to maltreated controls or non-maltreated MDD cases. If maltreated controls show a different inflammatory profile to maltreated MDD cases, this may offer a biological resilience mechanism for future research.

1.5 GENE-ENVIRONMENT INTERACTIONS AND RISK FOR PSYCHIATRIC DISORDERS

1.5.1 GENE-ENVIRONMENT STUDIES

Research into the predictors of psychiatric disorders has highlighted the importance of environmental stressors such as childhood trauma (9, 15, 150). However, since the use of twin studies in quantifying behavioural genetics, scientists have agreed that psychiatric disorder symptoms are the result of both genetic and environmental (GxE) influences (151, 152). In addition, the field of behavioural genetics has suggested that genetic and environmental factors interact with one another, as well as having independent main effects. For example, twins with a low genetic risk for MDD were found to have a 0.5% chance of developing the disorder if they were exposed to stressful life events, as opposed to 6.2% chance if they were exposed to childhood adversity (153). This was increased to 1.1% and 14.6% respectively in twins with a high genetic risk for MDD, showing that the genetic risk for MDD is moderated by environmental factors.

Advancements in technology in recent years have allowed scientists to move towards examining the gene-environment interaction at the level of an individuals' DNA rather than a statistical estimation, with a breakthrough study by Caspi and colleagues (2003) showing that a genetic polymorphism in the promoter region of the serotonin transporter (*5-HTTLPR*) gene in people who have stressful life events, is associated with depression (154). Since then, genes such as the Serotonin transporter (*SLC6A4*), the dopamine receptor D4 (155) and the serotonin-transporter-linked polymorphic region (*5-HTTLPR*) have been some of the most commonly studied candidate genes implicated in MDD, BD and SCZ (155-157).

However, there are a number of limitations regarding the candidate GxE studies in the field of psychiatry. Firstly, there is a risk of selecting inappropriate candidate genes, mainly due to the limited knowledge regarding the specific biological mechanisms at play in psychiatric disorders. Secondly, recent discoveries in psychiatric disorders have highlighted that psychiatric disorders manifest as a result of thousands of genetic variants with small effects rather than one gene with a large effect. Finally, there has been difficulty in replicating the candidate GxE findings, possibly due to small and underpowered sample sizes. In fact, a recent study suggested that the hypothesis regarding the promoter region of the *5HTTLPR* gene being associated with an increased risk of depression in individuals exposed to stressful situations may not be true. The interaction seems to not be generalisable, having a modest effect size in only limited situations (158). These studies have led to the transition of GxE research towards genome-wide approaches, using very large samples.

An alternative approach to candidate gene studies is investigating the polygenic score by environment interaction using GWAS results. For example, these studies are able to investigate the interaction between PRS for psychiatric disorders such as MDD and childhood maltreatment. Peyrot and colleagues (2014) showed that childhood trauma has a greater effect

on MDD risk in those with a higher PRS for MDD (159) and other studies have shown that stressful life events and PRS for MDD are associated with more depressive symptoms, especially in those who had lost a spouse and had a greater PRS for MDD (160). Interestingly, few studies to-date have used genome-wide GxE analyses to study biological mechanisms that may be responsible for psychiatric disorder symptoms, possibly because a large sample size is needed to perform interaction analyses.

1.5.2 EPIGENETICS

Twin studies and GxE interactions are known to play significant roles in the manifestation of mental illness, whereas epigenetic mechanisms provide biological evidence for how stable and lasting changes in gene expression following exposure to environmental stress can occur (161). Epigenetic changes describe a dynamic process whereby changes in gene expression can be elicited without altering the DNA sequence (162).

Chromatin is made up of DNA wrapped around a histone molecule, with a variety of chromatin remodelling enzymes able to influence gene activity by inducing either the active (via histone acetyltransferases) or the inactive (via histone deacetylases) chromatin state (163). Histone acetyltransferases add an acetyl group to lysine residues on the histone tail, causing the chromatin to be in a more relaxed state, promoting gene transcription. Histone deacetylases on the other hand promote transcriptional repression by removing the acetyl group from histone tails (164). Although these are the most prominent epigenetic modifiers, other posttranslational modifications include phosphorylation, methylation and ubiquitination (161). DNA methylation for example, is the addition of a methyl group to DNA by DNA methyltransferases, which are regarded as key players in silencing gene transcription (165).

Animal studies have shown that hippocampal-dependent fear-related memories ae dependent on DNA methylation and that inhibiting this can abolish fear-related memories altogether

(166). Other studies have shown that DNA methylation is necessary for memory formation and the storage of long-term memories in the cortex (166-168).

Regarding psychiatric disorders, epigenetic modifiers have emerged as mediators and moderators of long-term dysfunctions in biological processes as a result of environmental effects such as childhood maltreatment. For example, animal studies have shown that rat offspring of non-caring mothers have higher methylation in a region of the gene encoding the glucocorticoid receptor, a known risk mechanism for MDD (169). Human studies have also shown that exposure to childhood and *in utero* adversity is associated with hypermethylation in the gene encoding the glucocorticoid receptor, suggesting environmental stress has the same detrimental effect in humans (161, 170). Studies investigating post-traumatic stress disorder also shows that people with the disorder who have suffered childhood maltreatment, show different levels of DNA methylation and subsequent gene expression compared to people who have not suffered maltreatment (171).

Epigenetic modifications have also been shown to have positive effects. For example, exercise has been shown to increase the expression of genes involved in tumour suppression and decrease the expression of cancerous genes via DNA methylation (172). In addition, studies have shown that environmental enrichment in mice can lead to genome-wide transcriptional and methylation differences, which are associated with increased hippocampal neurogenesis and volume (173). Medication has also been shown to result in epigenetic changes in patients with BD, suggesting that understanding the epigenome and how drugs can affect epigenetic regulation is a crucial part for future drug design (174).

1.5.3 SUMMARY

It is evident that the GxE interaction plays a crucial part in the susceptibility for psychiatric disorders, with epigenetics bridging these two factors. Although epigenetic research is still in

its infancy, recent studies have highlighted the importance of epigenetics in many human disorders and hold much promise for the future of epigenetics research. Technological advancements are allowing scientists to analyse large amounts of data produced by high throughput sequencing tools, helping understand the role of epigenetics in different diseases, different tissues and different stages of development.

In psychiatry, it is generally accepted that epigenetic factors sit alongside DNA polymorphisms in mediating susceptibility to disorders such as MDD, as epigenetic research provides a biological explanation for how the environment can influence basic biology, and how that can lead to pathology. Given that the environment plays such a big role in mediating susceptibility in psychiatric disorders, this field holds much promise for the future in terms of both psychological and pharmacological intervention; as identifying the risk genes can help scientists develop better medication, potentially targeting those who have been subject to certain environmental stimuli.

1.6 PUTATIVE BIOLOGICAL MECHANISMS MODERATING THE RISK FOR PSYCHIATRIC DISORDERS

1.6.1 INFLAMMATION

1.6.1.1 CYTOKINES

There are a number of reasons why increased cytokine circulation has been associated with psychiatric disorder symptoms. Cytokines are large molecules and do not readily cross the blood-brain barrier; however, they can get into the brain via incomplete or permeable areas of the blood-brain barrier (142).

In addition, cytokines can bind to the vascular endothelium in the brain and facilitate the release of secondary messenger molecules, resulting in an increase in local cytokine activity; and

carrier proteins have also been shown to actively transport cytokines across the blood-brain barrier in rodents (142, 175, 176).

Cytokines may also cause damage to the blood-brain barrier, destroying the tight junctions of endothelial cells forming the barrier, exposing the brain to numerous damaging circulating factors (177, 178).

Once in the brain, cytokines are thought to have a number of functions. They are able to activate the brain's resident immune cells, called microglia, which are responsible for both pro-inflammatory and anti-inflammatory processes in the brain, playing a part in pathogen recognition, antigen presentation, phagocytosis as well as synapse remodelling, stem cell proliferation and repair (179, 180). Non-activated microglia (also known as resting microglia) survey their nearby environments and in response to changes in the environment, they are able to change shape and form to become activated microglia. Activated microglia vary from pro-inflammatory phenotypes (characterized by M1 cells) to immunosuppressive and anti-inflammatory phenotypes (characterized by M2 cells) (181, 182).

The M1-type activation of microglia can lead to increased local production of pro-inflammatory cytokines such as TNF-a and IL-6, as well as superoxide radicals and nitric oxide (182). They serve as the first line of defence in the brain and can recognise harmful stimuli using a full array of immune receptors (183). Their activation is responsible for killing off the offending foreign pathogen and clearing dysfunctional cells, and chronic exposure to glucocorticoids has been shown to prime microglia towards this pro-inflammatory M1 state (184). In contrast, the M2-type activation is in turn associated with the release of anti-inflammatory cytokines such as IL-10, IGF-1 and TGF-b (182). This activated state has been associated with positive influences such as tissue remodelling, inflammatory dampening, angiogenesis and immunoregulation (183), see **Figure 1.6.1**. The origin and magnitude of

injury or stressor can dictate the development of these two microglial phenotypes, with several studies showing that the number and the morphology of microglial cells is involved in cognitive and behavioural changes observed in psychiatric disorders (185).

Cytokines can also exert direct actions on neurons and glia, and indirect actions on brain vasculature, blood flow and temperature (186). Common pathways of neuronal cell death and signalling disruption have been attributed to neuronal excitotoxicity, impaired release and reuptake of neurotransmitters, and intracellular entry of calcium (186). Receptors for cytokines such as TNF-a, IL-6, IL-10 and IL-2 have been reported on most cell types throughout the brain, sharing many common signalling mechanisms (186).

It is become clear that pro-inflammatory cytokines can have a profound effect on the brain via a wide range of targets, and this has in turn been associated with an increased risk of developing certain psychiatric disorders.

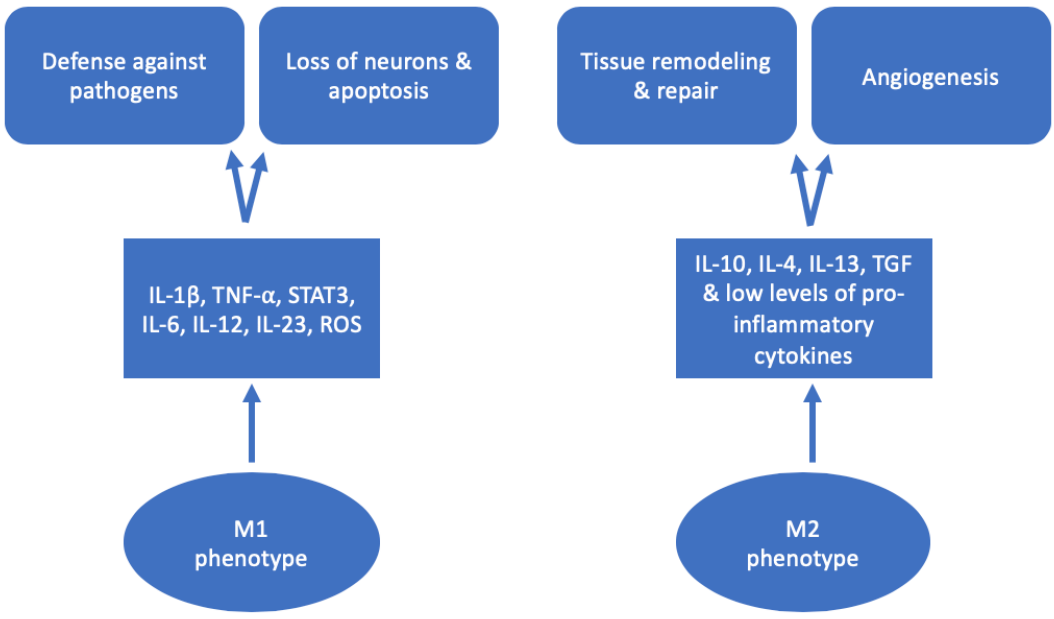

Figure 1.6.1 – Inflammatory cytokines released from different subtypes of microglia, and their roles. This figure shows the different types of cytokines that are released from the M1 and M2 types of microglia, and the types of biological activities they have been associated with. Adapted from Wang et al (2015).

1.6.1.2 CYTOKINES, MALTREATMENT AND PSYCHIATRIC DISORDERS

Environmental stressors such as childhood maltreatment have been associated with psychopathology in later life, increasing the risk for major depressive disorder, bipolar disorder and schizophrenia (34, 187-189). Environmental stress is collectively referred to as "allostatic load", which essentially describes stress in a neurobiological context. It refers to the gradual physiological decline of an individual as a result of repeated allostasis (maintenance of stability), ultimately leading to aberrant mechanisms to shut-off or turn-on responses to stressors (190). Repeated exposure to childhood trauma is likely to elicit a great allostatic load on an individual, leading to the gradual decline of the ability to regulate the physiological stress response.

A study by Heim and colleagues (2000) evaluated the endocrine response of women categorized into four groups, depending on their early-life trauma experiences. They found that women with a history of abuse showed an increased ACTH response to stress compared to controls. These women also showed a greater increase in heart rate and a higher cortisol response to stressors compared to women who had not experienced early-life trauma. Interestingly, the increase was greater in women with current major depression (191), see **Figure 1.6.2**. These findings suggest that early-life trauma can result in HPA axis hyperactivity, resulting in long lasting consequences and ultimately increasing the risk of depression in adulthood.

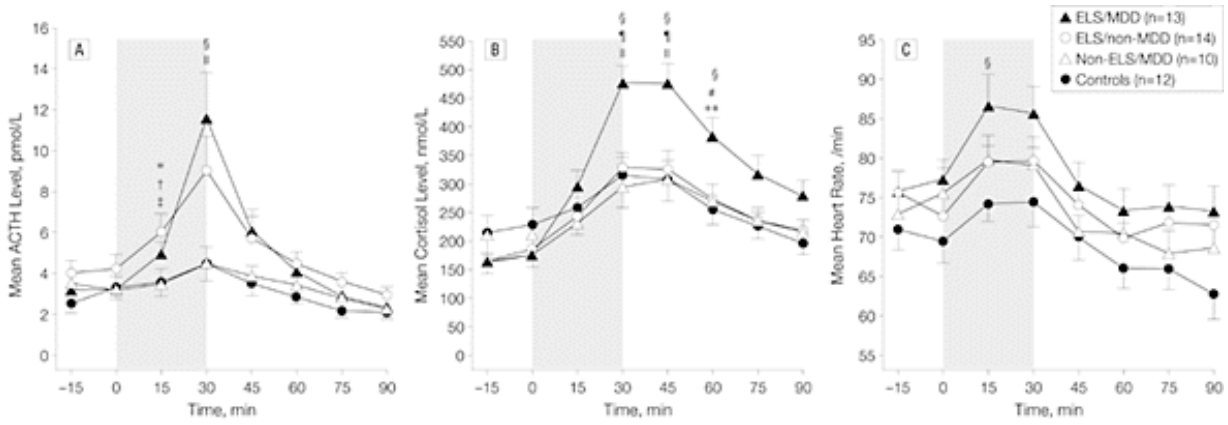

Figure 1.6.2 – HPA reactivity to a standardised laboratory stressor in women after childhood sexual and physical abuse.

These charts show the mean (A) adrenocorticotropin, (B) cortisol, and (C) mean heart rate, before, during (shaded area) and after psychological stress in women. Women were characterised as having no history of early life stress and no psychiatric disorder (controls), a history of sexual or physical abuse without MDD (ELS/non-MDD), a history of childhood sexual or physical abuse and MDD (ELS/MDD) and women without a history of early-life stress and MDD (non-ELS/MDD). The results show that women who have experienced early life stress and have MDD have the highest physiological reaction to laboratory stress. Taken from Heim et al (2000).

A wide range of studies have reported increased pro-inflammatory cytokines in psychiatric disorder patients compared to controls, and given that childhood trauma is associated with a greater risk of developing psychiatric disorders, cytokines may be the key mediators in this relationship (17, 18, 192), see **Figure 1.6.3**. In particular, studies have reported increased circulating levels of TNF-α, IL-6 and CRP in psychiatric disorders such as MDD, BD and SCZ (193-196). Indeed, cytokines have been associated with neurotransmitter regulation, HPA axis regulation and hippocampal neurogenesis, all effects that have the potential to contribute towards the development of psychiatric disorders (197).

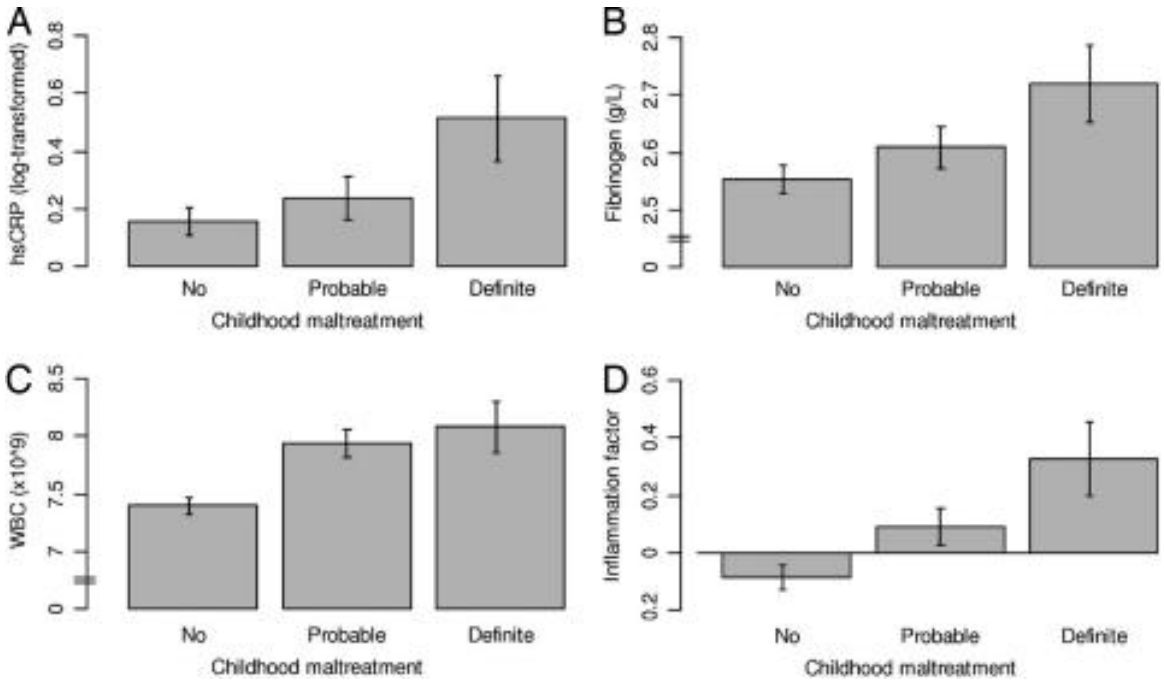

Figure 1.6.3 – Association of childhood maltreatment with biomarkers of inflammation.

(A) The mean and standard error of CRP levels according to maltreatment experiences. Analyses showed that definite maltreatment (but not probable maltreatment) was associated with a significant increase in CRP levels compared to no maltreatment. (B) Mean and standard error of fibrinogen according to maltreatment experiences. Analyses showed that definite maltreatment (but not probably maltreatment) was associated with an increase in fibrinogen levels. (C) Mean and standard error of white blood cell count according to maltreatment experiences. Both definite and probable maltreatment was associated with an increase in white blood cell count. (D) Mean and standard error of a factor score for inflammation according to maltreatment experiences. Both definite and probable maltreatment were associated with an increase in a factor score for inflammation. Taken from Danese et al (2007)

1.6.1.3 DISCREPANCIES IN THE LITERATURE

Although there is a great amount of literature supporting the association between childhood maltreatment an increased inflammatory response in depression, it should be noted that there are several studies that do not find a significant effect (198).

Many studies differ in their methodologies and suffer from small sample numbers (especially for the maltreatment subgroups) as well as ethnicity and ancestry biases (198). Perhaps a more striking limitation is that most studies solely investigate the effects of IL-6, CRPand TNF-a, providing little insight into the other pro-inflammatory and anti-inflammatory cytokines that may be involved in the immune response.

A recent large-scale study in this field carried out by Powell and colleagues (2018) reported no associations between 42 inflammatory markers and depression case or control status (199). This study was carried out on 1208 individuals from a diverse ethnical background and controlled for a large number of external variables such as smoking, age, BMI, antidepressant use and gender. Strikingly, this study showed that up to 18% of the variance for IL-6 expression, for example, can be explained by BMI alone, see **Figure 1.6.4**.

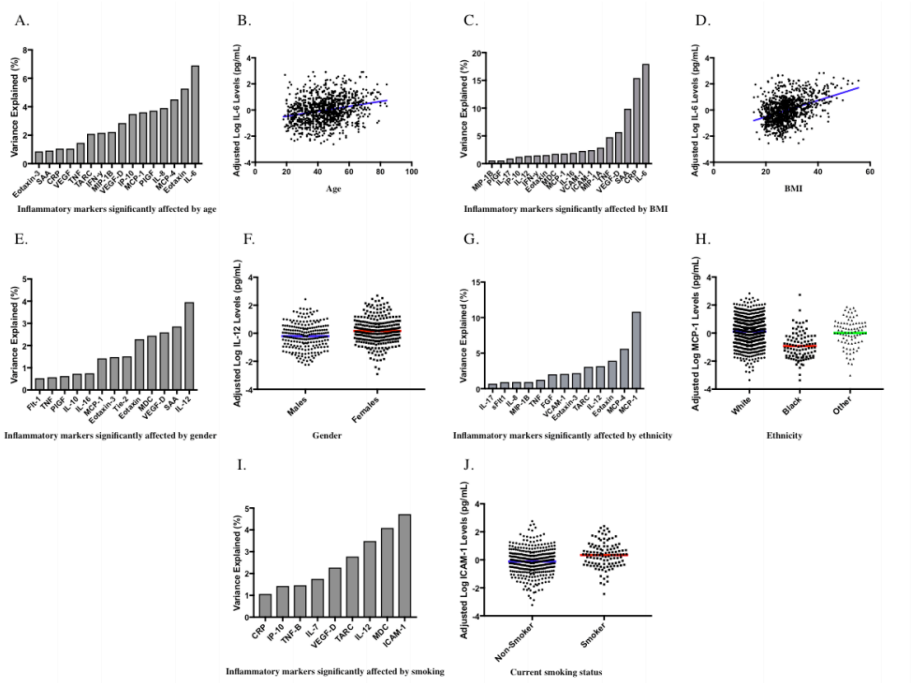

Figure 1.6.4 – Confounding factors and their influences on inflammatory marker expression.
(A) Inflammatory markers significantly affected by age on the x-axis, with the percentage of variance explained by age on the y-axis. (B) Scatterplot showing the significant positive relationship between age (x-axis) and IL-6 levels (y-axis), the line of best fit is shown in blue. (C) Inflammatory markers significantly affected by BMI on the x-axis, with the percentage of variance explained by BMI on the y-axis. (D) Scatterplot showing the significant positive relationship between BMI (x-axis) and IL-6 (y-axis), the line of bets fit is shown in blue. (E) Inflammatory markers significantly affected by gender on the x-axis, with the percentage of variance explained by gender on the y-axis. (F) A plot showing gender (x-axis) and IL-12 levels (y-axis), the mean of each group is shown in coloured lines. (G) Inflammatory markers significantly affected by ethnicity on the x-axis, with the percentage of variance explained by ethnicity on the y-axis. (H) A plot showing ethnicity (x-axis) and adjusted log MCP-1 levels (y-axis), the mean in each group is shown by a coloured line. (I) Inflammatory markers significantly affected by smoking status on the x-axis, with the percentage of the variance explained by smoking on the y-axis. (J) A plot showing smoking status (x-axis) and ICAM-1 levels (y-axis), the mean of each group is displayed by a coloured line. Taken from Powell et al (2018).

Adipose tissue may be partially responsible for systemic inflammation, with more than 50 different cytokines such as IL-6, IL-8 and TNF-a being produced and released from adipose tissue itself (200). Larsson and colleagues (2015) measured 92 cytokines in cerebrospinal fluid taken from 89 patients and found 19 markers to be significantly associated with BMI (200). Furthermore, Schmidt and colleagues studied 117 obese patients and 83 non-obese controls and reported that obesity is associated with elevated levels of IL-12, IFN-y and TNF-a (201). A study by Borges and colleagues (2018) reports very similar findings and states that obesity changes lipid profiles of individuals, increases pro-inflammatory molecule levels, decreases anti-inflammatory marker levels, and promotes a state of chronic inflammation (202).

Current literature suggests that the effects of BMI may need to be strongly considered when investigating the effects of inflammation and psychiatric disorder aetiology. Interestingly, some studies have suggested that childhood maltreatment is associated with increased adulthood BMI, which may be another driving factor for the increased circulating pro-inflammatory cytokines in adulthood.

Although there are relatively few studies investigating the effects of childhood maltreatment on adulthood BMI, a study carried out by Sacks and colleagues (2017) reports that people exposed to physical abuse and physical neglect are more likely to show an increase in BMI in adulthood compared to those who have not experienced abuse (203, 204). Another study using a 50-year follow-up to monitor the BMI trajectory of people who have been exposed to childhood maltreatment showed that in females, sexual abuse was associated with faster lifetime BMI gains (205).

Studies investigating associations between inflammation and MDD often struggle to discern cause and effect alongside external factors such as BMI, given that there is a case to be made for BMI as a potent mediator of the inflammatory response in adulthood, especially in people

who have experienced childhood trauma. This does not imply that the association between inflammation and psychiatric diagnosis is invalid, but rather that it needs to be considered alongside other factors such as BMI, age, smoking and mediation use. It is likely that there is a subset of psychiatric disorder patients that suffer from inflammation-induced pathology, as opposed to others who may show an increased pro-inflammatory phenotype due to other factors (e.g. BMI) (206).

1.6.1.4 ANTI-INFLAMMATORY THERAPIES

There is a pressing need for new therapies in the field of psychiatry, especially due to treatment resistance and a limited number of psychopharmacologic therapies currently available. Based on the current evidence anti-inflammatory drugs may possess some utility as an alternative or adjuvant treatment. Indeed, animal studies have showed that by treating mice with anti-inflammatory compounds, it prevents depressive behaviours in mice by reducing oxidative stress and neuroinflammation in the cortex and the hippocampus (207, 208). Furthermore, human studies have suggested that anti-inflammatory therapies show better symptom improvements compared to placebo as monotherapies as well as add-on treatments to antidepressants (209-212).

However, the inflammatory cascade is highly complex, and studies have demonstrated that inflammatory cytokines also play a pivotal role in learning, memory and neuronal integrity (213). Moreover, not all anti-inflammatory drugs are the same and some may have off-target effects, resulting in more harm than good (214). It may therefore be beneficial to identify those with the highest risk of developing psychiatric disorders as a result of increased inflammation and develop targeted anti-inflammatory treatments for this sub-group specifically, as opposed to giving a blanket treatment for everyone. Moreover, by using genetic risk scores we may be better able to offer precise and preventative treatments at an early age.

1.6.1.5 SUMMARY

In summary, there has been much research to support the notion that childhood maltreatment is associated with an increase in pro-inflammatory cytokines, and that this is in turn associated with an elevated risk of developing psychiatric disorders such as MDD. The primary biological mechanism involved in this is thought to be the HPA axis, which after sustained activation via childhood trauma is unable to initiate the negative feedback loop to dampen the immune response. As a consequence, the body is subject to elevated circulating pro-inflammatory cytokines throughout life.

Pro-inflammatory cytokines are known to infiltrate the brain and activate microglia, the resident immune cells, which are known to have a range of negative effects on synapse formation and the health of neurons and progenitor cells. This is thought to contribute toward risk of MDD, BD and anxiety disorder.

Due to the complexity of the immune response, few studies have investigated a wide range of cytokines in psychiatric disorder cases vs controls. In addition, studies often focus on clinical samples which suffer from a range of external factors that cannot easily be controlled for in statistical analyses (such as medication use and suboptimal lifestyles). For example, BMI has been shown to be a strong mediating factor in the association between MDD and circulating pro-inflammatory cytokines, as well as childhood trauma and circulating pro-inflammatory cytokines.

Nevertheless, there is a very strong relationship between childhood trauma, inflammation and psychiatric disorder aetiology. This relationship needs to be investigated further to establish cause and effect, the degree of influence of external factors.

1.6.2 TELOMERE SHORTENING

1.6.2.1 WHAT ARE TELOMERES?

Telomeres are TTAGGG nucleotide repeats at the end of chromosomes. During cell division, it is not possible to fully extend the newly synthesized chromosome (a phenomenon known as the 'end-replication problem'), resulting in a slight loss of DNA each time a cell divides. The word 'telomere' means 'end-part' and was first coined by Barbara McClintock and Hermann Muller in the 1930's, who hypothesized that telomeres form a 'buffer zone', protecting the ends of eukaryotic chromosomes during cell division and preventing valuable DNA from being lost (Gall, 1995; Blackburn, 2001). As we age and the number of cell divisions increase, telomere length progressively gets shorter, making it a useful biomarker for 'cellular age'.

The function of telomeres may be better understood if we further consider the end-replication problem. During mitosis, DNA molecules are unwound into single strands. Then, DNA polymerase moves in a 5' to 3' direction along the leading strand of DNA to create a new, complementary strand (215). However, due to the nature of DNA polymerase the lagging strand is made discontinuously, meaning that RNA primers must temporarily attach to the lagging strand of DNA and act as starting points for the synthesis of Okazaki fragments – newly synthesized DNA fragments on the lagging strand – in concordance with the 5' to 3' directionality rule (215). The RNA primers then get replaced with DNA and the Okazaki fragments are joined together by DNA ligase.

This process results in both strands being replicated to almost the same length, with the crucial difference lying at the ends of the newly synthesized lagging strand. Once the end-primer removes itself from the lagging strand, there is no more DNA regions left for the DNA polymerase to bind to, meaning there is no potential for the strand to be elongated fully and thus the new strand becomes shorter than the original strand (215). This is how the end replication problem occurs, and how telomeres shorten, see **Figure 1.6.5**.

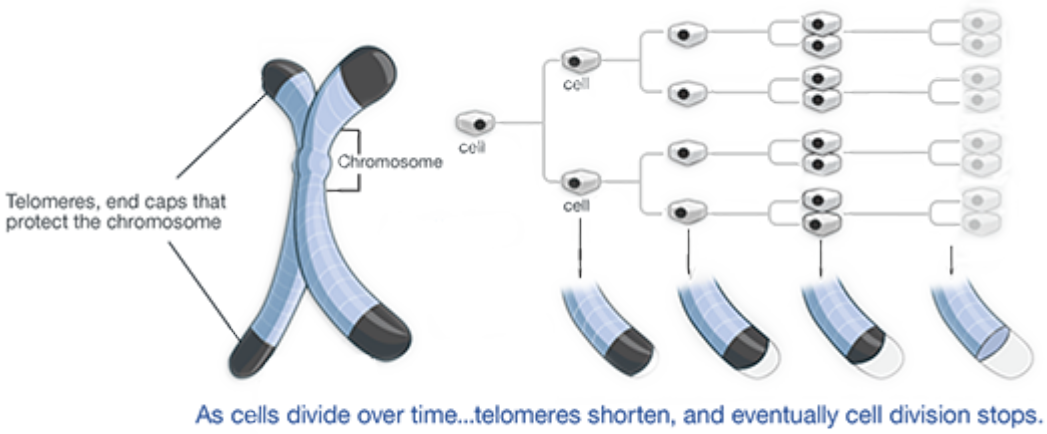

Figure 1.6.5 – Telomere shortening.
A diagram showing the location of telomeres as the ends of chromosomes as well as the progressive shortening of telomeres as the cell undergoes repeated cell division. telomeres (in black) get shorter each time a cell divides, but the valuable DNA (in blue) remains intact. However, after many rounds of cell division telomeres get too short and the cell can divide no further. Taken from: https://www.tasciences.com/what-is-a-telomere.html

1.6.2.2 GENETIC FACTORS INFLUENCING TELOMERE LENGTH

Telomere length shows high interindividual variability, and the largest meta-analysis on telomere length revealed lowest heritability estimates to be 65% and highest to be 86%, as well as a strong maternal inheritance effect (216).

However, the largest GWAS on telomere length to-date, consisting of 37,684 individuals, could only predict 1% of the variance in telomere length, using a genetic risk score that encompassed seven SNPs that surpassed the GWAS significance threshold (217), see **Figure 1.6.6**. This suggests a high degree of 'missing heritability' whereby the results from GWAS do not meet the number of SNPs expected based on the high degree of heritability estimated from twin studies. This might reflect an underpowered GWAS and highly polygenic trait, effects of copy number variants and rare variants on telomere length (not captured by GWAS), or gene-environment interactions inflating twin heritability estimates. Future research will need to

explore whether the cumulative effects of more common genetic variants (e.g. polygenic risk scores) can better predict risk for shorter telomere length.

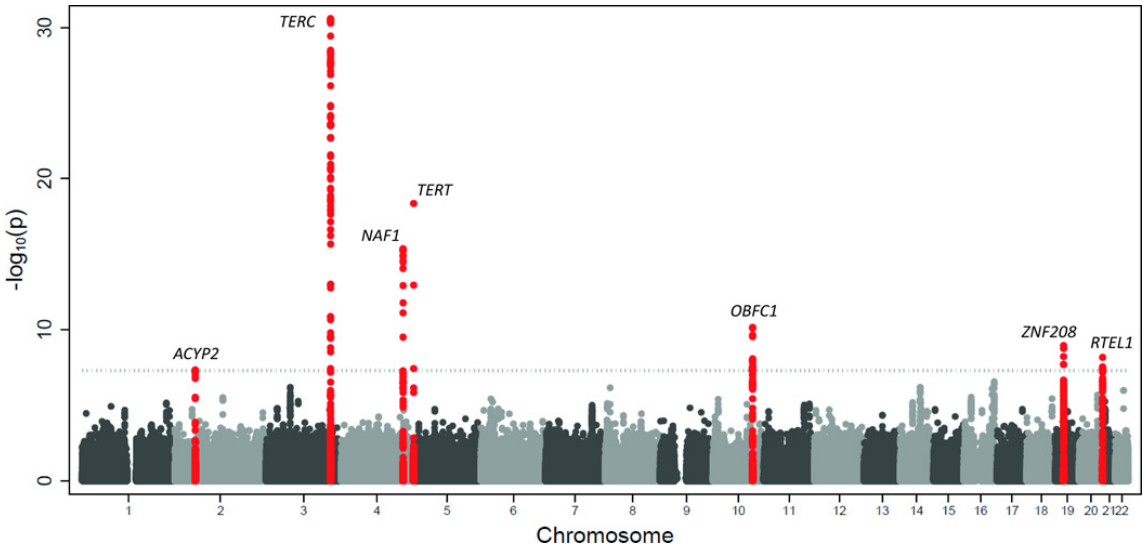

Figure 1.6.6 – Telomere GWAS.
The most recent GWAS on telomere length, displaying the data as -log10P values (y-axis) against chromosomal location (x-axis) for over 2 million SNPs tested. The dotted line represents genome-wide significance at P = 5x10-8. The 7 loci that showed a significant association at this level are displayed in red. Taken from Codd et al (2014).

1.6.2.3 TELOMERE SHORTENING

Most cells in the human body divide continuously throughout our lifetime. This means that telomeres get shorter with each cell division, and this progressive telomere shortening is thought to represent a 'molecular clock' which underlies cell ageing (218, 219). Although telomere shortening is highly variable between and within humans and is heavily influenced by factors such as diet and exercise, telomere length is estimated to decrease by around 24.8 – 27.7 base pairs per year (220, 221).

When a telomere reaches a critical length, it brings about a realization of the 'Hayflick limit', meaning the cell cannot divide any further, entering a state of 'cellular senescence' (218). Ultimately, this means that the body is less able to replace old and damaged cells as we age,

becoming more vulnerable to disease, with studies showing that progressive telomere shortening has consequences for age-related phenotypes (218, 219, 222-224).

Research suggests that shorter telomere length relative to one's chronological age is associated with all-cause mortality (225), as well as risk for age-related disease and associated risk factors, including: cardiovascular disease (226), coronary artery disease (227), type-2 diabetes (228), major depression (229), chronic low-grade inflammation ("inflamm-aging") (230), chronic obstructive pulmonary disease (231), and Alzheimer's disease (232). Association, however, is not equivalent to *causation*. So far, genetic studies employing Mendelian randomisation designs provide robust inference that genetic risk for shorter telomere length directly increases risk for Alzheimer's disease (233), early-onset major depression (234), coronary artery disease (235), and cognitive ageing (236).

1.6.2.4 ENVIRONMENTAL FACTORS INFLUENCING TELOMERE LENGTH

Environmental factors have also been shown to strongly influence telomere length, these include: stress, childhood maltreatment, pessimism, shorter sleep duration and poorer sleep quality (237-241). In terms of dietary intake, a higher intake of vitamins, cereal fiber and vegetable intake have been associated with longer telomeres; and higher total fat intake, linoleic acid and phenylalanine have been associated with shorter telomeres (242-245). In addition, studies have shown that exercise and meditation are both associated with longer telomeres, and that these activities are especially good at buffering the effects of chronic stress (246, 247). It should be noted that the biological mechanisms, inflammation and oxidative stress are thought to be primary contributors to diet-induced telomere shortening.

1.6.2.5 STRESS AND TELOMERE SHORTENING

Stress is a known risk factor for age-related diseases such as cardiovascular disease and stroke, possibly via a reduction in telomerase and advanced telomere shortening (248). Epel and

colleagues (249) report that women with the highest levels of perceived stress have leukocyte telomere length equivalent to being ten years shorter, relative to women with lower levels of perceived stress (248). Research on childhood trauma has consistently shown that individuals who report childhood maltreatment have significantly shorter telomeres compared to controls (241, 248, 250-252). In addition, this effect on telomeres may begin very early in life, with Entringer and colleagues (253) showing that exposure to maternal stress during foetal development is associated with shorter telomere length in adulthood (251). The biological repercussion of environmental stress could be increased inflammation and oxidative stress, as studies have shown that childhood maltreatment is associated with heightened levels of pro-inflammatory modulators and shorter telomeres in adulthood (250, 252, 254).

1.6.2.6 INFLAMMATION AND TELOMERE SHORTENING

Transient changes in inflammation due to environmental stress is a natural consequence of HPA axis activation (121, 255, 256). However, repeated exposure to environmental stressors such as childhood maltreatment means that the HPA axis remains active over long periods of time, damaging the negative feedback loop necessary to return cortisol and inflammatory modulators (257) to a basal level, and altering the stress response in the long-term (122, 258). This results in increased levels of circulating cortisol and pro-inflammatory cytokines, and it is this increase in pro-inflammatory cytokines that has been associated with telomere shortening and a decrease in telomerase activity (259, 260).

1.6.2.7 OXIDATIVE STRESS AND TELOMERE SHORTENING

Oxidative stress has also been named as a major risk factor for increased telomere shortening (261). Oxidative stress is caused by reactive oxygen species (ROS), which are generated by external factors such as UV radiation and pollution (261). Although the body produces antioxidant defenses to protect against oxidative damage, an imbalance between antioxidant

defenses and ROS production may result in DNA damage and telomere loss (261, 262). More specifically, ROS can cause breaks in the telomeric repeat region, meaning that DNA cannot be replicated beyond this break during the next round of cell division, losing the sequence beyond the break and making the new chromosome significantly shorter (261, 262). This ultimately means that cellular senescence is reached sooner.

1.6.2.8 TELOMERES AND PSYCHIATRIC DISORDERS

Psychiatric disorders such as MDD, BD and SCZ have been associated with an increased risk of severe age-related medical conditions throughout a person's life, including cardiovascular disease, diabetes, stroke and respiratory problems (11, 25, 263-268). Interestingly, psychiatric disorder patients also exhibit advanced telomere shortening (269-271), suggesting that psychiatric disorders may be associated with advanced cellular ageing, and several studies investigating telomeres support this notion.

The largest meta-analysis involving 14,827 people reported shorter LTL in patients with MDD, BD and SCZ, anxiety disorder and post-traumatic stress disorder (PTSD), supporting the notion that psychiatric disorders and risk for comorbid age-related conditions may be mediated by shorter telomere lengths (272, 273). Despite this association, further research is still needed to establish cause and effect, and to determine whether genetic or environmental factors explain the relationship between psychiatric disorder risk and telomere length.

1.6.2.9 TELOMERE LENGTH AND THE BRAIN

Given the relationship between telomere length and psychiatric disorder risk there have been several studies investigating the potential impacts telomere length has on the brain. For instance, a recent functional MRI study revealed that longer buccal telomere length predicted higher levels of activation in the amygdala and cuneus during a facial affect recognition task

(274), suggesting a relationship between telomere length and brain function. However, perhaps the most well studied relationship has been between telomere length and brain volume.

The largest telomere neuroimaging study to-date has been performed by King and colleagues (2014) and revealed strong positive correlations between telomere length and many individual brain areas. Several studies have now replicated the relationship between telomere length and hippocampal volume, an area of the brain strongly implicated in psychiatric disorder aetiology. For instance, Powell and colleagues (2018) revealed a strong association between shorter buccal telomere length and reduced hippocampal volume in adults, see **Figure 1.6.7**. The authors' suggested that shorter telomere length in the periphery may mirror what's found in hippocampal progenitor cells, corresponding to their reduced proliferative potential and subsequently their ability to maintain hippocampal volume (275, 276).

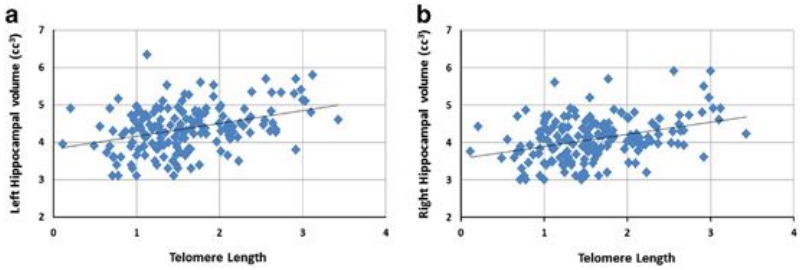

Figure 1.6.7 – The association between telomere length, hippocampal volume and memory. These scatter charts show that telomere length is positively correlated with both left (a) and right (b) hippocampal volume, in a study of 217 individuals. Taken from Powell et al (2018)

Postmortem studies have also investigated the relationship between telomere length and the hippocampus. For instance, Mamdani and colleagues (2015) reported significantly shorter telomere length in the hippocampi of MDD patients and showed that this was associated with altered levels of gene expression in neuroprotective genes (277). They hypothesize that MDD can be characterized by stress-mediated and accelerated ageing of the brain and their findings

further support the hypothesis that shorter telomeres in the hippocampus (as measured by shorter LTL) may be associated with MDD.

1.6.2.10 NEURAL STEM CELL NICHE

Although most somatic cells do not contain enough telomerase in order to counteract telomere shortening, stem cells are one type of cell which do exhibit detectable levels of telomerase activity. In germline stem cells the loss of telomeric DNA is counteracted by telomerase, which maintains telomeres at a maximal level throughout life. In contrast, most other adult stem cells only have enough telomerase to maintain telomeres indefinitely and although they display telomere shortening with age, although this happens at a much slower rate compared to somatic cells (278, 279).

Adult stem cells reside in stem cell niches and telomerase is carefully regulated in these regions, so not to be continuously expressed (280). These regions are responsible for the production of hematopoietic, epithelial, intestinal and neural stem cells, and aberrant telomere maintenance and telomerase regulation in these niches has been associated with a number of diseases and psychiatric disorders (281-284).

The adult neural stem cell niche in the brain is found in the sub-granular zone (SGZ) of the dentate gyrus in the hippocampus, and is responsible for hippocampal neurogenesis, a biological process implicated in learning, memory and emotional regulation (139, 285-287).

Given that hippocampal progenitor cells (HPCs in this part of the brain are thought to generate new neurons throughout life, it is perhaps unsurprising that telomere length in the hippocampus has been the focus of much research in recent years. A study by Zhou and colleagues (2011) showed that chronic mild stress can cause depressive-like behaviours in mice, impair hippocampal neurogenesis, lower telomerase activity and shorten telomeres (288). They also

showed that over-expressing intra-hippocampal telomerase upregulates hippocampal neurogenesis, increases telomere length and produces antidepressant-like behaviours in these mice (288). It should be noted that although studies investigating telomeres and neurogenesis using animal models have shown great potential, these studies have not yet been translated to humans, with most studies using either post-mortem tissue or brain tumor samples (289).

1.6.3 TELOMERE LENGTH AS A THERAPEUTIC TARGET

Given that telomere length has been implicated as a causal risk factor in several age-related diseases, there has been recent focus on whether therapies could target telomere length and in doing so elicit anti-ageing effects. One of the most well studied ways to elongate telomeres is via telomerase activation, though medications have also gained traction as potential telomere modulators.

1.6.3.1 TELOMERASE

In 1984, Carol Greider and Elizabeth Blackburn validated the existence of the enzyme telomerase, describing a protein domain able to synthesize DNA using a native RNA comprised of a complementary sequence to one DNA sequence of a telomere (290).

Telomerase is a reverse transcriptase enzyme comprised of an innate RNA molecule, which is used as a template when elongating telomeres (291). It is also organized into a ring configuration allowing it to move along the chromosome and bind new nucleotides together. Telomerase RNA component (TERC) and telomerase reverse transcriptase (181) are the most important subunits of telomerase, with TERC serving as a template for reverse transcription and TERT helping add new nucleotides to the TTAGGG sequence, see **Figure 1.6.8**. The TERT and TERC genes are located on different chromosomes and mutations in these genes have been associated with a number of conditions such as idiopathic pulmonary fibrosis, bone marrow failure and ischemic stroke (10, 292, 293). Ultimately these disorders are characterized

by very short telomeres, suggesting that short and dysfunctional telomeres play a big role in the pathogenesis of disease states.

It should be noted that a number of immortalized stem cell lines used in cell biology research (such as lymphoblastoid cell lines) achieve their endless replicative phenotype via continual maintenance of telomerase (294). These lineage restricted cells show overexpression of human telomerase, which allows them to divide indefinitely and give rise to phenotypically restricted cells (294).

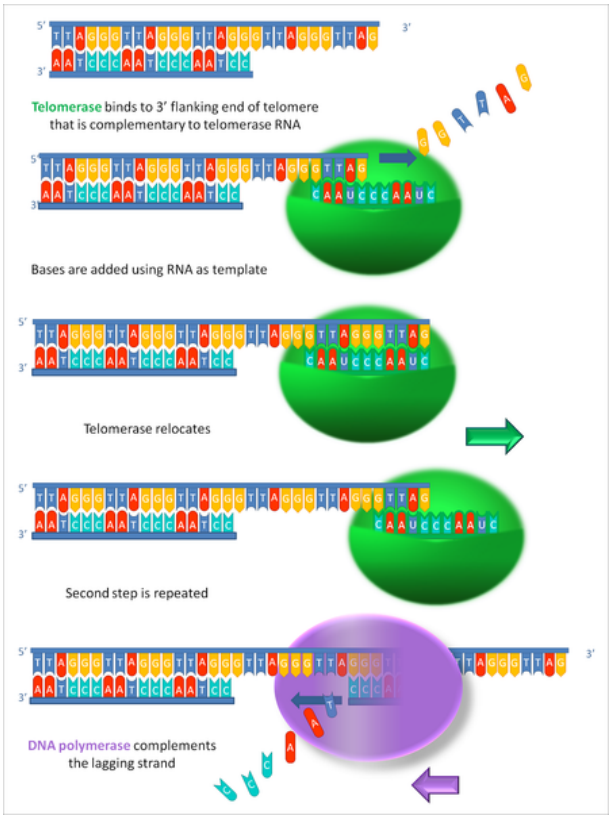

Figure 1.6.8 – A diagram showing how telomerase elongates telomeres.
This diagram shows that telomerase binds to the 3' end of the leading stand, and using its innate complementary RNA as a template, it adds bases to the leading strand. Once this is complete, telomerase detaches and moves progressively further along the leading strand, helping to elongate the DNA until it reaches the end of the strand. DNA polymerase then extends the lagging strand in a 5' to 3' direction and the fragments are joined together using DNA ligase. Taken from: https://www.khanacademy.org/science/biology/dna-as-the-genetic-material/dna-replication/a/telomeres-telomerase.

1.6.3.2 TELOMERASE AS A THERAPEUTIC TARGET

A recent study showed that *Terc*-deficient mice have shorter telomeres compared to wild-type, alongside reduced longevity, pre-mature appearances of different pathologies and a reduction in hippocampal neurogenesis (295). However, this study also showed that telomerase gene therapy can ameliorate the effects of neurodegeneration associated with shorter telomeres, treating these pathologies effectively and increasing hippocampal neurogenesis. This study suggests that telomerase has potential to be used as a therapeutic agent in the future, being able to increase hippocampal neurogenesis and treat associated disorders.

However, it should be noted that therapeutic utility of telomerase is not very straightforward. Although animal studies have shown that telomerase therapy can delay pathologies, prevent cognitive decline and delay age-associated conditions, off-target effects of compounds that activate telomerase are a major concern (296, 297). In particular, these compounds seem to lead to strong activation of mitogenic pathways that may drive cancer (298). In fact, it is important to study telomerase activation and telomere length regulation in compounds and drugs that promote cell division, in order to minimize off-target effects. For example, antidepressant use has been shown to increase cell division in the hippocampus as one of its major targets (299), with presumed off-target effects including increased proliferation in blood (300, 301). Given this proliferative effect, it would be useful to study the consequences this may have on telomere length and the long-term proliferative capacity of these cells (302).

However, direct delivery of telomerase activating agents via gene therapy does show promise for the future, as off-target effects are minimized (297). In addition, a recent study proposed mRNA as a way to activate telomerase in specific areas, without affecting the rest of the body, showing further promise for the future of telomerase therapy (303).

Nevertheless, there are strong correlations between telomerase activation and most cancers, highlighting the importance of safe strategies for controllable and transient telomerase activation in humans (304).

1.6.3.3 ANTI-AGEING DRUGS

It should be mentioned that drugs commonly used in the treatment of medical conditions may also possess anti-ageing properties either via their effects on telomerase or via unknown mechanisms. For instance, metformin has been shown to significantly extend lifespan in C. elegans, although there is no such evidence in human studies (305). The molecular mechanisms of metformin include a reduction in ROS, DNA damage and inflammation, as well as activation of AMP-activated kinase (AMPK) signaling, a signaling pathway often implicated in longevity (306). Although few studies have examined the effect of metformin on telomere length, a recent study by Garcia-Martin and colleagues (2018) reported that both metformin and insulin treatments prevent placental telomere attrition in boys exposed to maternal diabetes (307). More recently, attention is being drawn to the bipolar disorder drug, lithium, due to its apparent effects on telomere length, health and longevity.

1.6.3.4 LITHIUM AS AN ANTI-AGEING DRUG

Lithium is a metal naturally found in drinking water as well as a primary medication for the treatment of bipolar disorder. Animal studies have shown that lithium can extend longevity by around 25% in C. elegans and reduce amyloid-beta build up in Drosophila, increasing their lifespan, see **Figure 1.6.9** (308, 309).

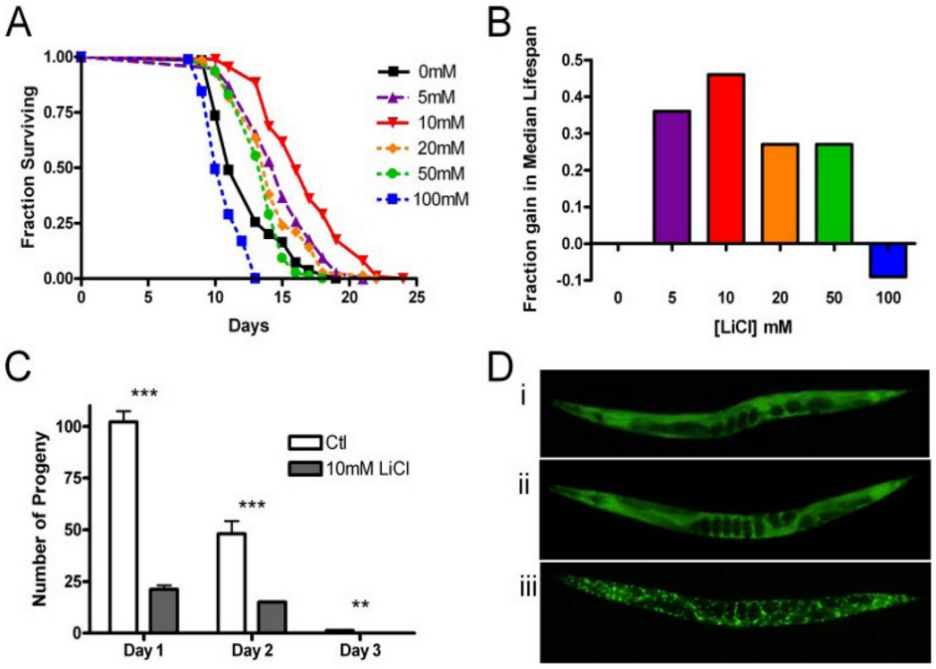

Figure 1.6.9 – The effects of lithium chloride on wild-type *C. elegans* lifespan.

(A) The survival curves of worm populations exposed to 0 – 100 mM of lithium chloride. All concentrations apart from the highest (100 mM) resulted in extended lifespan compared to the 0 mM group. (B) The fraction gain in median survival (in days) of wild-type population cultured on 0 – 100 mM lithium chloride, compared to the 0 mM group. Taken from McColl et al (2008).

Human population studies also support the beneficial effects of lithium. For instance, low-level lithium exposure in drinking water has been studied, with research revealing an inverse correlation between drinking water lithium concentrations and all-cause mortality in 18 Japanese neighborhoods consisting of 1,206,174 individuals, see **Figure 1.6.10** (310). Low level lithium exposure in drinking water has also recently been associated with a decrease in the incidence of dementia, an age-related disorder, see **Figure 1.6.11** (311); and animal studies have shown that lithium can increase hippocampal neurogenesis and increase cognitive functions in mice that suffer from Alzheimer's disease pathology (312).

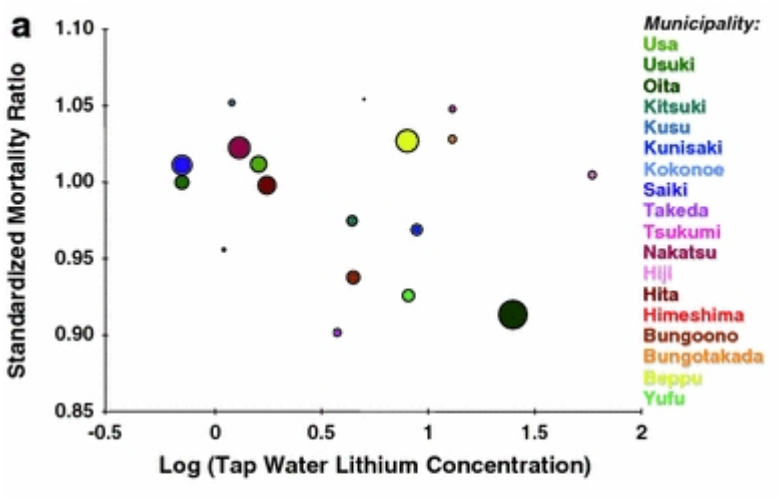

Figure 1.6.10 – Low-dose lithium exposure and mortality.

A chart showing data from 18 Japanese municipalities (coded on the right-hand side), displaying the numbers of inhabitants by the circle diameters. The x-axis shows log-median tap water lithium concentrations and the y-axis shows the standardized mortality ratio, which is defined as the number of deaths observed by the number of deaths expected. Mortality was found to be inversely correlated with lithium concentration. Taken from Zarse et al 2008.

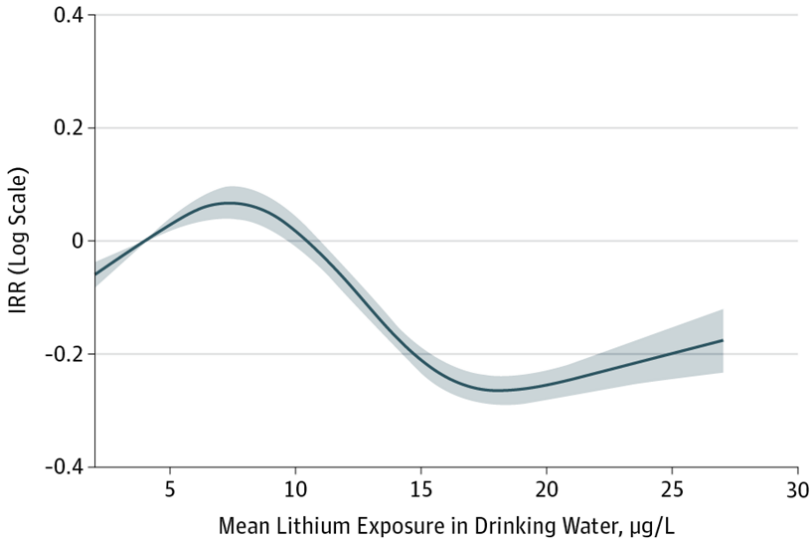

Figure 1.6.11 – Association of lithium in drinking water with the incidence of dementia.

Association between mean lithium exposure in drinking water on a continuous scale (x-axis) and the overall dementia rate (y-axis) data are displayed as the incidence rate ratio (IRR) in relation to the mean (4 µg/L) of the lowest group on a logarithmic scale. The shaded areas indicate a 95% confidence interval. The data shows that as the mean lithium exposure in drinking water increase, the IRR decreases. Taken from Kessing et al (2017).

Although the mechanism via which lithium confers these anti-ageing benefits is still unclear recent research implicates telomere length. For instance, Powell and colleagues showed that chronic lithium use is associated with longer telomere lengths in bipolar patients compared to non-lithium users, see **Figure 1.6.12** (313). Other studies have shown a similar effect, suggesting that long-term lithium use as well as lithium response is associated with longer telomeres in bipolar patients (314, 315).

Given that the hippocampus is one of the key brain regions involved in mood and cognition (316), and mood disorders are often associated with a reduction in adult hippocampal neurogenesis (317), it is possible that lithium is able to confer its mood-stabilising effects by increasing hippocampal neurogenesis over long periods of time by elongating telomeres and prolonging the proliferative potential of progenitor cells (313, 318). This is supported by neuroimaging studies which demonstrate younger 'brain age' and larger hippocampal volumes amongst bipolar disorder patients treated with lithium, relative to bipolar disorder patients not treated with lithium (319-321). Animal studies have further hinted that increases in telomerase activity in the hippocampus may be one mechanism via which lithium confers its effects (322). Nevertheless, further research is needed to fully understand the precise effects of lithium on hippocampal neurogenesis and telomere length restoration.

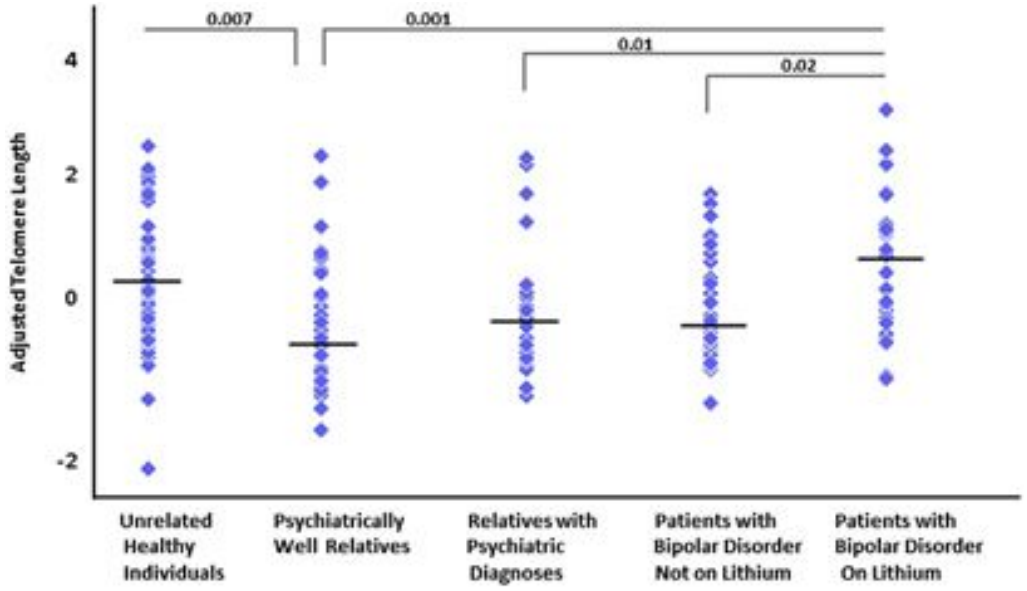

Figure 1.6.12 – Telomere length and bipolar disorder.

A scatterplot showing the distribution of adjusted telomere length in a sample of bipolar disorder patients, their relatives and unrelated healthy individuals. Telomere length is shown to be shorter in bipolar disorder patients not on lithium, psychiatrically well relatives and in relatives with psychiatric diagnoses compared to controls, suggesting that bipolar disorder is associated with shorter telomeres and that this phenotype may be heritable. Lithium-treated bipolar disorder patients show longer telomere length compared with patients with bipolar disorder not on lithium and relatives regardless of psychiatric status, suggesting that lithium may be associated with longer telomeres. Taken from Powell et al (2018).

1.6.3.5 Summary: What we know and what still needs to be studied

Telomeres have become the focus of much research in the fields of gerontology and psychiatry in recent years. Both genetic and environmental factors may mediate the association between telomere length and psychiatric disorder risk. Furthermore, the influence of telomere length on the proliferation of hippocampal progenitor cells, and subsequently hippocampal volume, could be one mechanism contributing to this aforementioned association. Lithium is an effective mood-stabiliser which may reverse some of the age-related alterations concurrent with psychiatric disorder diagnoses, including lengthening telomeres and increasing hippocampal neurogenesis.

However, we do not yet know whether genetic risk for MDD, BD and SCZ is directly associated with shorter telomere length, or whether other external environmental factors are involved in shortening telomeres amongst patients. In addition, telomere GWAS has not yet been explored using genetic correlations to study the relationship with age-related diseases. Nor have gene-level enrichment analyses been performed, which allow researchers to better understand the biological risk mechanisms associated with trait and identify possible treatment targets. Finally, no study has yet explored the direct effects of long-term lithium exposure on human hippocampal cells, studying lithium's effect on telomere length, telomere-associated transcripts, cell proliferation and differentiation.

1.6.4 HIPPOCAMPAL NEUROGENESIS

1.6.4.1 THE HIPPOCAMPUS AND MOOD CONTROL

The hippocampus is a complex structure located below the cerebral cortex, divided into the left and the right hippocampi, according to the two hemispheres (323). It is also comprised of a number of subfields, which have distinct cellular compositions and functions (324). The three core regions are the dentate gyrus, the cornu ammonis (CA), and the subicular complex, with the CA and the dentate gyrus being separated by the hippocampal fissure (324).

The dentate gyrus is at the core of the hippocampus, receiving input of electrical activity from the entorhinal cortex via the preforant pathway (which connects the entorhinal cortex with all hippocampal subfields) (325). It is often considered as the first step of information processing in the hippocampus, characterized by a lack of pyramidal neurons and instead consisting of small granule cells and other neuronal species (326). The granule cells are located in the granule cell layer of the dentate gyrus, with the granule cell dendrites residing in the molecular layer (327). Below the granule cell layer is the sub-granular zone (SGZ), which is where a pool of stem cells reside and is the location of adult hippocampal neurogenesis (AHN) (327).

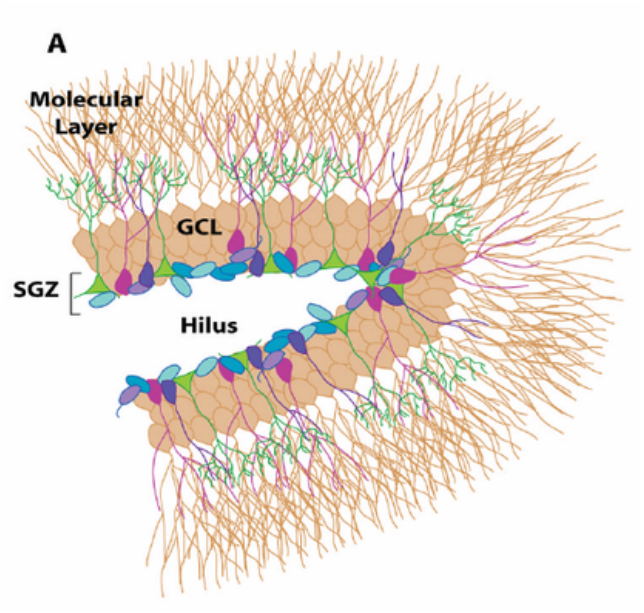

Figure 1.6.13 – The subgranular zone of the hippocampus.
The subgranular zone is located at the border of the hippocampal dentate gyrus granule cell layer and hilus. The neurogenic subranular zone contains cells at various stages of neurogenesis. New-born neurons migrate from the subgranular zone to the granule cell layer and develop the morphology of hippocampal granule cells, extending dendrites into the molecular layer of the dentate gyrus, growing axons into the CA3 region, and forming synaptic connections. Eventually, these neurons are integrated into the hippocampal circuitry as matured granule cells. Taken from Eisch et al (2008).

The subicular complex is the major output center of the hippocampus and has been shown to influence activity in a number of brain regions (328), whereas the CA regions are made of up densely packed pyramidal neurons, and are subdivided into CA1, CA2, CA3 and CA4. The regions are defined according to cell size and synaptic connections and are involved in information processing (323). For example, the CA3 and CA2 fields contain larger pyramidal cells compared to CA1 and CA4.

It has long been established that the hippocampus plays an important role in memory and cognition (329). In addition, the hippocampus is known to be one of the moderators of the HPA axis, acting as a feedback site for corticosteroids (330). More recently the hippocampus has been identified as a brain region responsible for mood regulation, with a number of studies

suggesting that aberrant hippocampal functioning is responsible for symptoms observed in disorders just as depression and bipolar disorder (331).

Human postmortem studies have shown changes in glial cell density and decreased neuronal size in brain tissue from patients with MDD (332). These findings coincide with MRI studies, which have shown that depressed patients and patients with bipolar disorder have significantly decreased left and right hippocampal volumes compared to controls (324, 333). Interestingly, stress and subsequent glucocorticoid exposure have been associated with changes in cellular morphology of hippocampal cells in both human and animal studies, as well as an increased risk of developing psychiatric disorders in humans (334-336).

It is therefore hypothesized that risk factors such as stress may increase the risk for depression via reducing hippocampal volume by decreasing cell density and neuronal size (337). Interestingly, hippocampal atrophy has also been correlated with increased severity, length and recurrence of MDD, which further supports this hypothesis (338, 339).

Overall, there is a great deal of evidence implicating the hippocampus in mood disorder etiology. In particular the dentate gyrus, the major neurogenic region of the adult human brain, has been shown to play an important role in the development of these disorders, possibly via adult hippocampal neurogenesis.

1.6.4.2 ADULT HIPPOCAMPAL NEUROGENESIS

Adult hippocampal neurogenesis describes a process by which radial-glia like precursor cells (also known as Type 1 cells), which divide rarely and express neural stem cell marker, give rise to intermediate progenitor cells (also known as Type 2 cells). These then go through a migratory neuroblast-like stage (Stage 3), after which the newborn cell matures and extends dendrites into the molecular layer of the dentate gyrus and the axon to the CA3 region. For

several weeks these cells show increased synaptic plasticity, finally becoming mature granule cell neurons indistinguishable from the older granule cells (285, 340).

There are a number of commonly used protein markers able to identify cells at each stage of neurogenesis. These include Nestin, glial fibrillary acidic protein (341), RY (sex determining region Y) – box 2 (SOX2), proliferating cell nuclear antigen (PCNA), doublecortin (DCX), microtubule-associated protein 2 (MAP2) and neuronal nuclei (NeuN) (342).

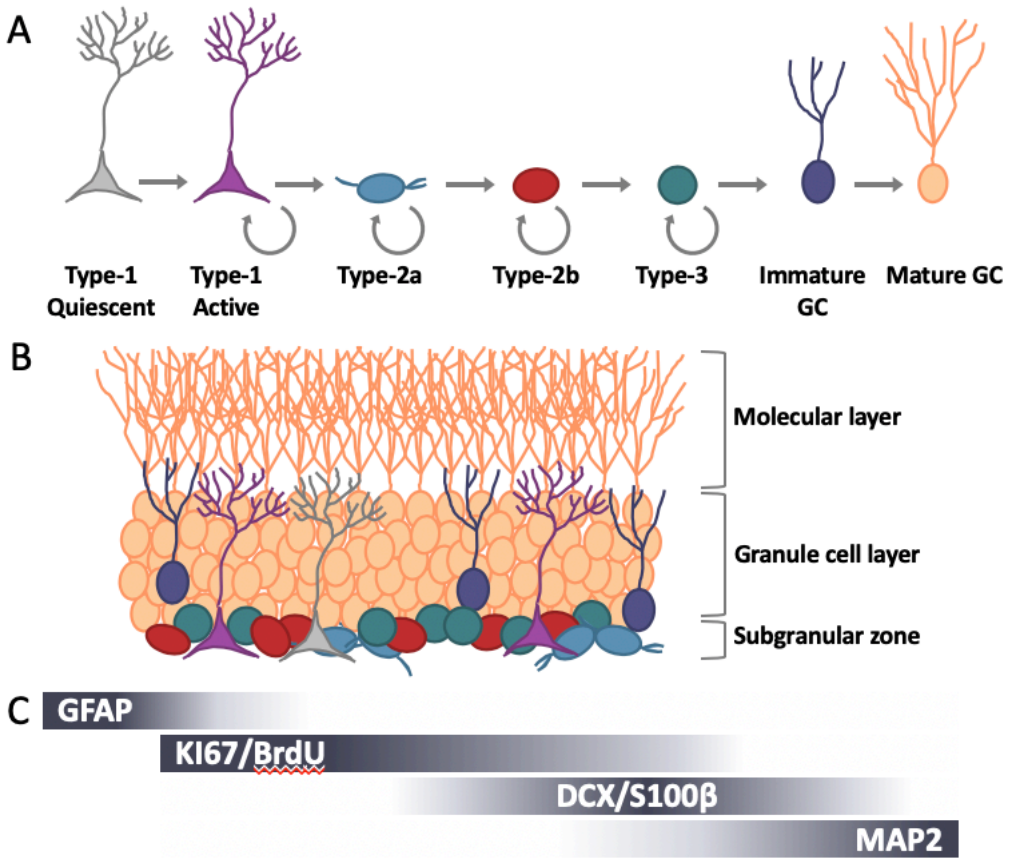

Figure 1.6.14 – Stages of cell maturation during hippocampal neurogenesis and associated markers. (A) Adult hippocampal neurogenesis originates from a population of radial glia-like precursor cells (also known as Type 1 cells) that have astrocytic properties and can switch between a quiescent and active state. They give rise to intermediate progenitor cells with first a glial (Type 2a) and then a neuronal (Type 2b) phenotype. They then go through a migratory neuroblast-like stage (Type 3), after which the new-born and lineage committed cell exits the cell cycle and enters the maturation stage. (B) It is here that they extend their dendrites into the molecular layer and their axons to the CA3 region. They go through a period of several weeks where they show synaptic plasticity, before becoming indistinguishable from other granule cells. (C) Various molecular markers have been developed to identify cells at various stages of hippocampal neurogenesis. Some of these have been used in this thesis. Taken from Smeeth (2019).

Estimates suggest that 35% of total hippocampal neurons are turned over throughout life, with around 700 new neurons being produced each day, in each hemisphere (343). This corresponds to an annual turnover of around 1.75% of cells in the dentate gyrus and implies that 100% of the dentate gyrus granule cells are replaced throughout a human lifespan (343, 344).

AHN is thought to be involved in learning, memory and pattern separation (345). The number of newborn neurons in the dentate gyrus is small, but given the plasticity, excitability and influence of new neurons, it is likely that they have a significant impact on hippocampal functioning. Indeed, the ablation of AHN through irradiation and genetic manipulation has been shown to result in learning and memory deficits in several animal studies (346-348). The animals are also less able to discriminate objects similar to each other and memorize location of food, suggesting a reduction in pattern separation ability (348, 349).

It is also thought that AHN plays an important role in tissue repair following injury. Animal studies using an induced excitotoxicity to model epilepsy, stroke and traumatic brain injury show that there is an increase in AHN following head trauma and that this repair mechanism may reverse some of the damage caused by injury (350, 351).

1.6.4.3 HIPPOCAMPAL NEUROGENESIS AND PSYCHIATRIC DISORDERS

The hippocampal stem cell niche has been implicated in psychiatric disorders such as major depressive disorder, bipolar disorder and schizophrenia (352, 353). Given that these disorders are also often associated with pro-inflammatory cytokines and abnormal hormone levels, it is therefore plausible that hippocampal neurogenesis may be one of the biological end-points for symptom development.

The neurogenic theory of depression states that environmental stress such as childhood maltreatment can impair AHN, resulting in depressive symptoms. Animal studies have shown

support for this theory, with rodents displaying depressive symptoms following chronic stressors, prenatal stress, chronic social defeat and glucocorticoid administration, as well as a reduction in hippocampal neurogenesis (354-356). It should be noted that stress has also been shown to negatively impact the hippocampus without implicating AHN (357). Interestingly, a recent study by Anacker and colleagues (2018) showed that the inhibition of adult-born neurons in the ventral dentate gyrus promotes susceptibility to social defeat stress, whereas increasing neurogenesis promotes resilience to chronic stress, in mice (358).

In humans, there is a strong positive association between childhood trauma and psychiatric disorder symptoms in adulthood (9, 150, 359). Although the effect is stronger for childhood trauma, traumatic events such as parental loss in adulthood are also associated with an increased risk for psychiatric disorders (360). Imaging studies have shown that these traumatic experiences are correlated with smaller hippocampal volume, suggesting long-lasting effects of developmental experiences on hippocampal structure (361, 362). Given that early-life stress has been shown to inhibit AHN, this has become the focus of much research in recent years.

Cellular studies investigating mood regulation have shown that stress-induced glucocorticoid levels are associated with a reduction in AHN, which could be responsible for the retraction of dendrites, the loss of glial cells and ultimately the reduction in hippocampal volume observed in psychiatric disorder patients (363-366). These cellular experiments further support the postmortem and MRI studies and suggest that increasing AHN may be one way to combat psychiatric disorders and show improvements in mood control.

Indeed, there is some evidence suggesting that environmental enrichment can lead to antidepressant-like effects in adults and that this is correlated with an increase in AHN (367-369). It should be noted that these results are mixed, with a debase as to whether it is the

positive events, or simply the lack of negative events during development that affect neurogenesis and behavior in adulthood.

Antidepressant drugs as well as therapeutic interventions have also shown positive effects on cell proliferation and neurogenesis in the dentate gyrus, first demonstrated in adult rodents (370-372). SSRIs such as citalopram and escitalopram, tricyclics such as imipramine and mood stabilizers such as lithium have been shown to increase neurogenesis and affect cell maturation and cell survival (312, 318, 373, 374). Animal studies suggest that following severe stress as a result of maternal separation, antidepressants can increase neurogenesis as well as increased levels of BDNF in the dentate gyrus and the CA3 region of the hippocampus and that these increases are associated with mood improvements (354, 375).

1.6.4.4 HIPPOCAMPAL NEUROGENESIS AND AGEING

Hippocampal neurogenesis has also been implicated in ageing, particularly with regards to cognitive decline and the etiology of neurodegenerative disorders. Studies have shown a decrease in the number of radial glial cells and intermediate progenitor cells in the DG of animals and humans as they age, which has in turn been associated with a decrease in cognitive function (343, 376, 377). Interestingly, the severity of cognitive decline with age has also been linked with the amount of adult neurogenesis in the hippocampi of rodents and non-human primates, suggesting a direct association (378). Although cognitive abilities linked to hippocampal neurogenesis have been shown to decline in humans as well, it is not clear whether this has a direct association with AHN.

It should be noted that the degree to which neurogenesis declines with age is still debated in the field. Given that most studies on hippocampal neurogenesis are conducted in carefully controlled animal experiments, it is perhaps unsurprising that some human post-mortem studies do not show concurrent results. For example, a recent study by Sorrels and colleagues (2018)

suggested that neurogenesis in humans drops to undetectable levels during childhood, whereas a study by Boldrini and colleagues (2018) stated the opposite and reported ongoing adulthood neurogenesis in human (379, 380). Nevertheless, leading scientists in the field argue that that hippocampal neurogenesis does occur throughout life, declines with age, and that different measurement tools may give different results in human tissue (381, 382).

What has become clear however, is that disorders associated with a loss of cognitive function, namely dementia, AD and Parkinson's disorder, are characterized by the dysregulation of hippocampal neurogenesis, possibly via functional deficits associated with the DG (383-385). In addition, pattern-separation ability, which is a robust measure associated with hippocampal neurogenesis, has been shown to diminish with increasing age and in particular in patients with disorders such as AD (386). Thus, understanding the dysregulation of AHN may be beneficial for a large population of patients suffering from a number of debilitating disorders.

Interestingly, voluntary running and environmental enrichment in rodents has been shown to increase cell proliferation in aged mice, suggesting that environmental cues can increase hippocampal neurogenesis in older brains (387, 388). In addition, exercise has been shown to reduce symptoms of AD and increase AHN in animal models, suggesting that the same effect could be seen in humans (389, 390).

Interestingly lithium has been associated with improvements in mood regulation, a lower incidence of AD and increased longevity in human and animal studies, possibly via increased AHN (312, 318, 322). Pharmacological treatments for boosting AHN are an important area of research as they may be beneficial for people who do not have the ability to exercise.

1.6.4.5 HIPPOCAMPAL NEUROGENESIS AND LITHIUM

Mood disorders have been associated with a reduction in hippocampal volume, possibly via aberrant AHN (317, 391-393). However, lithium, a mainstay in the treatment of mood disorders, has been associated with an increase in hippocampal neurogenesis in animal studies as well as increased hippocampal volume in humans (318). In addition, lithium has been shown to facilitate neurogenesis and cognitive functions in an animal model of AD (312).

Lithium has also been shown to increase telomere function and telomerase expression in the hippocampus of a well-defined animal model of depression. Given that AHN requires careful regulation of telomerase, this suggests lithium may play an important role in regulating telomerase in the hippocampus, which in turn can increase telomere length and replicative capacity of hippocampal progenitor cells, ultimately leading to increased AHN, mood improvements and increased cognition (322). Studies have also shown that lithium may promote the differentiation of neural stem cells towards dopaminergic neurons in a model of PD, suggesting that it may play a role in both proliferation and differentiation (394, 395).

1.6.4.6 SUMMARY

The hippocampus is a key region involved in learning, memory and mood regulation. It is also one of the only parts of the brain that is able to produce new cells throughout adult life, via a process known as adult hippocampal neurogenesis. Aberrant AHN has been associated with a number of psychiatric disorders such as MDD and BD as well as age-related neurological conditions such as AD and PD. This is perhaps unsurprising, as AHN has been shown to decrease with age, possibly increasing the risk for these disorders. However, environmental risk factors such as stress and poor lifestyle have been shown to severely decrease AHN independent of age, as well as increasing the risk for psychiatric disorders.

Together, this suggests that AHN may be a key region involved in psychiatric disorder etiology as well as age-related neurological conditions, especially given that psychiatric disorders in old

age have been associated with increased risk for AD and PD. Enriched environments have been shown to increase AHN in rodents, and antidepressants have been shown to increase AHN in both animal and cellular experiments, leading to antidepressant-like effects. This suggests that increasing AHN could be a key treatment strategy for mood disorders and age-related neurological conditions in animals and humans.

Lithium is a drug that is commonly used for treating mood disorders. It has also been associated with a reduction in AD risk and increased longevity in humans. To date, no study has investigated the effects of lithium using human hippocampal progenitor cells to determine whether chronic lithium treatment has a direct effect on hippocampal neurogenesis via cell proliferation and differentiation in older cells (396), and whether this coincides with an increase in telomere length.

1.7 AIMS AND HYPOTHESES

1.7.1 INFLAMMATION IN PSYCHIATRIC DISORDERS

AIM 1 (CHAPTER 2): To determine the effect of childhood maltreatment on circulating levels of 41 inflammatory markers in healthy individuals and patients suffering from major depressive disorder.

HYPOTHESIS 1: We hypothesize that patients suffering from major depressive disorder who have been subject to childhood maltreatment will show higher levels of circulating pro-inflammatory cytokines compared to non-maltreated patients and healthy individuals exposed to maltreatment, after controlling for a range of factors such as BMI, smoking, antidepressant use and age.

AIM 2 (CHAPTER 3): To examine whether having a high polygenic risk score for major depressive disorder or BMI is associated with higher circulating inflammatory marker levels, including those classically associated with MDD (IL-6, CRP, TNF).

HYPOTHESIS 2: Recent large-scale studies have revealed a stronger relationship between body mass index and inflammation, than MDD and inflammation. Consequently, we hypothesize a stronger positive association between polygenic risk for BMI and circulating levels of pro-inflammatory cytokines, than between polygenic risk for MDD and pro-inflammatory cytokines.

1.7.2 TELOMERE LENGTH IN PSYCHIATRIC DISORDERS

AIM 3 (CHAPTER 4): To determine if the relationship between shorter telomere length and psychiatric disorder risk is driven by shared genetic factors, or whether other non-genetic factors have a stronger effect on telomere length.

HYPOTHESIS 3: We hypothesize that a higher polygenic risk for major depressive disorder, bipolar and schizophrenia in an unaffected cohort of individuals will be associated with shorter leukocyte telomere length, after controlling for a wide range of external factors such as age, gender, smoking status, antidepressant use and BMI. Given the link between antidepressants and cell proliferation, we also hypothesize that antidepressant use may also be associated with shorter leukocyte telomere length.

AIM 4 (CHAPTER 5): To better understand the genetic regulation of telomere length

HYPOTHESIS 4: We hypothesize that by using a gene-level analysis of the biggest telomere GWAS, we can get a deeper understanding of the genetic regulatory mechanisms for telomere length. We also hypothesize that telomere length is a polygenic trait and that by using a

polygenic approach, more variance in telomere length can be predicted in an independent sample compared to the variance predicted by only the significant genes from the largest telomere GWAS. Finally, we hypothesize that by using genetic correlations, we can confirm a genetic relationship between telomere length and age-related phenotypes.

AIM 5 (CHAPTER 5): To confirm that long-term lithium use is associated with longer telomere length.

HYPOTHESIS 5: We hypothesize that the duration of lithium use in bipolar disorder patients is positively correlated with telomere length. In addition, we hypothesize that polygenic factors responsible for regulating telomere length will moderate the ability of lithium to lengthen telomeres.

1.7.3 HIPPOCAMPAL NEUROGENESIS IN PSYCHIATRIC DISORDERS

AIM 6 (CHAPTER 6): To better understand the relationship between telomere shortening and hippocampal cell proliferation, which may explain the reduction in hippocampal neurogenesis and hippocampal volume with age.

HYPOTHESES 6: We hypothesize that as hippocampal progenitor cells are aged (via increased passaging), they exhibit telomere shortening which is in turn associated with a reduction in hippocampal cell proliferation.

AIM 7 (CHAPTER 6): To better understand how lithium confers its anti-ageing and neuroprotective properties.

HYPOTHESIS 7: We hypothesize that lithium may have neuroprotective effects on hippocampal progenitor cells by elongating telomeres and increasing cell proliferation and differentiation.

2 – ASSOCIATIONS BETWEEN CHILDHOOD MALTREATMENT AND INFLAMMATORY MARKERS

Figure 1.7.1 – The childhood trauma art collection.
This artwork is taken from The Childhood Trauma Collection, which contains 250 artworks made by adults sho have experienced physical, mental and sexual abuse as children. It provides a platform to understand the experience and the impact of childhood trauma. Taken from: www.dxacentre.org/collection/childhood-trauma-collection

2.1 PREFACE

Several studies have reported an association between MDD and elevated levels of circulating pro-inflammatory markers such as IL-6 and CRP (194, 397). In addition, studies have shown that people who report experiences of childhood maltreatment are more likely to show higher levels of these circulating pro-inflammatory markers (17, 19, 398, 399). Given that childhood maltreatment is one of the strongest environmental risk factors for developing MDD (400, 401), an increase in levels of pro-inflammatory markers as a consequence of childhood maltreatment could be a putative biological mechanism for MDD aetiology. Interestingly, a recent large-scale analysis of 42 inflammatory markers in over 1000 MDD cases and non-MDD controls found no significant associations, instead revealing a very strong effect of BMI on circulating pro-inflammatory marker levels (199). Other studies have shown that BMI is indeed a strong confounding factor when studying inflammatory mechanisms and adipose tissue itself is a source of pro-inflammatory cytokine release (257, 402-405).

This study aimed to investigate whether individuals who have been subject to childhood maltreatment and who subsequently developed MDD are more likely to show increased levels of circulating pro-inflammatory cytokines compared to non-maltreated individuals or healthy controls, or whether BMI is present as a strong confounding factor in this relationship. We screened for 41 inflammatory markers in 164 patients clinically diagnosed with MDD and 301 controls taken from the general population, controlling for a wide range of factors such as BMI, age, ethnicity, gender, antidepressant use and smoking status, to investigate the moderating effect of childhood maltreatment.

The study was published in the British Journal of Psychiatry Open (doi: 10.1192/bjo.2018.80) on the 4th of January 2019. Demographic data and blood from MDD cases and healthy controls was collected by the antiDepressants in Depression and South East London Community Health

Study, respectively. Inflammatory markers were screened by Dr Timothy Powell. All other analyses were carried out by me.

Associations between childhood maltreatment and inflammatory markers

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Background

Childhood maltreatment is one of the strongest predictors of adulthood depression and alterations to circulating levels of inflammatory markers is one putative mechanism mediating risk or resilience.

Aims

To determine the effects of childhood maltreatment on circulating levels of 41 inflammatory markers in healthy individuals and those with a major depressive disorder (MDD) diagnosis.

Method

We investigated the association of childhood maltreatment with levels of 41 inflammatory markers in two groups, 164 patients with MDD and 301 controls, using multiplex electrochemiluminescence methods applied to blood serum.

Results

Childhood maltreatment was not associated with altered inflammatory markers in either group after multiple testing correction. Body mass index (BMI) exerted strong effects on interleukin-6 and C-reactive protein levels in those with MDD.

Conclusions

Childhood maltreatment did not exert effects on inflammatory marker levels in either the participants with MDD or the control group in our study. Our results instead highlight the more pertinent influence of BMI.

Declaration of interest

D.A.C. and H.W. work for Eli Lilly Inc. R.N. has received speaker fees from Sunovion, Jansen and Lundbeck. G.B. has received consultancy fees and funding from Eli Lilly. R.H.M.-W. has received consultancy fees or has a financial relationship with AstraZeneca, Bristol-Myers Squibb, Cyberonics, Eli Lilly, Ferrer, Janssen-Cilag, Lundbeck, MyTomorrows, Otsuka, Pfizer, Pulse, Roche, Servier, SPIMACO and Sunovian. I.M.A. has received consultancy fees or has a financial relationship with Alkermes, Lundbeck, Lundbeck/Otsuka, and Servier. S.W. has sat on an advisory board for Sunovion, Allergan and has received speaker fees from Astra Zeneca. A.H.Y. has received honoraria for speaking from Astra Zeneca, Lundbeck, Eli Lilly, Sunovion; honoraria for consulting from Allergan, Livanova and Lundbeck, Sunovion, Janssen; and research grant support from Janssen. A.J.C. has received honoraria for speaking from Astra Zeneca, honoraria for consulting with Allergan, Livanova and Lundbeck and research grant support from Lundbeck.

Keywords

Depressive disorders; inflammation; maltreatment.

Worldwide, an estimated 25% of adults have reported physical abuse in childhood. In the UK, the most comprehensive overview of child protection states that there were 47 008 sexual offences and 10 136 cruelty and neglect offences recorded against children under the age of 16 in 2014/15.[1] Childhood maltreatment has been associated with a wide range of negative health consequences, psychosocial outcomes and heightened risk for psychiatric disorders; including anxiety disorder, bipolar disorder, delinquent behaviour, impaired cognitive development and particularly depression.[2–4] Evidence suggests that numerous biological mechanisms become activated in response to maltreatment (for example epigenetic changes, telomere erosion, cortisol dysregulation, inflammation), which alone or in combination may explain the increased vulnerability to disorders such as depression, among adults who have been maltreated.[5]

Current understanding

Immunoinflammatory activation, and an increased release of proinflammatory cytokines, is one biological mechanism associated with childhood maltreatment and an area of growing interest in psychiatry. Cytokines play an important role in brain development and affect neurogenesis, synaptic remodelling and neurotransmitter systems to produce behavioural change.[6,7] Many studies have reported an increase in proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumour necrosis factor-alpha and increases in the acute phase protein C-reactive protein (CRP) in patients with major depressive disorder (MDD) and among those exposed to maltreatment.[8] Whereas, others have reported protective effects of anti-inflammatory cytokines such as IL-10.[9]

Aims

The current study investigated the association of inflammatory marker levels in response to childhood maltreatment. Among patients with MDD, we tested whether individuals who had been maltreated had specific differences in levels of inflammatory markers compared with those patients with MDD who had not experienced maltreatment. The rationale for this was to determine if individuals with MDD who had been maltreated represent those with an inflammatory subtype of depression; which could lead to differential diagnoses (for example depression with risk for inflammatory disease) and subsequently novel intervention strategies (such as anti-inflammatory adjuvants).

Among control participants, we tested whether individuals who had been maltreated had altered inflammatory marker levels, compared to those individuals who had not been maltreated. The rationale for this was to identify whether alterations to specific

components of the immune system might mark MDD resilience in response to maltreatment, and therefore hint towards a novel treatment strategy.

We addressed these aims by assessing 41 inflammatory markers in a homogeneous treatment-resistant MDD cohort recruited as part of the Antiglucocorticoid augmentation (metyrapone) of antiDepressants in Depression (ADD) study ($n = 164$), and screened controls recruited as part of the South East London Community Health Study (SELCoH, $n = 301$). In the case and control groups separately, we investigated the association of inflammatory markers with the presence of childhood maltreatment.

Method

Participants

Peripheral blood samples used in this study were obtained by venepuncture as part of two separate UK studies. Controls were recruited as part of SELCoH and participants with MDD were recruited as part of the ADD study. Childhood maltreatment information was collected within both studies. After collection, serum from both studies were stored at −80°C until required. Participant information relating to each study is detailed in Table 1 and a description of each study is given below.

Control group

The participants in the control group were recruited as part of SELCoH, which is a study in London, UK investigating mental and physical health in the general South East London population.[10] Participants in this study received detailed and repeated phenotypic assessments as part of three separate phases. The first phase was carried out to assess common health disorders and mental health disorders in South East London; the second phase aimed to examine the roles of historical social context and policy in shaping patterns of health inequalities; and in the third phase, a number of biological specimens were collected from a subset of participants including blood for serum separation. All phases collected information on psychiatric disorder symptoms. Control participants were defined as those who showed no MDD symptoms in any of the three phases of SELCoH, determined using the Clinical Interview Schedule-Revised,[11] and who had no previous diagnosis of depression, determined by a self-report questionnaire.

Table 1 Characteristics of the case and control groups

Characteristic	Case group, ADD participants	Control group, SELCoH participants
n	164	301
Age, years: mean (s.d.)	47.38 (9.63)	48.50 (16.14)
Gender, % men	40.9	47.5
Body mass index, mean (s.d.)	31.54 (6.87)	26.93 (5.24)
Ethnicity, %		
Black	1.8	20.9
White	94.5	75.4
Other	3.7	3.7
Current episode, *n*	164	0
On antidepressant, *n*	164	0
Current smoker, *n*	57	48
No maltreatment	56	256
Maltreatment	108	45

ADD, Antiglucocorticoid augmentation (metyrapone) of antiDepressants in Depression (ADD) study; SELCoH, South East London Community Health Study.

Case group

The participants with MDD (case group) were recruited as part of the ADD study, which was a clinical trial that aimed to assess the efficacy of metyrapone (a cortisol synthesis inhibitor) as an adjuvant to selective serotonin reuptake inhibitors (SSRIs) in treating MDD in those previously shown not to respond to at least two forms of treatment (treatment-refractory MDD).[12] Participants were recruited from multiple UK centres, which included Manchester, Leeds, Bradford and Newcastle.

Major depression diagnoses were defined using DSM-IV criteria[13] and assessed using the Structured Clinical Interview for DSM Disorders research version.[14] Further eligibility criteria required participants to have a Hamilton Rating Scale for Depression (HRSD) score greater than 18;[15] have a Massachusetts General Hospital Treatment Resistant Depression staging score of 2–10;[16] be currently taking an SSRI; be aged between 18 and 65; not have alcohol or drug dependence; be free of physical comorbidities (untreated hypothyroidism, disorders of steroid production, cardiac failure, angina, myocardial infarction, renal failure in the past 3 years); and not take a medication that would contraindicate metyrapone. We utilised blood serum collected during the screening phase of the study.

Mild and major depression symptoms at the time of blood collection, were differentiated using the HRSD, where HRSD scores of 18–19 were considered mild symptoms (no participants were recruited with a HRSD <18), and HRSD scores of 20 and above were considered moderate–severe symptoms, as described previously.[15]

Childhood maltreatment measure

The presence of childhood maltreatment was determined using the Childhood Trauma Questionnaire (CTQ).[17] CTQ data in our datasets were positively skewed and remained non-normal even after log-transformation. Consequently, we generated ordinal mean maltreatment measures,[17] which, because of the small numbers of individuals with severe maltreatment, we collapsed into 'no maltreatment' (0) and 'maltreatment' (mild, moderate or severe maltreatment) (1), (Table 1).

Ethics

For the ADD study, clinical trial authorisation was given by the Medicines and Healthcare products Regulatory Agency (MHRA: EudraCT: 2009-015165-31). Ethical approval was granted by the Sunderland Local Research Ethics Committee (REC reference number 10/H0904/9). The SELCoH study received approval from the King's College London research ethics committee, reference PNM/12/13-152. Participants from both studies provided written informed consent.

Inflammatory marker quantification

Upon use, serum was thawed at room temperature and 41 inflammatory markers were quantified simultaneously using multiplex enzyme-linked immunosorbent assay-based technology provided by the Meso Scale Discovery V-PLEX Plus Human Biomarker 40-Plex kit, and a customised human duplex kit assaying brain-derived neurotrophic factor and interferon-alpha, as described previously.[18] Seven-point standard curves were run in duplicate on each plate in order to calculate absolute pg/mL values for the 80 samples assayed per plate, and a no-template control was used to correct for background fluorescence. Case and control groups were randomised across batches, and plates were scanned on the Mesoscale Scale Discovery Meso Quickplex SQ 120 reader at the Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, Psychology and Neuroscience, King's College London.

Pilot studies revealed very high intraplate (r>0.99) and interplate (r>0.97) correlations, suggesting single measurements were acceptably reliable using this methodology. Furthermore, known quantities within the standard curves used on each plate, correlated very highly with quantities predicted by fluorescence intensity (r>0.99).

Statistical analysis

Data processing

Standard curves were used to determine absolute quantities (pg/mL) of each inflammatory marker. Absolute quantities (pg/mL) were then log-transformed to allow for parametric analyses. Subsequently, data points were removed if they exceeded plus or minus 2 standard deviations from the mean. We excluded inflammatory markers where greater than 30% of data was missing.

Maltreatment analyses

For the case and control groups separately, we performed linear regressions with log-protein level as the dependent variable and childhood maltreatment as the independent variable, alongside ethnicity, smoking, antidepressant use, study site (ADD study), plate/batch effects, gender, current depressive episode severity (ADD study), age and BMI as covariates. Within our analyses we applied the Bonferroni method of multiple testing correction, in order to minimise risk for false associations.

Sensitivity analyses

We individually tested the potential mediating/confounding effects of physical illness (type 2 diabetes, arthritis, cardiovascular disease, stroke, high blood pressure and cancer) and socioeconomic status, within the SELCoH study, where this data was available. Physical illness information was obtained via self-report. Socioeconomic status was determined based on an individual's type of employment: manual work, non-manual work, unemployed and economically inactive (such as retired, full-time parents, students or those unable to work because of disability). Based on our previous work (which included SELCoH),[18] we also attempted to replicate the effects of BMI on levels of CRP and IL-6 (commonly shown to be associated with maltreatment and MDD) in the ADD study, using the same model as above.

Results

Inflammatory markers adequately detected in serum

Using our methodology, 34 inflammatory proteins passed our quality control criteria. Seven inflammatory markers were found to have greater than 30% missing data from across the whole sample and were removed from any downstream analyses (macrophage inflammatory protein-1a, granulocyte-macrophage colony-stimulating factor , IL-1a, IL-13, IL-1b, IL-2, IL-4). See Fig. 1 for a summary of detectable inflammatory markers. Known quantities within the standard curves used on all plates, correlated very highly with quantities predicted by fluorescence intensity (r>0.99), results also showed acceptably low levels of intraplate and interplate variability based on coefficient of variation metrics. For further details on correlations between different inflammatory markers, and assay variability metrics, see supplementary Tables 1 and 2 available at https://doi.org/10.1192/bjo.2018.80.

Effect of maltreatment on inflammatory marker levels in case and control groups

We found no significant associations between childhood maltreatment and levels of inflammatory markers in controls (Table 2). We found one nominally significant association between maltreatment and levels of an inflammatory marker in the MDD case group, whereby, childhood maltreatment was associated with a reduction in circulating levels of serum amyloid A in adulthood ($F(1, 143) = 4.837$, $P = 0.029$, variance explained, 3.3%). This effect did not remain significant following multiple testing correction (Table 2).

Sensitivity analyses

We found no significant effects of physical illness, or socioeconomic status on inflammatory markers (P>0.05). We replicated our previous work, showing strong positive correlations between BMI and CRP levels ($F(1, 86) = 29.489$, $P = 5.137 \times 10^{-7}$, variance explained, 25.5%), and between BMI and IL-6 levels ($F(1, 85) = 32.994$, $P = 1.403 \times 10^{-7}$, variance explained, 28%), in the ADD study.

Discussion

Main findings

Inflammation is one putative risk mechanism linking childhood maltreatment to adulthood depression. This study sought to investigate whether childhood maltreatment evokes differential effects on inflammatory markers among participants with MDD and controls. We studied the effects of maltreatment on 41 inflammatory markers in a UK sample of individuals with MDD and screened controls while accounting for a broad range of confounding factors.

We found no significant associations between childhood maltreatment and inflammatory markers in the control group, suggesting that inflammation may not represent a mechanism conferring resilience to MDD in response to maltreatment. Likewise, we did not find a significant association between childhood maltreatment and inflammatory markers among the case group, suggesting there may not be an inflammatory subtype of MDD related to childhood maltreatment exposure.

Interpretation of our findings

Our negative results differ from the more common reports of higher CRP and IL-6 levels in those with a history of childhood maltreatment.[3,19] A lack of replication here could relate to the fact that not all studies have covaried for a broad range of confounding factors as we do. For instance, studies have revealed that childhood maltreatment is associated with higher adulthood BMI, and consequently this could partially mediate previously reported associations.[20–22] Indeed, we have recently reported major influences of BMI (as well as other factors) on inflammatory marker levels, namely CRP and IL-6, which once covaried for, removes MDD case–control differences in inflammatory marker levels.[18] Here, we replicate the effects of BMI on CRP and IL-6 levels in the ADD study, confirming the necessity to appropriately covary for BMI and other confounders in statistical models relating to inflammatory measures.

The effect of BMI on inflammatory levels likely results from the fact that adipose tissue is known to release adipokines and proinflammatory cytokines.[23–25] Therefore, regardless of what causes heightened inflammation among the participants with MDD or individuals who were maltreated, weight management via balanced

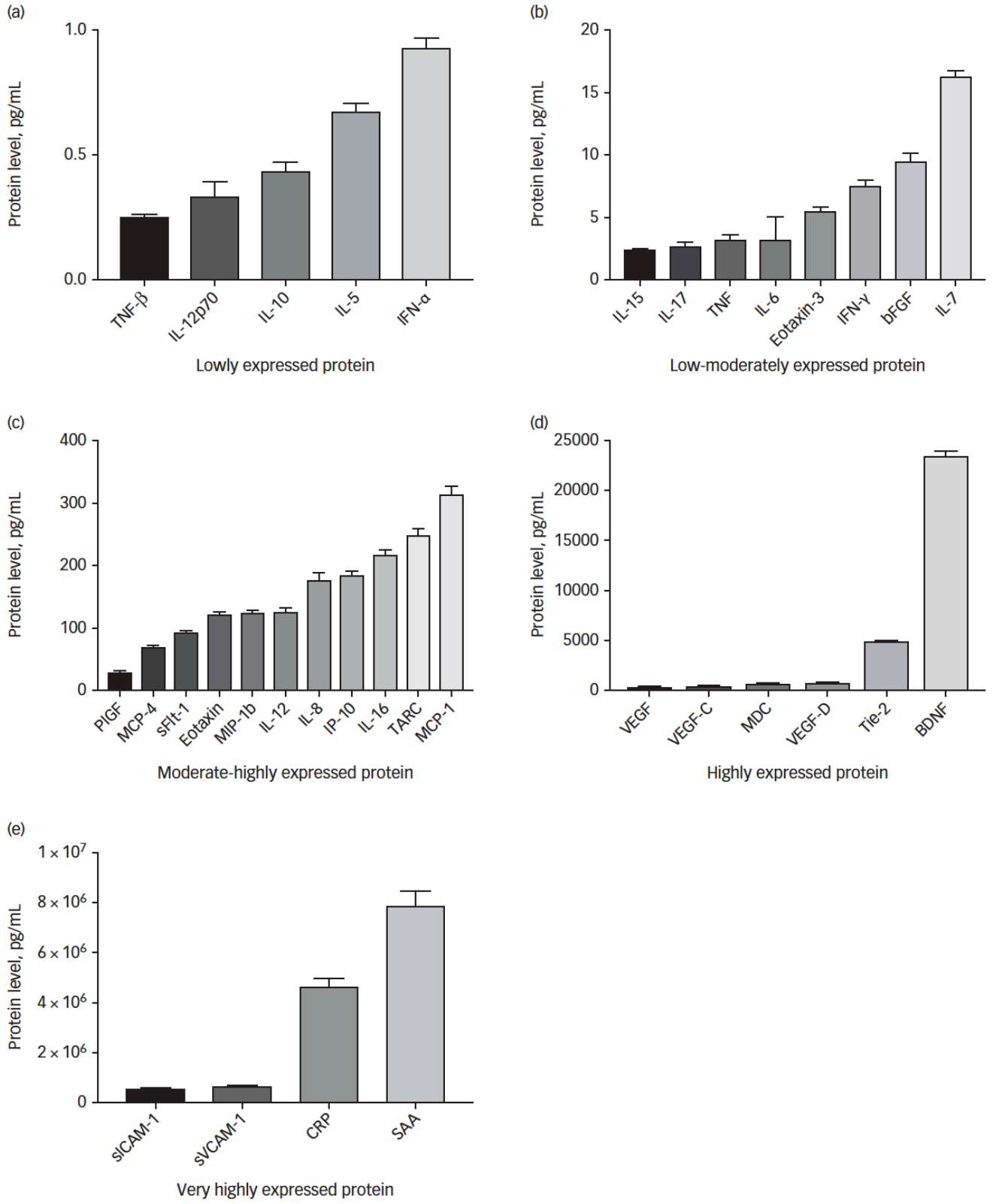

Fig. 1 Detectable inflammatory markers.
(a) Lowly expressed protein, < 1 pg/mL; (b) low–moderately expressed protein, 1 – 20 pg/mL; (c) moderate–highly expressed protein, 21 – 400 pg/mL; (d) highly expressed protein, 401 – 25,000 pg/mL; (e) very highly expressed protein, 25,001 – 100,000,000 pg/mL. Bars represent the mean and error bars represent the standard error of the mean. TNF, tumour necrosis factor; IL, interleukin; IFN, interferon; bFGF, basic fibroblast growth factor; PIGF, phosphatidylinositol glycan biosynthesis class F protein; MCP, monocyte chemoattractant protein 4; sFLT, soluble fms-like tyrosine kinase; MIP, macrophage inflammatory protein; IP, induced protein; TARC, chemokine (C-C motif) ligand 17 (also known as CCL17); VEGF, vascular endothelial growth factor; MDC, macrophage-derived chemokine; Tie, tyrosine kinases with immunoglobulin-like and EGF-like domains; BDNF, brain-derived neurotrophic factor; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; CRP, C-reactive protein; SAA, serum amyloid A.

Table 2 Analysis of associations between childhood maltreatment and levels of inflammatory markers in the case and control groups

	Control group				Case group			
Marker name and abbreviation	*F*	d.f.	*P*	Variance explained, %	*F*	d.f.	*P*	Variance explained, %
Phosphatidylinositol glycan biosynthesis class F protein (PIGF)	2.564	279	0.110	0.9	0.032	145	0.859	0.0
Tyrosine kinases with immunoglobulin-like and EGF-like domains (Tie)-2	2.705	281	0.101	1.0	1.399	147	0.239	0.9
Vascular endothelial growth factor (VEGF)	2.049	277	0.153	0.7	1.212	133	0.273	0.9
VEGF-C	0.056	283	0.813	0.0	0.001	133	0.979	0.0
VEGF-D	2.523	280	0.113	0.9	0.875	128	0.351	0.7
Basic fibroblast growth factor (bFGF)	0.003	281	0.955	0.0	0.144	122	0.705	0.1
Soluble fms-like tyrosine kinase (sFlt-1)	0.072	280	0.788	0.0	0.255	146	0.614	0.2
Eotaxin	0.594	275	0.442	0.2	0.014	142	0.906	0.0
Eotaxin-3	2.016	245	0.157	0.8	0.035	120	0.851	0.0
Induced protein (IP)-10	0.481	277	0.488	0.2	1.226	133	0.270	0.9
Monocyte chemoattractant protein (MCP)-1	2.830	283	0.094	1.0	0.022	143	0.882	0.0
MCP-4	0.955	275	0.329	0.3	0.509	137	0.477	0.4
Macrophage-derived chemokine (MDC)	0.365	276	0.546	0.1	0.024	134	0.876	0.0
Macrophage inflammatory protein (MIP)-1b	0.200	279	0.655	0.1	0.463	134	0.497	0.3
Chemokine (C-C motif) ligand 17 (TARC)[a]	0.174	282	0.677	0.1	0.812	137	0.369	0.6
Brain-derived neurotrophic factor (BDNF)	0.536	268	0.465	0.2	0.983	130	0.323	0.8
Interferon (IFN)-alpha	2.870	215	0.092	1.3	0.000	97	0.985	0.0
Interleukin (IL)-12	0.012	283	0.912	0.0	0.532	144	0.467	0.4
IL-15	0.763	281	0.383	0.3	0.137	145	0.712	0.1
IL-16	2.106	284	0.148	0.7	3.051	133	0.083	2.2
IL-17	2.039	265	0.154	0.8	0.031	140	0.861	0.0
IL-5	3.007	196	0.084	1.5	2.750	131	0.100	2.1
IL-7	1.015	284	0.314	0.4	0.141	136	0.708	0.1
Tumour necrosis factor (TNF)-beta	0.344	268	0.558	0.1	1.717	129	0.192	1.3
IFN-gamma	0.033	270	0.855	0.0	0.492	140	0.484	0.4
IL-10	0.103	265	0.749	0.0	0.230	133	0.632	0.2
IL-12p70	0.096	251	0.757	0.0	2.286	114	0.133	2.0
IL-6	0.030	265	0.864	0.0	1.693	138	0.195	1.2
IL-8	2.969	286	0.086	1.0	2.222	130	0.139	1.7
TNF	0.464	282	0.496	0.2	0.073	139	0.787	0.1
C-reactive protein (CRP)	0.002	272	0.960	0.0	3.770	141	0.054	2.6
Serum amyloid A (SAA)	1.301	266	0.255	0.5	4.837	143	0.029	3.3
Soluble intercellular adhesion molecule (sICAM)-1	1.717	272	0.191	0.6	1.363	147	0.245	0.9
Soluble vascular cell adhesion molecule (sVCAM)-1	0.530	269	0.467	0.2	0.858	148	0.356	0.6

a. Also known as CCL17.

diets and regular exercise, may be one method to reduce excessive inflammation.

Strengths and limitations

The strengths of the current study include the fact we assessed a broad range of inflammatory proteins using validated electrochemiluminescence methods; we used a well-characterised method of assessment for childhood maltreatment subtypes; we statistically corrected for a number of confounding factors and performed appropriate sensitivity analyses; and we performed analyses in a screened control group and a homogeneous treatment-resistant MDD cohort.

However, the study also has a number of limitations that should be acknowledged. First, and foremost, our study is of a cross-sectional design capturing inflammation levels only in adulthood. A longitudinal design would allow one to determine the temporal ordering of events, and measure how maltreatment has an impact on inflammation immediately, during adolescence and then in adulthood; identifying acute and persistent effects, if any, on inflammatory markers. Second, maltreatment was captured as a binary variable and we were underpowered to assess the effects of maltreatment severity. As the majority of a individuals who had been maltreated in our two samples experienced mild–moderate maltreatment as opposed to severe maltreatment, it is possible that the biological embedding effects of stress are less penetrant in our sample, which is why we did not observe broad effects on inflammatory marker levels.

Third, all the participants with MDD were currently on antidepressant treatment, as per the recruitment criteria. As antidepressants are known to possess some anti-inflammatory properties, it is possible this may be masking the long-term effects of maltreatment on immunoinflammatory function.[26] Fourth, the CTQ measure of childhood maltreatment is widely used and although reliable, arguably lacks validity and is subject to the biases of retrospective recall, especially in individuals with high neuroticism.[27] Despite this, it is important to note that recent research shows a moderate–strong positive correlation between CTQ and prospective measures of maltreatment, collected as part of longitudinal studies, validating the usefulness of the CTQ as a tool for assessing maltreatment severity.[28]

Fifth, there may be other confounding factors influencing inflammatory marker levels that we were not able to include here (such as time and season of blood collection) that may have affected our results. Finally, despite representing one of the larger single studies to date assessing disorder-specific effects of maltreatment, we may still be underpowered; therefore, future studies with even larger sample sizes may be able to confirm our largely negative findings.

In conclusion, our study does not support previous research revealing associations between childhood maltreatment and inflammatory markers in either participants with MDD or controls. Instead, our findings suggest that other factors such as BMI may be more pertinent in influencing inflammatory marker levels.

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Supplementary material

Supplementary material is available online at https://doi.org/10.1192/bjo.2018.80.

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2.2 CHAPTER 2 REFERENCES – ASSOCIATIONS BETWEEN CHILDHOOD MALTREATMENT AND INFLAMMATORY MARKERS

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2.3 POSTFACE

This study was designed to answer the first aim or this thesis, regarding inflammation as a biological mechanism for psychiatric disorder aetiology. We hypothesized that patients suffering from major depressive disorder who have been subject to childhood maltreatment will show higher levels of circulating pro-inflammatory cytokines compared to non-maltreated patients and healthy individuals exposed to maltreatment, after controlling for a range of factors such as BMI, smoking, antidepressant use and age. Our results revealed no significant association, instead showing that BMI is a strong confounding factor that may be having a robust effect on levels of circulating pro-inflammatory cytokines. This is in keeping with the findings by Powell and colleagues (2018), with the additional notion that after controlling for a range of external factors, childhood maltreatment may not be associated with an increase in circulating pro-inflammatory markers in MDD cases.

It is however possible that other confounding factors aside from BMI are also having a strong effect on inflammatory marker levels, as studies have shown that clinical populations suffering from psychiatric disorders are more likely to engage in unhealthy lifestyles (406). Given that we are not able to control for most of these in our clinical population, we decided to use polygenic risk scores as predictors for developing MDD in a healthy population and investigate the association with these 41 inflammatory markers. The same method was used to study the association between polygenic risk scores for BMI and inflammatory markers, given the mounting evidence of the strong confounding effect. By doing this, we are able to overcome many other external factors that could be influencing inflammatory marker levels, as is discussed in the next chapter.

3 – RECONSIDERING THE REASONS FOR HEIGHTENED INFLAMMATION IN MAJOR DEPRESSIVE DISORDER

Figure 2.3.1 – An image depicting inflammation in the brain.
This image represents the ongoing hypothesis that psychiatric disorder may be caused by elevated circulating inflammatory cytokine levels. Taken from:
http://emorymedicinemagazine.emory.edu/issues/2016/spring/briefs/the-big-idea-inflammation/index.html

3.1 INTRODUCTION

The total number of people with major depressive disorder (MDD) exceeded 300 million globally in 2015 and the World Health Organisation currently states that MDD is the single largest contributor to global disability worldwide (6). The pathophysiology of MDD is not yet fully understood, although numerous causal mechanisms have recently been proposed, with some studies suggesting that MDD could manifest as a result of stress-induced aberrant immune functioning in the body, often termed the 'cytokine hypothesis' (407, 408). According to this hypothesis, over-activation of inflammatory pathways can lead to a systemic increase in peripheral immune modulators known as cytokines, which have been shown to affect the brain and induce psychiatric symptoms in both humans and animal models (407, 409). This is supported by research that reveals approximately one third of patients given interferon therapy for the treatment of hepatitis develop depressive symptoms (410, 411). The cytokine hypothesis is also used to explain why MDD patients exhibit heightened levels of acute phase proteins and proinflammatory cytokines such as CRP, IL-6, and TNF-a (194, 412-414).

However, it could also be that inflammation only causes MDD in a small number of cases and that a heightened proinflammatory profile commonly reported amongst patients has a very different cause. For instance, heightened inflammation may be related to lifestyle and health differences that are more frequent amongst MDD patients but not necessarily causal, such as an unhealthy diet, smoking or being overweight (199, 415, 416). In particular, research highlighted in Chapter 1 indicates a strong confounding influence of BMI on inflammation levels (417). Having a better understanding of the effects MDD and heightened BMI risk have on inflammation may be important in reducing risk for comorbid inflammatory-related diseases, such as cardiovascular disease and arthritis amongst MDD patients (202, 418, 419).

High BMI and MDD often co-occur, and inflammation is usually assayed after an MDD diagnosis, making it difficult to study the moderating effects of BMI and to robustly establish whether MDD represents a cause or effect of inflammation. When exploring disease aetiology, one way the effect of confounding factors can be overcome is by using genetic risk scores as a proxy for susceptibility, and studying the impact of genetic risk on biological systems, in a healthy population. Genetic factors play a significant role in determining risk for MDD and adulthood BMI, with studies reporting heritability estimates of around 40% - 50% and 41% - 85% respectively (420, 421). MDD and BMI are both considered to be highly polygenic, meaning that many genetic polymorphisms of small effect size work in tandem to confer a proportion of risk. Individual polymorphisms may have little diagnostic value, but by using summary statistics taken from mega-GWASs such as the one carried out by the PGC or the GIANT consortium, it is now possible to calculate polygenic risk scores for MDD and BMI, for any given individual (85, 94, 111).

In this study we tested whether PRSs for MDD or BMI influence 34 inflammatory marker levels, including those classically associated with MDD diagnosis (IL-6, CRP, TNF). By applying polygenic risk scores for MDD and BMI to a largely disease-free population, we could study the causal effects MDD and BMI have on inflammatory marker levels and study their effect in isolation, without the confounding factors often present in clinical samples such as medication use, higher incidences of smoking, drug use, and various other factors known to be associated with MDD and obesity.

3.2 METHODS

3.2.1 THE SAMPLE

Peripheral blood samples used in this study were collected by venipuncture as part of the South East London Community Health Study (422). SELCoH is a population study in London, UK,

investigating mental and physical health in the general population (423). Participants have so far received detailed phenotypic assessments as part of three separate phases. The first phase was carried out to assess common mental and physical health disorders in South East London; the second phase examined the roles of social context and policy in shaping patterns of health inequalities; and the third phase included the collection of biological specimens including blood for DNA extraction and serum separation. All three phases collected information on psychiatric disorder symptoms. After collection, serum was stored at -80°C until required. Information relating to age, BMI, smoking status, depression status and ethnicity was collected in conjunction with blood samples. For this study we used participants for whom we had both genotype and inflammatory marker data available. See **Table 3.2.1** below for the full sample specification.

Table 3.2.1 – SELCoH Sample specification

Sample	N	Age (SD)	Sex (% male)	BMI (SD)	Ethnicity	Current smoker (N)
SELCoH	406	48.68 (15.04)	45.3	27.3 (5.54)	Black (19.2%) White (71.4%) Other (9.4%)	85

3.2.2 ETHICS

The SELCoH study received ethics approval from the King's College London research ethics committee, reference PNM/12/13-152. Participants all provided written informed consent to taking part in the study.

3.2.3 INFLAMMATORY MARKER QUANTIFICATION

Upon use, serum was thawed at room temperature and 41 inflammatory markers were quantified simultaneously using multiplex ELISA-based technology provided by the Meso

Scale Discovery V-PLEX Plus Human Biomarker 40-Plex kit, and a customised human duplex kit assaying brain-derived neurotrophic factor (BDNF) and IFN-α. Note however, that IL-87 is repeated twice on the 40-plex array (IL-8 and IL-8(HA)) alongside two different standard curves, allowing for a very wide range of IL-8 levels to be detected. We only utilized data from IL-8 (not IL-8(HA)) as our samples were detectable specifically within the range of this standard curve (0.0700 – 498 pg/mL). The 41 captured antibodies are etched to the bottom of five 96-well plates, each capturing between 2 and 10 inflammatory markers. Seven-point standard curves were run in duplicate on each plate in order to calculate absolute pg/mL values for the 80 samples assayed per plate, and a no-template control was used to correct for background fluorescence. Samples were randomised across batches, and plates were scanned on the Mesoscale Scale Discovery MESO Quickplex SQ 120 reader at the MRC SGDP Centre, Institute of Psychiatry, Psychology and Neuroscience, King's College London. Pilot studies revealed very high intra-plate ($r > 0.99$) and inter-plate ($r > 0.97$) correlations, suggesting single measurements were acceptably reliable using this methodology. Furthermore, known quantities within the standard curves used on each plate, correlated very highly with quantities predicted by fluorescence intensity ($r > 0.99$). As noted in Chapter 2, only 34 inflammatory markers were adequately expressed in serum samples and carried forward to subsequent statistical analyses.

3.2.4 DNA EXTRACTION

10 mL of blood was collected from subjects in tubes containing EDTA (BD Vacutainer; BD, NJ, USA) and stored at -80°C. DNA was then extracted using a standard in-house protocol (424) and stored at -80°C. All samples had 260/280 ratios of between 1.7 and 1.9, tested using the Nanodrop D1000 (Thermoscientific, Wilmington, DE).

3.2.5 GENOTYPING & QUALITY CONTROL (TARGET DATASET)

DNA samples were sent to the Affymetrix Research Services Laboratory in Santa Clara, California, USA. Genotyping for SELCoH was performed using the UK Biobank Axiom Array which comprises of 820,967 genetic markers (Affymetrix, California, United States). Genotype data was put through quality control measures as outlined by Coleman et al. (2016), using PLINK v1.07 (425).

3.2.6 POLYGENIC RISK SCORE QUANTIFICATION

3.2.6.1 PRSICE SOFTWARE

Individualised Polygenic Risk Scores (PRS) within our sample were calculated using PRSice, a PRS quantification software (94). The software uses summary results from previously performed, well-powered GWAS (the base dataset) to generate PRS in our sample, SELCoH (the target dataset). Briefly, PRSice works by first clumping SNPs in the genotype PLINK files corresponding to the target dataset and removing those in high linkage disequilibrium, as this can falsely inflate polygenic scores. Subsequently, within the target dataset the number of risk alleles at a particular SNP is multiplied by that SNP's effect size (established in the base dataset), and then all the SNP information is summed. The user can define which SNPs to include in the PRS.

For MDD PRS analyses we set a p-value threshold of p=0.1, as defined by the most recent Psychiatric Genetics Consortium (83) MDD GWAS (85), whereby we included all SNPs under this threshold from our base datasets to calculate polygenic risk scores in our target dataset.

For BMI PRS analyses, the p-value threshold was set to p=0.2, as defined by PRSice, which automatically detects the optimal p-value threshold predicting BMI in a user-defined phenotype file (adjusted for age, ethnicity and sex) corresponding to our cohort. PRSice repeats analyses at many thresholds in order to identify the most predictive threshold and model.

3.2.6.2 BASE DATASETS

The MDD base dataset (summary statistics) was obtained from the PGC website (https://www.med.unc.edu/pgc/results-and-downloads/downloads) and represents the largest GWAS for MDD to-date, consisting of 130,664 MDD cases and 330,470 controls (85).

The BMI base dataset was downloaded from the Giant Consortium website (the specific file is labelled BMI.SNPadjSMK.CombinedSexes.AllAncestry): (https://portals.broadinstitute.org/collaboration/giant/index.php/GIANT_consortium_data_files).

3.2.6.3 POPULATION COVARIATES

To reduce noise in our analyses as a result of ancestry differences within the sample, we generated population covariates (PCs) using multidimensional scaling in PLINK, via the PRSice software, which allowed us to detect and adjust for population structure in our analyses (426, 427). Population covariates were incrementally tested for association via scatter charts (e.g. PC1 vs PS2, PC2 vs PC3, PC3 vs PC4 etc.) until a normal distribution was achieved. A normal distribution was achieved after the first seven PCs, and thus this is what we used in our downstream analyses, see Supplementary Information for more details.

3.2.7 STATISTICAL ANALYSIS

3.2.7.1 DATA PROCESSING

Standard curves were used to determine absolute quantities (pg/mL) of each inflammatory marker. Absolute quantities (pg/mL) were then log-transformed to allow for parametric analyses. Subsequently, data points were removed if they exceeded +/- 2 standard deviations from the mean. We also excluded inflammatory markers where greater than 30% of the data was missing.

3.2.7.2 Major Depressive Disorder Analyses

To test the association between genetic risk for MDD and inflammatory marker levels, we performed general linear regressions with log-protein levels as the dependent variable and a PRS for MDD as the independent variable, alongside ethnicity, smoking, plate/batch effects, gender, age, antidepressant use, BMI and seven population parameters as covariates.

3.2.7.3 Body Mass Index Analyses

First, we tested whether BMI correlated with inflammatory marker levels. Log-protein level was set as the dependent variable and BMI was set as the independent variable, with age, plate/batch effects, gender, ethnicity and smoking as covariates. For those markers significantly affected by BMI, we determined if BMI was likely to be causally associated with levels of inflammatory markers by performing the same regression, but instead of BMI as the independent variable we included PRS for BMI, alongside seven population parameters.

3.2.7.4 Sensitivity Analyses

We performed additional sensitivity analyses to verify the validity of our results. First, because inflammation has been associated with depression and several individuals in our sample have self-reported depressive symptoms, where we found significance, we repeated models to include depression case/control status and depression severity at the time of blood collection as covariates.

Second, we tested whether PRS for MDD and BMI predicted lifetime depression risk in our sample by performing binary logistic regressions, covarying for seven population covariates, age, sex, ethnicity, and in the MDD PRS analysis, BMI.

Third, for any significant associations, we repeated the same model and individually tested the potential mediating/confounding effect of physical illness (type-2 diabetes, arthritis,

cardiovascular disease, stroke, high blood pressure and cancer), socioeconomic status (employment status, educational attainment level) and antidepressant use, all of which were available within the SELCoH study.

3.3 RESULTS

3.3.1 THE EFFECT OF A POLYGENIC RISK FOR MDD ON INFLAMMATORY MARKER EXPRESSION

The first part of our regression analyses investigated the main effect of PRS for MDD on inflammatory marker levels. Our findings revealed that an increase in the polygenic risk for MDD results in a nominally significant increase in IL-10 levels ($F(1, 348) = 5.829$, $P = 0.016$, variance explained = 1.6%); this finding did not survive multiple testing correction, see **Figure 3.3.1**. No other inflammatory markers were found to be significant. See Supplementary Information for a full table of results.

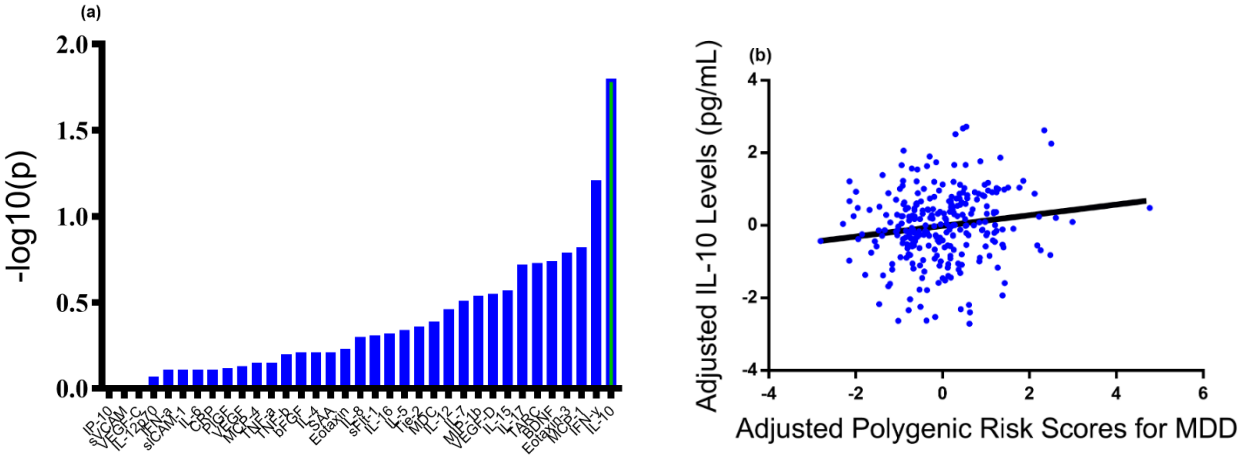

Figure 3.3.1 – The association between a PRS for MDD and inflammatory marker levels.

(a) Bar chart showing the main effect of PRS for MDD on inflammatory marker levels. Inflammatory markers are displayed on the x-axis and negative log(p-values) are displayed on the y-axis. Nominally significant inflammatory markers are displayed in green; (b) scatter chart showing the association between a polygenic risk for MDD and IL-10 levels. Adjusted polygenic risk scores are displayed on the x-axis and negative log(p-value) is displayed on the y-axis.

3.3.2 EFFECTS OF BMI ON INFLAMMATORY MARKER LEVELS

To narrow down which inflammatory markers should be the focus of our BMI PRS analyses, we first investigated the main effect of raw BMI scores on inflammatory marker levels. 16 inflammatory markers showed a significant association; nine of which survived multiple testing correction. See **Figure 3.3.2** (a). We subsequently tested which of these inflammatory makers are significantly affected as a result of a PRS for higher BMI. Our results show that PRS for higher BMI is nominally associated with two inflammatory markers, whereby higher BMI PRS predicts higher amounts of IL-6 ($F(1, 348) = 5.665$, $P = 0.018$, variance explained = 1.6%) and CRP ($F(1, 361) = 6.604$, $P = 0.011$, variance explained = 1.8%), see **Figure 3.3.2** (b). These findings did not survive multiple testing correction. See Supplementary Information for a full table of results.

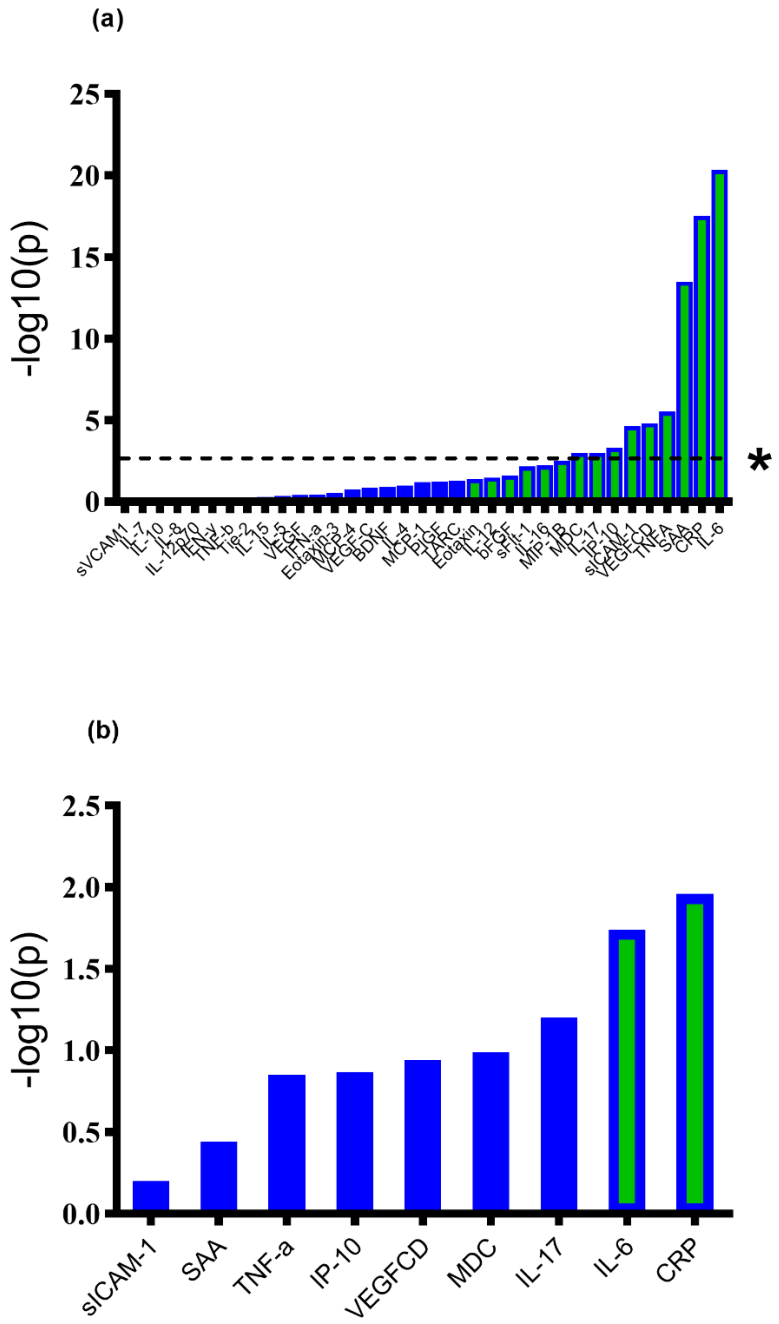

Figure 3.3.2 – Effects of PRS for higher BMI on inflammatory marker levels.

Bar charts showing the effect of BMI and polygenic risk for higher BMI on inflammatory marker levels. Inflammatory markers are displayed on the x-axes and negative log(p-values) are displayed on the y-axes. (a) inflammatory marker levels associated with having higher BMI; (b) inflammatory marker levels associated with PRS for having higher BMI. Inflammatory markers below the nominally significant p-value threshold are displayed in green and the multiple testing significance threshold is marked with a dotted line and an asterisk (*).

3.3.3 SENSITIVITY ANALYSES

Self-reported depression and depression severity had no influence on significant associations, nor did the occurrence of comorbid diseases or socioeconomic status. Neither PRS for MDD or BMI could predict depression caseness in the SELCoH sample (428).

3.4 DISCUSSION

The aim of our study was to discern whether genetic risk for MDD or BMI is causally associated with higher levels of circulating pro-inflammatory cytokines, by using polygenic epidemiology. Our findings indicate that polygenic risk for MDD has minimal effects on inflammatory profiles, only nominally affecting IL-10 levels. It is perhaps surprising that a genetic risk for MDD is not associated with adulthood inflammatory marker levels, in light of the cytokine theory, and given the high number of reports finding higher levels of proinflammatory cytokines in patient blood (429). A lack of significant associations here could relate to the fact that BMI (as well as other factors) can have a very strong influence of inflammatory marker levels, especially CRP and IL-6 (199, 417). This prompted us to study the effects of BMI on inflammatory marker levels, focusing specifically on the genetic risk for BMI as a predictor, to see whether there is shared genetic etiology between higher BMI and inflammatory marker levels.

In contrast to MDD polygenic risk, BMI and polygenic risk for BMI predicted heightened levels of the proinflammatory markers most commonly implicated in MDD, CRP and IL-6 (397, 430, 431). Studies have previously shown that high BMI and larger abdominal adiposity is associated with increased circulating levels of IL-6 and CRP (432, 433), but our study is one of the first to demonstrate this effect using polygenic risk scores for BMI as a predictors. This supports work in Chapter 2 but extends these findings by demonstrating a potential causal role

for BMI in affecting inflammation, above-and-beyond the genetic influences relating to MDD. Given that polygenic risk scores can be generated for individuals from a young age, BMI polygenic risk scores could be a useful tool in identifying those at risk of increased adulthood inflammation and subsequent inflammatory-related conditions.

It is important to note that our study has a number of limitations. First, the study is of cross-sectional design, meaning that we were unable to capture longitudinal changes in inflammatory marker levels. Second, although we controlled for a number of factors in our statistical models, we could be missing important variables that can influence inflammatory marker levels (such as seasonality or current infection status). Third, although our study utilizes a well characterized sample, larger sample sizes are still needed to validate our findings, as the PRS effects on CRP and IL-6 were nominal. Fourth, it should be noted that PRS for BMI may be inherently better at predicting inflammatory marker expression compared to PRS for MDD as BMI is more heritable and therefore captures more of the phenotype compared to MDD, which may explain why we see such robust findings related to pro-inflammatory cytokine release. Finally, although polygenic risk scores are commonly used to infer causality, they are subject to the effects of horizontal pleiotropy and reverse causality which may confound interpretations. Horizontal pleiotropy describes a concept whereby a genetic variant can influence multiple traits, without one trait being mediated by another (434). In the current study for example, we report that genetic variants associated with increased BMI are also associated with increased levels of CRP, although a higher BMI may not actually mediate levels of CRP. Reverse causality on the other hand would imply that increased levels of CRP may be casually affecting an increase in BMI, and not the other way around. Studies with larger sample sizes investigating the genetic risk for inflammatory marker levels may help to further dissect this relationship further.

To conclude, heightened levels of immune modulators such as pro-inflammatory cytokines are strongly associated MDD diagnosis, as well as inflammatory conditions such as cardiovascular disease. Our study is the first to demonstrate that genetic risk for MDD may not be responsible for increased inflammatory marker levels amongst patients; instead our results implicate the importance of BMI and genetic risk for BMI on inflammatory marker profiles.

4 – GENETIC RISK FOR PSYCHIATRIC DISORDERS AND TELOMERE LENGTH

Figure 3.4.1 – Artistic depiction of genetics.
This artwork was created by Julie Huang for the cover of a journal article. It intricately depicts how the double-helix DNA is the building block of humans and connects us all together. Taken from: http://www.uwomj.com/wp-content/uploads/2011/04/backcover1.jpg

4.1 PREFACE

Other than inflammation, this thesis also focuses on telomere length as a biological mechanism for psychiatric disorder aetiology. As described in Chapters 1.6.2 and 1.6.3, telomeres are small DNA repeats on the ends of chromosomes that protect valuable coding DNA from damage during each round of mitosis, but get shorter each time the cell divides (435). This eventually leads to the loss of telomere length, cellular senescence and the inability of the cell to repair old or damaged cells, which is a hallmark of biological ageing (435, 436). Interestingly, psychiatric disorders such as MDD, BD and SCZ are associated with a higher incidence of age-related disorders such as CAD and stroke, with advanced telomere shortening being a potential biological mechanism for both age-related disorder and psychiatric disorder aetiology (304, 437-440). As with the previous chapter, we chose to focus on a non-psychiatric population and use the polygenic risk for MDD, BD and SCZ as a proxy in order to overcome the multitude of external factors that could impact telomere length amongst cases (238, 244, 441).

However, we did wish to investigate the impact of antidepressants on telomere length in this population, as studies have shown that antidepressants work by stimulating cell division, meaning the cell may be subject to accelerated telomere shortening (299, 374). We made use of the fact that a subset of both non-depressed and depressed participants were taking antidepressants in a population cohort, allowing us to tease apart the effects of antidepressants on telomere length and age-related disease, irrespective of depression diagnosis.

The study was published in Frontiers in Genetics (doi: https://doi.org/10.3389/fgene.2018.00468) on the 16th of October 2018. Demographic data and blood were collected by the South East London Community Health Study. All other measures and analyses were carried out by me.

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Genetic Risk for Psychiatric Disorders and Telomere Length

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Background: Previous studies have revealed associations between psychiatric disorder diagnosis and shorter telomere length. Here, we attempt to discern whether genetic risk for psychiatric disorders, or use of pharmacological treatments (i.e., antidepressants), predict shorter telomere length and risk for aging-related disease in a United Kingdom population sample.

Methods: DNA samples from blood were available from 351 participants who were recruited as part of the South East London Community Health (SELCoH) Study, and for which whole-genome genotype data was available. Leukocyte telomere length was characterized using quantitative polymerase chain reactions. Individualized polygenic risk scores for major depressive disorder (MDD), bipolar disorder (BD), and schizophrenia (SCZ) were calculated using Psychiatric Genomics Consortium summary statistics. We subsequently performed linear models, to discern the impact polygenic risk for psychiatric disorders (an etiological risk factor) and antidepressant use (common pharmacological treatment) have on telomere length, whilst accounting for other lifestyle/health factors (e.g., BMI, smoking).

Results: There were no significant associations between polygenic risk for any of the psychiatric disorders tested and telomere length ($p > 0.05$). Antidepressant use was significantly associated with shorter telomere length and this was independent from a depression diagnosis or current depression severity ($p \leq 0.01$). Antidepressant use was also associated with a significantly higher risk of aging-related disease, which was independent from depression diagnosis ($p \leq 0.05$).

Conclusion: Genetic risk for psychiatric disorders is not associated with shorter telomere length. Further studies are now needed to prospectively characterize if antidepressant use increases risk for aging-related disease and telomere shortening, or whether people who age faster and have aging-related diseases are just more likely to be prescribed antidepressants.

Keywords: polygenic risk score, psychiatry, antidepressants, aging, telomeres

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INTRODUCTION

The complex and dynamic relationship between physical illness and psychiatric disorders was highlighted in a Chief Medical Officer's 2013 annual report, which stated that people with a psychiatric disorder experience worse physical health than those without (Davies, 2014). The comorbidity of a long-term physical illness and psychiatric disorder raises total health care costs by at least 45% per person (Naylor et al., 2012), and increases the risk of early mortality (Chang et al., 2011).

Psychiatric disorders such as major depressive disorder (MDD), bipolar disorder (BD), and schizophrenia (SCZ) have all been linked to an increased risk of severe medical conditions throughout a person's life (Kessler et al., 2005; Rai et al., 2014; Kang et al., 2015; Menear et al., 2015). The prevalence of MDD in patients with a physical illness is reported to be around twofold to threefold higher than in the general population and comorbid illnesses such as diabetes, pain, cancer, stroke, and cardiovascular disease have become an increasingly important global health issue (Kessler et al., 2005; Kang et al., 2015; Winkler et al., 2015). A cross-sectional report on patients with schizophrenia has stated that more than 50% of patients with schizophrenia possess at least one comorbid physical illness such as chronic pain, liver disease, and in particular, type-2 diabetes, which can often lead to microvascular and macrovascular complications such as neuropathy, coronary heart disease, and stroke (Chwastiak et al., 2006; Meeuwisse-Pasterkamp et al., 2008; Smith et al., 2013). BD is also associated with medical conditions likely to increase mortality, including respiratory, cardiovascular, and endocrine problems (Kemp et al., 2010; Forty et al., 2014).

Interestingly, medical conditions that show the highest prevalence of comorbidity across these three psychiatric disorders (MDD, BD, and SCZ) tend to be associated with aging; namely cardiovascular disease, stroke, obesity, and type-2 diabetes. This suggests that psychiatric disorders may be associated with faster biological aging, and some studies which assay 'telomeres' support this notion (e.g., Simon et al., 2006).

Telomeres are found on the ends of chromosomes and are special structures that are essential for protecting the chromatin from DNA damage during recombination (de Lange, 2002). Telomeres get shorter with each cell division as a result of the end-replication problem and this progressive telomere shortening is thought to represent a 'molecular clock' which underlies cell aging (Blackburn, 2001; Collins and Mitchell, 2002). Telomere shortening to a critical length, results in the realization of the "Hayflick limit" and a reduction in the ability of cells to divide (Blackburn, 2001). Ultimately, this means that new cells are less able to replace old, damaged cells, and thus the body becomes more vulnerable to aging-related diseases (Blackburn, 2001; Samani et al., 2001; Collins and Mitchell, 2002; Oh et al., 2003; Blasco, 2005).

Shorter telomeres have been demonstrated in patients suffering from MDD, BD, and SCZ compared to controls, leading to speculation that telomeres may play a role in psychiatric disorder etiology (Simon et al., 2006; Lung et al., 2007; Kao et al., 2008; Yu et al., 2008; Hartmann et al., 2010; Elvsåshagen et al., 2011; Wikgren et al., 2012; Lima et al., 2015; Lindqvist et al., 2015; Mamdani et al., 2015). However, understanding whether or not telomere shortening is directly related to the pathophysiology of psychiatric disorders is difficult to determine from classic case-control studies due to confounding factors. Specifically, psychiatric patients recruited to case-control studies are often already taking medications and are significantly more likely to be leading unhealthy lifestyles (e.g., poor diet, smoking), which may impact upon rates of telomere shortening. Subsequently, what may be more informative is to study a known causative risk mechanism for a psychiatric disorders and its association with telomere length, outside the context of the disorder itself and associated environments - polygenic risk scores (PRS) represent one option which may allow us to achieve this.

Polygenic risk scores represent the cumulative effect of many common risk variants for a given trait and are an effective way of quantifying genetic risk for a psychiatric disorder, even in non-clinical population cohorts (Sullivan, 2010; Clarke et al., 2016). PRS have previously been used to better understand the effect genetic risk mechanisms have on biological systems and clinical symptoms (e.g., Alloza et al., 2017). A recent study from our group has shown the importance of studying genetically at-risk, but clinically unaffected individuals, when investigating telomere length differences (Powell et al., 2017a). We previously found that unaffected first-degree relatives of BD patients have shorter telomeres compared to control participants, implying an association between familial risk for BD and shorter telomeres. This effect was not clear amongst BD cases, potentially as a result of lithium use, which was associated with longer telomeres within BD patients. Subsequently, PRS allows us to draw similar comparisons in any given population, with genetic risk being quantified empirically using genome-wide genotype data, as opposed to familial relatedness.

In addition to lithium use, other more commonly used pharmacotherapies have been implicated in affecting telomere length (Lindqvist et al., 2015; Monroy-Jaramillo et al., 2017). Recent studies have revealed that only depressed patients taking antidepressants have significantly shorter telomere lengths relative to controls; with the reports surmising that the effect is likely due to the more severe nature of depression in those requiring medication (Lindqvist et al., 2015; Needham et al., 2015). However, epidemiological studies independently reveal associations between antidepressant use and an increased risk for aging-related disease, which is irrespective of depression diagnosis (Hippisley-Cox et al., 2001; Gareri et al., 2002; Caughey et al., 2010). Thus, there is warrant for further investigation on the effects of antidepressant medication on telomere length, especially when used outside the context of depression, as this would allow one to tease apart the impact of psychiatric diagnosis from the impacts of medication.

Our study comprised of 351 participants from a South East London, United Kingdom population cohort. We aimed to investigate whether: (i) PRS for MDD, BD, or SCZ predicts shorter relative telomere length in the non-clinical majority of the sample; (ii) antidepressants impact on telomere length,

irrespective of depression diagnosis, and (iii) if any significant associations from (i) or (ii) are additionally associated with risk for aging-related disease.

MATERIALS AND METHODS

Participants

A cohort study design was used to address the research questions. A total of 351 participants including 167 males (mean age of 50 (16.6 S.D.)) and 184 females (mean age of 47 (14.1 S.D.)) had their blood samples collected for DNA extraction as part of the South East London Community Health study (SELCoH; Hatch et al., 2011, 2016), see **Table 1**. Depression was the only common clinical psychiatric diagnosis in SELCoH, based on self-report data (n = 61). Current depression severity (at the time of blood collection) was coded as an ordinal measure (0 = no depression symptoms, 1 = mild depression symptoms, 2 = moderate-severe depression symptoms), using the Clinical Interview Schedule-Revised (CIS-R; Lewis et al., 1992), which uses an algorithm to approximate ICD-10 diagnoses (World Health Organisation [WHO], 1993).

To examine the relationship between PRS and telomere length, independent from the confounders of disease factors and medication in SELCoH, we split our sample into those without a reported depression diagnosis (dep−), and those with a reported depression diagnosis (dep+). DNA from participants was extracted from blood samples and this was used to calculate RTL and assay common genetic variation.

Ethics Statement

The SELCoH study received approval from King's College London research ethics committee, reference PNM/12/13-152. Informed written consent was obtained from all participants at the time of sample collection.

TABLE 1 | The characteristics of our sample at the time of blood collection, including gender, age, BMI, smoking status, current antidepressant use and average polygenic risk scores (PRS), in depressed cases (Dep+) and non-depressed controls (Dep−).

	Dep− sample	Dep+ sample
n	290	61
Age	48.43 (15.58)	48.36 (14.39)
Sex (% male)	142 (49)	25 (41)
BMI	27.04 (5.39)	27.99 (6.25)
Ethnicity	White British: 165	White British: 35
	Black Caribbean: 24	Black Caribbean: 6
	Black African: 33	Black African: 3
	White other: 39	White other: 12
	Non-white other: 18	Non-white other: 4
	Mixed: 11	Mixed: 1
Smoking (n)	Never: 122	Never: 14
	Current: 53	Current: 24
	Ex-smoker: 115	Ex-smoker: 23
Currently taking antidepressants (n)	10	30
PRS MDD	−0.0029	0.0137
PRS BD	−0.0141	0.067
PRS SCZ	−0.0164	0.078

Data Availability Statement

Due to ethical restrictions SELCoH data is not publically available. Details on the SELCoH sample and requests to access phenotype data can be made here: http://www.slam.nhs.uk/research/selcoh/selcoh-projects. Access to genetic data requires local approval via the NIHR Bioresource (contact: bioresource@kcl.ac.uk).

Aging-Related Disease

We constructed an ordinal measure for aging-related disease, whereby 0 indicated no reported aging related disease, 1 indicated one reported aging-related disease, and 2 indicated two or more aging related diseases. Aging-related diseases included: type-2 diabetes, arthritis, cardiovascular disease, stroke, high blood pressure, and cancer.

DNA Extraction and Telomere Assessment

10 mL of blood was collected from participants in tubes containing EDTA (BD Vacutainer; BD, NJ, United States) and stored at −80°C. DNA was then extracted using a standard in-house protocol (Freeman et al., 2003) and stored at −80°C. All samples had 260/280 ratios of between 1.7 and 1.9, tested using the Nanodrop D1000 (Thermoscientific, Wilmington, DE, United States).

To assess relative telomere length (RTL), we performed a modified version of a quantitative polymerase reaction (qPCR) protocol by Cawthon (2009), as previously described (Powell et al., 2017a; Vincent et al., 2017). The protocol involves two separate qPCRs performed on separate 384-well plates with DNA samples pipetted into identical wells on each plate. In the first reaction, we assayed the telomere repeat region (TTAGGG). In the second reaction, we assayed a single copy gene, albumin, which we used as an internal control to correct for differences in DNA concentration between samples (Cawthon, 2009). The telomere/albumin ratio was used to calculate RTL.

On each plate, six negative controls consisting of RNase-free water were used to screen for any DNA contamination. An eight-point dilution series using human leukocyte genomic DNA (0.47, 0.94, 1.88, 3.75, 7.5, 15, 30, and 60 ng) was used on each plate to allow for absolute quantification of each sample and to account for any differences in efficiency between the telomere and albumin reactions. All reactions were performed using three technical replicates. Each qPCR mix for the telomere reactions consisted of 10 μL of 2x qPCR Mastermix with SYBR Green (Primer Design, Southampton, United Kingdom), 5 μL or RNase free water, 12 ng of DNA, 1000 nM of telg, 5′-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3′ and 800nM of telc, 5′-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3′. Four stages made up the thermocycling conditions as follows: Stage 1: 95°C for 15 min, Stage 2: 2 cycles for 15 s at 94°C and 49°C, Stage 3: 25 cycles at

94°C for 15 s, 10 s at 62°C, and 15 s at 73°C (data collection), Stage 4: dissociation curve (primer specificity detection).

The same reagents and quantities were used for the albumin reactions, apart from the albumin forward and reverse primers replaced the telomere primers. Quantities of the albumin forward and reverse primers were adjusted to 765 nM for the forward primer albu, 5′-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTT-3′ and 930 nM for the reverse primer albd, 5′-GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-3′. The thermocycling conditions for the albumin reaction consisted of four stages: Stage 1: 95°C for 15 min, Stage 2: 2 cycles for 15 s at 94°C and 49°C, Stage 3: 33 cycles at 94°C for 15 s, 10 s at 62°C, and 15 s at 88°C (data collection), Stage 4: dissociation curve (primer specificity detection).

Reactions were performed using either the ABI Prism 7900HT Sequence Detection System (Thermofisher Scientific, MA, United States) or the QuantStudio 7 Flex Real-Time PCR System (Thermofisher Scientific).

Genotyping and Quality Control (Target Dataset)

DNA samples were sent to the Affymetrix Research Services Laboratory in Santa Clara, California, CA, United States. Genotyping for SELCoH was assayed using the United Kingdom Biobank Axiom Array (r3) which comprises of 820,967 genetic markers (Affymetrix, California, CA, United States). Genotype data was put through quality control measures as outlined by Coleman et al. (2016), using PLINK v1.07 (Purcell et al., 2007) and mapped to genomic build hg19. Specifically, patient samples were excluded if there was greater than 5% missingness in genotype data, and individual SNPs were excluded if there was greater than 5% missingness. A minor allele frequency (MAF) threshold was set to 0.05, and a Hardy-Weinberg threshold of 0.00001, in keeping with what's recommended for smaller sample sizes (Coleman et al., 2016).

The absence of sample mismatching was confirmed using sex checks, where genetic sex was compared to phenotypic sex. The genome-wide Identity by Descent (IBD) analysis which is performed between pairs of samples, measured the probable number of shared alleles at any given marker, and was used to identify and exclude relatives within our sample. Relatives were identified as those with a PI-HAT (proportion of IBD) threshold of greater than 0.1875; where 0.5 represents first-degree relatives and 0.25 represents second-degree relatives. Only a single member from each family were retained post quality control. Following quality control, the sample consisted of 351 unrelated individuals, for which we had both genome-wide genotype data and telomere length data.

Polygenic Risk Score Quantification

PRSice Software

Individualized Polygenic Risk Scores within our sample were calculated using PRSice, a PRS quantification software (Euesden et al., 2015). The software uses summary results from previously performed, well-powered GWAS (the base dataset) to generate PRSs in our sample, SELCoH (the target dataset). Briefly, PRSice works by first clumping SNPs in the genotype PLINK files corresponding to the target dataset and removing those in high linkage disequilibrium, as this can falsely inflate polygenic scores. Subsequently, within the target dataset the number of risk alleles at a particular SNP is multiplied by that SNP's effect size (established in the base dataset), and then all the SNP information is summed. The user can define which SNPs to include in the PRS. For all analyses we set a p-value threshold of $p = 0.1$, whereby we included all SNPs under this threshold from our base datasets to calculate polygenic risk scores in our target dataset.

Base Datasets

The MDD base dataset (summary statistics) was obtained from the Psychiatric Genomics Consortium (PGC) and represents the largest GWAS for depression to-date, consisting of 135,458 MDD cases and 344,901 controls (Wray et al., 2018). The base dataset for BD consists of GWAS results for 7,481 cases and 9,250 controls (Sklar et al., 2011). For the BD GWAS, SNP positions were lifted over from hg18 to hg19 build using UCSC LiftOver tool (Kuhn et al., 2013). The base dataset for SCZ consists of insights from a multi-stage schizophrenia genome-wide association study of up to 36,989 cases and 113,075 controls (Ripke et al., 2014). All base datasets were downloaded from the PGC website[1].

Population Covariates

To reduce noise in our analyses as a result of ancestry differences within the sample, we generated population covariates (PCs) using multidimensional scaling in PLINK, via the PRSice software, which allowed us to detect and adjust for population structure in our analyses (Patterson et al., 2006; Price et al., 2006). Population covariates were incrementally tested for association via scatter charts (e.g., PC1 vs. PS2, PC2 vs. PC3, PC3 vs. PC4, etc.) until a normal distribution was achieved. A normal distribution was achieved after the first seven PCs, and thus this is what we used in our downstream analyses.

Statistical Analysis

RTL Calculation

A standard deviation of less than 0.5 was required for at least two of the three cycle threshold (C_t) technical triplicates for a sample to be included in downstream analysis. C_q values were then created from the remaining C_t values by relating them to absolute quantities as part of a standard curve. RTL was then calculated by dividing each sample's mean C_q value from the telomere reaction by each sample's mean C_q value from the albumin reaction. RTL was then log-transformed to allow for parametric analysis. Outliers were identified as those data points greater than two standard deviations from the mean and subsequently removed. As a final check, we performed a one-tailed Pearson correlation test to confirm there was a negative correlation between log(RTL) and age.

[1]http://www.med.unc.edu/pgc/results-and-downloads/results

PRS and its Relationship to Telomere Length (Dep−Sample Only)

To determine the effect of each PRS on telomere length we performed three independent linear regressions, whereby log(RTL) was selected as our outcome; age, BMI, PCs 1-7, plate batch, smoking status (former, current, never), gender, and ethnicity were selected as covariates; and PRS was selected as our independent variable.

Antidepressant Use and Telomere Length (Full Sample)

The relationship between antidepressant use and telomere length was investigated using a linear regression, whereby log-telomere length was selected as the outcome variable; age, BMI, PCs 1-7, plate batch, smoking status (former, current, never), gender, ethnicity, depression diagnosis were included as covariates; and antidepressant use was used as our independent variable.

Antidepressant Use and Aging-Related Disease (Full Sample)

An ordinal logistic regression was used to determine the relationship between antidepressant use and the number of aging-related diseases (0/1/2). Number of aging-related diseases was selected as our outcome variable; age, BMI, PCs 1-7, smoking status (former, current, never), gender, ethnicity, and lifetime depression diagnosis were selected as covariates; with antidepressant use selected as the independent variable.

Sensitivity Analyses

We performed a series of sensitivity analyses to determine the relationship between telomere length and physical illnesses/medication use, and the potential confounding effects of depression severity.

Power Calculation

Power calculations indicate we have 100% power to detect small-moderate effect sizes (effect size = 0.3) for analyses (i) to (iii), given our sample size, with an $\alpha = 0.05$.

RESULTS

Quality Control Checks

Standard curves from all reactions showed an $R^2 \geq 0.98$ between quantity of known DNA and C_t values. Negative controls showed no amplification on any of the plates and a single peak was detected for the dissociation curves (melting curves) across all plates, demonstrating that binding specificity of the primers to the DNA was achieved to a high degree, see **Supplementary Information**. The telomere reaction achieved a mean efficiency of 90% and the albumin reaction achieved a mean efficiency of 79%. Efficiencies were all corrected via a standard curve on each of the plates and all samples which didn't pass our quality control criteria were removed from any further analyses (17 samples).

Telomere Length and Chronological Age

A one-tailed Pearson's correlation showed that relative telomere length (adjusted for inter-plate variability) is negatively correlated with age in our whole sample, $r(351) = -0.223$, p 1.20E-05 (**Figure 1**) as expected.

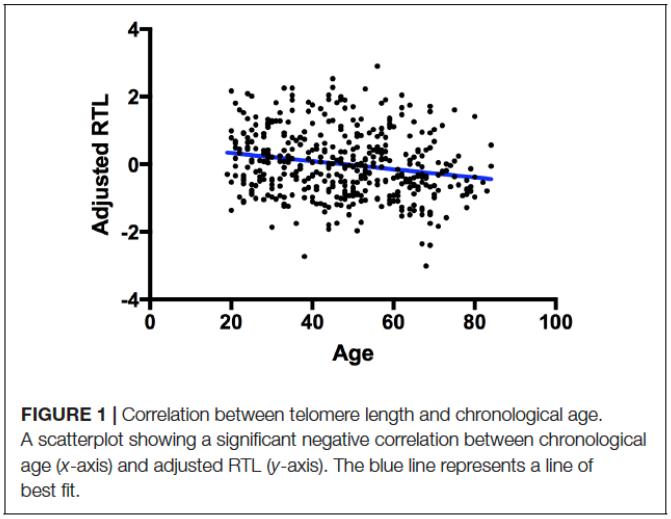

FIGURE 1 | Correlation between telomere length and chronological age. A scatterplot showing a significant negative correlation between chronological age (*x*-axis) and adjusted RTL (*y*-axis). The blue line represents a line of best fit.

Polygenic Risk for Psychiatric Disorders and Telomere Length

The regression model examining the effect of polygenic risk for SCZ on log(RTL) did not reveal a significant association ($F(1,265) = 1.622$, $p = 0.204$). Similarly, we did not find a significant association between the polygenic risk for BD and log(RTL; $F(1,265) = 1.872$, $P = 0.172$), nor between polygenic risk for MDD and log(RTL; $F(1,265) = 0.519$, $P = 0.472$), see **Figure 2**.

The Effect of Antidepressants Use on Telomere Length

Antidepressant use was significantly associated with telomere length in our total sample, which was irrespective of depression diagnosis ($F(1,325) = 6.575$, $P = 0.011$, variance explained = 2%), see **Figure 3**.

Antidepressant Use and Aging Related Disease

We found that those currently taking antidepressants also had a higher frequency of aging-related disease, relative to those not currently taking an antidepressant. (Estimate = −0.981 (95% *C.I.* = −1.878, −0.084), $p = 0.032$), see **Figure 4**.

Sensitivity Analyses

Telomere length and Aging-Related Disease

We performed ordinal logistic regressions to determine if log(RTL) predicts number of aging-related diseases (0,1,2) whilst covarying for age, BMI, PCs 1-7, smoking status (former, current, never), gender, ethnicity, lifetime depression diagnosis and antidepressant use. Log(RTL) did not predict number of ageing-related diseases ($p > 0.05$).

Telomere Length, Medication Use and Disease

We performed linear regressions which included age, sex, ethnicity and BMI as covariates, and log(RTL) as the outcome

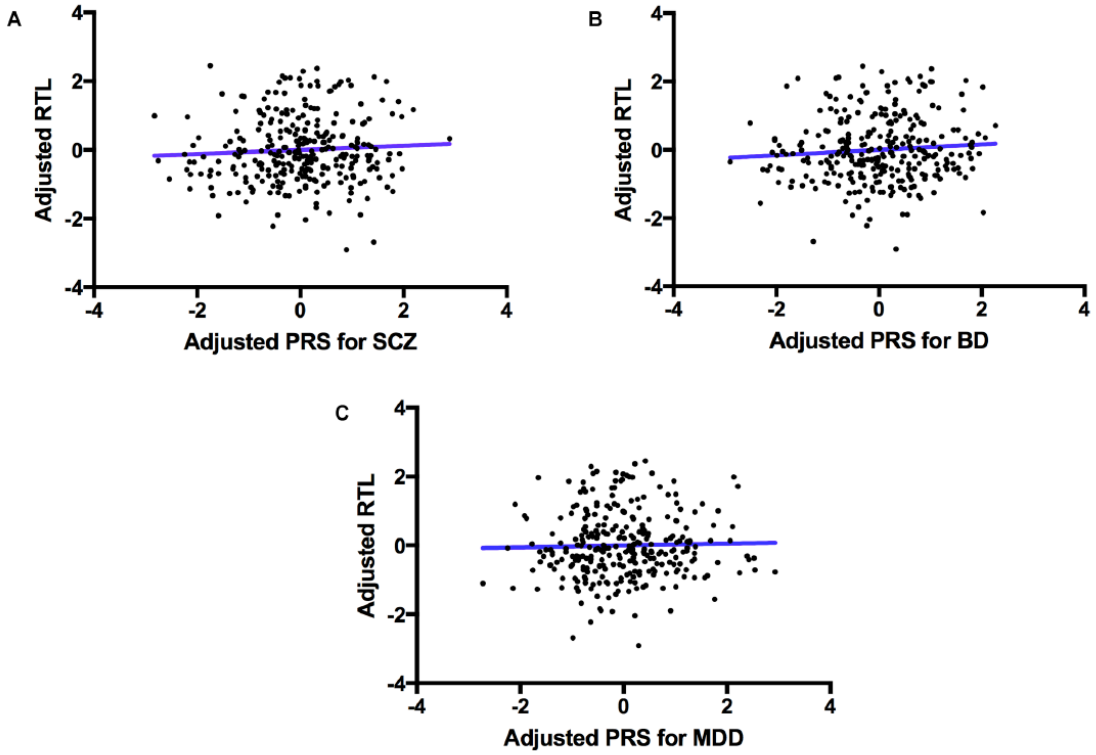

FIGURE 2 | The association between polygenic risk for psychiatric disorders and telomere length. A scatter plot showing PRSs for SCZ (**A**, top left), BD (**B**, top right) and MDD (**C**, bottom) adjusted for PCs 1–7 (*x*-axis), and log(RTL) adjusted for age, sex, ethnicity, BMI, smoking status and telomere plate batch (*y*-axis). The blue line represents the line of best fit.

variable, alongside self-reported disease or medication use. No diseases or medications predicted log(RTL), $p > 0.05$, see **Supplementary Information** for further details.

Telomere Length, Antidepressant Use and Aging-Related Disease

We further tested whether telomere length mediated the association between antidepressant use and risk for aging-related disease. So we repeated analysis (iv) as described above: An ordinal logistic regression was used to determine the relationship between antidepressant use and the number of aging-related diseases (0/1/2 +; outcome variable). However, we also included log(RTL) as a covariate. The relationship between antidepressant use and number of aging-related diseases remained significant ($p < 0.05$), suggesting the effect was not mediated by telomere length.

Depression Severity at Blood Collection, and Telomere Length

To confirm depression severity did not confound analyses investigating the effects of antidepressants on log(RTL), we repeated analysis (iii), however, we further included depression severity (0 = none, 1 = mild, 2 = moderate/severe) as a covariate, and found that antidepressant use still predicted log(RTL; $p < 0.05$), suggesting episode severity was not confounding our result.

DISCUSSION

Previous studies have revealed higher rates of aging-related diseases amongst psychiatric disorder patients, with some studies indicating that shorter telomere length (and faster aging) may be the cause (Hippisley-Cox et al., 2001; Gareri et al., 2002; Caughey et al., 2010). The first aim of our study was to clarify whether genetic risk for psychiatric disorders also carries risk for shorter telomere length. To achieve this aim we generated PRS for MDD, BD, and SCZ, and assessed the relationship between these PRS and telomere length measurements in a cohort of individuals with no history of psychiatric health problems. We found no evidence to suggest that genetic risk for psychiatric disorders also contributes to telomere shortening, **Figure 2**. In terms of translational medicine, our results suggest that although polygenic risk scoring may be useful in predicting those at risk for psychiatric disorders, current psychiatric polygenic risk scores alone may not be useful in predicting those who are also susceptible to shorter telomeres and aging-related diseases. Instead, our results support previous work indicating the importance of environmental factors associated with psychiatric diagnosis, in accelerating telomere shortening (Kessler et al., 2005; Whiteford et al., 2013).

Indeed, we found evidence that antidepressant use was associated with shorter telomere length, an effect which was independent of depression diagnosis. This partially corroborates

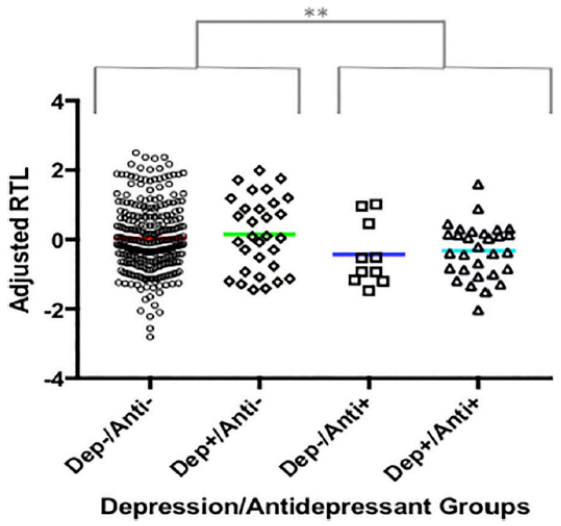

FIGURE 3 | The association between antidepressant use and RTL. A plot showing adjusted RTL in participants: (i) without depression and who are not currently taking antidepressants (Dep−/Anti−), (ii) with a depression diagnosis and who are not currently taking antidepressants (Dep+/Anti−), (iii) without depression who are currently taking antidepressants, (iv) with depression who are currently taking antidepressants. Participants currently taking antidepressants had significantly shorter RTL compared to those not taking antidepressants, irrespective of depression diagnosis. The symbol '**' indicates a difference of $p \leq 0.01$.

previous reports which found that only depressed patients currently taking antidepressants have shorter telomeres (Lindqvist et al., 2015; Needham et al., 2015). However, in contrast to previous studies which have suggested that antidepressant use is a proxy for current depression severity, and that this is what drives the association with shorter telomeres, our results show that even non-depressed participants who are taking antidepressants for other purposes (e.g., sleep) had similarly short telomere lengths, **Figure 3**. We further confirmed that current depression severity did not differ between depressed patients who were and were not taking antidepressants at the time of blood collection, suggesting the effect was not driven by current depression severity. One possible explanation is that antidepressants are increasing the proliferation of blood cells in users, and the knock-on effect is telomere shortening. Indeed, research using lymphoblastoid cell lines (resembling white blood cells), hippocampal progenitor cell lines, and *in vivo* studies of the hippocampus, support the notion that antidepressants increase proliferation (Manev et al., 2001; Breitfeld et al., 2017; Powell et al., 2017b). However, some work indicates that antidepressants may additionally increase the activity of telomerase, which is an enzyme involved in telomere maintenance and elongation (Bersani et al., 2015), therefore more work is needed to better understand the effects of antidepressants on telomere length and proliferation over time.

Interestingly, we additionally found an association between antidepressant use and risk for aging-related disease; with antidepressant use predicting a higher number of aging-related diseases, an effect which was independent of depression case/control status, **Figure 4**. This matches recent epidemiological reports that antidepressant use is associated with an increased risk for aging-related disorders such as cardiovascular disease (Musselman et al., 1998; Lichtman et al., 2008; Hamer et al., 2011). Interestingly, our sensitivity analyses suggest that the relationship between antidepressant use and aging-related disease is not mediated by telomere length variability, such that antidepressant use is independently associated with both risk for aging-related disease and shorter telomere length. These results indicate one of two things. First, that antidepressants increase telomere shortening and risk for aging-related disease via independent mechanisms. Second, and more likely, that antidepressant use, or prescription, is more common amongst those who suffer from depression (or related conditions such as sleep problems), and who also suffer

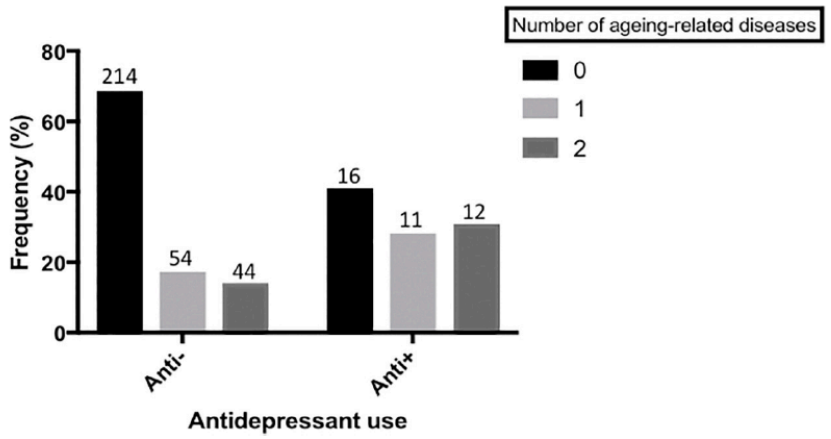

FIGURE 4 | Antidepressant use and aging-related disease. A bar chart showing the frequency of participants with 0, 1 or 2(+) aging-related diseases, split by those who are not currently taking antidepressants and those who are. Actual sample size within each group is shown on top of each bar. There is a significant difference in the frequency of aging-related disease between the two groups ($p < 0.05$). Aging-related diseases included: type-2 diabetes, arthritis, cardiovascular disease, stroke, high blood pressure, and cancer.

from chronic debilitating aging-related disease. Nevertheless, the combination of previously reported epidemiological data linking antidepressant use with aging-related disease, and molecular data reported here, warrants further consideration of the long-term impact of antidepressant use on aging-related phenotypes. In particular, studies recruiting antidepressant users (both depressed and non-depressed) as part of a longitudinal design may help in discerning causality.

The main limitations of our study are the small number of individuals taking antidepressants, and the fact we may be underpowered to detect small effect sizes. Nevertheless, our study is the first to suggest that: (i) genetic risk for psychiatric disorders does not predict faster biological aging, (ii) antidepressant use is associated with shorter telomeres independently of depression diagnosis, (iii) antidepressant use is associated with an increased number of aging-related diseases, independently of depression diagnosis. Our work suggests that the relationship between antidepressant use and risk for aging-related disease may need to be reconsidered.

CONCLUSION

We found no evidence to suggest that genetic risk for psychiatric disorders also contribute to faster telomere shortening, highlighting the potential importance of environmental factors in mediating physical disease comorbidity. We did, however, find an association between antidepressant use and telomere length, with antidepressant use being associated with shorter telomere length. In addition, we found antidepressant use to be associated with a higher number of aging-related diseases in participants, replicating previous epidemiological evidence. Further work is now needed to test whether antidepressants induce telomere shortening via their proliferative effects, and how antidepressant use relates to aging-related disease.

AUTHOR CONTRIBUTIONS

GB, LG, SF, SH, and MH did the sample collection, interpretation of results, final approval for publication. CL and ST interpreted the results and provided the final approval for publication. AP and TP created and designed the work, acquired the data, analyzed and interpreted the data, prepared and revised the manuscript, and provided the final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2018.00468/full#supplementary-material

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4.2 CHAPTER 4 REFERENCES – GENETIC RISK FOR PSYCHIATRIC DISORDERS AND TELOMERE LEGNTH

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4.3 POSTFACE

This study presents several interesting findings. First, this study demonstrates that an increased genetic risk for MDD, BD and SCZ does not result in advanced telomere shortening, which could be associated with a higher incidence of age-related diseases as well as psychiatric disorder aetiology. This suggests that other environmental factors are likely to be involved in telomere shortening observed in the psychiatric disorder population. Indeed, many studies have shown that poor lifestyle choices such as smoking, poor diet and drug use are associated with advanced telomere shortening (238, 244, 442), which could increase the risk for developing psychiatric disorders and age-related disease (226, 231, 437, 440, 443). This study, however, suggests that this association is not mediated by an increased genetic risk for MDD, BD or SCZ.

The study does however show that antidepressant use is associated with shorter telomere length, as well as a higher number of age-related diseases, even in people taking antidepressants for non-psychiatric purposes. Antidepressants are known to increase cell division and studies have shown that an increase in hippocampal neurogenesis (Chapter 1.6.4) following antidepressant treatment is associated with behavioural improvements in animals (374, 444). However, few studies have considered what effect this could be having on the rest of the body (445), with this study suggesting that antidepressant use is either associated with advanced cell ageing, or that people who age faster and have more age-related diseases are more likely to be prescribed antidepressants.

More work will need to be carried out to investigate this putative association, with the next two chapter aiming to disentangle the polygenic architecture of telomere length as a marker of cell ageing, and investigate the effects of lithium, a mood-stabilizer and a potential anti-ageing drug on hippocampal progenitor cells (310, 318).

5 – THE POLYGENIC NATURE OF TELOMERE LENGTH AND THE ANTI-AGEING PROPERTIES OF LITHIUM

Figure 4.3.1 – The beauty of ageing.
This photograph was taken by Raffaele Montepaone, who has a series of black and white photographs aimed at capturing the beauty of old age. Taken from: https://raffaelemontepaone.it/index.php/galleries/life/

5.1 PREFACE

As mentioned in previous chapters, telomere length could be a useful biomarker for age-related disease and could be used as a potential anti-ageing drug target. In addition, advanced telomere shortening has been suggested as a possible aetiological mechanism for developing psychiatric disorder such as BD, with a study by Powell and colleagues (2018) showing that telomere length is shorter in relation to familial risk for BD (313). Interestingly, the same study showed that patients with BD who are treated with lithium have longer telomeres than patients with BD who are not taking lithium. This suggests that telomere length is modulated by familial risk to psychiatric disease and that lithium may possess anti-ageing properties (217, 310, 321). However, the genetic, or polygenic nature, of telomere length has not been explored fully to-date, nor have the modulatory effects of lithium on telomere length.

In order to investigate this, the study utilised the largest telomere length GWAS to-date to estimate SNP-chip heritability via LD score regression, and applied polygenic risk scoring to estimate the variance in telomere length that could be explained in an independent BD cohort. To better understand how lithium might confer its effects, we first performed gene-level analyses to get a deeper understanding of which genes regulate telomere length, and second, we tested whether these genes transcripts' are affected in a lithium-induced model of longevity (309). Finally, we attempted to confirm whether patients with BD who are chronic lithium users have longer telomeres in an independent sample and meta-analysis, and test whether this effect is moderated by genetic background.

The study was published in Neuropsychopharmacology (doi: https://doi.org/10.1038/s41386-018-0289-0) on the 18th of December 2018. Demographic and patient samples were collected as part of the Bipolar Association Case-Control Study. Experiments and primary analyses were

performed by Palmos & Coutts (joint first authors). Secondary analyses were carried out on publicly available data (217, 309) and were carried out by Duarte & Powell.

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ARTICLE OPEN

The polygenic nature of telomere length and the anti-ageing properties of lithium

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Telomere length is a promising biomarker for age-related disease and a potential anti-ageing drug target. Here, we study the genetic architecture of telomere length and the repositioning potential of lithium as an anti-ageing medication. LD score regression applied to the largest telomere length genome-wide association study to-date, revealed SNP-chip heritability estimates of 7.29%, with polygenic risk scoring capturing 4.4% of the variance in telomere length in an independent cohort ($p = 6.17 \times 10^{-5}$). Gene-enrichment analysis identified 13 genes associated with telomere length, with the most significant being the leucine rich repeat gene, *LRRC34* ($p = 3.69 \times 10^{-18}$). In the context of lithium, we confirm that chronic use in a sample of 384 bipolar disorder patients is associated with longer telomeres ($p = 0.03$). As complementary evidence, we studied three orthologs of telomere length regulators in a *Caenorhabditis elegans* model of lithium-induced extended longevity and found all transcripts to be affected post-treatment ($p < 0.05$). Lithium may therefore confer its anti-ageing effects by moderating the expression of genes responsible for normal telomere length regulation. This is supported by our bipolar disorder sample, which shows that polygenic risk scores explain a higher proportion of the variance in telomere length amongst chronic lifetime lithium users (variance explained = 8.9%, $p = 0.01$), compared to non-users ($p > 0.05$). Consequently, this suggests that lithium may be catalysing the activity of endogenous mechanisms that promote telomere lengthening, whereby its efficacy eventually becomes limited by each individual's inherent telomere maintenance capabilities. Our work indicates a potential use of polygenic risk scoring for the prediction of adult telomere length and consequently lithium's anti-ageing efficacy.

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INTRODUCTION

'Aging is not lost youth but a new stage of opportunity and strength' [1].

Our population is ageing [2]; with increased longevity and decreased fertility rates, the median age of populations within more economically developed countries has risen from 28 in 1950 to 40 in 2010 [3]. Although longer life span has clear benefits, when it is associated with an increased proportion of the population suffering from age-related diseases, it can pose an economic burden [4]. Consequently, there has been an international effort to identify factors that can both increase longevity and delay the onset of morbidity [5].

One predictor of age-related disease, including coronary artery disease and obesity, as well as all-cause mortality and longevity, is telomere length [6]. Telomeres are stretches of TTAGGG nucleotide repeats at the ends of chromosomes [7]. They represent sacrificial DNA elements that protect vital coding DNA from being lost, as a result of the 'end replication problem'; which is the loss of genetic material at the end of chromosomes (i.e. telomeres) each time a cell divides [8]. When a critical telomere length is reached, a cell loses the ability to divide [9]. This ultimately means that as we age, we are less able to replace old or damaged cells, and this can increase risk for age-related disease. Indeed, a direct relationship between telomere shortening and disease risk has been highlighted recently by Mendelian randomisation studies revealing that robust genetic predictors of telomere length also predict risk for coronary artery disease [10]. Therefore, telomere length represents both a biomarker for cellular age, and a potential anti-ageing drug target.

Psychiatric disorder patients exhibit high rates of comorbid age-related disease and frequently exhibit shorter telomere length relative to non-affected controls of a similar age [11]. Consequently, they represent a useful group in which to better understand the genetic and environmental contributions to shorter telomere length. Our recent work suggests a familial transmission of shorter telomere length, whereby even non-affected relatives of psychiatric disorder patients exhibit shorter telomeres compared to those with no family history [12]. Based on twin studies that reveal blood (leukocyte), telomere length is a highly heritable trait [13], and genome-wide association studies that reveal numerous loci as being involved in the regulation of its length [10], shared genetics could underlie this familial

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association. Indeed, we have previously shown that a genetic risk factor for shorter telomeres also confers risk for childhood-onset major depressive disorder [14]. However, in the more frequent cases of adult-onset psychiatric disorders, it appears that environmental factors (e.g. childhood stress) play a more important role in explaining shorter telomeres, than common genetic risk factors [15, 16]. Consequently, intervention strategies that focus on the environment may be particularly useful in preventing excessive telomere shortening amongst high-risk groups for age-related disorders, such as psychiatric disorder patients or those exposed to environmental trauma, and perhaps even more broadly for the general population.

In addition to stress, diet and medications can also affect rates of telomere shortening, suggesting that to an extent, we can actively moderate how we age [15–18]. This has led to the realisation that we may be able to pharmacologically reduce telomere shortening via the creation of anti-ageing (or anti-telomere shortening) medications. However, one of the pitfalls faced so far in targeting telomeres, pharmacologically, has been that excessive telomere length, and activity of the telomere-lengthening enzyme, telomerase, is associated with an increased risk of cancer [19]. Therefore, it's likely that effective anti-ageing strategies would need to evoke subtle effects to telomeres across the life course, rather than rapid effects that may simultaneously increase cancer risk.

On a population scale, perhaps one of the most wide-reaching and effective ways to implement anti-ageing benefits across the life course would be by altering diet, and recent research suggests that even the water we drink may be important. Specifically, reports indicate that a higher level of lithium, naturally found in drinking water, is associated with fewer incidences of all-cause mortality, a reduced number of individuals committing suicide, increased longevity, and a reduced risk of neurodegenerative disease [20–23]. The anti-ageing benefits of lithium are not limited to humans either, with the effects being replicated in the worm *C. elegans*, and in the fly *Drosophila melanogaster*, where it extends lifespan [23–26].

In addition to being a metal naturally found in drinking water, lithium also has clinical applications, and is currently a first-line treatment for bipolar disorder (BD), where it acts as an effective mood-stabilizer [27]. BD patients are therefore a useful cohort to study the effects of lithium on anti-ageing mechanisms as these individuals are often taking relatively high, controlled doses for long periods of time. Indeed, we have shown in previous research that current lithium use is associated with longer telomere length amongst BD patients [12], and others have shown that lithium treatment duration amongst BD patients positively correlates with telomere length, specifically amongst chronic lifetime users [28, 29]. Lithium is further of interest, as its use is associated with longer telomeres, but with a decreased risk of cancer [30]. This accumulation of findings has sparked interest regarding the repositioning potential of lithium as an anti-ageing drug, and even the utility of lithium supplementation in drinking water (similarly to the use of fluoride for teeth), as a way to keep people healthier for longer [20, 31].

Due to lithium's ability to affect a multitude of biological systems [32], more research is needed to understand its anti-ageing mechanism of action, and how generic its effects are in humans. For instance, research in *C. elegans* has shown a strong mediation of lithium's anti-ageing effects by genetic factors [24], suggesting it may not be effective at preventing telomere shortening in a one-size-fits-all fashion across different genetic backgrounds. In humans, we know that variation in telomere length is moderated by a multitude of factors, such as oxidative stress and inflammation [33, 34], with perhaps the most pertinent factor being the activity of the telomerase enzyme, which adds TTAGGG repeats to telomere ends in dividing cells [35]. At least some of this inter-individual variation moderating telomere length is captured at the genetic level, for instance, single nucleotide polymorphisms (SNPs) within, or upstream of the telomerase genes represent the strongest predictors of leukocyte telomere length [10, 14]. In the case of lithium however, it's currently unknown whether its telomere-lengthening effects work similarly for everyone (i.e. in a one-size-fits-all fashion), or whether variation in genes regulating baseline telomere length maintenance also contribute to variation in its anti-ageing benefits.

In this report, we determine: (i) the heritability of telomere length and confirm its genetic relationship to age-related disease and cancer, (ii) a polygenic risk score (PRS-TL) capable of predicting telomere length in an adult population, (iii) that chronic lithium use is associated with longer telomeres in an independent bipolar disorder sample, (iv) that genetic regulators of telomere length are affected in a *C. elegans* model of lithium-induced extended longevity and (v) that polygenic risk scores for telomere length explain a substantial proportion of inter-individual variability in telomere length amongst chronic lithium users, suggesting that lithium's anti-ageing efficacy may be moderated by polygenic factors.

MATERIALS AND METHODS

LD score regression: heritability and genetic correlations

LD score regression via LD Hub (http://ldsc.broadinstitute.org/ldhub/) was used to estimate the SNP-chip heritability of telomere length, i.e. the proportion of variance in telomere length explained by common genetic differences [36]. To achieve this, we obtained genome-wide summary statistics directly from Codd and colleagues who performed the largest GWAS of telomere length to-date, using data from 37,684 individuals [10]. SNPs were merged to the recommended SNP list in LD Hub which excludes the major histocompatibility complex (MHC). In LD hub we further performed genetic correlations to test whether age-related phenotypes robustly associated with telomere length at the molecular level were mirrored at the genetic level. We limited our phenotypes to: (i) any cancer diagnosis (UK Biobank), (ii) body mass index (UK Biobank), (iii) coronary artery disease [37], (iv) low density lipoprotein [38] and high density lipoprotein [38].

Bipolar association case-control study

Within this study we utilise 384 recurrent bipolar disorder patients recruited as part of the Bipolar Association Case-Control Study (BACCS) [39]. For full details on recruitment criteria, see S1 Supplementary information. Detailed phenotype data were also collected during the interview which included information on current lithium use, lifetime lithium use, duration of lithium treatment and lithium dose. Based on previous reports showing that lithium's telomere-lengthening effects only correlate with duration of treatment amongst chronic lifetime users, (i.e. after several years of taking the drug) [28, 29], and because the treatment duration data was negatively skewed, we split our lifetime user group by the median treatment duration into two equally sized (and normally distributed) subgroups consisting of "short-term lifetime lithium users" (<4.5 years, $n=84$), and "chronic lifetime lithium users" (4.5–30 years, $n=84$). See Table 1 for sample characteristics. Access to molecular and clinical data related to BACCS is available upon request via a local access procedure, in accordance with the ethics agreement.

BACCS DNA extraction and preparation

25 mL of whole blood was taken from each participant at the time of interview and stored in EDTA blood tubes at −20 °C. Genomic DNA was then extracted using an inhouse protocol, previously described [40]. All DNA samples had 260/280 ratios of between 1.7 and 1.9, tested using the Nanodrop D1000 (Thermo Fisher Scientific, Massachusetts, United States), indicating good DNA purity.

Table 1. Demographic data within the bipolar disorder sample

	Li-naive BD patients	Short-term lifetime Li users	Chronic lifetime Li users	Full sample
n	83	84	84	384
Age (mean, (SD))	44.48 (12.18)	44.06 (9.99)	52.14 (10.34)	48.42 (11.57)
Sex, n (% males)	22 (26.50)	30 (35.29)	35 (41.67)	126 (32.81)
BMI (mean, (SD))	26.20 (5.57)	28.24 (7.06)	28.08 (4.92)	27.42 (5.67)
Age of onset (mean, (SD))	21.08 (11.58)	20.69 (9.04)	22.31 (10.93)	21.63 (10.57)
Illness duration, years (mean, (SD))	24.26 (13.66)	22.63 (10.96)	30.26 (11.45)	21.21 (12.29)
Number of depressive episodes (mean, (SD))	11.60 (20.62)	11.96 (18.74)	14.08 (22.84)	12.22 (19.69)
Number of manic episodes (mean, (SD))	11.49 (22.70)	10.45 (18.85)	11.56 (19.94)	10.86 (19.30)
Number of mixed episodes (mean, (SD))	2.69 (12.25)	4.35 (16.03)	3.66 (13.29)	3.12 (12.72)
Current lithium use (n)	0	33	59	170
Ever taken other mood-stabilizers (n)	45	61	47	215
Ever taken antidepressants (n)	63	76	71	303
Ever taken antipsychotics (n)	49	64	63	250
Ever taken anxiolytics (n)	24	38	34	147

Information is based on available self-report data

Telomere protocol

Relative telomere length (RTL) was quantified using a modified version of the quantitative Polymerase Chain Reaction (qPCR) protocol described by Cawthon and colleagues [41], as used by our lab previously [12, 16]. First, the protocol assayed the telomere variable repeat region (TTAGGG), and the cycle threshold (C_t) required to reach a predetermined level of fluorescence: this correlated with the number of telomere repeats present in the individual samples. Second, and in parallel, a single-copy gene (albumin) was assayed in the same way, except the C_t now correlated with the number of copies of the genome in that individual DNA sample. Finally, a telomere-to-single-copy-gene ratio was used to determine RTL, where the number of telomere repeats in each sample was corrected for the total number of copies of that individual's genome in the DNA sample being tested. See S2 for further details on the protocol and S3 and S4 in Supplementary information for the quality control procedures and results.

BACCS genetic data

Genotype data was generated using Illumina HumanHap550 BeadChip (Illumina Inc., San Diego, CA, USA). Quality control was performed including the removal of SNPs with minor allele frequencies below 1%, and those not in Hardy-Weinberg equilibrium ($p < 1 \times 10^{-5}$), as described previously [42]. Multi-dimensional Scaling (MDS) in PLINK [43] was used to construct three population covariates (PCs), which were used in all analyses to correct for minor differences related to ancestry.

Individualised polygenic risk scoring for telomere length

PRSice version 1.25 software [44] was used to determine the optimal *p*-value threshold (P_T) where the polygenic risk for telomere length from the GWAS summary statistics [10] predicted telomere length in the BACCS cohort. To achieve this, our RTL data was initially adjusted for age, sex and BMI by taking the standardized residuals (z-scores); this phenotype was then modelled in PRSice with three ancestry PCs as covariates, for p-value thresholds from $p = 0.001$ to $p = 0.5$, increasing in 0.001 increments.

Gene-enrichment analysis

MAGMA was applied to genome-wide summary statistics from the Codd et al. [10] GWAS, using the online tool FUMA [45]. MAGMA maps SNPs to genes in order to prioritise genes of functional relevance to a given trait. It generates a gene-wide statistic (and weighted *p*-value) from the GWAS results files, adjusting for gene size, single nucleotide polymorphism (SNP) density and linkage disequilibrium effects. We used a 10 kb 5′ and 3′ window around protein coding genes, as recommended by the authors, where genes surpassing genome-wide significance ($P = 0.05/18879 = 2.648 \times 10^{-6}$) were then investigated in datasets from a *C. elegans* model of lithium-induced longevity.

eQTL analysis

We tested the effects of the most significant SNP associated with telomere length, rs10936599, on gene expression across multiple tissues using the online interface provided by the Genotype-Tissue Expression (GTEx) project [46].

Lithium and C. elegans longevity microarray

Previous research in *C. elegans* assayed genome-wide expression changes associated with lithium-induced longevity [24]. Specifically, work by McColl and colleagues, revealed that a 10 mM dose of lithium increased the median lifespan of *C. elegans* by 46%. They subsequently assayed the genome-wide expression effects of a two-day 10 mM lithium treatment using a purpose-built *C. elegans* microarray (Genome Sequencing Center, University of Washington School of Medicine; Platform GPL5367 in GEO) to better understand the molecular mechanism conferring longevity. They found overlapping molecular effects of a two-day lithium treatment in C. elegans with effects observed in human cells treated in the same conditions. Microarray data is publically available from the Gene Expression Omnibus (GDS3140). OrthoList was used to identify *C. elegans* orthologs of human genes [47].

Statistical analysis

Effects of lithium on telomere length. We tested the effect of lifetime lithium duration on RTL amongst short-term and chronic lifetime users separately, using a linear regression where RTL was the outcome, age, sex, BMI, current lithium use and three PCs were included as covariates, with lifetime lithium duration (weeks) as the independent variable. In the full bipolar disorder sample, we performed sensitivity analyses to test for the effects of (i) number of episodes (depressed/manic/mixed), (ii) other medications used, (iii) duration of illness and (iv) lithium dose, on RTL, with all models including age, sex, BMI and three PCs as covariates.

We additionally used data summary techniques to expand our current sample size and draw support from previous work on the

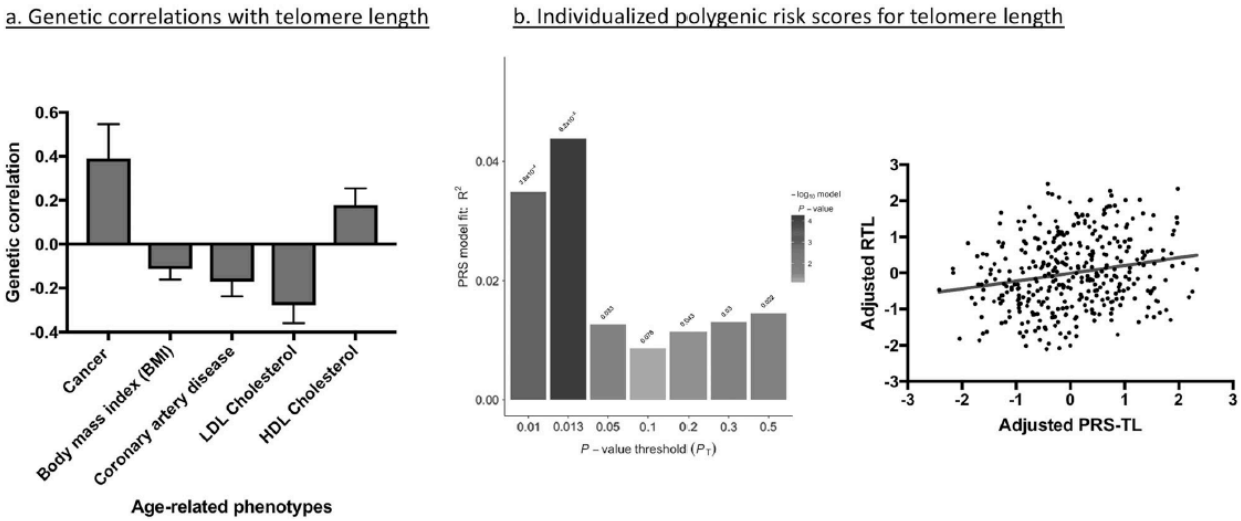
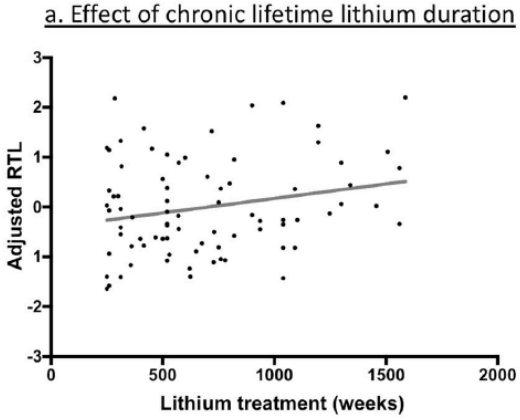


Fig. 1 Genetic correlations with telomere length and individualised risk prediction. **a** Genetic correlations between single nucleotide polymorphisms predictive of increased telomere length and age-related phenotypes. **b** Left: Output from PRSice displaying a range of p-value thresholds (P_T) tested, including the optimal P_T as shown in the tallest bar at threshold $P_T = 0.013$, which explained ~4.4% of the variance ($p = 6.174 \times 10^{-5}$). Right: A scatterplot showing the positive correlation between polygenic risk scores for telomere length (PRS-TL; adjusted for 3 PCs) and relative telomere length (RTL; adjusted for age, sex and BMI), Pearson (r) = 0.205, $p \leq 0.0001$



b. Data summarisation across multiple studies

Study	Effect of lithium	n	p
Martinsson et al. (2013)	+	72	0.031
Squassina et al. (2016)	+	150	0.037
Current study	+	84	0.033
Weighted effect	**+**	***306***	***0.001***

Fig. 2 Lithium affects telomere length. **a** Scatterplot showing a positive association between lithium treatment duration and relative telomere length (RTL; adjusted for age, sex, BMI, PCs 1-3 and current lithium use) in chronic lifetime lithium users. **b** Data summarisation results using Stouffer's sum of z method. Table includes previous studies assaying the effect of chronic lithium duration on RTL, the direction of effect observed (effect), sample size (n) and *p*-value (p), as well as a weighted effect combining results from all studies

effects of chronic lithium use on RTL. We included all primary human studies where the effects of lithium alone had been considered in the context of telomere length, by searching for "lithium" + "telomere" in PubMed (https://www.ncbi.nlm.nih.gov/pubmed). We conducted the data summarisation in R (https://www.r-project.org) using the package "metap" [48] and Stouffer's "sumz" method [49], which allows for an estimation of z-scores and weighted average *p*-values in the absence of effect sizes. Weights of *p*-values from each study were defined as the square root of the sample size, as recommended by the authors.

Effect of PRS-TL on telomere length amongst lithium users and non-users. To compare the impact of PRS-TL in lithium-naive BD patients, short-term lifetime lithium users, and chronic lifetime lithium users, we performed a linear regression for each group of patients separately. RTL was selected as the outcome variable, with age, sex, BMI, current lithium use, lifetime lithium duration (weeks) and three PCs as covariates, with PRS-TL as the independent variable.

Effects of lithium on genetic regulators of telomere length in a model of extended longevity. Following MAGMA analyses, paired sample *t*-tests were used to compare whether implicated genes were affected in *C. elegans* following lithium treatment.

Multiple testing correction. For hypothesis-driven tests (effects of lithium on telomere length, genetic correlations on ageing traits) we considered $p < 0.05$ to be significant as these were replications of previous work. For all remaining analyses, we applied the Bonferroni method of multiple testing correction.

RESULTS

Telomere length is polygenic and associated with risk for age-related disease and cancer

LD score regression was applied to the largest telomere length GWAS to-date [10] in order to establish the proportion of variance in telomere length explained by common genetic differences. Results revealed a significant polygenic component to telomere length regulation, whereby there was a SNP heritability estimate of 7.29% (S.E. = 1.54). Polygenic risk for longer telomere length was associated with increased risk for cancer and HDL cholesterol, and decreased risk for coronary artery disease, high BMI and high levels of LDL cholesterol (all $p \leq 0.02$) replicating epidemiological reports, Fig. 1. Polygenic risk scoring revealed that 3634 SNPs under the *p*-value threshold, $P_T = 0.013$ significantly predicted 4.382% of the variance in adjusted RTL ($p = 6.174 \times 10^{-5}$) in an independent sample of 384 bipolar disorder individuals, see Fig. 1.

This effect remained significant after correcting for the 500 thresholds tested (adj. $p = 0.03$).

Lithium use is associated with longer telomere length

Within our UK bipolar disorder sample (BACCS; see Table 1), short-term lifetime lithium duration did not predict telomere length ($F(1, 75) = 0.47$, $p = 0.829$, variance explained = 0.1%) as expected, but chronic lithium treatment duration did predict longer telomere length ($F(1, 75) = 4.733$, $p = 0.033$, variance explained = 6.3%), replicating previous findings [28, 29], Fig. 2. The effect of lithium treatment duration amongst chronic lifetime users was further validated using data summary methods (Stouffer's $z = 3.120$ $p = 9.061 \times 10^{-4}$), Fig. 2. There were no effects of number of episodes, illness duration, other medications, or current lithium dose in the full BACC sample (see S5 Supplementary information). Amongst chronic lifetime lithium users, daily doses ranged from 120–1800 mg, but similarly to the full BACC sample, there were no effects of dose on RTL.

Lithium targets genetic regulators of telomere length in a model of extended longevity

Our gene-enrichment analysis, using MAGMA and GWAS summary statistics from Codd and colleagues [10], revealed 13 genes significantly implicated in telomere length regulation, Fig. 3. eQTL analysis using GTex [46] confirmed an effect of the top telomere SNP rs10936599 on the expression of the most significantly enriched gene *LRRC34*, in transformed fibroblasts, adipose tissue, tibial nerve, heart, arteries, oesophagus and adrenal gland (all $p < 3 \times 10^{-5}$), whereby the risk allele for shorter telomere length (T-allele) was consistently associated with reduced *LRRC34* expression. There was no effect of rs10936599 on *TERC* expression levels. Of the 13 genes identified by our gene-enrichment analysis, three had orthologs in *C. elegans* that were also assayed in the McColl et al. study of lithium-induced extended longevity [24]. All three genes were differentially expressed upon lithium treatment in the model, including: *Y55F3AM.14* (human ortholog: *ZNF257*; $t(5) = -3.884$, $p = 0.012$), *F25H8.2* (human ortholog: *NAF1*; $t(5) = 4.973$, $p = 0.004$), and Y54E10BR.2 (human ortholog: ARFRP1; $t(5) = 2.597$, $p = 0.048$), Fig. 3. The effect on *Y55F3AM.14* and *F25H8.2* remained significant after correcting for three tests (adj. $p < 0.05$).

Polygenic risk explains more inter-individual variability in telomere length amongst lithium users

To understand how PRS-TL behaves in lithium users and non-users, we tested its effect in lithium-naive BD patients, short-term lifetime lithium users and chronic lifetime lithium users, separately. The rationale for this was to understand if lithium works in a one-size-fits-all manner, or whether variation in PRS-TL explains inter-individual variation in telomere length amongst lithium users. In subsamples of just over 80 patients (Table 1), PRS-TL did not predict a significant amount of variance in RTL amongst BD patients who were naive to lithium ($F(1, 72) = 0.250$, $p = 0.619$, variance explained = 0.3%), nor in those who were short-term lifetime users ($F(1, 75) = 0.85$, $p = 0.771$, variance explained = 0.1%). In contrast, PRS-TL explained a relatively high proportion of the variance in RTL amongst chronic lifetime users ($F(1, 75) = 6.802$, $p = 0.011$, variance explained = 8.9%), see Fig. 4. The effect of PRS-TL in chronic lifetime users remained significant following multiple testing correction (adj. $p = 0.033$).

DISCUSSION

Telomere length represents a promising biomarker for age-related disease and a potential anti-ageing drug target. In this study we examined the genetic basis of telomere length regulation and explored the repositioning potential of lithium as an anti-ageing medication. First, our study revealed that telomere length is a polygenic trait with SNP heritability estimates of 7.29%. Using polygenic risk scoring we identified a genetic score which explained 4.4% of the variance in telomere length in an independent sample, which is an improvement to the previously reported genetic risk score consisting of only genome-wide significant SNPs that explained just over 1% [10]. These findings further support twin research suggesting telomere length is a highly heritable trait, but our work also suggests that a significant amount of variation remains missing (up to 74%), which may indicate that even larger sample sizes and more powerful GWAS are required, or that rare variants, gene-environment interactions or epigenetic modifications also add significantly to twin heritability estimates [13]. Genetic correlations corroborate previous reports that indicate a higher risk for cancer amongst individuals with very long telomeres [19]. In terms of age-related disease phenotypes, we found genetic risk for longer telomeres was associated with higher levels of high density lipoprotein (the 'good cholesterol') and reduced levels of low density lipoprotein (the 'bad cholesterol'), alongside a reduced risk for coronary artery disease and high body mass index. This supports a multitude of studies that indicate a strong relationship between telomere length and age-related risk for coronary artery disease [10, 50, 51].

To better understand what genes are functionally important in regulating telomere length, we performed gene-level enrichment analysis on GWAS summary data. We found that the top five genes associated with telomere length were all clustered around the same genomic location on chromosome 3. These adjacent genes fall upstream of the telomerase gene *TERC*, and consequently it's possible that a range of SNPs exerting long range cis-regulatory effects on *TERC* are inflating signal in this genomic area. To gain a better grasp on what SNPs in this area affect which genes, we performed expression quantitative trait loci (eQTL) analysis on the most significant SNP associated with telomere length (rs10936599). This analysis did not reveal any effect of rs10936599 on *TERC* but did reveal an effect of the SNP on the most significantly enriched gene, leucine rich repeat containing 34 (*LRRC34*), across multiple tissue types, whereby the T-allele (associated with shorter telomere length) was consistently associated with reduced expression. Although the exact role of *LRRC34* is unclear, it is predicted to act as a ribonuclease inhibitor [52]. As a key component of telomerase's mechanism is the temporary incorporation of a non-coding RNA template to the lagging strand of DNA at our chromosome ends, it's possible that ribonuclease inhibitors help to preserve the RNA primer pivotal to telomere restoration. Consequently, its plausible that the LLR genes proximal to *TERC* are independently important in the regulation of telomere length, however further functional studies (e.g. CRISPR) will be needed to gain a definite understanding of how SNPs in these regions exert their effects. Other genes identified from our analyses included previously implicated regulators of telomere length (*TERT*, *NAF1*, *OBFC1*, *ZBTB46*, *ZNF257*) and some novel genes (*AL158091.1*, *RP4-583P15.15*), which will require further work to better understand their function [10].

Next, we confirmed that chronic lifetime lithium use is associated with longer telomere length in an independent sample of 384 BD patients, and in an expanded sample [12, 28, 29]. This supports epidemiological data which has shown that lithium in our water supply has beneficial effects on health and longevity and suggests that lithium's effect on telomere length may be one mechanism by which it confers its anti-ageing properties [20, 23]. To corroborate this theory, we tested whether lithium affects the expression of genes responsible for telomere length maintenance (identified from our gene-enrichment analyses) in a relevant model system that recapitulates the drug's anti-ageing effects. We found that 3 out of the 13 genes identified from the gene-enrichment analysis had an assayed ortholog in a *C. elegans* model of lithium-induced extended longevity, where we found that lithium had an effect on all three genes. This subsequently supports the notion that genes responsible for normal telomere

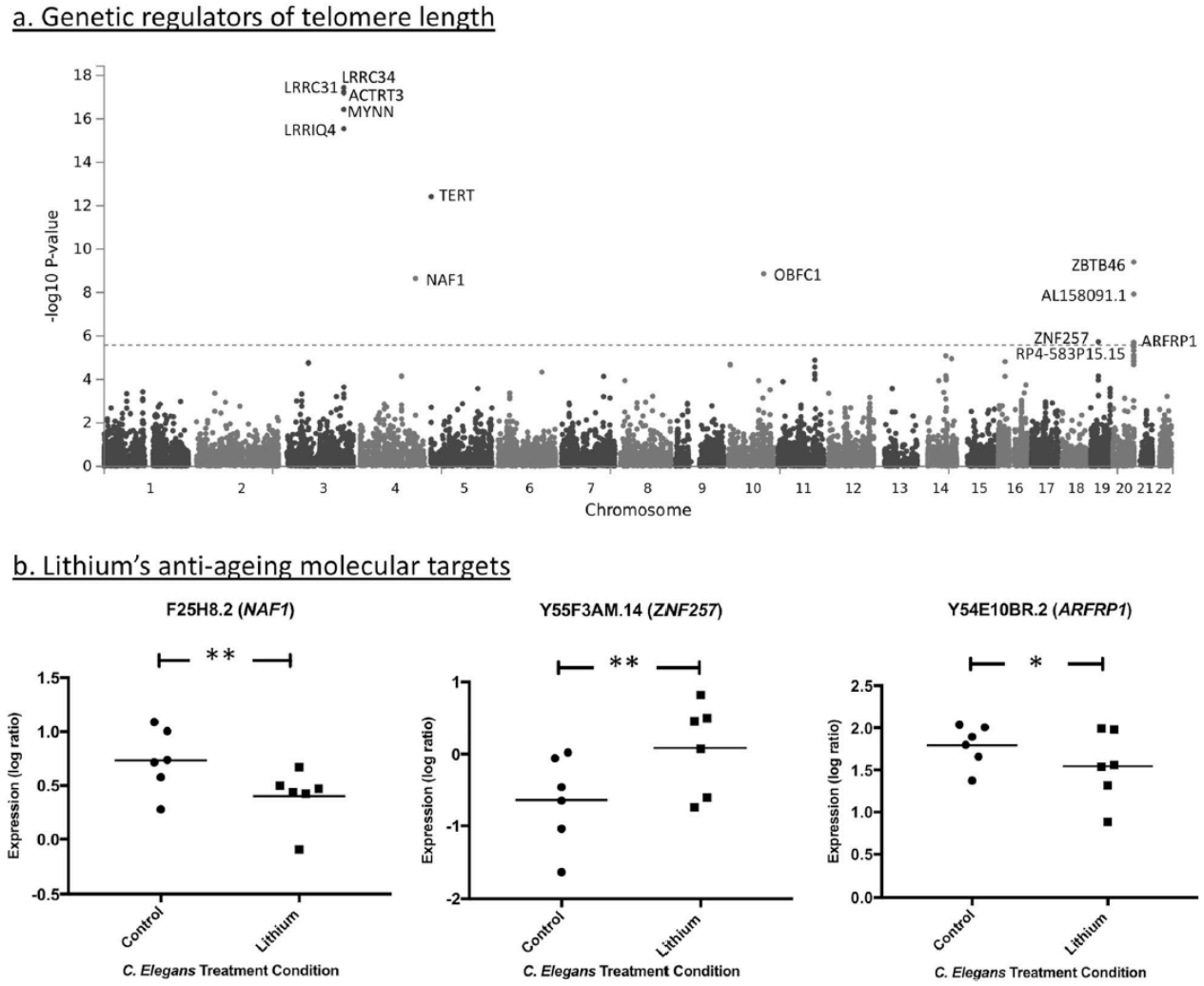


Fig. 3 Genetic regulators of telomere length and effects of lithium. **a** Manhattan plot showing results from telomere length gene-enrichment analyses, indicating which genes are most important in affecting telomere length. The dashed line represents the threshold for genome-wide significance. **b** Three orthologs were assessed at the mRNA level in a *C. elegans* model of lithium-induced extended longevity, $*p \leq 0.05$, $**p \leq 0.01$

length regulation may play a role in mediating lithium's anti-ageing mode of action.

Finally, we used PRS-TL to better understand whether SNPs involved in telomere maintenance contribute to inter-individual variation amongst lithium users, or whether lithium's telomere-lengthening effects work the same for everyone. Our results revealed variation in telomere length amongst lithium users, with a substantial proportion being explained by PRS-TL. In fact, far greater variance in telomere length was explained by PRS-TL in chronic lifetime lithium users (8.9%) relative to lithium-naive BD patients (0.3%) and short-term lifetime users (0.1%). This disparity suggests that lithium is not simply extending telomere length in a one-size-fits-all fashion, with residual baseline differences between individuals remaining, rather that lithium is increasing the penetrance of genetic differences in telomere length. In light of the results from the *C. elegans* model, it further suggests that lithium may be catalysing the activity of endogenous mechanisms responsible for telomere lengthening via its effects on gene transcription, whereby it eventually approaches a plateau and its efficacy becomes limited by each individual's inherent telomere maintenance capabilities, as captured using PRS-TL.

In sum, our findings have several potential implications. Our polygenic risk scoring result suggests that common genetic differences can predict over 4% of the variance in adult telomere length. This supports the possibility that PRS-TL may eventually represent a useful way of predicting those at risk for age-related disease (or cancer), though this will need to be verified in independent studies. It also adds support for further larger telomere GWAS to be performed in order to observe whether we can increase the predictive power of our PRS. Our comparative genomics work revealed that lithium can moderate the expression of genes governing telomere length, and this might be one mechanism via which it extends telomeres amongst bipolar disorder patients. Consequently, lithium may have repositioning potential for its anti-ageing effects in susceptible individuals. For instance, studies have shown that childhood maltreatment can shorten telomeres, which is a possible mechanism via which these individuals are also at higher risk for age-related disease [16]. Therefore, if telomere length was confirmed to be shorter amongst a maltreated individual, lithium might be a treatment option to prevent further premature ageing. Our results also suggest that lithium would likely be most effective if that individual also has a genetic predisposition to having longer telomeres in the first place (captured by PRS-TL). Thus, a combination of information on an individual's exposure to telomere-shortening environmental risk factors (e.g. by a

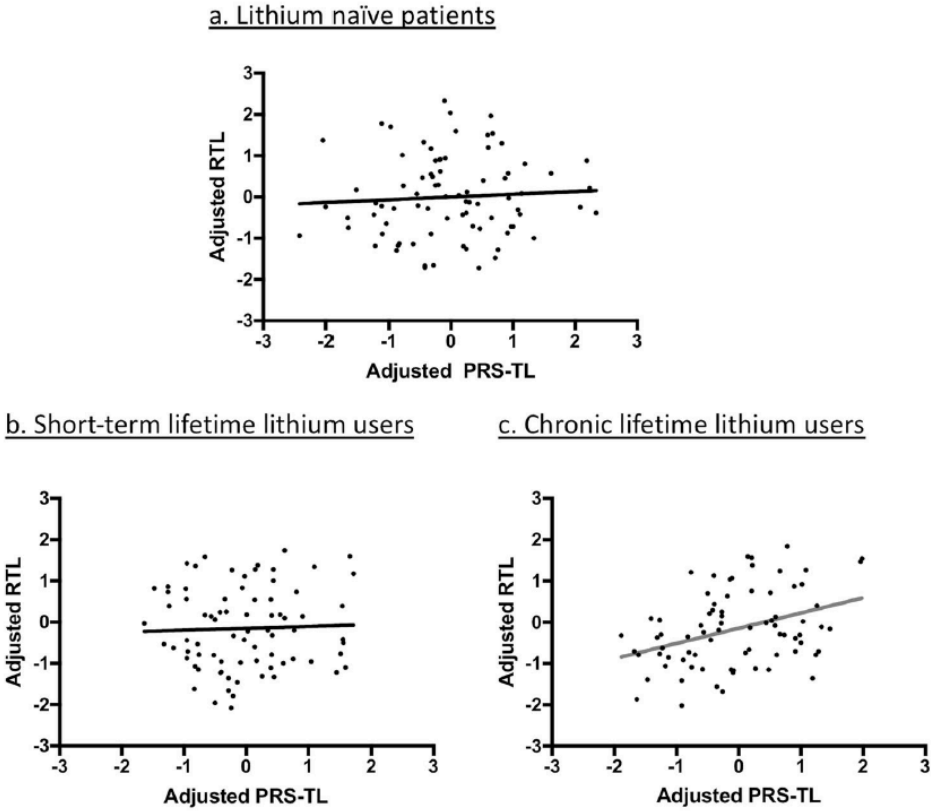


Fig. 4 Lithium use and the effect of PRS-TL. Scatterplots showing the effects of polygenic risk scores for telomere length (PRS-TL; adjusted for PC's 1-3), on relative telomere length (RTL; adjusted for age, sex, BMI and current lithium use), in **a** lithium-naive BD patients, **b** short-term lifetime lithium users and **c** chronic lifetime users. Significant correlations are indicated with a red line of best fit

childhood trauma questionnaire), confirmation of shorter telomere length via molecular probing (e.g. qPCR), and quantification of genetic risk for telomere length (e.g. PRS-TL), could be useful in identifying individuals who will need, and respond best, to the anti-ageing benefits of lithium.

Although the work in BD patients reported here represents a microcosm of how lithium supplementation may act on a population level, the results are encouraging, and adds support to epidemiological data which finds associations between higher lithium levels in water supplies and lower risk for age-related disease [20, 23]. We now need to study how lithium acts in a far larger, non-clinical population setting and confirm that the genetic factors restricting lithium's benefits identified here, replicate in other contexts. Furthermore, although, chronicity of treatment seems to be more important than lithium dose based on our analyses, we still need to consider how comparable low lithium levels are to the high clinical levels used to treat BD. Moreover, when considering doses of lithium for repurposing we should be mindful that high doses can be toxic, and are related to thyroid dysfunction, kidney injury, blood dyscrasias, and polydipsia, all of which can shorten lifespan [53]. Therefore, careful consideration of upper dose limits and further refinement of the optimal therapeutic range of lithium for anti-ageing purposes will need to be considered in the future.

There are a number of other limitations in this report that should also be acknowledged. First, the study makes a number of inferences about the effects of lithium based on associations and the use of genetic predictors, but ultimately prospective longitudinal data and functional studies are required to confirm our findings and to better understand how lithium mediates its telomere-lengthening effects in the context of different genetic backgrounds. Second, our BD sample size is relatively small and our study utilises samples from a severe clinical population on high doses of lithium, and therefore the results may not be representative of the wider unaffected population. Third, analysis using the *C. elegans* model may not reflect what is observed in humans. For instance, the 10 mM dose applied is ten times that which is found in the serum of BD patients and would be considered toxic for humans [54]; although the authors found that this dose was not toxic in their model, and it is generally accepted that smaller organisms require higher doses of drugs due to their faster metabolisms [55]. Future longitudinal studies assessing the effects of lithium in the context of telomere length and age-related disease risk will be best placed to confirm which gene transcripts are important in mediating lithium's telomere-lengthening effects. Fourth, although our polygenic predictor captures a significant amount of the variance in adult telomere length, the effect is still small, and may not be clinically useful in predicting age-related disease risk, or it may only be valuable when combined with disease-specific environmental risk factors [56]. Despite these limitations, our results extend previous work on the genetics of telomere length and confirms the potential utility of lithium as an anti-ageing compound, though we acknowledge that lithium's effects may be limited by the same polygenic factors responsible for baseline telomere length maintenance.

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ADDITIONAL INFORMATION

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5.2 CHAPTER 5 REFERENCES – THE ANTI-AGEING AND NEUROGENIC PROPERTIES OF LITHIUM

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5.3 POSTFACE

This study provides a deep insight into the polygenic nature of telomere length as well as the anti-ageing properties of lithium. First, the study confirms that there is a genetic correlation between telomere length and an increased risk for age related disease such as CAD. Second, the study shows that SNP-chip heritability estimates of 7.29% can be achieved for telomere length using the largest telomere GWAS, with polygenic risk scoring capturing 4.4% of the variance in an independent cohort. Third, by using gene-level analyses the study has provided a deeper understanding of the genetic regulatory mechanisms behind telomere length maintenance and has shown that lithium may be promoting longevity in an animal model by acting on the gene expression of some telomere related genes. This suggests that telomere length may be vital in promoting lithium-induced longevity as seen in some human and animal studies (317, (314, 315). Finally, the study shows that there is an association between telomere length in BD patients and the duration of lithium use. Moreover, the study shows that people who have a higher PRS for telomere length show longer telomere lengths if they have been taking lithium for long periods of time. This suggests that lithium may be catalysing endogenous telomere length promoting mechanisms and suggests that polygenic risk scoring could be used as a predictor for adult telomere length as well as for the anti-ageing efficacy of lithium treatment.

This study is the first to delve deeper into the polygenic nature of telomere length, a possible biological mechanism related to the aetiology or pathophysiology of psychiatric disorders (438, 443). It is also the first study to suggest that lithium-induced longevity is associated with telomere length regulation, and findings from this study were subsequently used to inform an *in vitro* model of cell ageing and long-term lithium treatment using a human hippocampal progenitor cell line.

6 – THE ANTI-AGEING AND NEUROGENIC PROPERTIES OF LITHIUM IN HUMAN HIPPOCAMPAL STEM CELLS

Figure 5.3.1 – Slowing down ageing.
An image depicting the efforts to slow down the ageing clock and increase the number of healthy years lived, rather than chase the quest for immortality. Taken from:
https://www.sciencenews.org/article/healthy-old-age-may-trump-immortality

6.1 INTRODUCTION

Telomeres are DNA repeat structures found at the ends of chromosomes, comprised of a six-base, TTAGGG, repeat sequence. They are vital in maintaining genomic stability and regulating cellular replicative capacity (446).

Telomere length gets shorter with each somatic-cell division due to the inability of DNA polymerase to fully replicate the 3' end of the new DNA strand. Once the telomere reaches critically short lengths, the cell is said to reach a 'Hayflick limit' and enter replicative senescence, meaning the cell is no longer able to divide and replace old and damaged cells (446, 447). Due to this, telomere length is considered to be a hallmark of biological ageing, with many age-related diseases such as cardiovascular disease (CAD) and diabetes being associated with shorter telomeres (254, 448-450).

Stem cells, germs cells and other cells that require continual renewal are usually able to maintain telomere length via the enzyme telomerase (451). Telomerase is a ribonucleoprotein that contains an endogenous telomerase reverse transcriptase (181) and a telomerase RNA component (TERC) (451). The latter component provides the template for nucleotide addition by TERT, as the enzyme travels along the newly synthesized strand. Mutations and common variants in the TERT or TERC genes are known risk factors for both physical and psychiatric disorders (234, 440, 452).

Previous studies have reported shorter leukocyte telomere length (LTL) in patients suffering from major depressive disorder (MDD), schizophrenia (SCZ) and bipolar disorder (BD), suggesting that these disorders may be associated with advanced cell ageing. Indeed, studies have shown that patients suffering from MDD, BD and SCZ are more likely to suffer from age-related disorders such as coronary artery disease (CAD), diabetes and dementia (265, 268,

453). This suggests that shorter telomere length may somehow be linked to psychiatric disorder aetiology or pathophysiology and could explain the high rates of age-related comorbidity.

Although neurons in the brain are non-mitotic (and therefore would be expected to have similar telomere lengths), a recent post-mortem study by Mamdani and colleagues (2015) reported significant telomere shortening in the cortices of all patient samples compared to other brain regions (277). In addition, they reported greater telomere shortening in the hippocampi of MDD subjects compared to controls, suggesting hippocampal stress-mediated accelerated cellular ageing in depression. Given that the hippocampus exhibits continual cell division throughout life, via a process called adult hippocampal neurogenesis (AHN), it is very likely that this process involves careful regulation of telomere length and telomerase activity (454). Aberrant telomerase maintenance and shorter telomeres may therefore lead to a reduction in AHN (284, 455), and smaller hippocampal volumes which subsequently increases risk of psychiatric and age-related neurological disorders. Indeed, a multitude of neuroimaging studies show a relationship between shorter LTL and smaller hippocampal volumes (313, 456), supporting a potential relationship between telomere length and adult hippocampal neurogenesis. However, association is not equivalent to causation, and there may be indirect moderators affecting both of these factors (e.g. cortisol) which drives the association. Consequently, further work is needed to confirm how telomere shortening in hippocampal progenitor cells affects rates of cell proliferation and their propensity to differentiate.

Interestingly, telomere length has been shown to increase in response to environmental stimuli such as exercise, a healthy diet and some pharmacological compounds (315, 457, 458). Lithium is one such compound, with some studies suggesting that it may promote anti-ageing effects in animals and humans (309, 459). A study by McColl and colleagues (2008) was one of the first to report this in C. elegans, showing that lithium can extend the lifespan of these organisms by

up to 30% (309). Another study by Zarse and colleagues showed that lithium in drinking water is associated with a reduction in all-cause mortality in 18 Japanese neighbourhoods (310). Furthermore, one study recently reported that lithium in drinking water is associated with a lower incidence of age-related dementia (311). Together these findings suggest that lithium may promote anti-ageing effects, and subsequent, complementary research hints that telomere lengthening may play a role.

Indeed, animal studies have suggested that lithium can increase telomerase activity and promote telomere elongation in mice (460). This has been shown to take place in the hippocampi of mice, suggesting that this may be the mechanism by which lithium can prevent the onset of dementia (given that the hippocampus is heavily implicated in learning and memory) (322, 335, 345). Lithium has of course been a long-standing treatment for mood disorders such as BD, and studies by our team (461) agree that lithium may have the ability to prevent or even elongate telomeres. What is observed in blood may similarly reflect lithium's effects on telomere length in the brain, as imaging studies have reported that long-term lithium use in BD patients is associated with a "younger brain age" and increased hippocampal volumes (321). This effect could theoretically be mediated by lithium's ability to promote telomere elongation in this brain region, maintaining or even increasing the replicative capacity of adult hippocampal stem cells, giving rise to its mood-stabilising and neuroprotective effects (319, 462). Alternatively, lithium's effects in blood and brain may be independent.

In summary, current research suggests that as well as being a mood stabilizer, lithium may also exert anti-ageing effects. The exact biological mechanism by which this occurs is not fully known, but studies suggest that telomere elongation and telomerase regulation may be key targets of lithium. This would support current findings which show that lithium is associated

with longer telomeres, larger hippocampal volumes and extended longevity in both humans and animals.

This study first aimed to investigate the effects of telomere shortening on human hippocampal progenitor cells. A better understanding of the cellular effects of telomere shortening on neurogenic processes could shed light on the cellular and molecular mechanisms contributing to the association between telomere length and hippocampal volume. Second, we aimed to test whether lithium treatment could lengthen telomeres in aging hippocampal progenitor cells and promote their proliferative capacity, or whether lithium's neurogenic mechanism was related to increased rates of progenitor differentiation.

6.2 METHODS

6.2.1 HUMAN HIPPOCAMPAL PROGENITOR CELL LINE

The work was carried out on a human foetal hippocampal progenitor cell (HPC) line, HPC007/03 (ReNeuron, UK). This cell line has been used as an *in vitro* model of human hippocampal neurogenesis in multiple studies (374, 463, 464). The cells were extracted from the hippocampal region of a terminated 12-week old female foetus and grown as a primary culture. They were then infected with a retroviral vector pLNCX2, carrying a c-MycER^{tam} transgene, which was constructed to generate a conditionally immortalised cell line. This means that the transgene can be activated by 4-hydroxytamoxifen (4-OHT), and with the additional presence of basic fibroblast growth factor (294) and epidermal growth factor (EGF), the cells will maintain cell proliferation without triggering the differentiation process. Upon the removal of 4-OHT, bFGF and EGF, the cells are allowed to differentiate into neurons, oligodendrocytes and astrocytes.

After 7 days of differentiation, characterization experiments show that the population is composed of around 35% DCX-positive cells, 25% MAP2-positive cells, 27% S100β-positive cells and 2% O1-positive cells (374). The rest of the cells remain as neural progenitors.

Given the established effects of c-myc activation on telomerase activation (465, 466), we removed 4-OHT from the media in all experiments described here, and grew cells only in the presence of the proliferative growth factors bFGF and EGF. Prior to the start of experiments, cells had been grown for at least four passages (~10 days) without 4-OHT.

6.2.2 CELL CULTURE CONDITIONS

Upon revival, HPCs were cultured in a T25 flask for one passage, after which they were routinely cultured and passaged in T75 flasks (Thermo Scientific, 156367/156499). All flasks were coated with 23 μg/ml laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma, L2020) in phosphate buffered saline (PBS; Gibco, 18912-014) for 1-24h to aid cell adhesion. Cells were maintained in a very specific, chemically-defined medium (**Table 6.2.1**) and incubated at 37^{O}C, 5% CO_2 with sufficient humidity in order to prevent evaporation.

Table 6.2.1 – Cell culture medium components.
The following table lists the contents of the cell culture medium used in all experiments, created by supplementing Dulbecco's modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM/F12).

Material names	***Concentration***	***Supplier***	***Catalogue number***
Dulbecco's modified Eagle's Medium	-	Sigma	D6421 or D6343
Human albumin solution	0.03 % (v/v)	Zenlab	20
Apotransferin	100 μg/ml	Sigma	T1147
Putrescine dihydrochloride	16.2 μg/ml	Sigma	P5780
Human recombinant insulin	5 μg/ml	Sigma	I9278
Progesterone	60 ng/ml	Sigma	P8783
L-glutamine	2 mM	Sigma	G75313
Sodium selenite	40 ng/ml	Sigma	S9133
Absent in differentiation medium (present in proliferation medium)			
Epidermal growth factor (EGF)	10 ng/ml	Peprotech	AF 100-15-500
Basic fibroblast growth factor (294)	20 ng/ml	Peprotech	EC 100-18B

6.2.3 CELL BANK AND REVIVAL

For all experiments, HPCs originated from a common cell bank that was created within the Thuret lab group. For these particular experiments, a Passage 13 cryovial was removed from storage and expanded so that up to 15 cryovials at Passage 16 could be stored. Subsequently, each Passage 16 cryovial was revived as an experimental condition. The cryovials were stored by resuspending in 10% dimethyl sulfoxide (DMSO; Sigma, D2650) in proliferation medium at a density of 1 x 10^6 cells/ml. The cells were frozen in cryovials (Corning, 430487) with the use of a Mr Frosty (Nalgene, C1562) at -80^{O}C for 24-48h before being transferred to liquid nitrogen for long-term storage. When reviving the cryovials for each experimental condition,

the cryovials were thawed at 37^{O}C for 1-2 minutes, followed by resuspension in 10 ml of prewarmed proliferation medium. Subsequent resuspension and centrifugation were carried out to remove any residual DMSO.

6.2.4 PASSAGING CELLS

When passaging, media was aspirated and cells were exposed to warm accutase (Sigma, A1110501) to lift them from monolayer, resuspended in warm medium, and washed twice via centrifugation at 900 rpm for 5 minutes with subsequent resuspension.

The cells were passaged approximately every 72h, at which point they were deemed to be 80-90% confluent, as confirmed by cell count. The average cell count across all passages was 3.6 x 10^6, SD = 0.5. When passaging, cells were reseeded at a density of 2 x 10^6 cells/T75 flask. The rest of the cells were removed for storage (for future DNA/RNA extraction) at -80^{O}C. Cells divided an average of 1.8 times across all passages, SD = 0.3.

Medium was changed 24h after passaging to remove residue accutase and clear dead cell and debris. The cells were regularly checked (daily via light microscopy and twice via mycoplasma detection) to make sure they were growing without any infections.

6.2.5 LONGITUDINAL HPC CULTURE

Each cryovial revived at Passage 16 was treated as a separate biological replicate (n=4). Each experimental condition involved reviving a cryovial at Passage 16, into a T25 flask supplemented with proliferation medium. Once confluent, the cells were passaged to a T75 supplemented with proliferation medium. The cells were passaged in this way for at least four passages (passage 20 or 21), each time reseeding 2 x 10^6 cells into a fresh T75 flask. This initial 'washout step' was to ensure that 4-OHT removal had no confounding impact on telomere length on the initiation of experiments.

6.2.5.1 OUR MODEL OF 'YOUNG' CELLS

Cells taken from passage 20 and 21 to model *relatively* 'young cells' (see **Figure 6.2.2**). These cells were then either isolated for DNA/RNA extraction (telomere and gene expression assessment), seeded onto two 96-well plates (Nunclon, Denmark) for proliferation and differentiation assays (see **Figure 6.2.4** and **Figure 6.2.5**) (467), or passaged onto another T75 for subsequent *in vitro* telomere experiments.

6.2.5.2 MODELLING TELOMERE SHORTENING & THE MODERATING EFFECTS OF A CHRONIC LITHIUM TREATMENT

Young cells were then grown under the conditions described above and systematically expanded to seed three T75s by passage 25 (corresponding to three treatment conditions). According to our pilot work, four passages is sufficient to evoke telomere shortening in cells (see **Figure 6.2.1**). A subset of cells (which we term "older cells") were collected from passage 25 for DNA/RNA extraction and to confirm telomere loss (see **Figure 6.2.3**).

At passage 25, after 24 hours of seeding the cells, we also initiated lithium drug treatments. Our three T75 flasks were entered into either a control (0 mM), low (0.75 mM) or high (2.25 mM) lithium chloride (LiCl) condition. Cells were grown in the same manner as described above and for a further four passages in T75 flasks; 24 hours of lithium-free media during seeding, followed by lithium treatment (0, 0.75 or 2.25 mM) until cells reached 80-90% confluency, at approximately 48 hours. On the final passage (passage 29), cells were pelleted for DNA/RNA extraction, or were plated onto two 96-well plates, for final proliferation (see **Figure 6.2.4**) and differentiation assays (see **Figure 6.2.5**). We term these cells as "old cells".

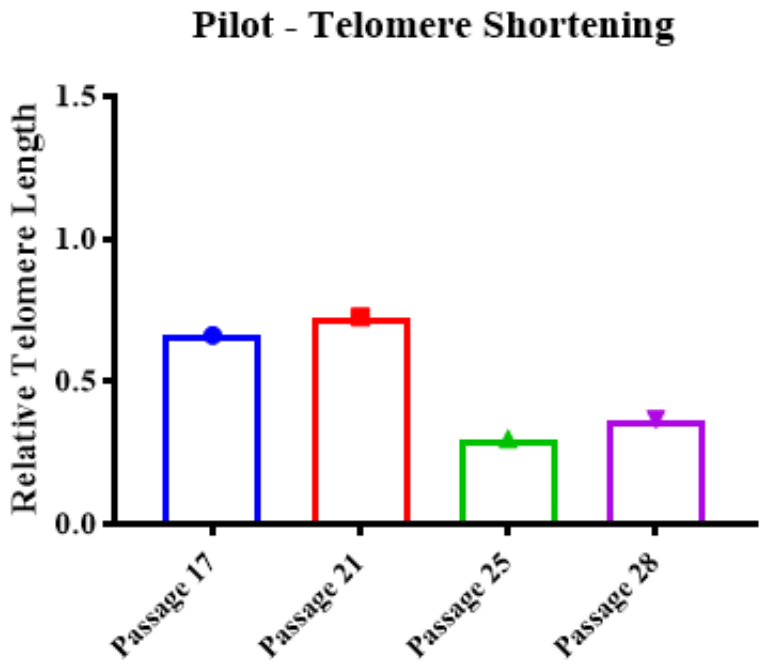

Figure 6.2.1 – Pilot experiment on telomere shortening.

This figure shows the relative telomere length of HPCs grown for 10 passages. The passage numbers are displayed on the x-axis and the relative telomere length is displayed on the y-axis. The results show that after Passage 24 relative telomere length is greatly reduced and remains so for a number of passages. This confirmed that growing cells without 4-OHT can shorten telomere length of HPCs over time and use them for a cell ageing model.

6.2.6 LITHIUM TREATMENT

Lithium chloride was obtained from Sigma (Sigma, Gillingham, UK) and reconstituted in sterile phosphate buffered saline to produce a 1 M stock concentration in 50 ml. This was split into five 10 ml working volumes and stored at 4 ^{O}C. Each tube was used for each biological replicate and used within 6 months of reconstitution. Relevant concentrations of LiCl were made by dilution in cell media and chosen to reflect a biologically relevant "low dose" (0.75 mM (468)), and a "high dose" (2.25 mM (three times that of the biologically optimal dose)) that falls within a range previously used in *in vitro* work (469). Both lithium's therapeutic effects and anti-ageing properties are reported to be more penetrant after chronic treatment,

and as such cells underwent a chronic treatment with lithium across five passages. As opposed to some studies which focus on studying the effects of drugs over a set period of time, our study primarily wanted to model the effects of long-term lithium over a set number of cell divisions. This allowed us to compare whether lithium could prevent telomere loss in cells undergoing approximately equal numbers of cell division. Consequently, this study focuses on a chronic lithium treatment in relation to cells reaching 80-90% confluency over the course of five passages (i.e. to undergo approximately 1.8 cell divisions per passage or 9 divisions in total). Nevertheless, a 48 hr treatment duration did consistently correlate with cells reaching confluency at each passage, with the exception of two instances (across all biological replicates/passages) where an extra 24 hr of treatment was required for cells to reach confluency. The differences in the length of treatment did not deviate significantly between conditions ($P > 0.05$).

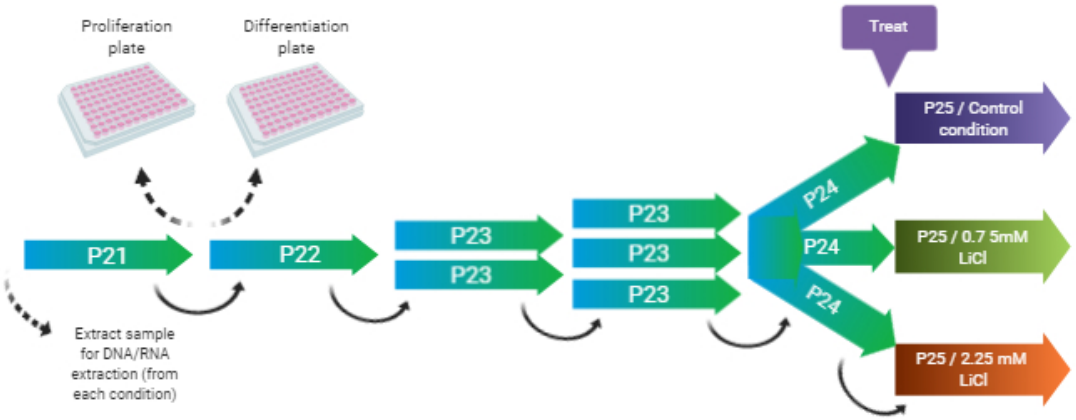

Figure 6.2.2 – A schematic of the assay performed on young cells prior to lithium chloride treatment. This schematic shows how at Passage 20 & 21, the young cells extracted, or were seeded into two 96-well plates (one plate for a proliferation assay and one plate for a differentiation assay). Following this, cells were expanded for three passages, so that one T75 flask could be split into three T75 flasks. Once in three separate flasks, the cells were randomly allocated a treatment condition (Control, 0.75 mM LiCl or 2.25 mM LiCl) and remained in this condition for the remainder of the assay.

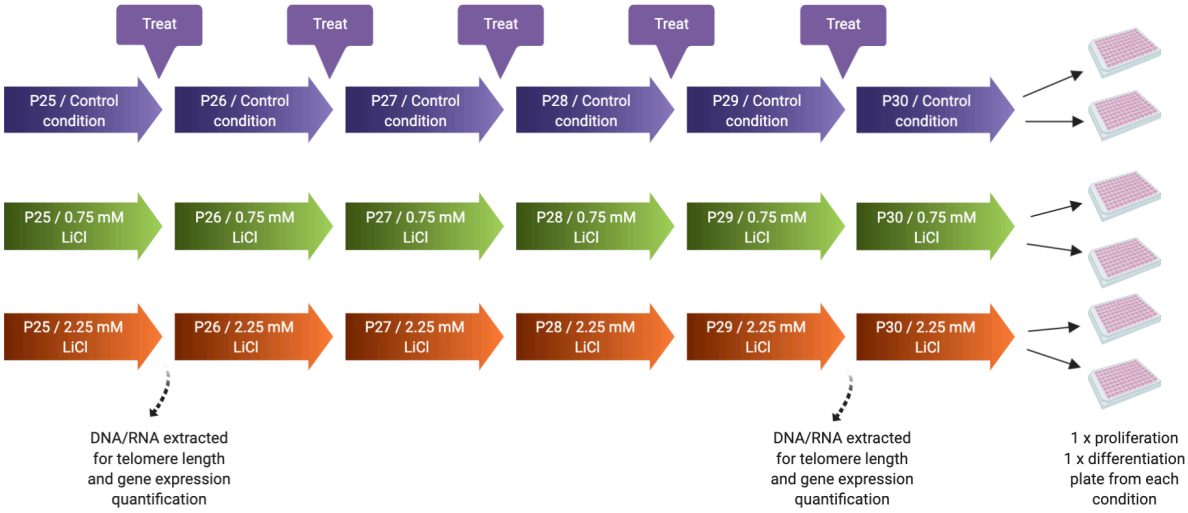

Figure 6.2.3 – A schematic of the assay performed on older cells.

This schematic shows the assay that was performed on older cells, following cell expansion. Once cells were allocated a condition (Control, 0.75 mM LiCl and 2.25 mM LiCl), they were grown in that condition across all remaining passages. After the first and fifth passages, subsets of cells were collected for DNA/RNA extraction. After the sixth passage, cells were plated into two 96-well plates (one for a proliferation assay and one for a differentiation assay), for each condition.

6.2.7 PROLIFERATION ASSAYS

6.2.7.1 SEEDING

Cells were seeded on laminin-coated wells on a 96-well plate at a density of 1.2 x 10-4 cells/well in 100 uL of proliferating cell media. Three technical replicates (wells) were generated in relation to each staining marker assayed per condition.

6.2.7.2 YOUNG CELLS

24h after seeding cells in a 96-well plate, the proliferation media was aspirated and replaced entirely. Cells were then grown in concentrations of lithium corresponding to their condition (control, low, high LiCl groups).

6.2.7.3 OLD CELLS

24h after seeding cells in a 96-well plate, the proliferation media was aspirated and replaced entirely. Cells were then grown in concentrations of lithium corresponding to their condition (control, low, high LiCl groups) (see **Figure 6.2.4**).

6.2.7.4 BROMODEOXYURIDINE (BRDU) INCORPORATION

One of the antibodies used to mark proliferation, binds Bromodeoxyuridine (BrdU) (BrdU; Sigma, B9285), and involves a BrdU incorporation step consisting of a 10 μM BrdU treatment for the last 4h prior to fixing (3 wells for each treatment condition). As cells divide and synthesize new DNA during each round of mitosis, they are able to incorporate synthetic nucleotide analogues such as BrdU during the S-phase (470). BrdU can then be detected using fluorescent microscopy and is only present in cells that recently divided.

At the end of both proliferation protocols, cells were fixed and immunostaining was performed to assay cell markers.

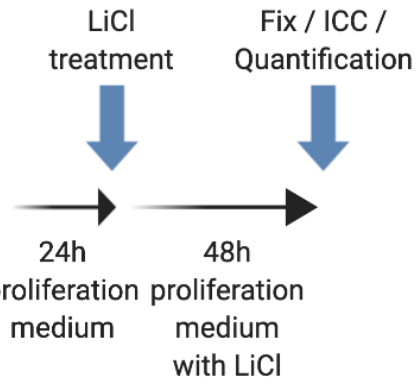

Figure 6.2.4 - A schematic showing the proliferation assay.
Following 24h incubation, the cells had a full medium change, corresponding to their allocated condition. They were left in this medium in the incubated for 48h, after which they were washed and fixed for immunocytochemistry and high throughput screening.

6.2.8 DIFFERENTIATION ASSAYS

The differentiation plates followed the same protocol as the proliferation plates (seeding, followed by 24-hour incubation, followed by 48-hour treatment with the relevant

concentrations of LiCl). After 48 hours, instead of fixation, the cells were washed twice with differentiation medium (proliferation medium without EGF or bFGF) in order to remove remaining growth factors and cell debris. Cells were then treated with the concentrations of LiCl relevant to their treatment condition (control, low or high doses) in differentiation media. These cells were then incubated for a further 7 days with no media changes, as previously done using this cell line (464) (see **Figure 6.2.5**).

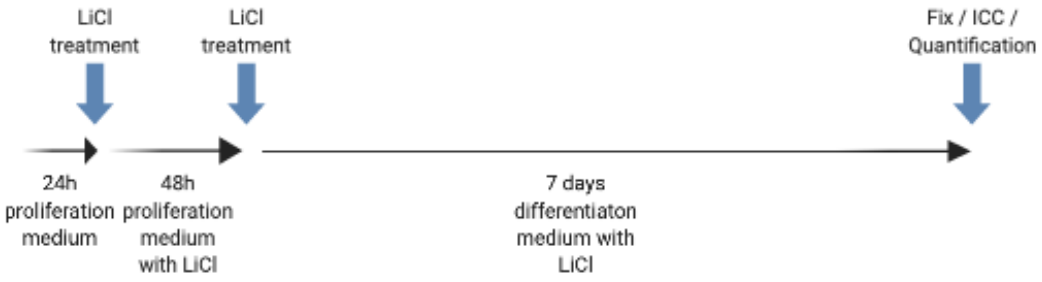

Figure 6.2.5 - A schematic showing the differentiation assay.

Following 24h incubation, the cells had a full medium change, corresponding to their allocated lithium treatment condition. They were left in this medium in the incubated for 48h, after which they were washed with the normal differentiation medium and finally left in the differentiation medium supplemented with their allocated lithium treatment condition for 7 days. After 7 days, the cells were washed and fixed for immunocytochemistry and high throughput screening.

6.2.8.1 Cell fixing

At the end of the proliferation/differentiation protocols, the culture medium was aspirated and HPCs were washed with warm cell media to remove any cell debris and any remaining treatment. The cells were then fixed with 50 µl/well of freshly thawed 4% paraformaldehyde (PFA, Alfa Aesar 43368) in PBS and incubated for 20 mins at room temperature and in the dark. The PFA was removed after 20 mins and the fixed cells were washed three times with PBS. The plates were then stored in 0.05% Sodium Azide (Sigma, S8032) in PBS, wrapped in parafilm (Sigma, P7793) at 4^{O}C.

6.2.9 Cellular Analysis

6.2.9.1 IMMUNOCYTOCHEMISTRY

Immunocytochemistry (ICC) was carried out on control and lithium treated cells in order to analyse the fate, viability and health of these cells, based on morphological and protein localisation quantification. For BrdU cell only, following fixation the cells were incubated with 2N hydrochloric acid for 40 mins in order to denature the DNA strands. Cells were then neutralised with 0.1 M sodium borate buffer for 10 mins and washed with PBS.

For all cells, blocking solution comprised of 5% normal donkey serum (Sigma, D9963) and 0.3% Triton X-100 (Sigma, T9284) in PBS was used to block cells for 1h at room temperature.

Primary antibodies were diluted to the appropriate concentration in the blocking solution (see **Table 6.2.2**) and added to the cells for overnight incubation at $4^{O}C$. Antibodies were carefully chosen to identify cells at varying degrees of proliferation, differentiation and apoptosis. First, the cells were washed with PBS and incubated with blocking solution for 30 mins. During these 30 mins, secondary antibodies were diluted in blocking solution at the appropriate concentrations (see **Table 6.2.3**) and added to cells for 2h. Cells were then washed twice with PBS and the nuclei were stained with 300nM DAPI for 5 mins. Following this, cells were washed twice with PBS and stored in 0.05% sodium azide in PBS at $4^{O}C$, wrapped in tin foil to block sunlight and prevent photobleaching.

Table 6.2.2 – A table listing all the primary antibodies used.

Primary Antibody	*Dilution*	*Supplier*	*Catalogue #*	*Marker usage*
Rat anti-**BrdU**	1:500	Serotec	OBT0030CX	5-bromo-'2'-deoxyuridine, marker of proliferation by labelling cells during DNA synthesis
Mouse anti-**Ki67**	1:500	Abcam	Ab15580	Marker of proliferation, labels cells active in the cell cycle
Rabbit anti-**S100β**	1:500	Dako	Z0311	S100 calcium-binding protein β, a marker of astrocytes
Mouse anti-**MAP2**	1:500	Abcam	Ab11267	Microtubule associated protein 2, a marker of neurons
Rabbit anti-**CC3**	1:500	Cell Signalling	9964	Cleaved caspase 3, marker of caspase-dependent apoptosis
Rabbit anti-**DCX**	1:500	Abcam	Ab18723	Doublecortin, marker of neuroblasts and immature neurons

Table 6.2.3 – A table listing all the secondary antibodies used.

Secondary Antibody	*Dilution*	*Supplier*	*Catalogue #*
Alexa Fluor 488 Donkey anti-rat IgG	1:500	Life Technologies	A-21208
Alexa Fluor 488 Donkey anti-mouse IgG	1:500	Life Technologies	A-21202
Alexa Fluor 555 Donkey anti-rabbit IgG	1:500	Life Technologies	A-31572
Alexa Fluor 555 Donkey anti-mouse IgG	1:500	Life Technologies	A-31570

Every plate was designed to contain unstained control cells which were used as a benchmark for background fluorescence in downstream analyses. Antibody combinations were kept the same for all experiments and can be viewed alongside their representative images in **Figure 6.2.6** for proliferation and **Figure 6.2.7** for differentiation.

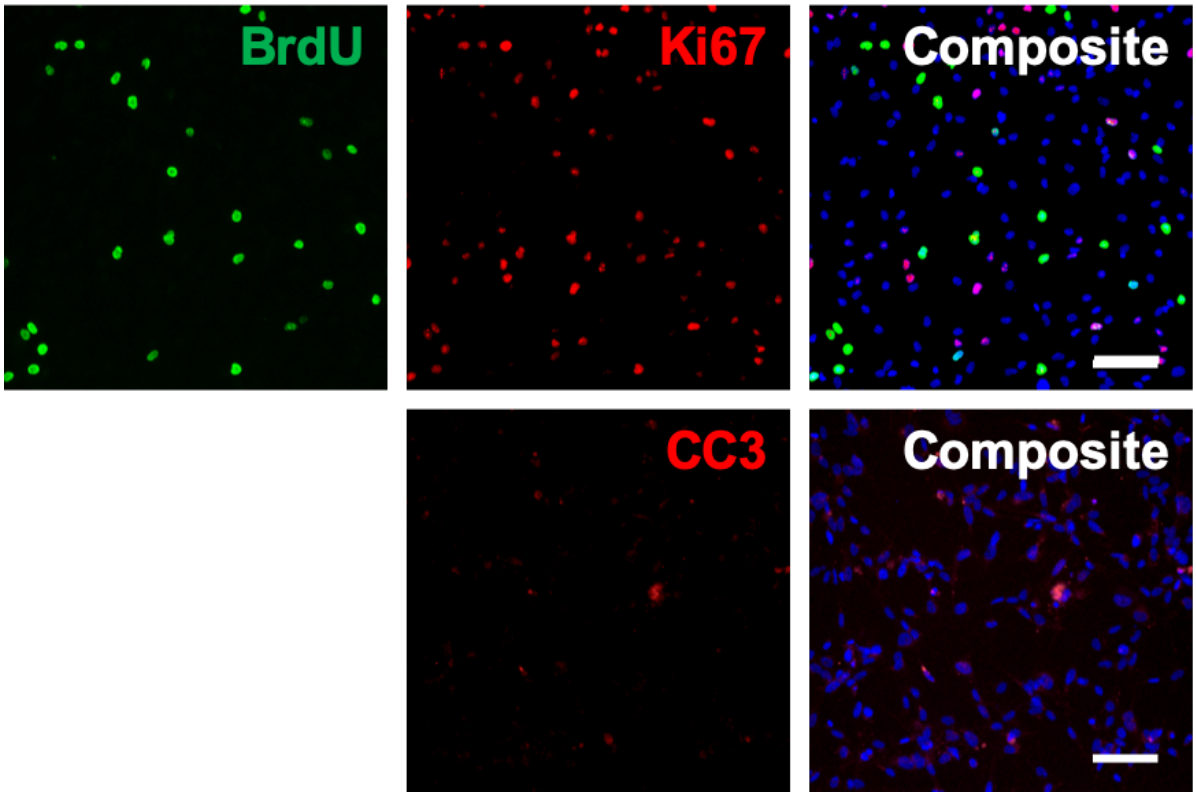

Figure 6.2.6 – Representative images of the markers used in the proliferation assays.
Each of the above images is representative of a field of immune-stained cells, taken using a 10X objective with the CellInsight High Content Screening Platform. East composite image includes the nuclear marker DAPI in blue, which is not displayed on its own. The proliferation, survival and hippocampal progenitor cell numbers can be seen using a variety of markers. Row 1 shows the proliferation markers bromodeoxyuridine (BrdU – in green) and Ki67 (in red). Row 2 shows the apoptotic maker cleaved caspase 3 (CC3- in red). Scale bar = 100μm.

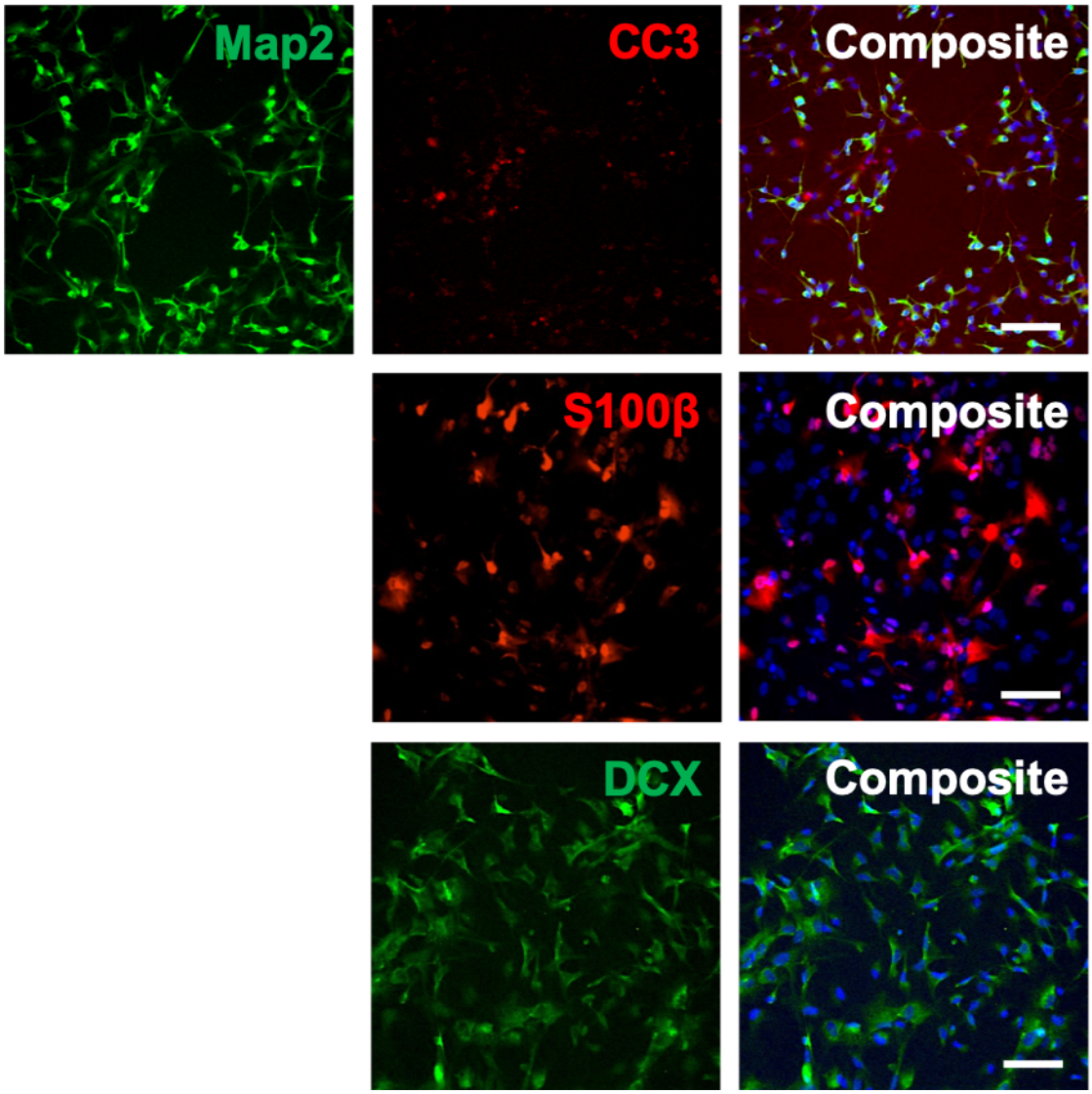

Figure 6.2.7 – Representative images of the markers used in the differentiation assays.

Each of the above images is representative of a field of immune-stained cells, taken using a 10X objective with the CellInsight High Content Screening Platform. East composite image includes the nuclear marker DAPI in blue, which is not displayed on its own. The differentiation and survival of cells can be seen using a variety of markers. Row 1 shows the neuronal marker microtubule-associated protein 2 (MAP2 – in green) and the apoptotic maker cleaved caspase 3 (CC3 – in red). Row 2 shows the astrocytic marker S 100 calcium-binding protein β (S100β - in red). Row 3 shows the neuronal stem cell marker doublecortin (DCX – in green). Scale bar = 100μm.

6.2.9.2 QUANTIFICATION OF CELL MORPHOLOGY AND PROTEIN LOCALISATION

Quantification of cell antibody staining was performed using an unbiased and semi-automated high-throughput Thermo Scientific Cell-Insight CX5 High Content Screening Platform (Thermo Scientific) alongside the native HCS Studio Cell Analysis Software (Thermo Scientific). Separate protocols were developed for each antibody using the Cell Health Profiling BioApplication, which first identifies cells based on their nuclear staining and then quantifies the intensity of fluorescent staining in user-defined regions. Once a protocol was developed, it was kept constant across all the other plates and biological replicates. On rare occasions the parameters were changed to account for differences in the brightness of immunofluorescence between biological replicates.

The Cell-Insight can detect fluorophores at three different wavelengths: 386/440 (e.g. blue / DAPI), 485/521 (e.g. green / Alexa 488) and 560/607 (e.g. red / Alexa 555) using 10x magnification. Using DAPI as a proxy, the Cell-Insight autofocuses on the DAPI stained nuclei and records images for the other two channels on the same plane.

The protocol involved manually calculating the exposure times to ensure a good signal-to-background ratio, using the unstained control cells as the background. This was performed using representative images from two stained and two unstained wells.

Images then underwent a background removal step using pre-defined software parameters. These parameters automatically identify and correct for the outline of individual nuclei, in relation to the background. Following this, smoothing, threshold and segmentation parameters were adjusted manually to ensure individual nuclei are identified correctly. These nuclei were then filtered by size to make sure that small debris and unsegmented nuclei were removed from downstream analyses. In addition, any nuclei which touched the outer border of the visual field were removed from downstream analyses.

The next step involved locating and quantifying cellular markers. A target location (**Table 6.2.4**) was defined for each marker with a defined distance from the nuclear outline (for identifying nuclear proteins), or a ring with the inner and outer boundary defined by its distance from the inner (nuclear) boundary (for identifying cytoplasmic proteins). Staining for each marker was identified based on the average fluorescent intensity, determined by a manually defined threshold.

Altogether this method calculates the proportion of DAPI-positive cells which have a positive stain for each marker (reported as a percentage). 15 fields were imaged per well, across three wells (technical replicate) to provide a single data point per marker, per biological replicate.

Table 6.2.4 – Cellular marker identification and localisation parameters used during high-throughput screening.

Immunocytochemical staining of all cellular markers was quantified in predefined cellular regions, all relative to the cell nucleus. These target regions are either a circle or a ring, depending on whether the marker is nuclear or cytoplasmic, respectively. The target sizes are all relative to the nucleus boundary. Distance described the distance from the nuclear boundary that the circle or ring lies, and Width describes how wide the ring target is. Measurements are given in μm.

Marker	*Cellular localisation*	*Target shape*	*Relative target size*
Ki67	Nucleus	Circle	Distance = 0
BrdU	Nucleus	Circle	Distance = 0
CC3	Near the nucleus	Circle	Distance = 0
DCX	Cytoplasm	Circle	Distance = 6
MAP2	Cytoplasm	Ring	Distance = 0 Width = 6
S100β	Nucleus	Circle	Distance = 0

6.2.10 TELOMERE QUANTIFICATION AND GENE EXPRESSION ANALYSES

6.2.10.1 DNA / RNA EXTRACTION

Approximately 50% of the cell suspension medium at each passage was pelleted and stored for future DNA/RNA/Protein extraction. During the passaging process, after the first spin at 900 rpm for 5 mins, the cells were resuspended in 5ml of medium. 2.5 ml of this cell suspension medium was split into two 1.5 ml Eppendorf tubes each (FisherScientific, 12380343). The remaining cell media was diluted (according to cell count data) and used for reseeding. The passaging process then continued with a second spin and the cells were resuspended in 10 ml of culture medium. The Eppendorf tubes were spun in a microcentrifuge at 900 rpm for 5 mins, the excess medium was removed, and the tubes were snap frozen for storage at -80 ^{O}C.

DNA and RNA were extracted using the Qiagen AllPrep DNA/RNA/Protein Mini Kit (Qiagen, 80004). The standard manufaturer's protocol was followed to extract DNA, RNA and protein from each cell pellet. The RNA and DNA concentration and purity were measured following the extraction process. DNA was deemed suitable if the 280/260 ratios were between 1.7 and 1.9, and the 260/230 ratios above 1.7. RNA was deemed suitable if the 280/260 ratios were between 1.9 and 2.1 and the 260/230 ratios above 1.7. If the purity was low, an ethanol precipitation was carried out to clean up the DNA and RNA. All samples were stored at -80^{O}C and used for experiments within 6 months.

6.2.10.2 TELOMERE QUANTIFICATION

Telomere quantification using DNA was carried out as described previously (see Chapters 4 and 5). All samples were run on the sample plate to reduce variability.

6.2.11 GENE EXPRESSION

6.2.11.1 GENES OF INTEREST

To investigate whether there were gene expression differences between old and young cells, and whether this was moderated by lithium treatment, we performed candidate gene expression analysis using quantitative real-time PCR (qPCR). Four genes (*NEK6*, *NCDN*, *SBNO2*, *GAB1*) were selected based on analysing the effects of age on human hippocampal dentate gyrus granule cells, using a publicly available RNA-sequencing dataset (471), see Table 1.2.6 and *Supplementary information* for further details.

The other genes were chosen based on our previous research. *TERT, LRRC34, ZNF257,* and *NAF1* were all genes that we found to be implicated in telomere length regulation from our gene-level enrichment analyses (461). Gene expression was normalised to both a genomic standard curve and to the reference gene Vimentin (*VIM*), as part of the relative quantification method (275). *VIM* is a gene that shows very low levels of variation in the hippocampal cell line as identified from microarray studies, and has previously been used as a reference gene both in relation to drug treatment and ageing related research (464, 472), see **Table 6.2.5** for a full list of the genes used.

Table 6.2.5 – A table listing the genes used I the gene expression analysis.

The table below lists the gene names, gene symbol and justification for all of the genes used in the gene expression analysis.

Gene name	***Gene symbol***	***Justification for using the gene***
NIMA Related Kinase 6	*NEK6*	One of top two downregulated genes with age in the human dentate gyrus
Neurochondrin	*NCDN*	One of top two downregulated genes with age in the human dentate gyrus
Strawberry Notch Homolog 2	*SBNO2*	One of top two upregulated genes with age in the human dentate gyrus

GRB2-associated-binding-protein 1	*GAB1*	One of top two upregulated genes with age in the human dentate gyrus
Telomerase Reverse Transcriptase	*TERT*	Found to be significant in the largest telomere GWAS as well as the MAGMA gene-set analysis on telomere length
Leucine-rich-repeat-containing protein 34	*LRRC34*	The most significant gene found in the MAGMA gene-set analysis on telomere length
Zinc Finger Protein 257	*ZNF257*	A gene found to be significant in the MAGMA gene-set analysis on telomere length, and a gene that is associated with lithium-induced longevity
Nuclear Assembly Factor 1	*NAF1*	A gene found to be significant in the MAGMA gene-set analysis on telomere length, and a gene that is associated with lithium-induced longevity
Vimentin	*VIM*	Reference gene

All primers were designed systematically by exploring the gene of interest using UCSC genome browser (https://genome.ucsc.edu), selecting an exon within each gene that incorporates all transcript versions, and outputting the nucleotide sequence. This genomic sequence was then input into Primer3 (http://primer3.ut.ee; product range of between 80-150 BP), where primers were designed. Specificity of primers was confirmed *in silico,* using the 'in silico PCR' tool in UCSC genome browser, and validated in pilot experiments by assessing qPCR melting curves.

Each primer was specifically designed to generate a single amplicon, and to fall within a single exon, so that it would create the same product size for either gDNA or cDNA, allowing for the

accurate use of a gDNA standard curve. Primers were supplied by Integrated DNA Technologies (IDT, London, UK).

6.2.11.2 QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

6.2.11.3 GENOMIC DNA WIPEOUT

1 µg of RNA from each sample was submitted to gDNA wipeout using the DNase Max Kit (Qiagen, Crawley, UK). This was carried out to increase the purity of RNA, prevent non-specific binding of primers and achieve optimal qPCR results.

6.2.11.4 REVERSE TRANSCRIPTION

gDNA-free RNA was the submitted to reverse transcription using the Invitrogen SuperScript III Reverse Transcriptase kit (Invitrogen, Warrington, UK), to generate complementary DNA (cDNA). cDNAamples were then diluted to a working concentration of 5ng/µl.

6.2.11.5 QPCR

qPCRs were performed on 384-well plates with each sample plated in triplicate. Per gene of interest, we used an eight-point standard curve consisting of DNA samples at 0.47 ng, 0.94 ng, 1.88 ng, 3.75 ng, 7.5 ng, 15 ng, 30 ng, 60 ng amounts. This allowed us to correct for any minor differences in PCR efficiency between our target and reference gene (*VIM*). We also included a no-template control consisting of RNase free water to check for nucleic acid contamination.

The final 12.5 µl qPCR reaction consisted of 25 ng of cDNA – 5 µl, 1x EvaGreen – 2.5 µl of 5x mix, 200 nM of forward and reverse primers – 0.25 µl, and 4.75 µl of water, to make up 12.5µl total volume. The thermocycling conditions were as follows: 15 min at 95 ^{O}C (denaturation and Taq enzyme activation), then 40 cycles of: 30 sec at 95 ^{O}C, 30 sec at 60 ^{O}C, and 30 sec at 72 ^{O}C (data collection), followed by a melting curve. qPCRs were ran on the QuantStudio 5 Realtime PCR system (Thermo Fisher Scientific, Massachusetts, USA). Cycle

threshold (C_t) values were the primary data produced, and corresponds to how many PCR cycles were required for a sample to reach a predefined level of fluorescence.

6.2.12 STATISTICAL ANALYSIS

6.2.12.1 QUALITY CONTROL

Quality control for telomere length quantification and gene expression analyses were almost identical and described previously (Chapters 4 and 5). Briefly, single outliers were identified and removed if C_t values corresponding to technical triplicates generated a standard deviation (SD) of above 0.5. If the C_t SD remained above 0.5 upon outlier removal, that sample was removed from further analyses. For samples that survived quality control, C_t values were related to absolute quantities as part of a standard curve to generate C_q values. The average C_q value was then generated across replicates, for each sample.

6.2.12.2 NORMALISATION

To generate relative telomere length, the average C_q value pertaining to the telomere repeat sequence was divided by the C_q relating to the single copy gene, albumin. For relative gene expression analyses the average C_q pertaining to the target gene was divided by the C_q relating to the reference gene, *VIM*.

6.2.12.3 COMPARING YOUNG AND OLD CELLS

(i) Telomere experiments: To confirm telomere shortening, we used a one-way ANOVA to compare young cells, older cells collected at the midpoint of the ageing protocol and at the start of the lithium treatment protocol, and old cells.

(ii) Immunostaining & gene expression experiments: We compared immunofluorescent cell marker measures and gene expression differences between young and old cells

using two-tailed t-tests. Statistically significant differences were considered when $p < 0.05$.

6.2.12.4 COMPARING THE EFFECTS OF CHRONIC LITHIUM TREATMENT IN OLD CELLS

For both telomere length comparisons and immunoflorescene cell marker measures, we used a one-way ANOVA. All statistical analyses were carried out using GraphPad Prism (version 6.07, GraphPad Software Inc.).

6.2.12.5 MULTIPLE TESTING CORRECTION

As multiple staining markers were assessed in either proliferating or differentiating cells, we applied the Bonferroni method of multiple testing correction on t-tests and ANOVA. For ANOVA results which remained significant after correction, the Tukey posthoc correction was applied to determine pair-wise group differences.

6.3 RESULTS

6.3.1 HIPPOCAMPAL PROGENITOR CELLS SHOW TELOMERE SHORTENING AND NEUROGENIC DIFFERENCES WITH AGE

6.3.1.1 HIPPOCAMPAL PROGENITOR CELLS SHOW TELOMERE SHORTENING IN ASSOCIATION WITH THE END REPLICATION PROBLEM

We compared relative telomere length from young cells, older cells that correspond to the midpoint (P25) and the start of the lithium treatment experiments, and old cells from the end of our cell protocol. ANOVA revealed significant differences in telomere length between the groups ($F(4, 9) = 9.349$, $p = 0.013$), whereby telomeres were significantly shorter in older cells ($p < 0.05$), and old cells ($p < 0.05$) relative to young cells, see **Figure 6.3.1**.

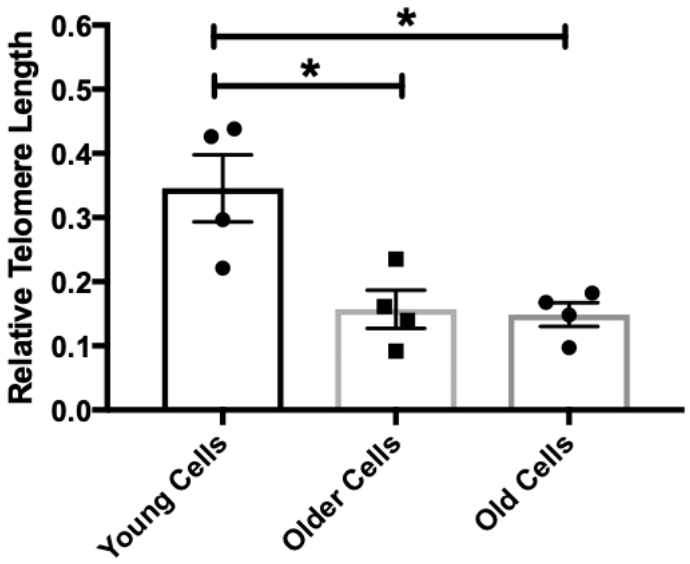

Figure 6.3.1 – A bar chart comparing the relative telomere length of young, older and old cells.

This bar chart shows the relative telomere length of young cells, older cells and old cells. Cells were not treated with lithium at any point. Relative telomere length was calculated by dividing the quantity of telomeric repeats by albumin present in the DNA of each sample. The results show that there is a significant reduction in telomere length in both the older and old cells, relative to young cells. Group differences were detected using a one-way ANOVA with Tukey's post-hoc test. Significant differences were considered when $p < 0.05$, indicated by *. N = 4 for all groups.

6.3.1.2 TELOMERE SHORTENING IS ASSOCIATED WITH REDUCTIONS IN CELL PROLIFERATION

Our results in proliferating cells revealed a significant difference in cell proliferation between young and old cells. Old cells show a significant reduction in the percentage of cells positive for BrdU (two-sample $t(6) = 6.663$, $p < 0.001$), a difference which remained significant after multiple testing correction ($p < 0.05$). This difference is also supported by the proliferation marker Ki67, which revealed a nominally significant difference, with young cells proliferating more than old cells (two-sample $t(6) = 2.959$, $p = 0.025$), see **Figure 6.3.2**. Amongst proliferating cells we also observed a higher percentage of young cells stained with CC3 compared to old cells (two-sample $t(6) = 2.481$, $p = 0.047$), which is indicative of lower rates of cell death, however this effect did not survive multiple testing correction ($p > 0.05$).

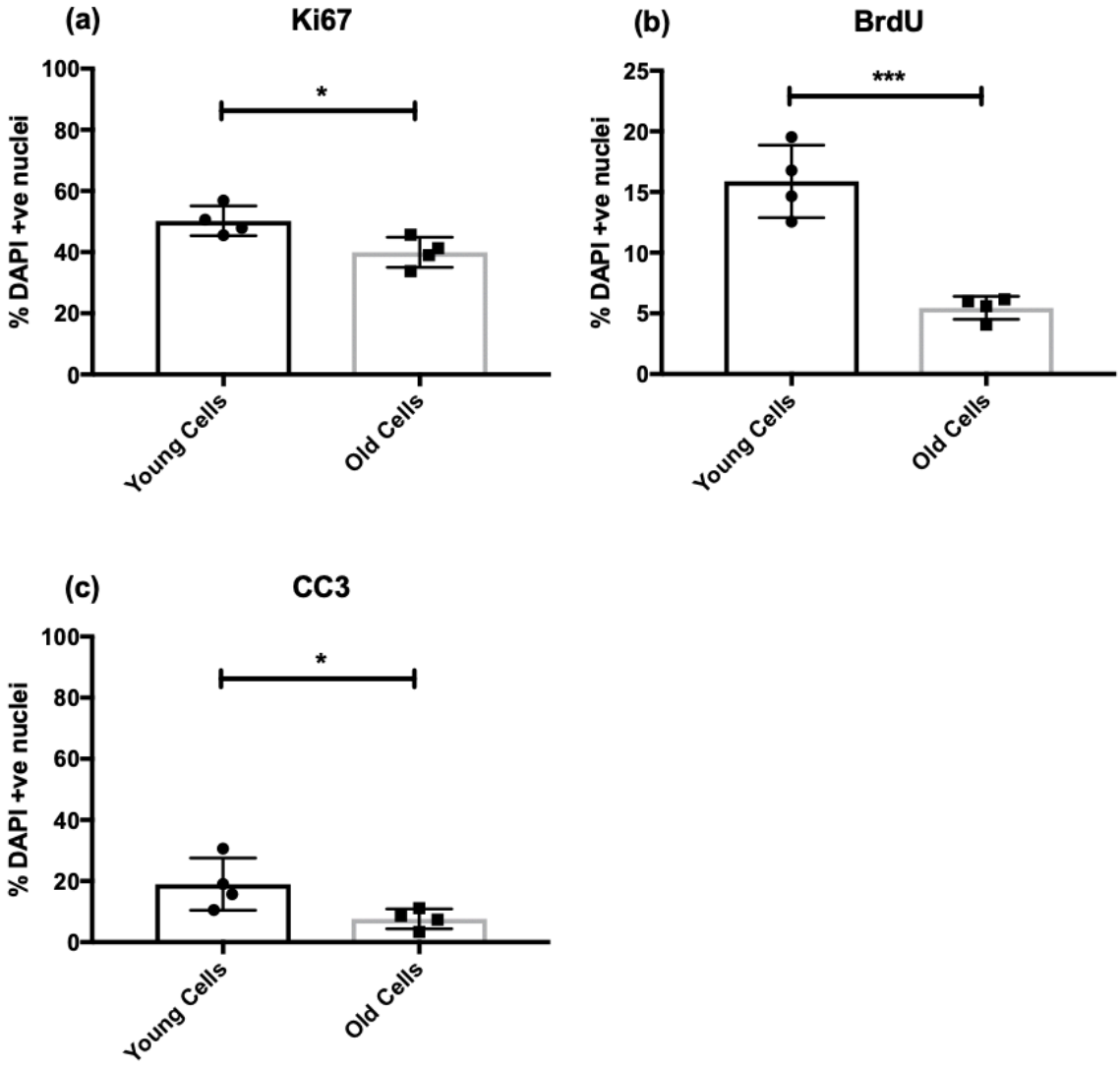

Figure 6.3.2 – Bar charts showing the percentage of Ki67, BrdU and CC3 positive cells as well as the total cell number in young and old proliferating cells.

These bar charts show the percentage of Ki67, BrdU and CC3 positive cells relative to the percentage of DAPI stained nuclei (y-axis) in young cells and old cells (x-axis). Each data point represents one biological replicate. (a) The percentage of Ki67 positive cells decreases in older cells compared to younger cells, as detected by a two-sample t-test, $p = 0.025$. (b) The percentage of BrdU positive cells decreases significantly in older cells compared to younger cells, as detected by a two-sample t-test, $p < 0.001$. (c) The percentage of CC3 positive cells decreases in old cells compared to young cells, as detected by a two-sample t-test, $p = 0.047$.

CELL DIFFERENTIATION

While we observed no differences ($p>0.05$) in markers of glial cells (S100ß) or more mature neurons (MAP2) in young versus old cells, there was a small increase in the number of

doublecortin positive neurons in older cells (two-sample t(6) = 3.097, p = 0.021), though this difference did not survive multiple testing correction (p > 0.05), see **Figure 6.3.3**.

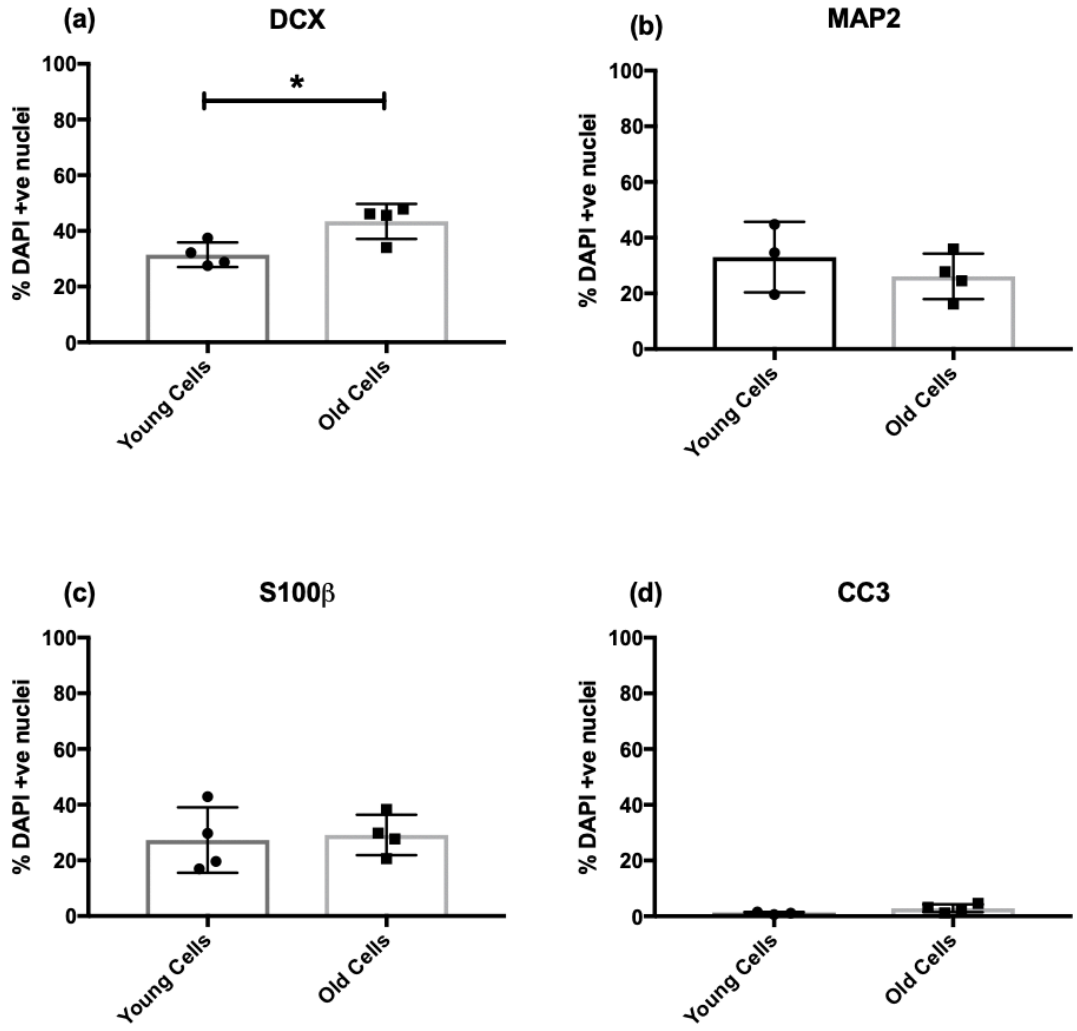

Figure 6.3.3 – Bar charts showing the percentage of DCX, MAP2, S100ß and CC3 positive cells in young and old cells.

These bar charts show the percentage of DCX, MAP-2, S100ß and CC3 positive cells relative to the percentage of DAPI stained nuclei in young cells and old cells. Each data point represents one biological replicate. Passage numbers are indicated on the x-axis and the percentage of immune-stained cells relative to DAPI positive nuclei is displayed on the y-axis. (a) The percentage of DCX positive cells increases in older cells compared to younger cells, as detected by a two-sample t-test, p = 0.021. (b) There is no significant difference in the percentage of MAP2 positive cells in older and younger cells. (c) There is no significant difference in the percentage of S100β positive cells in older and younger cells. (d) There is no significant difference in the percentage of CC3 positive cells in older and younger cells.

6.3.1.4 THERE ARE NO SIGNIFICANT CHANGES IN TELOMERE-RELATED OR AGE-RELATED GENES WITH INCREASING PASSAGE NUMBER

We did not find any significant differences in telomere-related or age-related gene expression between young and old cells ($p>0.05$), see **Figure 6.3.4**.

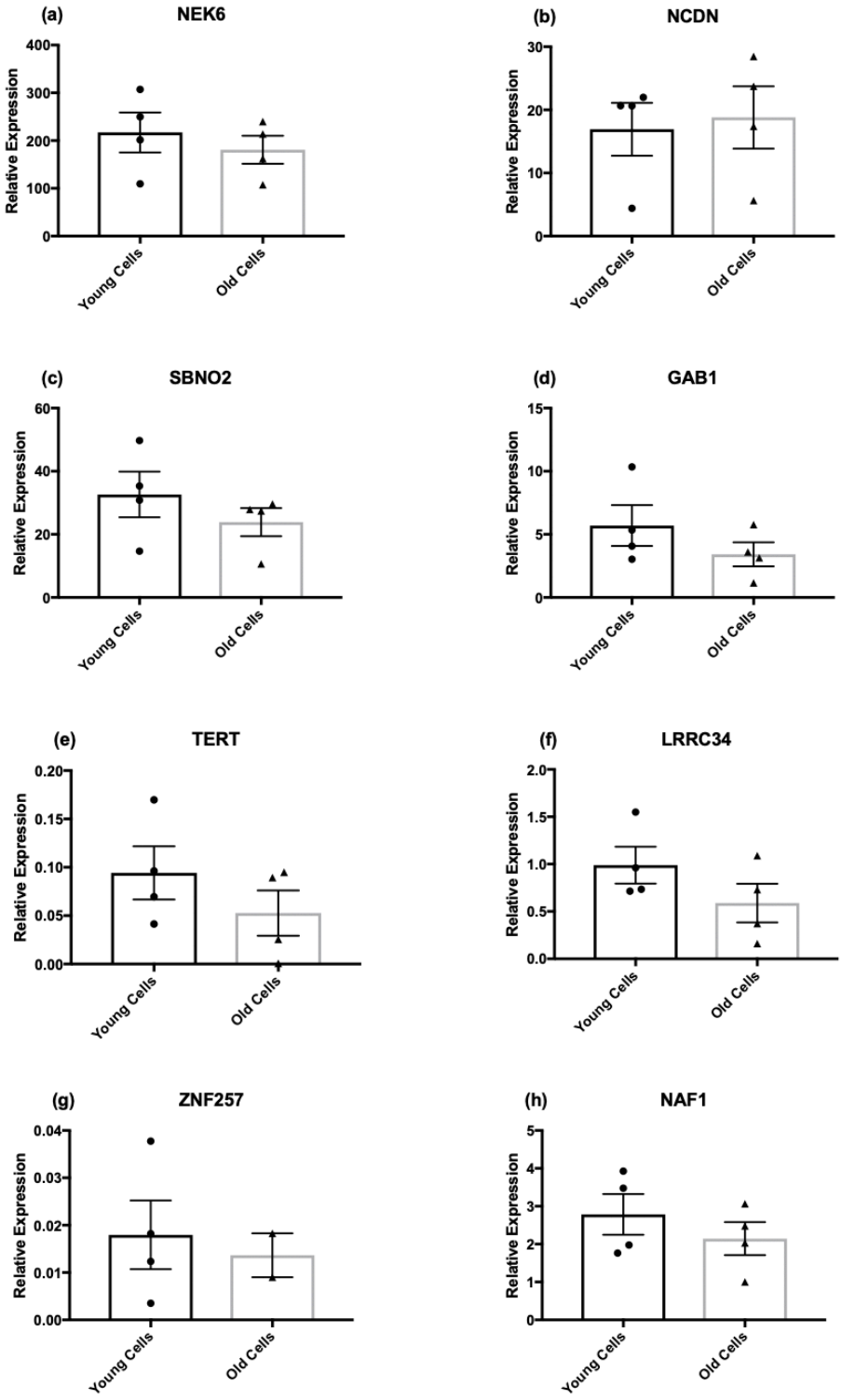

Figure 6.3.4 – Bar charts showing the differences in gene expression between young and old cells.

These bar charts show the relative gene expression (y-axis) of telomere-related and age-related genes in young cells and old cells (x-axis). Relative expression of each gene to VIM (housekeeping gene) was calculated by dividing the average mRNA quantities of each sample by the average quantity of VIM. Each data point represents one biological replicate. (a) Relative gene expression of NEK6. (b) Relative gene expression of NCDN. (c) Relative gene expression of SBNO2. (d) Relative gene expression of GAB1. (e) Relative gene expression of TERT. (f) Relative gene expression of LRRC34. (g) Relative gene expression of ZNF257. (h) Relative gene expression of NAF1. No significant differences in gene expression were found between younger and older cells after a two-sample t-test ($p > 0.05$).

6.3.2 LONG-TERM LITHIUM TREATMENT INCREASES HIPPOCAMPAL PROGENITOR CELL DIFFERENTIATION TOWARDS A NEURONAL AND GLIAL FATE, RATHER THAN INCREASING TELOMERE LENGTH OR INCREASING CELL PROLIFERATION

6.3.2.1 THERE IS NO SIGNIFICANT EFFECTS OF CHRONIC LITHIUM TREATMENT ON TELOMERE LENGTH

The results from long-term LiCl treatments reveal no significant effects on telomere length ($F(2, 9) = 2.767$, $p = 0.116$, see **Figure 6.3.5**. These results suggest that long-term lithium use may be conferring neuroprotective effects by mechanisms other than telomere regulation.

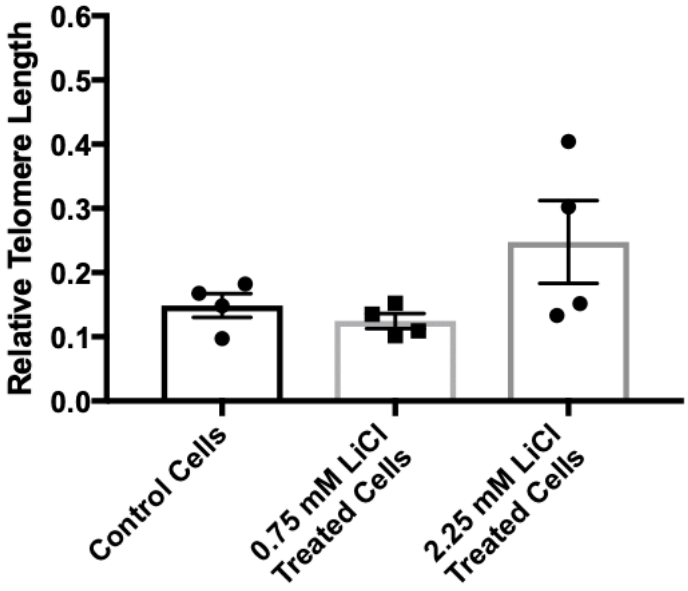

Figure 6.3.5 – Bar charts comparing the relative telomere length of old control cells, 0.75 mM LiCl treated cells and 2.25 mM LiCl treated cells.

These bar charts show the relative telomere length of old cells, subject to either the control, the 0.75 mM long-term LiCl or the 2.25 mM long-term LiCl treatment conditions. Relative telomere length was calculated by dividing the quantity of telomeric repeats by albumin present in the DNA of each sample. The results show no significant difference between any of the conditions based on a one-way ANOVA. N=4 for all groups.

6.3.2.2 THERE ARE NO SIGNIFICANT CHANGES IN TELOMERE-RELATED OR AGE-RELATED GENES FOLLOWING CHRONIC LITHIUM TREATMENT

One-way ANOVA did not reveal any significant changes to gene expression of our eight candidate genes following lithium treatment ($p > 0.05$), see **Figure 6.3.6**.

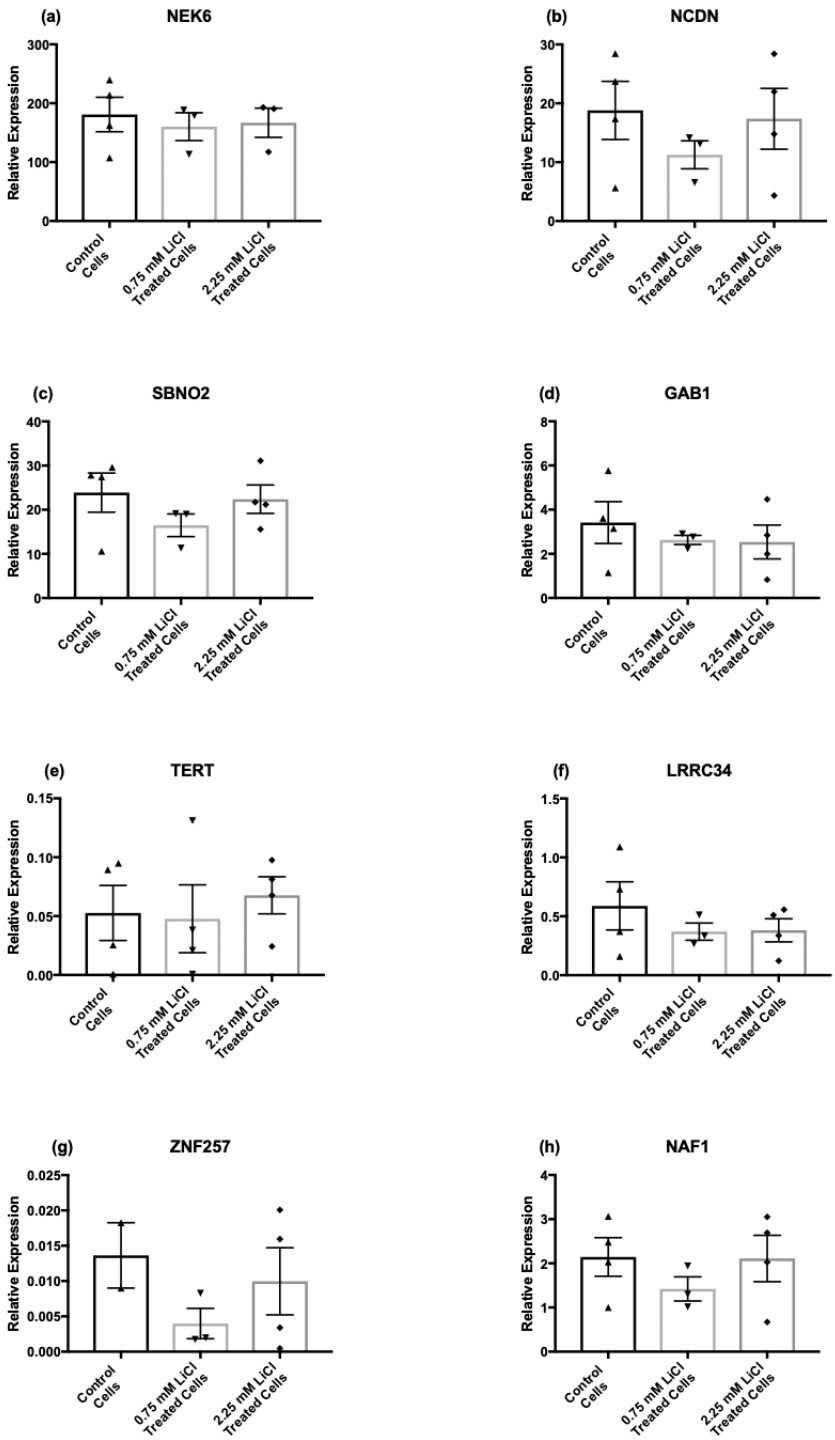

Figure 6.3.6 – Bar charts showing the difference in gene expression between old control, 0.75 mM LiCl, or 2.25 mM LiCl treated cells.

These bar charts show the relative gene expression of telomere-related and age-related genes in old cells, 0.75 mM long-term LiCl treated cells, and in 2.25 mM long-term LiCl treated cells. Relative expression of each gene to *VIM* (reference gene) was calculated by dividing the average mRNA quantities of each sample by the average quantity of *VIM*. Each data point represents one biological replicate. Passage numbers are indicated on the x-axis and the relative gene expression is indicated on the y-axis. (a) Relative gene expression of *NEK6*. (b) Relative gene expression of *NCDN*. (c) Relative gene expression of *SBNO2*. (d) Relative gene expression of *GAB1*. (e) Relative gene expression of *TERT*. (f) Relative gene expression of *LRRC34*. (g) Relative gene expression of *ZNF257*. (h) Relative gene expression of *NAF1*. No significant differences in gene expression were found between groups ($P > 0.05$).

6.3.2.3 THERE ARE NO SIGNIFICANT EFFECTS OF LITHIUM ON HIPPOCAMPAL CELL PROLIFERATION

ANOVA revealed significant differences in the proliferation marker Ki67 between conditions ($F(4, 9) = 8.384$, $p < 0.01$), with pairwise comparisons revealing that the 0.75 mM LiCl treated condition had a higher percentage of Ki67-positive cells relative to the 2.25 mM LiCl treated condition ($p < 0.01$). This effect survived multiple testing correction ($p < 0.05$) but was not supported by BrdU staining ($p > 0.05$), see **Figure 6.3.7**. There were no significant effects of lithium treatments relative to control conditions, in relation to either proliferation marker. There were also no effects of lithium on cell death as marked by CC3 ($p > 0.05$).

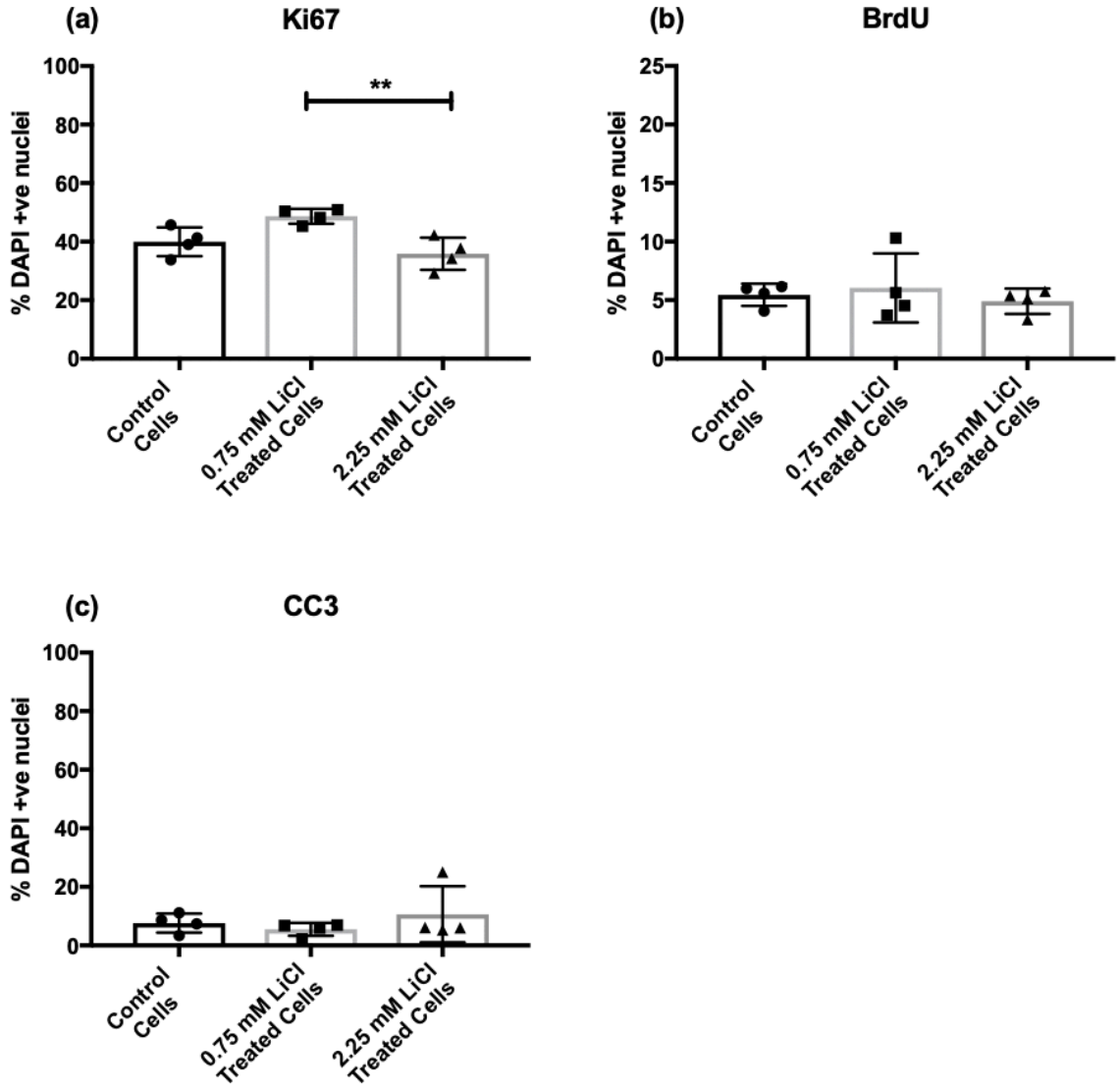

Figure 6.3.7 – Bar charts showing the percentage of Ki67 and BrdU positive cells in old cells that have been growth without LiCl, with 0.75 mM LiCl or 2.25 mM LiCl.

These bar charts show the percentage of Ki67 and BrdU positive cells relative to the percentage of DAPI stained nuclei in old cells. Each data point represents one biological replicate. The condition is indicated on the x-axis and the percentage of immunostained cells relative to DAPI positive nuclei is displayed on the y-axis. (a) The percentage of Ki67 positive cells shows a significant decrease if cells are grown in 0.25 mM LiCl compared to 0.75 mM LiCl, as detected by a one-way ANOVA, $p < 0.01$, as indicated by **. (b) There is no difference between any groups in the percentage of BrdU positive cells. (c) There is no significant difference between any groups in the percentage of CC3 positive cells. (d) There is a higher number of total cells in the 0.75 mM LiCl condition compared to the control condition, $p = 0.047$, and compared to the 2.25 mM LiCl condition, $p < 0.01$.

6.3.2.4 THERE ARE SIGNIFICANT EFFECTS OF LITHIUM ON HIPPOCAMPAL CELL DIFFERENTIATION

Our results revealed a significant increase in the percentage of cells stained with DCX ($F(4, 9) = 7.616$, $p < 0.01$), the percentage of cells stained with MAP2 ($F(4, 9) = 10.55$, $p < 0.01$) and the percentage of cells stained with S100β ($F(4, 9) = 17.48$, $p < 0.001$) in cells treated with the high (2.25 mM) dose of lithium relative to untreated control cells, see **Figure 6.3.8**. This suggests that a prolonged 2.25 mM LiCl treatment results in increased cell differentiation of hippocampal progenitor cells towards a neuronal (DCX, MAP2) and astrocytic fate (S100β).

The results also show that there is a significant increase in the percentage of cells stained with MAP2 in the 2.25 mM long-term LiCl treatment condition, compared to the 0.75 mM long-term LiCl treatment condition, $p < 0.01$, see **Figure 6.3.8**. This suggests that a 0.75 mM dose of LiCl may be insufficient for increasing the number of MAP2 positive cells, and that a higher dose of 2.25 mM is required to see this effect. In addition, our results show that there is a significant increase in the percentage of old cells stained with S100β following a 0.75 mM long-term LiCl treatment compared to old untreated control cells, $p = 0.027$, see **Figure 6.3.8.** This suggests that long-term LiCl treatment can push hippocampal progenitor cells towards an astrocytic fate, both via a lower and a higher dose of LiCl, although the effect is greater in the higher dose.

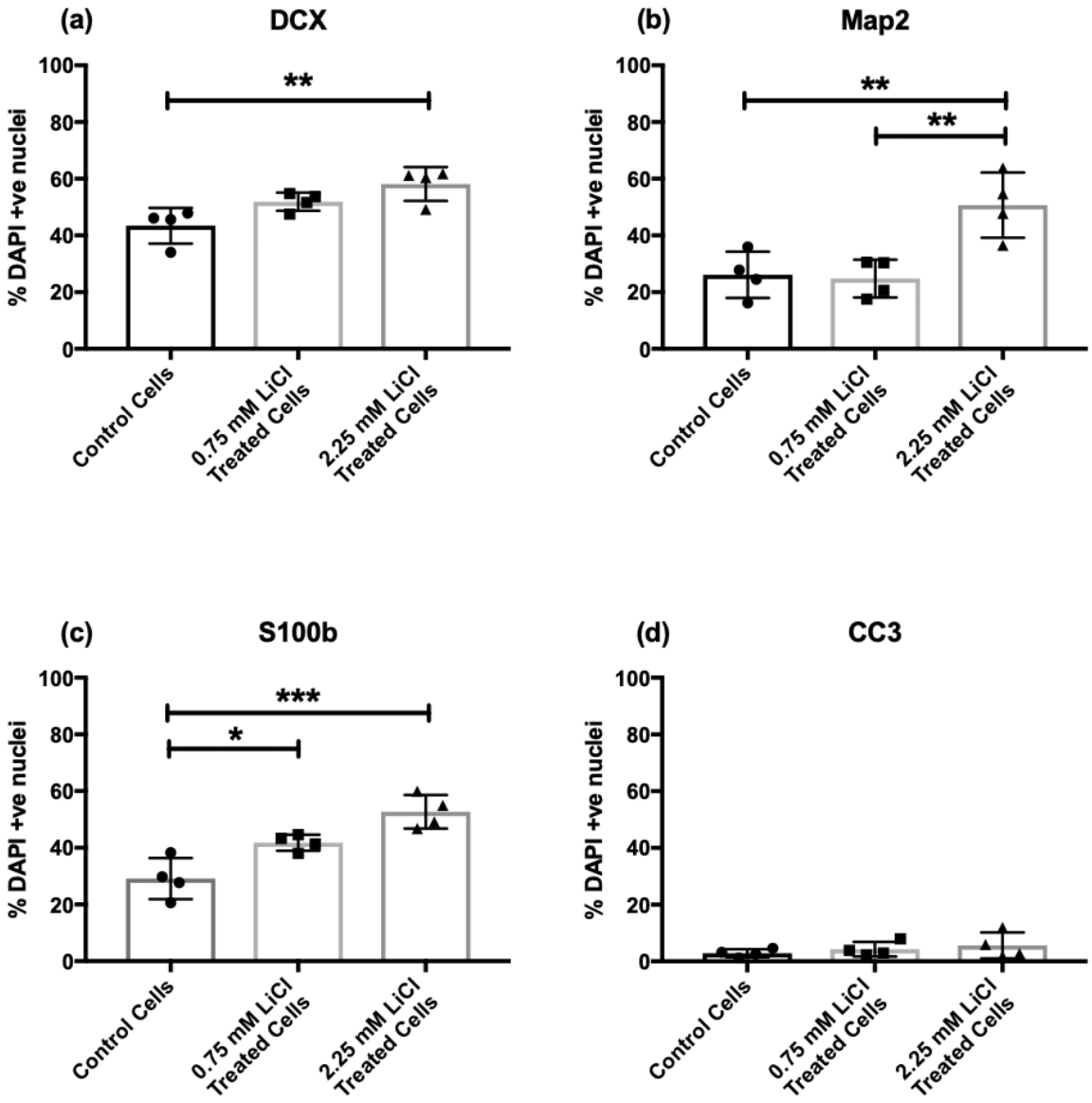

Figure 6.3.8 – Bar charts showing the percentage of DCX, MAP2 and S100β positive cells in old cells that have been gown with no LiCl, 0.75mM LiCl or 2.25mM LiCl.

These bar charts show the percentage of DCX, MAP2 and S100β positive cells relative to the percentage of DAPI stained nuclei (y-axis) in old cells grown chronically with no LiCl, 0.75mM LiCl or 2.25mM LiCl (x-axis). Each data point represents one biological replicate. (a) The percentage of DCX positive cells increases in 0.25mM long-term LiCl treated cells compared to control cells, as detected by a one-way ANOVA, $p < 0.01$, indicated by **. (b) The percentage of MAP2 positive cells increases in 0.25mM long-term LiCl treated cells compared to control cells and compared to 0.75mM long-term LiCl treated cells, as detected by a one-way ANOVA, $p < 0.01$, indicated by **. (c) The percentage of S100β positive cells increases in the 0.75mM long-term LiCl treated cells, as detected by a one-way ANOVA, $p = 0.027$, indicated by *, and in the 2.25mM long-term LiCl treated cells, as detected by a one-way ANOVA, $p < 0.001$, indicated by ***, compared to the control cells. There is no significant difference between any groups in the percentage of CC3 positive cells.

6.4 DISCUSSION

6.4.1 OVERVIEW

The first aim of this study was to investigate whether it is possible to model telomere shortening in a conditionally immortalised human hippocampal progenitor cell line, and subsequently whether telomere shortening is associated with a decrease in hippocampal neurogenesis and the differential expression of genes associated with ageing and telomere length regulation. The second aim of this study was to determine whether long-term lithium treatment in older cells is associated with an increase in telomere length, and whether this increase is associated with changes in hippocampal neurogenesis and the differential expression of genes associated with ageing and telomere length regulation.

6.4.2 HIPPOCAMPAL PROGENITOR CELLS SHOW TELOMERE SHORTENING AND NEUROGENIC DIFFERENCES WITH PASSAGING

With regards to the first aim, we found that telomere length gets significantly shorter as HPCs are passaged more (**Figure 6.3.1**), which suggests that by passaging these cells we are able to model telomere shortening related to the end-replication problem (the incomplete synthesis telomere ends during mitosis). Telomere shortening was also associated with a significant reduction in cell proliferation (**Figure 6.3.2**). This supports a multitude of studies relating to other cell types which show that cells approach the 'Hayflick limit' of mitotic potential when telomere length becomes very short (473).

Our findings also support animal studies which have showed a significant decline in BrdU labelled cells in the hippocampi of older rats compared to younger rats (376). A number of subsequent animal studies have shown similar results, hypothesizing that there is a progressive

decline in neuronal precursor cell proliferation with age (474). This is also supported by post-mortem work which reveals lower levels of adult hippocampal neurogenesis in brains from older individuals (380). In addition, a number of studies have shown that brain volume decreases with age, especially in the hippocampus (475-478), with our results suggesting that the reduction in brain volume in the hippocampus could be the consequence of telomere shortening in hippocampal progenitor cells.

More recent animal studies have suggested that a reduction in hippocampal cell proliferation is associated with cognitive decline, and rodent models of psychiatric disorders have shown that a decrease in hippocampal cell proliferation is associated with symptoms of MDD (479, 480). Given that for continual cell division there needs to be careful regulation of telomerase and telomere length in this region, it is unsurprising that studies have found shorter telomeres in the hippocampi of animals who show a reduction in neurogenesis (288, 481). More intriguingly, it has been shown that telomerase therapy can restore telomere length and increase the rate of hippocampal neurogenesis (288, 322). Together this suggests that people with psychiatric disorders may experience a reduction in hippocampal cell proliferation and subsequent neurogenesis, which may be caused by advanced telomere shortening, and that by restoring telomere length an increasing neurogenesis some of the psychiatric symptoms may be reduced.

Although it is very difficult to model this in living humans, a study by Mamdani and colleagues (2015) showed that people with MDD have shorter telomere lengths in their hippocampi compared to healthy controls (277). Our study is the first to demonstrate that telomere shortening in hippocampal progenitor cells directly affect their ability to proliferate. By establishing this link in a human cell model, we may be better able to design pharmacological

treatments for increasing hippocampal neurogenesis, possibly by targeting telomere length regulating mechanisms.

We should mention that we did not find any significant differences in telomere-related or age-related gene expression between younger and older cells. This could be because different genes are responsible for telomere length regulation in the hippocampus, as opposed to blood. Most of the genes we tested were derived from GWAS datasets, which collect blood samples from participants for telomere assessment, which could explain why we did not see significant gene expression changes in hippocampal cells. Indeed, studies have shown that a subset of genes are correlated, whereas another subset of genes are uncorrelated between the brain and the blood (482, 483). Another reason for the absence of gene expression changes could be that it is not possible to observe these differences in one cell line. It could be that these genes solely explain inter-individual variation and not intra-individual variation. It is therefore possible that we would observe significant gene expression differences if we were to use multiple cell lines or human brain tissue from a large number of people.

6.4.3 LONG-TERM LITHIUM TREATMENT MAY HAVE NEUROPROTECTIVE EFFECTS ON THE HIPPOCAMPUS BY INCREASING HIPPOCAMPAL PROGENITOR CELL DIFFERENTIATION TOWARDS A NEURONAL AND GLIAL FATE, RATHER THAN BY INCREASING TELOMERE LENGTH OR INCREASING CELL PROLIFERATION

With regards to the second aim, we did not find that long-term lithium treatment (a drug with mood-stabilising and anti-ageing properties) is associated with an increase in telomere length in older hippocampal progenitor cells. This was contrary to what we hypothesized, given lithium has been associated with increased telomere length in blood cells (Chapter 5) and given its association with extended longevity in human and animal models (309, 310).

One reason for this could be that the HPCs need to be treated with lithium for longer periods of time. Although we modelled a chronic treatment effect of lithium in cells, given our findings in Chapter 5, patients with BD who take lithium usually show longer telomere length after many years of taking it (Chapter 5).

Another reason for the absence of an effect could be that long-term lithium does not increase telomere length in the hippocampus directly, instead having a stronger effect on cell differentiation. Lithium has a complex pharmacology and studies have revealed significant effects of lithium on β-catenin, which is a key effector of the Wnt pathway and a target of glycogen synthase kinanse-3 β (GSK3β) (484). Activating the Wnt pathway has in turn been associated with the restoration of stem cell function (485) and GSK3β inhibition has been associated with increased cell differentiation, which could contribute to the results found in our study (459, 486).

A final reason for not seeing an effect could be whereas we modelled telomere shortening as a consequence of cell ageing, lithium is in fact protective against telomere shortening as a result of stressors such as cortisol and reactive oxygen species. Indeed, studies have shown that telomere length shortens following exposure to oxidative stress and other studies have shown that lithium acts by reducing reactive oxygen species (261, 487, 488). Together, this suggests that we may have seen a significant effect of lithium on telomere length if we had stressed our cells with oxidative stress or cortisol prior to treating them with lithium.

Indeed, we did not observe a change in telomere length when older cells were chronically treated with lithium, but we did find a change in hippocampal cell differentiation. Our findings show that cells which have been treated long-term with a 2.25mM dose of lithium are more likely to be DCX, MAP2 and S100β positive. DCX and MAP2 are neuronal markers and S100β

is an astrocytic marker, which suggests that chronic lithium use may be associated with hippocampal progenitor cells differentiation into neurons and astrocytes.

Animal studies have shown that lithium is associated with an increase in hippocampal cell differentiation, in particular towards cells labelled with MAP2 (395, 489). Several other animal studies have shown that lithium is associated with an increase in hippocampal neurogenesis and that this is in turn associated with improvements in mood and cognition (312). Our study is the first to demonstrate this effect in human hippocampal cells, in particular ones with signs of advanced cell ageing. This suggests that long-term lithium use may promote mood stabilising effects by increasing the number of new-born neurons and astrocytes in the hippocampus. Neuroimaging studies have shown that lithium is associated with an increase in brain volume (321) and we have shown that this increase could be the result of increased cell differentiation. It has been shown that of the proliferating cells, only a small number survive and integrate into the network of the dentate gyrus for long periods of time (285). It is possible that lithium promotes the differentiation of more progenitor cells towards a neuronal and astrocytic fate, increasing the number of cells that integrate and therefore increasing the total hippocampal volume. Although we showed this to be the case in older cells with shorter telomeres, it would be interesting to study the effects of long-term lithium treatment in younger cells with longer telomeres to test whether increased differentiation could occur at an earlier stage.

6.4.4 LIMITATIONS

We should note that our study has a number of limitations. We used an *in vitro* model of hippocampal neurogenesis, which may disregard many biological mechanisms involved in lithium metabolism, circulation and hippocampal maintenance. In addition, we did not measure longitudinal changes in cell proliferation and differentiation, which may have provided a

deeper understanding of the changes that occur over many passage numbers. We also treated cells with lithium once telomere length was significantly decreased, thus aiming to reverse telomere shortening. It is possible that lithium acts by preventing rather than reversing telomere shortening, which could be modelled by treating young cells with lithium. Another limitation is in our use of a single cell line rather than multiple patient derived cell lines. It is possible that lithium affects telomere length, neurogenesis and gene transcription differently in people (e.g. polygenic risk), which cannot be modelled using our current approach. Furthermore, we observed a significant effect of lithium on neurogenesis at the 2.25 mM dose, which is a dose that is three times that of a biologically relevant dose in humans. It is difficult to fully translate serum drug metabolite levels to equivalent *in vitro* drug doses, so ultimately further work *in vivo* will be needed to translate which dose safely evokes a neurogenic effect. Finally, although we deemed growing the cells for 10 passages enough to see significant differences in telomere length and lithium-induced neurogenic effects, is it likely that 10 passages is not enough to model the long-term lithium use in BD patients who often take lithium for many years.

6.4.5 CONCLUSION

Our study is the first to model telomere shortening in a conditionally immortalised human hippocampal stem cell line, demonstrating that telomere shortening is associated with a reduction in hippocampal cell proliferation. We did not find that lithium can rescue telomere shortening in older hippocampal progenitor cells, which may suggest that lithium slows down telomere shortening as opposed to elongate telomeres, although this requires more experiments. Our study is the first to show that long-term lithium treatment is associated with an increase in human hippocampal cell differentiation towards a neuronal and astrocytic fate, which may explain why lithium use is associated with larger hippocampal volumes and confers mood improvements in patients with bipolar disorder.

7 – GENERAL CONCLUSION AND DISCUSSION

7.1 SUMMARY OF FINDINGS AND IMPLICATIONS FOR FUTURE RESEARCH

In this thesis we aimed to investigate the role of inflammation, telomere length, and hippocampal neurogenesis in the aetiology and treatment of psychiatric disorders.

7.1.1 PATIENTS WITH MAJOR DEPRESSIVE DISORDER WHO HAVE EXPERIENCED CHILDHOOD MALTREATMENT DO NOT SHOW ELEVATED LEVELS OF CIRCULATING INFLAMMATORY MARKERS

The first study, investigating the effects of childhood maltreatment on circulating inflammatory markers in MDD cases and healthy controls revealed no significant effects of maltreatment in either group. Our hypothesis stipulated that MDD cases who have experienced childhood maltreatment are more likely to show increased levels of circulating pro-inflammatory markers compared to healthy controls or those who have not experienced childhood maltreatment. This hypothesis stems from a multitude of research findings which have shown that childhood maltreatment is associated with an increase in circulating pro-inflammatory markers (18, 198, 490), which in turn is associated with an increased risk of MDD (17, 36, 142). The lack of a significant association could be attributed to several factors.

Our study screened inflammatory markers in a comparatively large sample, which made sure we obtained enough statistical power when investigating a large number of inflammatory markers. Several studies that have found significant results in the past are made up of much smaller samples (491-494). In addition, we controlled for a large number of confounding factors such as antidepressant use, age, gender, smoking status and BMI, which could be

accounting for more variance than childhood maltreatment, and could actually be contributing to the 'effect' of maltreatment observed in previous studies. BMI in particular has been shown to be significantly associated with elevated levels of pro-inflammatory markers such as IL-6 and CRP (109, 199, 433, 495), an effect which we replicate in our study.

It is becoming apparent from recent studies that BMI is one of the strongest predictors of increased inflammation in individuals, requiring further study, especially in the field of psychiatry (53, 496-498). For example, it is crucial to investigate what it means to have high BMI in relation to age, sex, ethnicity, exercise and muscle mass, as all these factors can influence total BMI of an individual (499-504). Adipose tissue has been shown to secrete some pro-inflammatory cytokines, so another approach could be to measure the amount of adipose tissue instead of BMI to get a more precise 'fat' variable (257, 505, 506). This study also confirms the importance of using large sample sizes and screening a multitude of inflammatory molecules, first highlighted by a large-scale analysis of 42 inflammatory markers in 321 control subjects and 887 MDD cases, carried out by Powell and colleagues (2018). The inflammatory system is highly complex and involves a multitude of protein interactions; by increasing sample sizes it will become possible in the future to study many of these inflammatory markers without losing a substantial amount of statistical power.

7.1.2 A GENETIC RISK SCORE FOR HIGHER BMI IS ASSOCIATED WITH AN INCREASE IN PRO-INFLAMMATORY MARKERS IL-6 AND CRP, WHEREAS A GENETIC RISK SCORE FOR MDD SHOWS NO EFFECT

Following on from these findings, we sought to investigate the association between a higher genetic risk (measured by PRS) for MDD and inflammatory marker expression in a healthy population sample (422). This was carried out in order to overcome some of the confounding factors described above, often present in a psychiatric disorder sample (507-509) and to better

tease apart causal risk mechanisms implicated in higher adult inflammation. In addition, we sought to investigate the association between genetic risk for higher BMI and inflammatory marker levels in the same population, given the strong confounding effect of BMI (199). We controlled for the same confounding factors as in the previous study, in order to minimise the effects of external variables.

Contrary to the 'cytokine hypothesis' of depression, our results showed no significant associations between the genetic risk for MDD and circulating inflammatory marker levels. For those inflammatory markers strongly affected by BMI, we tested whether this likely represents a causal relationship by testing whether polygenic risk for higher BMI influences levels of the same inflammatory markers. In short, we found that those with either a higher BMI or with a genetic risk for higher BMI are more likely to show elevated levels of the pro-inflammatory markers IL-6 and CRP. This is the first study to suggest that genetic risk for higher BMI could be useful in predicting those at greater risk of suffering from heightened inflammation. These individuals may be advised to take preventative measures to reduce their BMI (if they have a higher than average BMI) or be advised on ways to reduce the risk of elevating the levels of circulating pro-inflammatory markers such as medications, specialized diets or exercise.

7.1.3 A GENETIC RISK SCORE FOR MAJOR DEPRESSIVE DISORDER, BIPOLAR DISORDER OR SCHIZOPHRENIA IS NOT ASSOCIATED WITH TELOMERE SHORTENING, BUT ANTIDEPRESSANT USE IS ASSOCIATED WITH BOTH TELOMERE SHORTENING AND NUMBER OF AGE-RELATED DISEASES

Our third study utilised polygenic risk score modelling in order to study the effects of a higher genetic risk for MDD, BD and SCZ on advanced cell ageing, characterised by shorter leukocyte

telomere length. MDD, BD and SCZ are associated with a greater risk of age-related medical disorders such as cardiovascular disease and stroke, which are often characterised by shorter telomeres (254, 435, 440, 510-512). Our aim was to investigate whether there is shared genetic aetiology between MDD, BD or SCZ and shorter telomere length, which could be driving this association.

The results showed no significant association, suggesting that environmental factors may be driving the association between MDD, BD, SCZ and shorter telomere length. Indeed, environmental factors such as smoking, drug use, stress and childhood maltreatment have all been associated with shorter telomeres and are more prevalent in the psychiatric population (441, 513). Interestingly, our study replicated the finding that antidepressant use is associated with shorter telomere length (428). Antidepressants have been shown to stimulate cell division as one of their target mechanisms (299). Hippocampal progenitor cells are one target of antidepressants linked to their therapeutic mechanism of action, whereby antidepressants stimulate the proliferation of new cells. However, unlike neural stem cells which have a heightened proliferative capacity (due to higher levels of telomerase), somatic cells like blood are likely even more susceptible to the effects of the end-replication problem (514). Thus, off-target effects of antidepressants on blood cell proliferation might result in telomere shortening.

In addition to telomere shortening, we sought to investigate whether antidepressant use is associated with age-related medical conditions, as further evidence of advanced cell ageing. Our results show that antidepressant use is associated with telomere shortening in healthy individuals who are taking them for purposes other than depression (e.g. sleep). In addition, we show that antidepressant use is associated with a higher incidence of physical illness such as coronary artery disease, which itself is associated with telomere shortening. Together these results may suggest that antidepressant use could increase the risk for age-related diseases,

possibly by promoting advanced cell ageing. These results will need to be replicated in a larger sample, as the subset of individuals taking antidepressants without MDD was considerably small. However, these results do suggest that the knock-on effects of antidepressant use need further investigation. Antidepressants are prescribed more and more, often needlessly (515, 516), which may lead to some unrealised negative consequences. As mentioned previously, it is likely that increased cell division is one effect of antidepressants, but this effect should be restricted to the areas of the brain such as the hippocampus and not the rest of the body (517, 518). If this effect is found in a larger sample, scientists may need to find a way to maintain telomere length in non-neuronal cells or engineer antidepressants so to only affect the brain.

7.1.4 TELOMERE LENGTH IS A POLYGENIC TRAIT MODERATED BY LITHIUM

Given the recent technological advancements in being able to carry out large-scale GWAS on a wide range of traits, such as telomere length, our fourth study aimed to investigate the polygenic nature of telomere length using publicly available data and probe the "anti-ageing" properties of lithium using telomere length as a proxy. A linkage disequilibrium (LD) score regression was applied to the largest telomere GWAS (217), which revealed a significant polygenic component of telomere length and a SNP heritability estimate of 7.29%. In addition, genetic correlations for telomere length revealed an increased risk for cancer and HDL cholesterol, and a decreased risk for coronary artery disease, high BMI and high levels of LDL cholesterol. These results support previous findings that shorter telomere length is associated with age-related diseases and BMI (226, 437, 519), and that longer telomeres are associated with "good cholesterol" (520), but also suggest that there is a great deal of missing heritability, which may indicate that larger sample sizes, rare variant assessment or gene-environment interactions are needed to bridge the gap between twin heritability estimates and SNP-chip estimates (521). Indeed, environmental factors such as smoking or childhood maltreatment

could be having a strong confounding effect on telomere length, as has been suggested by some studies (68, 250, 442) meaning even larger sample sizes are required to obtain sufficient power to identify risk SNPs and provide a better estimate of SNP-chip heritability . Nonetheless, these findings show support for the notion that telomere length is a heritable trait.

Following this, we wanted to perform a gene-level analysis on the same GWAS data (217) to identify significant SNP-associated genes. Our results revealed that 13 genes were significantly implicated in telomere length regulation, with the top five genes all clustering upstream of the telomerase gene, *TERC*. *TERC* itself was not found to be significant, which may mean *TERC* is less functionally important than previously expected, or that the associated SNPs located in neighbouring genes exert long-range regulatory effects on *TERC*. Nevertheless, the most significant SNP was associated with *LRRC34*, which is a ribonuclease inhibitor, likely to be involved in preserving the RNA primer necessary for telomere elongation, although this needs further investigation. Five other genes are known regulators of telomere length and three novel genes require further work to understand their effects.

In order to investigate the anti-ageing properties of lithium, we used microarray data from a study carried out by McColl and colleagues (2008), which found that lithium can extend the lifespan of *C. elegans* (309). We identified three ortholog genes in the microarray carried out by McColl and colleagues (2008), that corresponded to the genes we identified from our gene-level enrichment analysis, with the lithium treated *C. elegans* showing differential gene expression in all three genes compared to non-treated controls. Studies have shown that lithium may have anti-ageing properties in humans too (310), and our study suggests that lithium may partly achieve this by moderating the expression of telomere genes. Further study into this topic may help develop treatments that target telomere genes and subsequently prevent premature ageing.

To explore the polygenicity of telomere length and to study the effects of lithium on telomere length in humans, we measured LTL in a BD cohort (N = 384), which consisted of patients having been taking lithium for varying lengths of time. Using polygenic risk scoring, we identified a genetic risk score that was able to explain 4.4% of the variance in telomere length in our BD sample. Only 1% of the variance in telomere length could be explained using significant SNPs from the GWAS carried out by Codd and colleagues (2014), which suggests that with larger GWAS datasets more SNPs will approach genome-wide significance, and the amount of variance explained in telomere length may increase even further. This could be used as a useful predictor for identifying people who may be predisposed to advanced cell ageing and age-related disease, as indicated in our study, and has been suggested by some studies using polygenic risk score modelling for other traits (95, 522). Once identified, these individuals may be advised to take certain health precautions or medications to slow down premature ageing.

Another utility for telomere polygenic risk score modelling could be with regards to the anti-ageing effects of lithium. To investigate this, we first confirmed that chronic lifetime lithium use is associated with longer telomere length in our BD cohort as well as in an extended sample consisting of three separate cohorts. This supports previous epidemiological data showing that lithium can elongate telomeres in both human and animal models (314, 322). We then used polygenic risk scores for telomere length to understand whether SNPs involved in telomere length regulation are involved in lithium-induced inter-individual telomere length differences. Our results showed that more variance in telomere length can be explained by the PRS for telomere length in chronic lithium users compared to short-term lithium users or lithium-naïve patients. This suggests that chronic lithium may not work in a one-size-fits-all fashion on telomeres, instead increasing the penetrance of genetic differences in telomere length in some patients. Alongside the data from McColl and colleagues (2008), these results suggest that

lithium may be responsible for telomere lengthening effects via gene transcription, whereby the telomere lengthening efficacy of lithium becomes limited by each individual's genetic telomere maintenance capabilities, as captured by PRS for telomere length. These findings are important as they suggest that lithium would be most effective as a potential telomere lengthening compound in individuals who have a genetic predisposition for having longer telomeres in the first place, allowing for a more tailored treatment for patients who need and respond best to, the anti-ageing effects of lithium. Further study into the psychiatric and anti-ageing effects of lithium can help to better define the lowest therapeutic dose needed to achieve optimal anti-ageing or dementia-preventing (311) effects.

7.1.5 TELOMERE LENGTH SHORTENS IN HUMAN HIPPOCAMPAL PROGENITOR CELLS AS A RESULT OF THE END REPLICATION PROBLEM, WITH LONG-TERM LITHIUM TREATMENTS IN OLDER CELLS RESULTING IN MORE NEURONAL AND ASTROCYTIC CELL POPULATIONS

The final study was designed based on the findings described above. We aimed to replicate chronic lithium use using an *in vitro* hippocampal stem cell model and investigate the mood-stabilising and potential anti-ageing properties of lithium. Bipolar disorder has been strongly associated with hippocampal dysfunction and a reduction in hippocampal neurogenesis, with studies suggesting that lithium increases hippocampal neurogenesis as a mode of action (312, 318, 523). Long-term lithium uptake through drinking water has also been associated with a reduction in the incidence of dementia, another disorder known to affect the hippocampus (311). Together with the notion that lithium is associated with longer telomeres, it is plausible that long-term lithium use can increase telomere length in the hippocampus, which in turn increases hippocampal neurogenesis and exerts beneficial mood-stabilising and anti-ageing effects in people.

This study is the first to demonstrate cell ageing, characterised by telomere shortening, in a conditionally immortalised hippocampal stem cell model. Our results show that as hippocampal progenitor cells are passaged more, telomere length becomes shorter. This telomere shortening is in turn associated with a reduction in cell proliferation, whereby 'young' hippocampal progenitor cells have a higher proliferative potential compared to 'old' cells. These findings suggest that cell ageing by telomere shortening can be modelled using this hippocampal progenitor cell line and that future studies could study this biological phenotype (alongside others), to identify new ways of slowing down age-related changes to the brain.

The effects of lithium on telomere length did not yield significant results, as we found no difference in telomere length in older cells that have been treated long-term with no lithium, 0.75mM of lithium or 2.25mM of lithium. This reflects findings from our gene expression experiments, which showed no significant differences in gene expression between the lithium treated and untreated older cells, for the top genes taken from our telomere GWAS gene-level analysis. Together, these findings suggest that lithium may not have a direct effect on telomere length in human hippocampal progenitor cells, as most studies thus far have focused on animal models. Indeed, lithium is often described as a "dirty" drug that has a multitude of target effects, which could mean that other cell types or a more system-based approach is needed to capture lithium-induced telomere length elongation (524-526). Another reason could be that cells need to be treated with lithium for a much longer period of time. Epidemiological studies that have shown lithium to be anti-ageing, and studies into lithium and peripheral telomere length in bipolar disorder cohorts, consist of individuals who have been exposed to lithium for many years (313, 527). It is therefore possible that longer exposure to lithium would promote telomere elongation in the hippocampal progenitor cells. A study by Wei and colleagues (2015) showed that lithium can increase telomerase and telomere length in animals that have been exposed to lithium in their chow for 6-weeks, which supports this notion (322). It is also

possible that the increase in telomerase is not a direct target of lithium, as studies have shown that lithium upregulates β-catenin and BDNF levels, which in turn upregulates *TERT* expression (322, 528, 529). Wei and colleagues (2015) also showed that a 6-week treatment with lithium significantly increases telomerase, whereas the increase in telomere length was nominal, which suggests that perhaps lithium can increase telomerase in the short-term, but telomere length changes occur at a much slower rate (322). Finally, it is possible that lithium specifically prevents accelerated telomere loss induced by stressors such as cortisol and reactive oxygen species. Several studies have shown that telomere length is significantly affected by oxidative stress and other studies have indicated that a reduction in reactive oxygen species is one of the protective effects of lithium (261, 487, 488). Together, this suggests that we may have seen a significant effect of lithium if we had stressed our cells with oxidative stress or cortisol.

We did however find that a long-term lithium treatment is associated with increased differentiation of hippocampal progenitor cells towards a neuronal and astrocytic fate. Our findings revealed that in old cells, long-term lithium treatment (in particular the 2.25mM lithium treatment) increases the proportion of DCX, MAP2 and S100β positive cells. DCX and MAP2 are both microtubule-associated proteins that are widely expressed by neurons, and S100β is a calcium binding protein that is glial-specific and is primarily expressed by astrocytes (530-532). This finding expands on previous studies such as the one by Powell and colleagues (2017), which showed that genes responsible for hippocampal cell differentiation are associated with brain volume, and Powell and colleagues (2018), which showed that lithium is associated with an increase in hippocampal volume, together suggesting that this increase could be the result of more differentiating cells (313, 464). Lithium has been previously associated with an increase in neurogenesis (312, 318); and an increase in neurogenesis has been associated with a reduction in depressive symptoms (354, 533) and increased performance on

cognitive tasks (340, 534) in animal models, but this study is the first to demonstrate that lithium may promote hippocampal neurogenesis using human cells. Future studies in this field could investigate whether lithium can be used to stave of age-related memory decline and dementia, and whether the duration or the dosage of lithium treatments can alter cell differentiation.

7.2 NOTABLE RESEARCH APPROACHES

This thesis consisted of several novel research tools. Firstly, we highlight the importance of using large sample sizes and screening for many inflammatory markers, given the complexity of the inflammatory response (535, 536). Future studies would benefit from having even larger samples and screening for even more inflammatory markers in order to understand the interplay between environmental stressors, inflammatory markers and psychiatric disorder aetiology. This will of course greatly inflate the cost of research, although with current technological advances in screening tools and growing patient sample databases, this could become common practice. Using larger samples can increase statistical power when studying large numbers of inflammatory markers and reduce type 2 error.

Secondly, our study highlights the utility of polygenic risk scores when studying the biological mechanisms deemed to be responsible for the aetiology of psychiatric disorders. Although studies are able to control for some of the confounding factors, when investigating biological mechanisms in patients with psychiatric disorders, most confounding factors are not included in the analyses as they are difficult to tease apart from case-status, complex to screen for, or are unreported (406, 509). Confounding factors such as drug use, smoking and stress are often present in the psychiatric population and can have strong effects on the biological mechanisms being investigated, as has been shown by numerous studies (537-539). One way to circumvent this is to study biological mechanisms that may be responsible for psychiatric disorders in a

healthy population and use genetic risk scores as predictor variables. Those with a higher genetic risk score for MDD for example, are more likely to show a biological phenotype that resembles the aetiology of MDD, without showing actual symptoms or concurrent behaviours that may confound results. Conversely, those with a lower genetic risk score for MDD are more likely to show less of the biological phenotype. Using this methodology, researchers are able to place individuals on a continuous scale and measure the degree to which genetic risk confers an effect on a biological phenotype. This sort of approach may allow for a more direct assessment of aetiological risk mechanisms and allow researchers to develop effective treatments faster.

Thirdly, our study highlights the utility of polygenic risk score modelling when investigating certain traits. By applying polygenic modelling to telomere length GWAS data, we were able to gain valuable insights into shared genetic aetiology between telomere length and other traits, predict the telomere length of individuals from an independent cohort, and identify those who might respond best to the anti-ageing properties of lithium. This is particularly useful, as recent studies have shown that the interaction between genes and the environment occurs at a whole-genome level, rather at a single-gene level (540-542). This methodology can be used to explore a vast number of traits, allowing scientists to study the heritability, genetic predictors and gene by environment interactions of these traits.

Finally, our study has for the first time shown that it is possible to model cell ageing, characterised by telomere shortening, in a conditionally immortalised human hippocampal progenitor cell line. By removing 4-OHT from the cell culture medium, we showed that hippocampal progenitor cells continue to divide and show telomere length decline as they do so. Our study was designed to investigate whether lithium can rescue telomere shortening in these cells, whereas other studies may wish to take a different approach. For example, studies

have shown a reduction in hippocampal telomere length in dementia and MDD, with our *in vitro* ageing model potentially serving as a way to test the effectiveness of drugs on reversing this phenotype.

7.3 LIMITATIONS

We should note that there were limitations in the projects carried out in this thesis. Firstly, MDD cases used in our first study were taken from a clinical trial, whereas the controls were taken from a population sample. This means that the participants were not put through the same screening methods and might differ in some of their responses regarding history of childhood maltreatment or negative health habits, particularly as the MDD cases were screened by a medical professional whereas the controls were recruited as part of an invitation letter. Nevertheless, future studies would need to combine large cohorts collecting both cases and controls from different parts of country and the world in order to delve deeper into understanding the role of inflammation in MDD aetiology.

Secondly, polygenic risk scores used as predictors of MDD, BD, SCZ and BMI rely on publicly available GWAS datasets to gain insight into which SNPs are likely to be associated with a given phenotype. These datasets are constantly expanding, and it is possible that as datasets for these traits grow larger, we will not see the same associations between the PRS and certain biological phenotypes. For example, we could see an association between PRS for BD and telomere length in the future, as the BD GWAS datasets grow larger and predictive capabilities of PRS become more powerful.

Another limitation of PRS is population stratification. There are detectable differences in allele frequencies throughout the genome, making it possible to distinguish populations based on their genotype, which is a process referred to as population stratification (543). However, this

also means that LD patterns shaped by the human past are different in populations, which could result in false positive associations between a genotype and a trait of interest, whereby the associations may arise from differences in local ancestry that are unrelated to disease risk. This is especially true in more ancient gene pools, such as the African gene pool, which has greater amount of variation and a finer LD structure (544, 545). SELCoH consists of a population that is highly diverse in terms of ethnicity, and although this is representative of the UK population, our results may be impacted by population stratification. To overcome this, we used multidimensional scaling in PRSice, which calculates the proportion of alleles shared between any pair of individuals within our sample to generate quantitative components of genetic variation for each individual. These can then be used as covariates in analyses but are not always fully capable of correcting for population differences.

Finally, there were a number of limitations regarding our *in vitro* hippocampal progenitor cell aging model. By using an *in vitro* model, we disregard the rest of the brain and body. Telomere length is carefully regulated and is moderated by a multitude of genetic and molecular factors, including hormones secreted from different parts of the body (546). By reducing the process down to a cell model, we fail to capture external influences that may have a significant effect on both telomere length regulation and the effects of lithium. Another limitation regarding the use of an *in vitro* cell model is scale. Telomere shortening occurs over many years, whereas our ageing model was carried out over the course of two months. Although we captured a degree of telomere shortening, it is possible that we are not seeing the full effects of long-term cell ageing. Lastly, patients suffering from bipolar disorder take lithium orally and chronic users report taking the drug for many years (313, 527). Taking lithium orally may have a different effect on the brain, as there could be a number of molecules that interact with lithium systemically before it enters the brain. Simply adding lithium to culture medium may not be sufficient for the uptake of the drug and treating cells with lithium for 5 passages may be

insufficient for capturing its effect. Nevertheless, given the difficulty of accessing human brain tissue and studying biological phenotypes without any external influences, this cell model provides a novel and reliable way of measuring telomere shortening in hippocampal progenitor stem cells.

7.4 IMPACT ON PSYCHIATRY

This thesis has a notable impact on the field of psychiatry both in terms of the findings and the techniques used throughout. This thesis highlights the importance of using large datasets when studying the effects of heritable phenotypes on biological processes. Smaller sample sizes are more likely to lead to false positive results, especially when investigating complex biological processes such as inflammation, with larger sample sizes being able to increase the power of such studies and control for a wide range of confounding factors that are often present in psychiatric populations.

This thesis also adds to the growing consensus that the inflammation hypothesis for MDD needs to be considered alongside confounding factors such as BMI. One of the largest studies looking at differences in circulating inflammatory markers between MDD cases and healthy controls reported no significant results and instead pointed towards BMI as a major confounder (199, 433, 547). This thesis goes a step further to suggest that childhood maltreatment, one of the strongest environmental predictors of MDD, may also be confounded by BMI when considering its effects on inflammatory marker levels, whereby BMI explains a large proportion of the variance. Future research in the field of psychiatry may wish to take a different approach when studying inflammation in the context of childhood maltreatment and MDD and investigate BMI in more detail.

The lack of an association between an increased genetic risk for MDD, BD, SCZ, and telomere shortening, probes the field of psychiatry to focus more on environmental factors that are associated with telomere shortening and a higher incidence of age-related diseases. We highlight the importance of studying the effects of antidepressants on telomere length and consequently age-related diseases, although there are many other factors that could have the same effect. For example, childhood maltreatment and stress are known risk factors for psychiatric disorders, and telomere shortening has been implicated in psychiatric disorder aetiology (250).

Another contribution to the field of psychiatry is the deeper understanding of telomere genetics and the anti-ageing effects of lithium. This thesis demonstrates that people with a higher polygenic risk for longer telomeres who take lithium for a long period of time, show longer leukocyte telomere lengths compared to those who take lithium short-term or are lithium naïve. This suggests that it may be possible to predict the anti-ageing effects of lithium (based on cell ageing) in people, and that lithium may work by enhancing the innate genetic mechanisms for telomere elongation in some people but not others. In practical terms, this offers a way of identifying people who may be genetically primed for lithium-induced telomere elongation. In addition, this thesis suggests that lithium may be associated with longer telomere length, which has in turn been associated with slower cell ageing and longevity (309-311). There is growing interest around using lithium as an "anti-ageing" compound, and we add to this by suggesting that genes implicated in telomere length regulated are affected in lithium-induced models of longevity. These two findings together suggest that lithium could be used as an anti-ageing compound in some people, by way of slowing down or reversing cell ageing.

Finally, this thesis provides an *in vitro* model for telomere shortening in the hippocampus, which could be used in future studies. In addition, this thesis shows that lithium may be

promoting larger hippocampal volumes by promoting hippocampal progenitor cell differentiation, which could also explain its mood-stabilising effects. By understanding the cellular mechanisms which occur as a result of lithium treatment, we may get better at using lithium as a targeted treatment for not only BD patients, but other patients suffering from psychiatric disorders associated with hippocampal atrophy. In addition, further research could help us understand whether lithium could be used as a treatment for other disorders associated with age-related cognitive decline such as dementia.

7.5 CONCLUSION

In conclusion, this inter-disciplinary thesis uses a range of tools to study the roles of genetic risk, telomere length and inflammation in the aetiology of psychiatric disorders, and how treatments such as lithium could help reverse disorder progression. This thesis presents novel genetic analyses to study biological phenotypes, a novel cell model to study cell ageing in the hippocampus and novel findings in the field of inflammation and MDD. Our work highlights the importance of studying confounding factors in large cohorts studying inflammation, MDD and telomere length. Our work also provides a deeper insight into the polygenic architecture behind telomere length regulation and the anti-ageing properties of lithium, which as shown by our cell model, could also be harnessed to increase hippocampal neurogenesis and possibly restore hippocampal atrophy in some disorders. This thesis highlights the importance of using an interdisciplinary approach to study biological processes in psychiatric disorders and demonstrates that a combination of research tools can be useful in advancing and developing existing hypotheses.

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9 APPENDIX

9.1 CHAPTER 2 APPENDIX

9.1.1 CORRELATION MATRIX FOR ALL INFLAMMATORY MARKERS TESTED

Table 9.1.1 - Correlation matrix show in Pearson, r, values. Significant correlations (p<0.05) are indicated in green.

	PlGF	Tie2	VEGF	VEGFC	VEGFD	bFGF	sFlt1	Eotaxin	Eotaxin3	IP10	MCP1	MCP4	MDC	MIP1B	TARC	BDNF	IFNa	IL12	IL15	IL16	IL17	IL5	IL7	TNFB	IFNy	IL10	IL12p70	IL6	IL8_2	TNF	CRP	SAA	sICAM1	sVCAM1
PlGF	1	0.087	0.125	0.164	0.249	0.08	0.14	-0.038	-0.068	0.038	0.082	-0.117	-0.053	-0.024	-0.025	0.03	0.118	-0.028	0.023	0.078	-0.027	-0.156	-0.023	-0.13	0.169	-0.065	-0.01	0.005	0.003	0.053	0.049	0.102	0.217	0.106
Tie2	0.087	1	0.157	0.229	0.267	0.144	0.36	0.197	0.205	0.062	0.088	0.057	0.135	-0.014	0.087	0.108	0.117	0.112	0.229	0.221	0.034	0.192	-0.022	0.264	-0.006	0.074	0.072	0.012	0.074	0.036	-0.063	-0.055	-0.043	-0.095
VEGF	0.125	0.157	1	0.316	0.235	0.231	0.084	0.101	0.065	0.077	0.211	0.092	0.029	0.18	0.047	0.166	0.024	0.01	0.044	0.354	-0.046	0.041	0.108	0.062	0.089	0.164	0.24	0.219	0.225	0.251	0.073	0.086	0.07	0.035
VEGFC	0.164	0.229	0.316	1	0.31	0.198	0.257	0.159	0.12	0.018	0.184	0.079	0.127	0.067	0.001	0.307	0.158	-0.02	0.027	0.319	-0.075	0.048	0.239	0.054	0.093	0.198	0.211	0.184	0.2	0.235	0.077	0.065	0.015	-0.097
VEGFD	0.249	0.267	0.235	0.31	1	-0.011	0.05	0.02	0.063	-0.065	0.163	0.05	-0.011	-0.016	0.007	0.309	0.185	-0.096	-0.001	0.095	-0.143	-0.26	0.114	-0.085	0.084	0.08	0.079	0.11	0.089	0.144	-0.093	-0.012	-0.039	-0.014
bFGF	0.08	0.144	0.231	0.198	-0.011	1	0.086	0.267	0.115	0.042	0.258	0.036	0.182	0.244	0.023	-0.029	-0.098	0.174	0.031	0.672	0.075	0.175	-0.215	0.064	0.083	0.353	0.443	0.192	0.438	0.241	0.215	0.143	0.124	0.077
sFlt1	0.14	0.36	0.084	0.257	0.05	0.086	1	0.022	0.034	0.156	-0.064	0.08	-0.024	-0.018	0.067	0.123	0.08	0.099	0.307	0.132	0.115	0.152	0.104	0.216	-0.036	-0.039	-0.025	-0.052	-0.024	-0.019	0.022	-0.004	-0.007	-0.123
Eotaxin	-0.038	0.197	0.101	0.159	0.02	0.267	0.022	1	0.389	0.322	0.486	0.39	0.455	0.207	0.204	0.016	-0.051	0.185	0.188	0.381	0.155	0.265	0.078	0.173	0.13	0.419	0.358	0.296	0.312	0.293	0.041	-0.016	0.101	0.085
Eotaxin3	-0.068	0.205	0.065	0.12	0.063	0.115	0.034	0.389	1	0.109	0.379	0.219	0.168	0.136	0.094	-0.008	0.087	0.009	0.056	0.273	0.028	0.097	-0.013	0.21	0.025	0.227	0.297	0.285	0.289	0.268	-0.056	-0.05	-0.078	-0.065
IP10	0.038	0.062	0.077	0.018	-0.065	0.042	0.156	0.322	0.109	1	0.151	0.396	0.264	0.171	0.316	0.006	0.014	0.406	0.216	0.141	0.076	0.124	0.169	0.23	0.201	0.142	0.102	0.005	0.094	0.013	0.06	0.075	0.18	0.145
MCP1	0.082	0.088	0.211	0.184	0.163	0.258	-0.064	0.486	0.379	0.151	1	0.231	0.242	0.298	0.049	0.126	-0.007	-0.004	-0.017	0.459	-0.028	-0.075	0.002	-0.031	0.198	0.638	0.8	0.877	0.764	0.879	0.027	0.03	0.058	0.057
MCP4	-0.117	0.057	0.092	0.079	0.05	0.036	0.08	0.39	0.219	0.396	0.231	1	0.405	0.29	0.356	0.261	0.157	0.089	0.151	0.133	0.003	0.036	0.177	0.039	0.045	0.143	0.11	0.072	0.111	0.054	0.03	0.134	0.082	0.119
MDC	-0.053	0.135	0.029	0.127	-0.011	0.182	-0.024	0.455	0.168	0.264	0.242	0.405	1	0.115	0.444	0.072	0.029	0.142	0.185	0.191	0.06	0.17	0.073	0.175	0.064	0.188	0.152	0.129	0.123	0.121	0.155	0.059	0.169	0.183
MIP1B	-0.024	-0.014	0.18	0.067	-0.016	0.244	-0.018	0.207	0.136	0.171	0.298	0.29	0.115	1	0.077	0.175	-0.02	-0.032	-0.059	0.317	-0.04	-0.051	0.01	-0.043	0.121	0.239	0.328	0.164	0.352	0.197	0.044	0.021	-0.002	0.019
TARC	-0.025	0.087	0.047	0.001	0.007	0.023	0.067	0.204	0.094	0.316	0.049	0.356	0.444	0.077	1	0.122	0.042	0.157	0.111	-0.01	0.028	0.042	0.06	0.212	-0.017	0.069	-0.046	-0.024	-0.052	-0.023	0.074	0.103	0.051	0.127
BDNF	0.03	0.108	0.166	0.307	0.309	-0.029	0.123	0.016	-0.008	0.006	0.126	0.261	0.072	0.175	0.122	1	0.209	-0.162	-0.028	0.082	-0.104	-0.289	0.124	-0.116	0.075	-0.005	0.073	0.102	0.086	0.131	-0.097	-0.047	-0.174	-0.164
IFNa	0.118	0.117	0.024	0.158	0.185	-0.098	0.08	-0.051	0.087	0.014	-0.007	0.157	0.029	-0.02	0.042	0.209	1	-0.062	0.01	-0.041	0.024	-0.106	0.085	0.03	-0.006	-0.08	-0.073	-0.053	-0.065	-0.043	-0.009	0.034	-0.051	-0.096
IL12	-0.028	0.112	0.01	-0.02	-0.096	0.174	0.099	0.185	0.009	0.406	-0.004	0.089	0.142	-0.032	0.157	-0.162	-0.062	1	0.222	0.079	0.277	0.195	0.058	0.496	0.196	0.182	0	-0.053	-0.016	-0.033	0.133	0.092	0.125	0.179
IL15	0.023	0.229	0.044	0.027	-0.001	0.031	0.307	0.188	0.056	0.216	-0.017	0.151	0.185	-0.059	0.111	-0.028	0.01	0.222	1	0.125	0.16	0.201	0.111	0.176	0.167	0.018	0	-0.053	-0.02	-0.057	0.108	0.154	0.151	0.078
IL16	0.078	0.221	0.354	0.319	0.095	0.672	0.132	0.381	0.273	0.141	0.459	0.133	0.191	0.317	-0.01	0.082	-0.041	0.079	0.125	1	0.056	0.222	-0.111	0.043	0.124	0.467	0.671	0.433	0.632	0.471	0.255	0.138	0.173	0.079
IL17	-0.027	0.034	-0.046	-0.075	-0.143	0.075	0.115	0.155	0.028	0.076	-0.028	0.003	0.06	-0.04	0.028	-0.104	0.024	0.277	0.16	0.056	1	0.203	-0.022	0.189	0.121	0.087	0.001	-0.023	-0.003	-0.024	0.207	0.048	-0.02	0.021
IL5	-0.156	0.192	0.041	0.048	-0.26	0.175	0.152	0.265	0.097	0.124	-0.075	0.036	0.17	-0.051	0.042	-0.289	-0.106	0.195	0.201	0.222	0.203	1	-0.044	0.235	-0.091	0.033	0.045	-0.045	0.018	-0.048	0.072	-0.033	0.011	-0.003
IL7	-0.023	-0.022	0.108	0.239	0.114	-0.215	0.104	0.078	-0.013	0.169	0.002	0.177	0.073	0.01	0.06	0.124	0.085	0.058	0.111	-0.111	-0.022	-0.044	1	0.029	0.119	-0.088	-0.131	-0.07	-0.129	-0.084	0.025	0.056	0.025	0.066
TNFB	-0.13	0.264	0.062	0.054	-0.085	0.064	0.216	0.173	0.21	0.23	-0.031	0.039	0.175	-0.043	0.212	-0.116	0.03	0.496	0.176	0.043	0.189	0.235	0.029	1	0.03	0.098	-0.009	-0.067	-0.029	-0.068	0.023	0.024	-0.006	0.03
IFNy	0.169	-0.006	0.089	0.093	0.084	0.083	-0.036	0.13	0.025	0.201	0.198	0.045	0.064	0.121	-0.017	0.075	-0.006	0.196	0.167	0.124	0.121	-0.091	0.119	0.03	1	0.164	0.165	0.177	0.152	0.205	0.105	0.09	0.16	0.17
IL10	-0.065	0.074	0.164	0.198	0.08	0.353	-0.039	0.419	0.227	0.142	0.638	0.143	0.188	0.239	0.069	-0.005	-0.08	0.182	0.018	0.467	0.087	0.033	-0.088	0.098	0.164	1	0.762	0.643	0.761	0.656	0.142	0.149	0.172	0.165
IL12p70	-0.01	0.072	0.24	0.211	0.079	0.443	-0.025	0.358	0.297	0.102	0.8	0.11	0.152	0.328	-0.046	0.073	-0.073	0	0	0.671	0.001	0.045	-0.131	-0.009	0.165	0.762	1	0.827	0.98	0.844	0.13	0.063	0.085	-0.006
IL6	0.005	0.012	0.219	0.184	0.11	0.192	-0.052	0.296	0.285	0.005	0.877	0.072	0.129	0.164	-0.024	0.102	-0.053	-0.053	-0.053	0.433	-0.023	-0.045	-0.07	-0.067	0.177	0.643	0.827	1	0.775	0.981	0.041	-0.005	0.003	-0.026
IL8_2	0.003	0.074	0.225	0.2	0.089	0.438	-0.024	0.312	0.289	0.094	0.764	0.111	0.123	0.352	-0.052	0.086	-0.065	-0.016	-0.02	0.632	-0.003	0.018	-0.129	-0.029	0.152	0.761	0.98	0.775	1	0.794	0.118	0.062	0.076	-0.015
TNF	0.053	0.036	0.251	0.235	0.144	0.241	-0.019	0.293	0.268	0.013	0.879	0.054	0.121	0.197	-0.023	0.131	-0.043	-0.033	-0.057	0.471	-0.024	-0.048	-0.084	-0.068	0.205	0.656	0.844	0.981	0.794	1	0.047	0.011	0.011	-0.026
CRP	0.049	-0.063	0.073	0.077	-0.093	0.215	0.022	0.041	-0.056	0.06	0.027	0.03	0.155	0.044	0.074	-0.097	-0.009	0.133	0.108	0.255	0.207	0.072	0.025	0.023	0.105	0.142	0.13	0.041	0.118	0.047	1	0.601	0.52	0.487
SAA	0.102	-0.055	0.086	0.065	-0.012	0.143	-0.004	-0.016	-0.05	0.075	0.03	0.134	0.059	0.021	0.103	-0.047	0.034	0.092	0.154	0.138	0.048	-0.033	0.056	0.024	0.09	0.149	0.063	-0.005	0.062	0.011	0.601	1	0.517	0.565
sICAM1	0.217	-0.043	0.07	0.015	-0.039	0.124	-0.007	0.101	-0.078	0.18	0.058	0.082	0.169	-0.002	0.051	-0.174	-0.051	0.125	0.151	0.173	-0.02	0.011	0.025	-0.006	0.16	0.172	0.085	0.003	0.076	0.011	0.52	0.517	1	0.794
sVCAM1	0.106	-0.095	0.035	-0.097	-0.014	0.077	-0.123	0.085	-0.065	0.145	0.057	0.119	0.183	0.019	0.127	-0.164	-0.096	0.179	0.078	0.079	0.021	-0.003	0.066	0.03	0.17	0.165	-0.006	-0.026	-0.015	-0.026	0.487	0.565	0.794	1

9.1.2 INTER-ASSAY COEFFICIENT OF VARIATION

Table 9.1.2 – Inter-assay coefficient of variation between for all inflammatory markers.

Inter-assay coefficient of variation (CV) was calculated by dividing the S.D of marker levels on each run by the mean, and then calculating the mean CV across all plates. Intra-assay CV was estimated by assessing the CV for duplicates in the standard curve, specifically the fourth dilution as it represents the midpoint and closest to levels expressed in our samples.

Inflammatory marker	Inter-assay CV	Intra-assay CV	Highest detectable concentration (Standard 1; pg/mL)	Lowest detectable concentration (Standard 7; pg/mL)
PlGF	0.361	5.030	3460	0.845
Tie-2	0.329	5.477	77320	18.877
VEGF	0.833	1.336	1970	0.481
VEGF-C	0.427	7.474	23350	5.701
VEGF-D	0.520	3.213	21910	5.349
bFGF	1.124	1.859	1780	0.435
sFlt1	0.397	1.736	8170	1.995
Eotaxin	0.532	3.727	1440	0.352
Eotaxin-3	0.998	4.583	4760	1.162
IP-10	0.702	2.751	2470	0.603
MCP-1	0.761	8.388	445	0.109
MCP-4	0.511	2.444	623	0.152
MDC	0.448	1.962	10100	2.466
MIP-1β	0.586	3.870	982	0.240
TARC	0.749	3.114	1550	0.378
BDNF	0.465	2.480	100000	24.414
IL-12	0.624	1.228	3310	0.808
IL-15	0.449	0.931	767	0.187
IL-16	0.735	3.624	2250	0.549
IL-17	1.244	4.586	5850	1.428
IL-1α	1.399	6.360	394	0.096
IL-5	0.860	3.121	881	0.215
IL-7	0.527	2.073	871	0.213
TNF-β	0.639	1.736	578	0.141
IFN-γ	1.032	1.520	1270	0.310
IL-10	1.336	2.014	307	0.075
IL-12p70	1.233	3.106	389	0.095
IL-6	1.764	1.686	621	0.152
IL-8	1.730	2.461	504	0.123
TNF-α	0.966	1.900	313	0.076
CRP	1.258	0.932	195000	12.480
SAA	1.254	2.741	218000	13.952
sICAM-1	0.456	3.600	61400	3.930
sVCAM-1	0.411	1.678	51700	3.309

9.2 CHAPTER 3 APPENDIX

9.2.1 POPULATION STRUCTURE BY GENETIC RELATEDNESS

In order to reduce confounding variables in the form of population structure, we performed multidimensional scaling in plink (as part of PRSice), where we generated 7 population covariates. These were then incrementally correlated against each other until the population structure achieved a normal distribution. Following this, we could decide which population covariates to include in subsequent statistical analyses. Figure 2 shows that a correlation between population covariates 7 and 8 generated a normal distribution, which meant that we included population covariates 1, 2, 3, 4, 5, 6 and 7 as covariates in our regression analyses. The same structure was applied in Chapters 3 and 4.

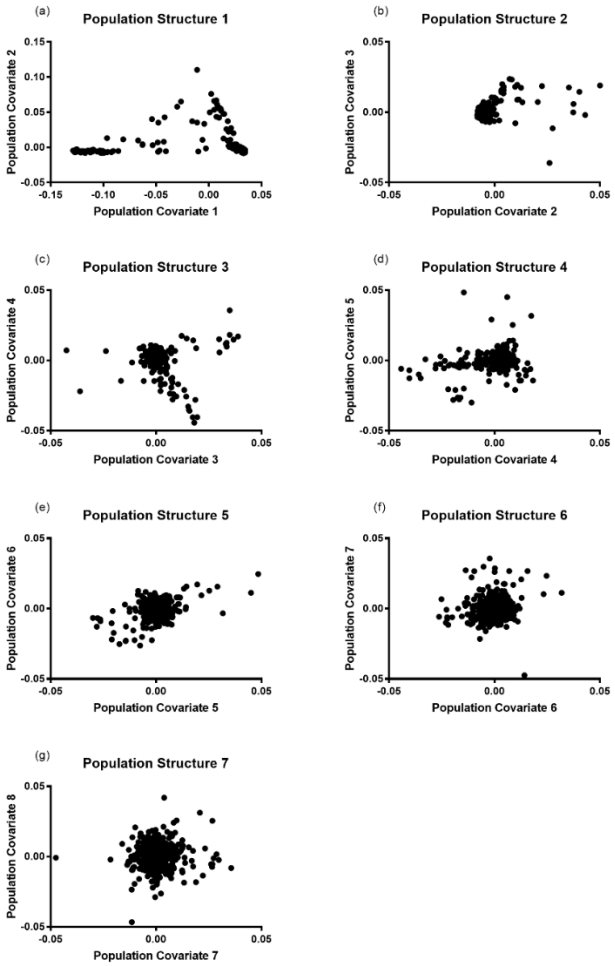

Figure 9.2.1 – Population structure correlation charts.

Correlation between population covariates are displayed incrementally, with both the x-axes and the y-axes labelled according to the population covariate number. (a) population covariate 1 correlated against population covariate 2; (b) population covariate 2 correlated against population covariate 3; (c) population covariate 3 correlated against population covariate 4; (d) population covariate 4 correlated against population covariate 5; (e) population covariate 5 correlated against population covariate 6; (f) population covariate 6 correlated against population covariate 7; (g) population covariate 7 correlated against population covariate 8.

9.2.2 INFLAMMATORY MARKERS ADEQUATELY DETECTED IN SERUM

Using our methodology, 34 inflammatory proteins passed our quality control criteria. 7 inflammatory markers were found to have greater than 30% missing data from across the whole sample and were removed from any downstream analyses (MIP-1a, GMCSF, IL-1a, IL-13, IL-1b, IL-2, IL-4). Figure 1 shows inflammatory markers that were expressed in >70% of our sample.

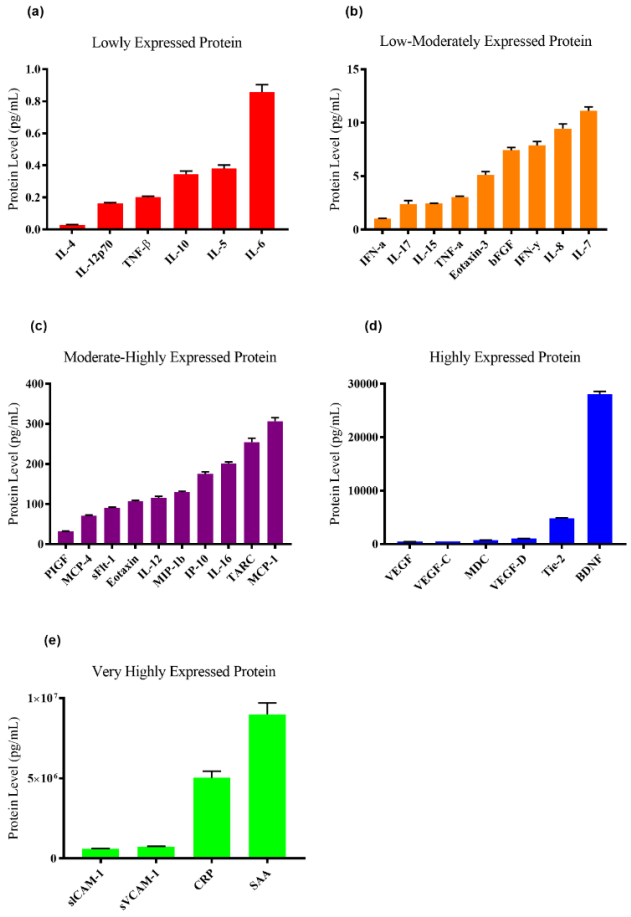

Figure 9.2.2 – Inflammatory markers were adequately expressed in our sample.

A summary of inflammatory markers lowly expressed in our sample. (a) < 1 pg/mL, lowly expressed in our sample; (b) 1 – 20 pg/mL, low-moderately expressed in our sample; (c) 21 – 400 pg/mL, moderate-highly expressed in our sample; (d) 401 – 25,000 pg/mL, highly expressed in our sample; (e) 25,001 - 100,000,000 pg/mL. Bars represent the mean and error bars represent the standard error of the mean. Inflammatory markers in 1a include: Tumour Necrosis Factor beta (TNF-b), Interleukin 12 heterodimer (IL-12), Interleukin 10 (IL-10), Interleukin 5 (IL-5) and Interferon alpha (IFN-a). Inflammatory markers in 1b include: Interleukin 15 (IL-15), Interleukin 17 (IL-17), Tumour Necrosis Factor (TNF), Interleukin 6 (IL-6), Eotaxin-3, Interferon gamma (IFNy), basic Fibroblast Growth Factor (bFGF), Interleukin 7 (IL-7). Inflammatory markers in 1c include: Placental Derived Growth Factor (PlGF), Monocyte Chemoattractant Protein 4 (MCP-4), vascular endothelial growth factor receptor 1 (sFlt-1), Eotaxin, Macrophage Inflammatory Protein 1 beta (MIP-1b), Interleukin 12 (IL-12), Interleukin 8 (IL-8), Interferon inducible protein 10 (IP-10), Interleukin 16 (IL-16), Chemokine (C-C motif) ligand 17 (CCL-17) and Monocyte Chemoattractant Protein 1 (MCP-1). Inflammatory markers in 1d include: Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial Growth Factor C (VEGF-C), Macrophage-Derived Chemokine (MDC), Vascular Endothelial Growth Factor D (VEGF-D), Tyrosine kinases with Ig and EGF homology domains-2 (Tie-2) and Brain-Derived Neurotrophic Factor (BDNF). Inflammatory markers in 1e include: soluble Intercellular Adhesion Molecule 1 (sICAM-1), soluble Vascular Cell Adhesion Molecule 1 (sVCAM-1), C-Reactive Protein (CRP) and Serum Amyloid A (SAA).

9.2.3 THE RELATIONSHIP BETWEEN BMI AND POLYGENIC RISK SCORES FOR BMI

To validate the relationship between raw BMI scores and PRS for BMI, we performed correlation analyses for these two variables. Our analyses reveal that there is a significant positive correlation between BMI and PRS for BMI (Pearson's correlation = 0.26, P = 1.13E-7).

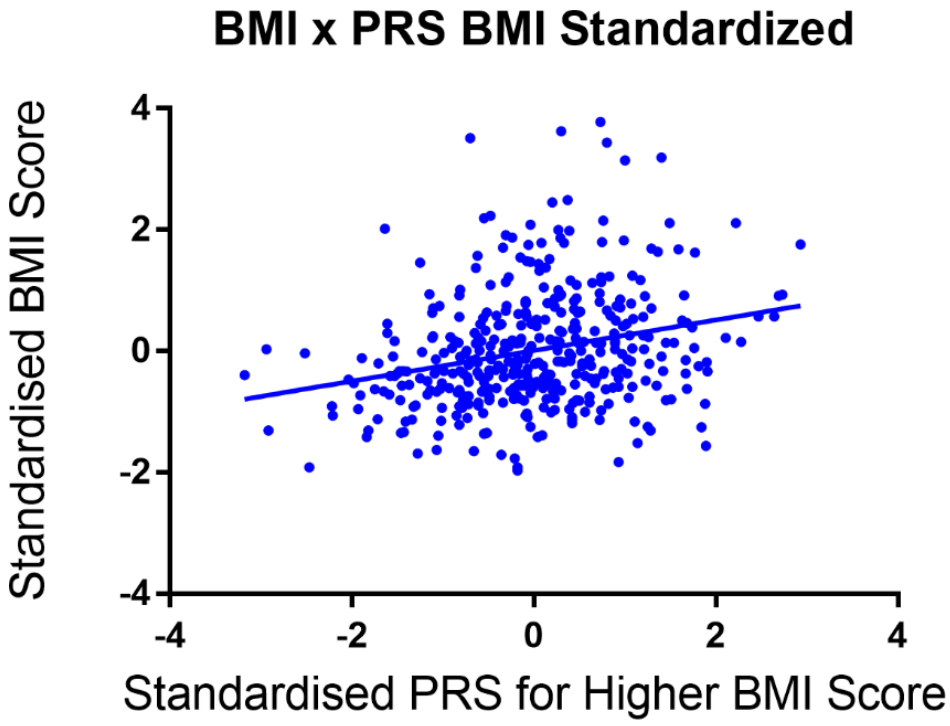

Figure 9.2.3 – The relationship between BMI and PRS for BMI

Scatter chart showing the relationship between standardised raw BMI scores and standardised PRS for BMI. Standardised PRS for BMI are displayed on the x-axis and standardised BMI scores are displayed on the y-axis.

9.2.4 MAIN EFFECTS OF A PRS FOR MDD ON INFLAMMATORY MARKER EXPRESSION

Table 9.2.1 – A table of the ANOVA results from the main effects analysis of a PRS for MDD on inflammatory marker levels.

	PRS for MDD - main effects on cytokine levels			
Cytokine name	**F Statistic**	**d.f.**	**p-value**	**effect size**
IP-10	0.000	360	1.00	0.00
sVCAM	0.000	351	0.99	0.00
VEGF-C	0.000	359	0.98	0.00
IL-12p70	0.033	327	0.86	0.00
IFN-a	0.081	287	0.78	0.00
sICAM-1	0.082	354	0.78	0.00
IL-6	0.083	347	0.77	0.00
CRP	0.086	360	0.77	0.00
PlGF	0.950	364	0.76	0.00
VEGF	0.106	359	0.75	0.00
MCP-4	0.135	363	0.71	0.00
TNF-a	0.143	365	0.71	0.00
TNF-b	0.231	345	0.63	0.00
bFGF	0.244	354	0.62	0.00
IL-4	0.245	264	0.62	0.00
SAA	0.246	352	0.62	0.00
Eotaxin	0.297	358	0.59	0.00
IL-8	0.462	360	0.50	0.00
sFlt-1	0.479	366	0.49	0.00
IL-16	0.508	358	0.48	0.00
IL-5	0.544	256	0.46	0.00
Tie-2	0.597	368	0.44	0.00
MDC	0.690	359	0.41	0.00
IL-12	0.890	361	0.35	0.00
IL-7	1.049	365	0.31	0.00
MIP-1b	1.132	356	0.29	0.00
VEGF-D	1.145	362	0.29	0.00
IL-15	1.230	371	0.27	0.00
IL-17	1.714	354	0.19	0.01
TARC	1.750	368	0.19	0.01
BDNF	1.797	331	0.18	0.01
Eotaxin-3	1.951	327	0.16	0.01
MCP-1	2.079	374	0.15	0.01
IFN-y	3.524	341	0.06	0.01
IL-10	5.829	348	0.02	0.02

9.2.5 MAIN EFFECTS OF BMI ON INFLAMMATORY MARKERS LEVELS AND OF THE SIGNIFICANT MARKERS, THE MAIN EFFECTS OF A PRS FOR BMI ON INFLAMMATORY MARKER LEVELS

Table 9.2.2 – This table displays the main effects of BMI on inflammatory marker levels, and of those significant associations, this table also displays the main effects of a PRS on BMI on inflammatory marker levels.

Main Effect of BMI on Inflammatory Marker Expression								Main Effect of PRS for Higher BMI on Inflammatory Marker Expression						
Cytokine Name	F Statistic	D. F.	p-value	Effect Size	q-value	Bonferroni Significant?	FDR Significant?	F Statistic	D. F.	p-value	Effect Size	q-value	Bonferroni Significant?	FDR Significant?
sVCAM1	0.010	445	9.2E-01	0.000	0.919	N	N							
IL-7	0.011	458	9.2E-01	0.000	0.919	N	N							
IL-10	0.019	437	8.9E-01	0.000	0.919	N	N							
IL-8	0.020	457	8.9E-01	0.000	0.919	N	N							
IL-12p70	0.050	419	8.2E-01	0.000	0.919	N	N							
IFN-y	0.187	434	6.7E-01	0.000	0.777	N	N							
TNF-b	0.321	439	5.7E-01	0.001	0.689	N	N							
Tie-2	0.348	465	5.6E-01	0.001	0.689	N	N							
IL-15	0.443	469	5.1E-01	0.001	0.656	N	N							
IL-5	0.566	329	4.5E-01	0.002	0.608	N	N							
VEGF	0.719	456	4.0E-01	0.002	0.556	N	N							
IFN-a	0.834	362	3.6E-01	0.002	0.528	N	N							
Eotaxin-3	1.460	414	2.9E-01	0.004	0.438	N	N							
MCP-4	1.775	460	1.8E-01	0.004	0.291	N	N							
VEGF-C	2.212	455	1.4E-01	0.005	0.230	N	N							
BDNF	2.317	425	1.3E-01	0.005	0.226	N	N							
IL-4	2.703	334	1.0E-01	0.008	0.186	N	N							
MCP-1	3.428	472	6.5E-02	0.007	0.126	N	N							
PlGF	3.544	462	6.0E-02	0.008	0.124	N	N							
TARC	3.851	464	5.0E-02	0.008	0.109	N	N							
Eotaxin	4.212	452	4.1E-02	0.009	0.096	N	Y							
IL-12	4.448	454	3.5E-02	0.010	0.088	N	Y							
bFGF	5.015	448	2.6E-02	0.011	0.070	N	Y							
sFlt-1	7.214	464	7.0E-03	0.015	0.020	N	Y							
IL-16	7.682	450	6.0E-03	0.017	0.019	N	Y							
MIP-1B	8.984	451	3.0E-03	0.020	0.011	N	Y							
MDC	11.276	452	1.0E-03	0.024	0.004	Y	Y	2.690	360	0.102	0.007	0.232	N	N
IL-17	11.316	447	1.0E-03	0.025	0.004	Y	Y	3.489	354	0.063	0.010	0.232	N	N
IP-10	12.323	455	4.9E-04	0.026	0.002	Y	Y	2.253	361	0.134	0.006	0.232	N	N
sICAM-1	18.298	447	2.3E-05	0.039	0.000	Y	Y	0.229	355	0.632	0.001	0.722	N	N
VEGFCD	19.003	454	1.6E-05	0.040	0.000	Y	Y	2.513	363	0.114	0.007	0.232	N	N
TNFA	22.703	460	3.0E-06	0.047	0.000	Y	Y	2.188	366	0.140	0.006	0.232	N	N
SAA	61.445	444	3.4E-14	0.122	0.000	Y	Y	0.824	352	0.365	0.002	0.531	N	N
CRP	82.753	452	3.0E-18	0.155	0.000	Y	Y	6.604	361	0.011	0.018	0.144	N	Y
IL-6	98.421	441	4.5E-21	0.182	0.000	Y	Y	5.665	348	0.018	0.016	0.144	N	Y

9.2.6 RESULTS FROM SENSITIVITY ANALYSES INVESTIGATING THE EFFECTS OF POTENTIAL CONFOUNDERS ON CYTOKINE EXPRESSION

Table 9.2.3 – A table showing results from sensitivity analyses for physical illnesses and CRP

Illnesses	F	d.f.	p-values	Effect Size
Diabetes	.601	457	.439	.001
Rheumatic Disorders/Arthritis	.001	457	.979	.000
Heart Trouble	.253	457	.615	.001
Stroke	.929	457	.336	.002
High Blood Pressure	1.722	457	.190	.004
Cancer	.016	457	.901	.000

All results are based on univariate linear regressions which included the physical illness and an independent variable; age and sex as covariates; and protein expression as the dependent variable.

9.3 CHAPTER 4 APPENDIX

9.3.1 RESULTS FROM SENSITIVITY ANALYSES INVESTIGATING THE EFFECTS OF POTENTIAL CONFOUNDERS ON LOG(RTL)

All results are based on univariate linear regressions which included age, sex and BMI as covariates, and log(RTL) as the outcome variable.

Table 9.3.1 – A table of results from sensitivity analyses on smoking and drug use

Smoking and Drugs	F	d.f.	p-values	Effect Size
Smoking Status	.918	1	.400	.004
Drug Dependency	.034	1	.855	.000
Drug Use	3.552	1	.060	.008

Table 9.3.2 – A table of sensitivity analyses on physical illness

Illnesses	F	d.f.	p-values	Effect Size
Asthma	.013	1	.909	.000
Depression/other Nervous Illnesses	.336	1	.563	.001
Diabetes	.533	1	.466	.001
Stomach/Digestive Disorders	.179	1	.672	.000
Rheumatic Disorders/Arthritis	.513	1	.474	.001
Heart Trouble	.450	1	.503	.001
Stroke	.015	1	.903	.000
High Blood Pressure	.195	1	.659	.000
Migraines	.064	1	.801	.000
Epilepsy	2.970	1	.086	.007
Gynaecological Problems	.210	1	.647	.001
Cancer	.666	1	.415	.002
Kidney Problems	2.171	1	.141	.005
Other	1.942	1	.164	.005
Long Lasting Illnesses	.135	1	.713	.000
Number of Long Lasting Illnesses	.286	1	.593	.001

Table 9.3.3 – A table of sensitivity analyses on medications and supplements

Medications	F	d.f.	p-values	Effect Size
Pain medication	.067	1	.796	.000
Antacid Medication	.662	1	.416	.002
Cold Medication	.232	1	.630	.001
Allergy Medication	.636	1	.426	.002
Antibiotic Medication	.001	1	.973	.000
Birth Control Medication	.006	1	.940	.000
Chest Medication	.057	1	.811	.000
Diabetes Medication	.007	1	.933	.000
Depression/Anxiety Medication	4.095	1	.044	.010
Heart/blood pressure Medication	.296	1	.587	.001
Thyroid Medication	.140	1	.709	.000
Other Medication	.150	1	.699	.000
Vitamin Supplements	.098	1	.754	.000
Herbal Medication	1.139	1	.286	.003
Any Medication	.417	1	.519	.001

9.3.2 MELT CURVE RESULTS CHARTS FROM THE TELOMERE AND ALBUMIN REACTIONS

These charts show the melt cure plots from the telomere and the albumin reactions. Note that all other experiments using the same protocol achieved the same melt curves.

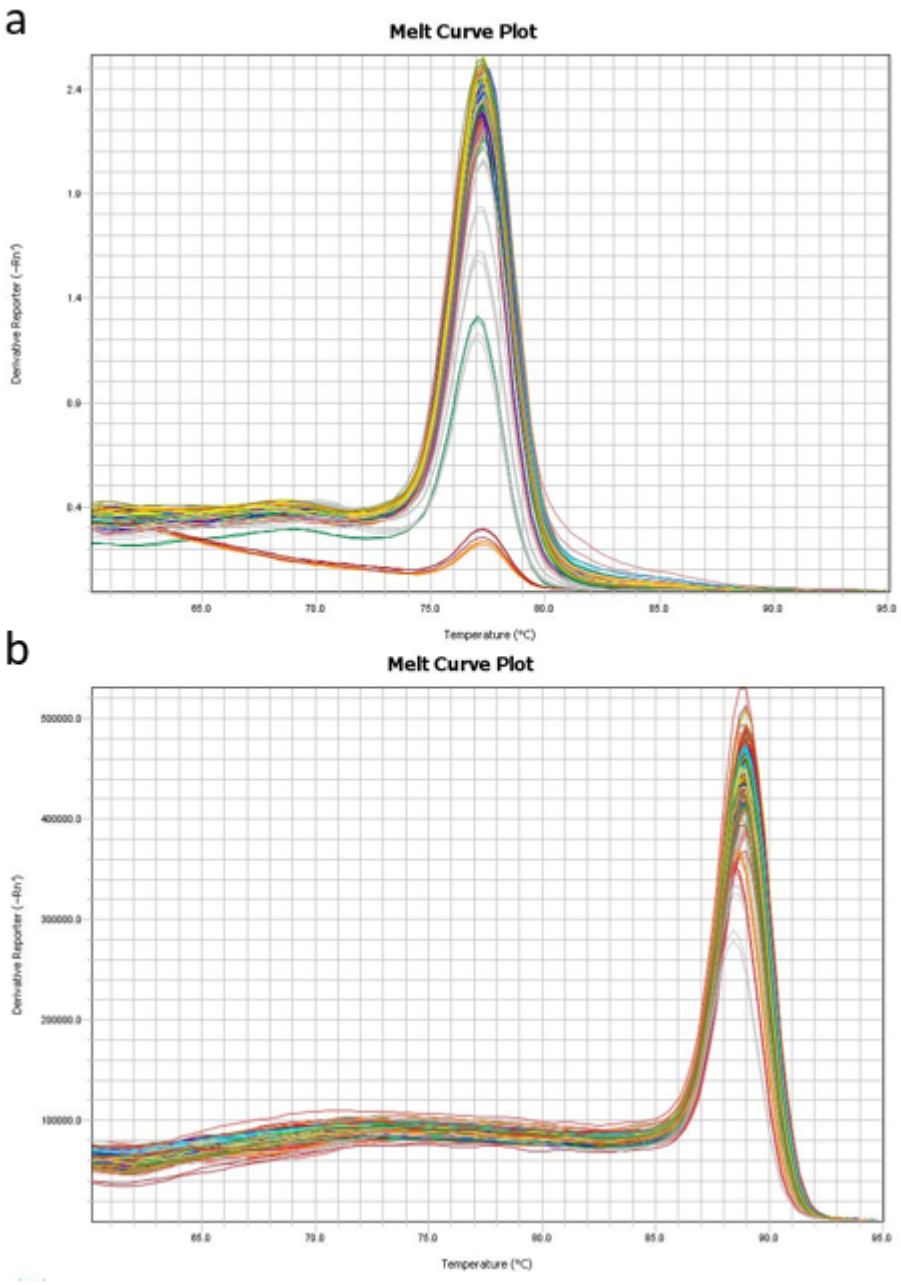

Figure 9.3.1 – Melt curve plots from the telomere and the albumin reactions.
These figures show the melt curve plots taken from one of the qPCR plates. (a) A melt curve plot for the telomere reactions. (b) A melt curve plot for the albumin reaction.

9.4 CHAPTER 5 APPENDIX

9.4.1 BACC STUDY RECRUITMENT CRITERIA

All participants had been diagnosed with Bipolar I or Bipolar II disorder as defined by the Diagnostic and Statistical Manual 4th edition operational criteria (DSM-IV (APA, 1994)). The exclusion criteria were: i) if a first degree relative met criteria for schizophrenia, ii) if the BD patient presented psychotic symptoms with no link to mood, iii) if intravenous drug use or drug dependency had ever occurred, iv) if mania/depression ever occurred solely due to alcohol or substance use or medical illness, v) if the patient was related to an individual already in the study. BD patients were identified from psychiatric clinics, hospitals, primary care physicians, patient support groups, and from volunteers responding to media advertisements. All participants were interviewed in person using the Schedules for Clinical Assessment in Neuropsychiatry (SCAN). Blood was taken from each participant for DNA extraction and subsequent genetic analyses. All patients were euthymic (not in a current mood episode) at the time of recruitment. Written informed consent was obtained from all participants and the study was approved by the Joint South London and Maudsley Ethics Committee.

9.4.2 TELOMERE PROTOCOL

Per 96 samples assayed, two 384-well plates were prepared in parallel; one to assay the telomere repeat region, and one to assay the albumin gene. A seven-point standard curve of doubling concentrations (0.47 ng, 0.94 ng, 1.88 ng, 3.75 ng, 7.5 ng, 15 ng, 30 ng) was set up by serial dilution to allow for absolute quantification of the amplified DNA, and to determine and control for the efficiency of the qPCR reactions. Four negative controls containing RNase-free water instead of DNA were used to test for DNA contamination, and five positive controls containing leukocyte DNA from five separate individuals were included on every plate to confirm successful PCR amplification. For all samples, the seven-point standard curve and the positive and negative controls, three technical replicates were used. The same sample well positions were used for both the telomere and albumin plates.

Each telomere reaction was made up to 15 μL per well, containing 10 μL SYBR green Primer Design Mastermix (548) (Primer Design, Southampton, UK), 5 μL of RNase-free water, 1000 nM of the telomere forward primer (5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'), 800 nM of the telomere reverse primer (5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'), and 12 ng of DNA. The thermocycling reaction (performed on the Quantstudio 7 Flex Real-Time PCR System) was set up as shown in **Figure 1a**. The albumin qPCRs were set up in the same way but instead using 765 nM of the albumin forward primer (5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTT-3') and 930 nM of the albumin reverse primer (5'-GCCCGGCCCGCCGCGCCCGTCCCGCCGG AAAAGCATGGTCGCCTGTT-3'), and using the thermocycling conditions seen in the figure below.

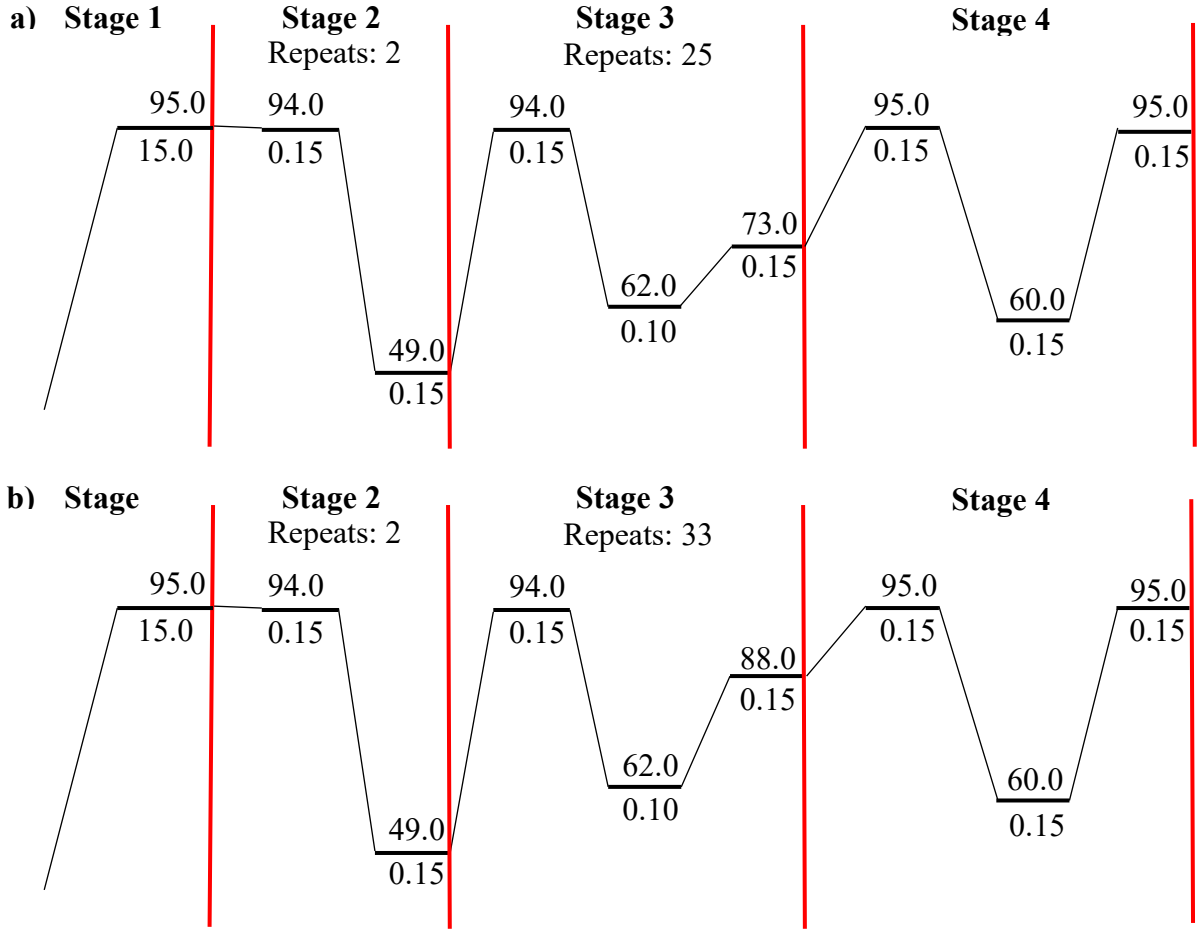

Figure 9.4.1 – Thermocycling conditions.

a) The telomere reaction, and b) the albumin reaction. The upper values represent temperature (°C) and the lower values represent time (minutes.seconds). Data was collected in the last step of Stage 3 for both reactions. Stage 4 represents the dissociation curve.

9.4.3 TELOMERE QUALITY CONTROL CRITERIA

Initial analysis of the standard curve, melt curve and controls was performed using QuantStudio™ Real-Time PCR Software. Subsequently, any replicates with C_t standard deviations > 0.5 from the other two were excluded from further analysis. Samples that still had a mean C_t standard deviation of greater than 0.5 after exclusion of one replicate were removed from downstream analysis. Remaining mean C_t values were then related to the standard curve in order to obtain absolute quantities of DNA for each sample (C_q). The RTL values were then calculated for each sample by dividing the mean C_q for the telomere reaction by the mean C_q for the albumin reaction. The RTL values were log-transformed for parametric use and adjusted for plate batch by taking the standardized residuals to control for the effects of minor inter-plate variability. Outliers were defined as those that were ±2 standard deviations from the mean.

9.5 CHAPTER 6 APPENDIX

9.5.1 DIFFERENTIAL GENE EXPRESSION OF RNASEQ DATA FOR SELECTING CANDIDATE GENES

In order to select candidate genes for qPCR experiments aimed at finding the differential expression of genes related to ageing, we downloaded a dataset made up of transcriptomic profiling of human hippocampal dentate gyrus granule cells.

The transcriptomic data was analysed using the Applied Biosystems SOLiD 4 high-throughput sequencer was available to download online.

The dataset was downloaded from the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42546) as cleaned raw counts (https://www.ncbi.nlm.nih.gov/geo/download/?acc=GSE42546&format=file&file=GSE42546%5FCleanedRawCounts%2Etxt%2Egz).

Differential expression analysis for sequence count data was carried out in R (The R Project for Statistical Computing) using the DESeq2 package (549). A likelihood ratio test was carried out controlling for diagnosis and gender, to measure the log-fold change in gene expression with age, in 79 people. The top two downregulated and top two upregulated genes were picked for gene expression experiments, see tables below.

Table 9.5.1 – A table listing the top 10 genes upregulated in the dentate gyrus as a result of age.

Rank	Gene Symbol	Ensembl Gene ID	Gene description
1	SBNO2	ENSG00000064932	strawberry notch 2
2	GAB1	ENSG00000109458	growth factor receptor bound protein 2-associated protein 1
3	TP63	ENSG00000073282	transformation related protein 63
4	TRPM6	ENSG00000119121	transient receptor potential cation channel, subfamily M, member 6
5	LHCGR	ENSG00000138039	luteinizing hormone/choriogonadotropin receptor
6	ITPKB	ENSG00000143772	inositol 1,4,5-trisphosphate 3-kinase B
7	ABTB2	ENSG00000166016	ankyrin repeat and BTB (POZ) domain containing 2
8	ARHGAP28	ENSG00000088756	Rho GTPase activating protein 28
9	ANTXR2	ENSG00000163297	anthrax toxin receptor 2
10	PCCB	ENSG00000114054	propionyl Coenzyme A carboxylase, beta polypeptide

Table 9.5.2 – A table detailing the top 10 genes downregulated in the dentate gyrus as a result of age.

Rank	Gene Symbol	Ensembl Gene ID	Gene description
1	NCDN	ENSG00000020129	neurochondrin
2	NEK6	ENSG00000119408	NIMA (never in mitosis gene a)-related expressed kinase 6
3	COG3	ENSG00000136152	component of oligomeric golgi complex 3
4	ARHGEF7	ENSG00000102606	Rho guanine nucleotide exchange factor (GEF7)
5	TFCP2	ENSG00000135457	transcription factor CP2
6	PGM2L1	ENSG00000165434	phosphoglucomutase 2-like 1
7	NISCH	ENSG00000010322	nischarin
8	CEP70	ENSG00000114107	centrosomal protein 70
9	NAV1	ENSG00000134369	neuron navigator 1
10	ATP2B1	ENSG00000070961	ATPase, Ca++ transporting, plasma membrane 1