Epigenetic Regulation of Stress-Related Genes in Dementia Brains: A Study

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Epigenetic Regulation of Stress-Related Genes in Dementia Brains: A Study

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Contents

Abstract

Dementia, particularly Alzheimer's disease (AD), is a prevailing cause of cognitive decline worldwide and presents a growing public health and economic burden. The underlying mechanisms of dementia are not fully understood, though they are believed to be multifactorial. Epigenetics, particularly DNA methylation, has gained attention for its involvement in neurodegenerative diseases, with alterations in key stress-related genes being implicated. Chronic stress and elevated cortisol levels, regulated by the hypothalamic-pituitary-adrenal (HPA) axis, are strongly associated with both depression and dementia. Depression is a widely recognised risk factor for dementia, with overlapping pathophysiological features such as neuroinflammation, glucocorticoid dysregulation, and amyloid-beta (Aβ) accumulation. However, the epigenetic link between depression and dementia remains underexplored.

This study aimed to investigate the relationship between DNA methylation in stress-related genes Glucocorticoid Receptor (coded by NR3C1) FKBP Prolyl Isomerase 5 (coded by FKBP5) and the Mineralocorticoid Receptor (coded by NR3C2) and cortisol levels, sleep quality and depression in individuals with dementia. Specifically, it sought to determine whether methylation levels in these genes correlate with the longitudinal factors, offering insights into the epigenetic mechanisms that might connect chronic stress and dementia.

We conducted an epigenetic analysis on 125 post-mortem frontal cortex brain samples from dementia and healthy patients. DNA methylation levels were measured in key CpG sites within the promoter regions of NR3C1, FKBP5, and NR3C2 genes using bisulfite pyrosequencing. Longitudinal data was collected by University of Manchester Longitudinal Ageing Study for depression, that was assessed through longitudinal depression scores, salivary cortisol and sleep measures obtained through self-reported sleep diaries. Statistical correlations were performed between methylation and cortisol levels, sleep quality and depression to investigate potential associations.

Significant correlations were observed between methylation levels and cortisol levels, sleep quality and depression measures. NR3C1 and NR3C2 showed multiple significant relationships

with both sleep quality and cortisol levels vs methylation. Depression showed significant relationships with NR3C1, FKBP5 and NR3C2 methylation. No gene-level differences emerged between neuropathologically defined dementia and control brains.

This study has identified multiple statistically significant relationships between the epigenetic regulation of stress-related genes and sleep, cortisol and depression which aligns with the supporting literature. Taken together, these findings suggest that site-specific methylation changes in HPA-axis receptors accumulate alongside decades-long alterations in cortisol rhythm, sleep quality and mood, potentially coupling chronic stress to neurodegeneration. Stress plays a significant role in dementia and a comprehensive understanding of its molecular mechanisms are crucial for disease management through lifestyle modifications, stress management, or pharmacological interventions.

Introduction

Dementia is a complex age-related neurological disorder identified by a progressive cognitive impairment affecting a range of cognitive domains such as memory loss and judgement, which introduces difficulties with everyday tasks (Malik et al., 2022). Dementia is the UK's leading cause of death and a major driver of dependency and disability (Giebel et al., 2025). Alzheimer's disease (AD) is the most common form of dementia. In the near future, neurodegenerative disorders like AD and Parkinson's disease (PD) are expected to overtake cancer and emerge as the second leading cause of mortality worldwide, behind cardiovascular disease (Kumar et al., 2022). At present, an estimated 944,000 people in the UK have dementia. This number is expected to surge to 1.6 million by 2050, driven by a mix of neurodegenerative lifestyle factors and longer life expectancy. It is estimated that 1 in 2 people will be affected by dementia in their lifetime whether by developing it themselves or caring for someone with dementia, or both (Luengo-Fernandez and Landeiro, 2022). The rising incidence of those living with dementia places significant extra strain on the social care system and poses a major economic challenge, largely because of the greater need for carers (Jönsson and Wimo, 2009). A recent study calculated the total societal cost of dementia in 2019 to be £1011 billion worldwide (Wimo et al., 2023). Despite the large burden dementia imposes on global public health and economies, the exact cause is still not fully understood, but it is accepted to be multifactorial (except in the familial forms) such as genetics, lifestyle and environment (Seifert et al., 2022). Therefore, it is clear there is a pressing need to investigate the potential risk factors of dementia in order to identify new therapeutic approaches and lifestyle changes, to help combat the rapidly rising incidence.

Epigenetics is a field of study that investigates changes in gene expression without altering the DNA sequence, including changes in phenotype via DNA methylation, histone acetylation/deacetylation as well as post-transcriptional/translational modifications. Epigenetics has emerged as a vital area of research for uncovering the underlying mechanisms of dementia related to lifestyle and environmental factors. DNA methylation consists of methyl group being covalently transferred to the C-5 position on the cytosine ring and has been amongst the most prevalent and significant epigenetic modifications found in disease research (Mire etal., 2023). DNA methylation can affect gene expression and activity and therefore may contribute to the development and progression of diseases. Various factors, such as age, environmental

influences, and lifestyle decisions, can influence these modifications (Walker et al., 2020). Research has shown that alterations in DNA methylation patterns can occur in the brains of individuals affected by AD, notably in genes involved in crucial brain function such as such as neuroplasticity (Younesian et al., 2022), inflammation (Giallongo et al,. 2022), and synaptic function (Younesian et al., 2022).

The body's stress response system is a dynamic mechanism capable of maintaining homeostasis during real or perceived stress conditions (Russell and Lightman, 2019). Numerous studies have shown that chronic stress can lead to dysregulation of the body's stress response system, resulting in elevated levels of stress hormones like cortisol. It is well-documented that sustained high cortisol levels can negatively impact the brain, affecting memory and cognitive function (Jones and Gwenin, 2021). The hypothalamus-pituitary-adrenal (HPA) axis is a classical neuroendocrine axis that is a crucial regulatory pathway responsible for the stress response cascade (Russell and Lightman, 2019). The hypothalamus releases corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), ultimately resulting in signals to the adrenal glands to produce and release glucocorticoids into the bloodstream, including the stress hormone cortisol. Increased glucocorticoid levels trigger the negative feedback loop via the hippocampal and hypothalamic corticosteroid receptors to suppress CRH expression. The negative feedback mechanism plays a vital role in halting HPA axis activation, which is essential in the short term but becomes detrimental if prolonged due to the catabolic implications of continued elevated cortisol levels (Magri et al., 2006). When the body is experiencing acute stress cortisol is secreted in a pulsatile pattern following the release of ACTH. However, if inflammatory stress persists for a prolonged duration then despite ACTH levels falling to near basal levels, cortisol remains elevated due to heightened adrenal sensitivity. Chronic stress leads to a reduction in the beneficial effects of cortisol such as the fight or flight response, while prolonged cortisol presence it becomes maladaptive, leading to a wide array of problems, including metabolic syndrome, obesity, cancer, mental health disorders, cardiovascular disease, and cognitive decline (Russell and Lightman, 2019). Impaired regulation of the HPA axis and elevated cortisol levels are commonly observed in people with dementia and have been found to cause a significant contribution to the disease progression. Hence, it is widely recognised that chronic stress is a risk factor for dementia development and progression (Milligan Armstrong et al., 2021). Alongside the direct physical impacts of chronic stress on the body, another possibility that studies have been unable to control for is the possibility that chronic stress might indirectly

contribute to the onset of AD by promoting unhealthy coping behaviours. Individuals under prolonged stress may be more inclined to adopt habits harmful to brain health, such as poor nutrition, lack of physical activity, insufficient sleep, or increased consumption of alcohol or tobacco. These lifestyle factors have been linked to a higher risk of cognitive decline and AD (Yang et al., 2022). This raises the question of whether changes in the function and expression of stress related genes (including the receptor for cortisol) in the brain relate to dementia.

Multiple meta-analyses have shown that late-life depression is associated with an elevated risk of subsequent cognitive decline and dementia, suggesting a possible aetiological link (Fernández et al., 2024). Conversely, depressive symptoms can also emerge during the prodromal phase of dementia, making the relationship bidirectional rather than strictly causal. The two disorders share several overlapping pathophysiological mechanisms, such as hippocampal atrophy, cerebrovascular disease, neuroinflammation, and hypothalamic–pituitary–adrenal-axis dysregulation, which further complicates efforts to disentangle cause from consequence in this comorbidity (Yin et al., 2024). Elevated cortisol levels have been observed in around 70% of depressed individuals alongside its high presence in the dementia community. Additionally there is evidence that cerebrovascular lesions lead to the onset of both depending on the location of the lesions. As well as neuroinflammation - which can be caused by elevated cortisol - playing a crucial part in the aetiology of both syndromes. Further overlapping processes include upregulation in microglial activation, alterations in Transforming Growth Factor-beta1 (TGF-beta1) signalling, synthesis of pro-inflammatory cytokines and a concurrent decrease in anti-inflammatory molecule production. A key etiological factor is increased plasma β-amyloid42 (Aβ) as an independent predictor for both as well as for the development of depression and then the potential conversion to dementia (Linnemann and Lang, 2020). This multitude of overlapping pathophysiological processes indicates the strong associations and link between these two syndromes however the exact relationship and to what degree they impact and influence each other remains unclear. Highlighting the need for further research and the importance of the potential impacts from this study. Research shows that addressing depression as a modifiable risk factor could contribute to preventing or delaying the onset of dementia which is considered a global public health priority. Recent findings show that antidepressant treatment stimulates neurogenesis in the human hippocampus and prevents Aβ oligomer-induced aggregation. Additionally, it may offer an effective approach for mitigating tau pathology. Furthermore, antidepressant treatment exhibits substantial anti-inflammatory effects and curtails the inflammatory activities of microglia and astroglia, which are both established

pathways within dementia progression. Early diagnosis of depression followed by antidepressive treatment could play a crucial role in minimising the neurotoxic effects of depressive episodes and preventing dementia onset. This is of particular importance as dementia has a notably long pre-clinical phase with pathophysiological processes being present 20-30 years before symptoms arise. Hence a more robust and thorough understanding of the relationship between these two syndromes could allow for early dementia detection and prevention as depression typically has a significantly faster patient diagnosis time. Thus, it is a plausible hypothesis that long-term antidepressant treatment could reduce the risk of developing dementia, not only in individuals with severe and recurrent depression but also in those with milder forms of depression, particularly in those at high risk for developing dementia (Dafsari and Jessen, 2020). This study investigates the relationship between dementia and depression further through the epigenetic analyses of stress-related genes which play a role in both.

It is also widely accepted that people with dementia and individuals with depression experience sleep disruption including decreased length of sleep and increased fragmentation, however more recently studies have been showing that there is evidence that altered quantity, quality and timing of a person's sleep is a causal risk factor for dementia (Anderson et al., 2021). Studies have shown that poor quality sleep can result in modifications in Aβ metabolism as well as stimulate neuroinflammation and oxidative stress (Fernandes et al., 2022). Neuroinflammation is characterised by the activation of immune cells, such as microglia, and the production of pro-inflammatory cytokines in the brain. This leads to persistent inflammation, which can intensify neuronal damage, cause synaptic dysfunction, and accelerate the accumulation of Aβ plaques and neurofibrillary tangles seen in dementia (Heneka et al., 2015). Research has shown that inflammation disrupts the ubiquitin-proteasome system and the autophagy-lysosomal pathway, which are vital for clearing abnormal proteins, thereby contributing to their buildup in AD (Nandi et al., 2006 and Zhang et al., 2022). The commonly observed Aβ plaques seen in dementia initially appear as a decrease in soluble Aβ levels in the cerebrospinal fluid (CSF) (Ju et al., 2014). Recent cross-sectional studies have discovered that patients with sleep disruptions have lower CSF Aβ levels and elevated plaque formation. Further studies have highlighted the importance of sleep in its role of clearing the cerebral metabolic products that accumulate during wakefulness, with Aβ being found amongst these catabolic products in the interstitial space (Liguori et al., 2019). Additionally the glymphatic system, a sleep-related clearance pathway, responsible for the removal of potentially neuro-damaging waste products, including Aβ, from the brain interstitial space can become

impaired in those with disrupted sleep or sleep conditions, ultimately resulting in an increase in the formation of cerebral neuritic plaques (Mestre et al., 2020). Overall research has shown that there is both a causal risk factor and a potential pathophysiological link between poor sleep disruptions and dementia (Perez-Cabezas et al., 2020), as well as sleep being a risk factor for depression. Hence this project seeks to investigate this link further through epigenetic analysis of DNA methylation and longitudinal sleep and depression data.

The relationship between DNA methylation, ageing, memory, cognition, and AD has received significant attention in research, particularly within the amyloidogenic pathway and neurochemical processes (Poon et al., 2020). However, to our knowledge, no published work has yet combined DNA-methylation profiles from post-mortem dementia brains with more than two decades of prospectively collected cognitive and medical data, such as those available from the University of Manchester Longitudinal Ageing Study (Rabbitt et al., 2004). In this research paper we investigate DNA methylation patterns in 125 dementia brains (frontal cortex) across three stress related genes: NR3C1, FKBP5 and NR3C2. NR3C1 codes for the glucocorticoid receptor which is the receptor for the stress hormone cortisol (Mendonça et al., 2021), FKBP5 which acts as a co-chaperone protein to NR3C1 by mediating its sensitivity to cortisol (Galbally et al., 2020), and NR3C2 which codes for the mineralocorticoid receptor and also binds to cortisol within the HPA axis (Mendonça et al., 2021). Methylation in the promoter region of genes has been shown to cause gene silencing and transcriptional repression which impacts expression regulation and disease susceptibility (Giallongo et al., 2022). What is therefore unclear is if changes in the regulation of stress related genes in the brain relates to dementia, sleep cortisol levels and depression. This study will perform epigenetic analyses through pyrosequencing to produce the percentage methylation from all samples and incorporate over 20 years of longitudinal data on dementia scores and depression scores in order to perform complex statistical analysis to understand the relationships between DNA methylation, stress, depression, cortisol levels, pathology and dementia, to identify new therapeutic targets for treatment developments as well implementing appropriate lifestyle changes.

Methods

Prior to the start of this study, ethical approval to perform this study and epigenetic analyses on the brains at MMU was granted through Manchester Brain Bank and EthOS, MMU's online research-ethics application and governance system.

Meta-Analysis

Study design

The systematic review and meta-analysis conducted in this study was carried out according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement by Page et al. (2021). The initial research question of "Do changes in cortisol relate to dementia?" was used to conduct a preliminary systematic literature search in the electronic database PubMed in order to identify the appropriate search terms. A further manual search was also conducted to retrieve additional studies which were not retrieved in the automated search. The manual search was conducted across academic resources through Manchester Metropolitan University Libraries and Manchester Central Library, however, no additional literature was retrieved.

Search strategy

The hypothesis was identified as 'Changes in cortisol predict cognitive decline and dementia development'. The three key aspects of the hypothesis were 'cortisol', 'cognitive decline' and 'dementia'. The building block approach was then used to create search terms to ensure the correct syntax was being used before the final search. MeSH terms for the main concepts of the search were identified to ensure literature was retrieved despite differences in terminology used by authors. Exclusions in the MeSH hierarchy were made where appropriate. Keywords were also incorporated into the search alongside MeSH terms to ensure more recent studies that are still awaiting indexing by the National Library of Medicine were still included in the search. Once all MeSH and keywords were identified, Boolean operators 'AND' and 'OR', truncation and the 'tw' field tag were applied. The final search syntax was as follows:

("Alzheimer Disease"[Mesh] OR "Alzheimer Disease*"[tw] "Alzheimer's Disease*"[tw] OR "Alzheimer Dementia*"[tw] OR "Alzheimer Type Dementia*"[tw] OR "Alzheimer-Type Dementia (ATD)"[tw] OR "Alzheimer Sclerosis"[tw] OR "Lewy Body Disease"[Mesh] OR "Lewy Body Disease"[tw] OR "Dementia, Vascular"[Mesh] “Vascular Dementia*”[tw] OR "Mixed Dementias"[Mesh] OR “Mixed Dementia*”[tw] OR "Senile Dementia"[tw] OR "Dementia Alzheimer-Type (ATD)"[tw] OR "Cognition"[Mesh] OR "Cognitive Dysfunction"[Mesh:NoExp] OR "Cognitive Function*"[tw] "Cognitive Dysfunction*"[tw] OR "Cognitive Impairment*"[tw] OR "Mild Cognitive Impairment*"[tw] OR "Cognitive Decline*"[tw] OR "Mental Deterioration*"[tw] OR "Cognitive Disorder*"[tw]) AND ("Hydrocortisone"[Mesh:NoExp] OR "Receptors, Mineralocorticoid"[Mesh] OR “NR3C2”[tw] OR "Mineralocorticoid* Receptor*"[tw] OR "Receptors, Glucocorticoid"[Mesh] OR “NR3C1”[tw] OR “Glucocorticoid* Receptor*”[tw] OR “Cortisol”[tw]) AND ("Longitudinal Studies"[Majr] OR “Longitudinal Stud*”[tw])

Selection criteria

The automated search was conducted on the 2nd of August 2024 and obtained 43 relevant papers whilst the manual search revealed no additional studies. All studies retrieved were required to meet the following criteria: 1) participants must have a clinical diagnosis of any form of dementia; 2) the study must carry out original research exploring the link between cortisol and cognitive function within dementia patients; 3) studies must include a control group with participants with no dementia diagnosis; 4) the research must be conducted with human adults as the study population; 5) the study must utilise objective neuropsychological cognitive tests such as Mini- Mental State Examination (MMSE) to assess participant cognitive function; 6) the research must have a longitudinal study design; 7) publications must be in the English language.

All papers which met the above inclusion criteria were then subject to the following exclusion criteria: 1) studies which had inadequate sample populations (>4 participants in control and dementia group); 2) research that had incorporated subjective measures of cognitive function; 3) both case studies and case reports; 4) research utilising a cross-sectional study design; 5) meta-analyses or systematic reviews to avoid duplicated data.

Given the high specificity of this research question the publication date was not restricted in this meta analysis to allow for all relevant publications to be identified and included. In cases where

the same study population was investigated the publication with the largest data set was included and the other papers were excluded to remove data duplications.

Following the literature search 43 studies were obtained. Studies were screened on their titles and 24 were removed because the title alone showed that they breached at least one mandatory criterion: animal or in-vitro studies (n = 9), paediatric or adolescent samples (n = 4), cross-sectional or case-series designs (n = 7), narrative reviews or commentaries (n = 3), or an absence of any cortisol-related term (n = 1). 19 papers were screened on their abstracts and 7 were removed; 3 lacked a dementia diagnosis group, 2 had no cognitively healthy control group, and 2 used only subjective or surrogate cognitive measures. The remaining 12 papers were screened across the full text against the exclusion and inclusion criteria, 4 papers were removed for having no dementia diagnosis group, 3 papers were removed for having no control group, and 1 paper was removed for having no measure of cortisol levels. The PRISMA flowchart that was produced is shown below as Figure 1 (Page et al., 2021).

Figure 1

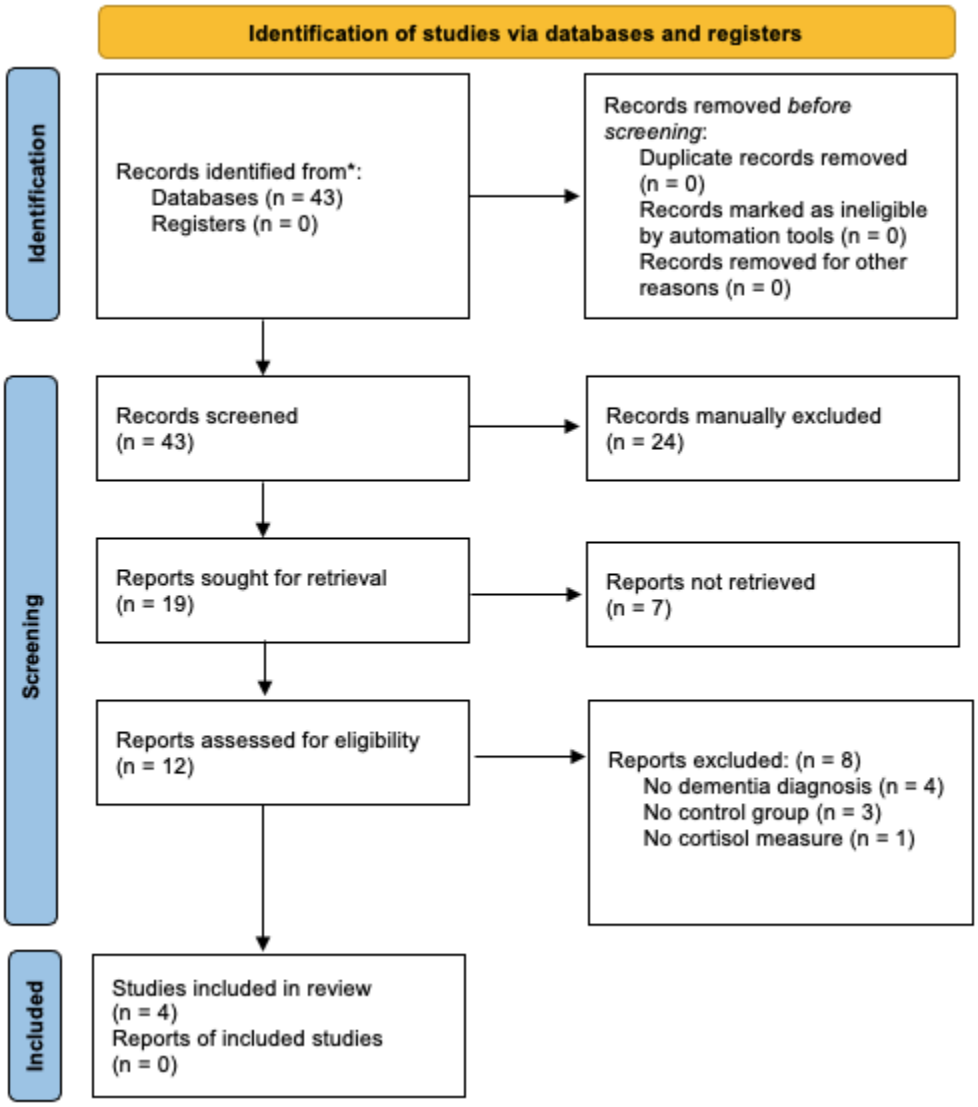

Figure 1: PRISMA flowchart (Source: Page et al., 2021:online)

Sample Collection

Prior to the start of this project, 125 fresh frozen superior frontal gyrus (SFG) (Brodmann area 8) samples were obtained from the Manchester Brain Bank at Salford Royal Foundation Trust, which received ethical approval from the Newcastle and North Tyneside 1 Research Ethics Committee on 6th May 2014. Ethical approval for this project was granted by the Manchester

Brain Bank Committee. The samples were stored at -80°C. The donors were participants from a large prospective cognitive ageing study known as The University of Manchester Age and Cognitive Performance Research Cohort (Rabbitt et al., 2004; Robinson et al., 2018). The SFG was selected because the metabolic and structural changes in this region appear early in AD and predict executive-function decline, and its connectivity with limbic stress circuitry is consistently altered in major depressive disorder, making it a logical substrate for cortisol-related epigenetic effects. For each donation (2004 – 2024), Brain-Bank technicians cryosectioned the SFG block at 50 µm on a Leica CM-series cryostat, following the BrainNet Europe/UK Brain Banks Network frozen-tissue protocols routinely used at Manchester. Alternate sections were employed for diagnostic BRAAK and CERAD staining, while adjoining ~50 mg grey-matter punches were archived at −80 °C for downstream molecular analyses; the present study utilised these pre-existing punches, and no additional sectioning was required. Samples were obtained from all individuals who had brain material and neuropathological data available. The samples were classified into dementia neuropathology and control groups, using the BRAAK stage to measure neurofibrillary tangle stages and the CERAD score to evaluate neuritic plaque scores, in accordance with The Consortium to Establish a Registry for Alzheimer's Disease guidelines (Mirra et al., 1991).

DNA Extraction

The samples were sectioned inside the Leica CM3050 S Cryostat at -20°C. 25mg of sample was collected and placed into 2.5 mL Eppendorf tubes. The tubes were labelled with an MMU sample number which corresponded to the sample ID, as shown by Table 1.

Table 1

MMU Sample No.	ID
1	22272
2	11426
3	10954
4	10719
5	20935
6	12033
7	12284
8	10772
9	22625
10	20402
11	21493
12	12221
13	11662
14	22738
15	20382
16	10118
17	10540
18	11427
19	22091
20	12022
21	20428
22	21337
23	22340
24	12755
25	11299
26	22083
27	11322
28	21664
29	11508
30	22105
31	20429
32	11341
33	11176

MMU Sample No.	ID
33	11176
34	22708
35	11550
36	11845
37	12504
38	12413
39	10664
40	22691
41	20845
42	22110
43	22867
44	10640
45	11383
46	21179
47	22194
48	10192
49	21297
50	21092
51	10132
52	11618
53	11379
54	23096
55	10544
56	11802
57	20274
58	21683
59	11971
60	20088
61	11052
62	11060
63	12762
64	10502
65	10004

MMU Sample No.	ID
66	12698
67	11240
73	21596
74	23136
75	11187
76	22626
77	10321
78	12208
79	11496
80	21984
81	12075
82	20753
83	10760
84	23281
85	21862
86	10997
87	20522
88	22378
89	11896
90	10187
91	11234
92	40003
93	22467
94	12800
95	22683
96	21288
97	22682
98	12545
99	10790
100	11233
101	22109
102	10280
103	11897

MMU Sample No.	ID
104	12509
105	23354
106	11937
107	21794
108	22751
109	23155
110	11044
111	11565
112	11785
113	11042
114	21766
115	21998
116	12241
117	23350
118	22781
119	20922
120	11789
121	12715
122	22809
123	20302
124	11207
125	11899
126	10884
127	22603
128	N/A
129	12045
130	11936

Table 1: shows a running table of the MMU sample number corresponding to the brain sample ID obtained from the University of Manchester Longitudinal Ageing Study.

The DNA extraction was carried out according to the Bioline ISOLATE II Genomic DNA Kit Product Manual by following the standard protocol for purifying DNA from human tissue. Prior to starting the Wash Buffer GW2 and Proteinase K were prepared by adding 200 mL of 99% ethanol and 6.7 ml of Proteinase K Buffer PR respectively. 180 *µ*l Lysis Buffer GL followed by 25 *µ*l Proteinase K solution were added to each sample followed by vortexing then a 3 hr incubation at 56°C. Samples were agitated with a p200 pipette prior to incubation by drawing the sample up and down repeatedly to break it down. This was repeated throughout the incubation at intervals 0:30, 1:00, 1:30, 2:00 and 2:30 hrs. After the incubation was complete all samples were vortexed then 200 *µ*l Lysis Buffer G3 was added then vortexed. Next, the

samples were incubated at 70°C for 10 min. After incubation was complete 210 µl ethanol was added then all samples were vortexed. A collection tube was added to each ISOLATE II Genomic DNA Spin Column and then each sample was then transferred into the columns. The samples were then centrifuged at 11,000 x g for 1 min. The flow-through was discarded from the collection tube and then the collection tube was reused. 500 µl of Wash Buffer GW1 was added then samples were centrifuged for at 11,000 x g for 1 min, the collection tube was emptied as previously described. Next, 600 µl of Wash Buffer GW2 was added and samples centrifuged at 11,000 x g for 1 min and collection tubes were emptied. Samples were then centrifuged again at 11,000 x g for 1 min. Following this, the columns were placed into 1.5 microcentrifuge tubes prior to adding 100 µl preheated Elution Buffer G (70°C) onto the silica membrane. Samples were incubated at room temperature for 1 min then centrifuged at 11,000 x g for 1 min. The microcentrifuge tubes containing collected isolated DNA were then labelled with the appropriate sample number.

DNA Quantification

The Thermo Scientific Nanodrop Spectrophotometer was used for DNA quantification, 1 µl of nuclease-free water water was added to blank the apparatus, then 1 µl of the isolated DNA samples were pipetted onto the Nanodrop Spectrophotometer and the results were tabulated and shown in the appendix section as Table 18. The average sample concentration was found to be 26.31 ng/ml with a lowest concentration of 10.4 ng/ml and a highest concentration of 81.8 ng/ml.

Further quantification was conducted by utilising the Qubit® Fluorometer using the Invitrogen Qubit 1X dsDNA HS Assay Kit. First the standards were made using 190 µl of Qubit® 1X dsDNA HS Working Solution followed by 10 µl of Qubit® 1X dsDNA HS Standard #1 (0 ng/µl in TE buffer) and 190 µl of Qubit® 1X dsDNA HS Working Solution along with 10 of Qubit® 1X dsDNA HS Standard #2 (10 ng/µl in TE buffer) into Qubit® assay tubes. For each sample, 1 µl of isolated DNA sample was loaded into the Qubit® assay tubes with 199 µl of Qubit® 1X dsDNA HS Working Solution. All tubes were vortexed and incubated for 3 minutes in the dark to improve their fluorescence. After incubation the standards were loaded first followed by all samples. The results are shown in the appendix as Table 18. The average sample concentration

was found to be 62.2 ng/ml with a lowest concentration of 18.12 ng/ml and a highest concentration of 151.3 ng/ml.

Bisulfite Conversion

The bisulfite conversion of the sample DNA was conducted according to the QIAGEN EpiTect Fast 96 DNA Bisulfite Kit protocol. Prior to the start: 120 ml of 98% ethanol was added to Buffer BW, 27 ml of 98% ethanol was added to Buffer BD, the sample DNA was thawed and the bisulfite solution was checked to ensure the solution was completely dissolved.

The reaction was prepped in the EpiTect 96 Conversion Plate that was provided. The setup was carried out according to the high concentration samples protocol. 20 *µ*l of sample DNA was pipetted into the conversion plate then 85 *µ*l of bisulfite solution was added along with 35 *µ*l of DNA Protect Buffer using reservoirs and a multichannel pipette. The reaction was mixed using the multichannel pipette then sealed using the EpiTect 96 Cover Foil and centrifuged briefly at 650 x *g* at room temperature. The plate was then added to the Blue-Ray Biotech TurboCycler thermal cycler and was programmed according to the following Table 2.

Table 2

Stage	Table (min)	Temperature (℃)
Denaturation	5	95
Incubation	10	60
Denaturation	5	95
Incubation	10	60
Hold	Indefinite	20

Table 2: shows the individual times and temperatures that were programmed into the thermal cycler for each stage of the reaction.

Following the completion of the incubation on the thermo cycler, the plate was briefly centrifuged at 650 x *g.* The EpiTect 96 Plate was placed onto a vacuum manifold and 310 *µ*l Buffer BL was pipetted into the wells. The completed reactions from the EpiTect 96 Conversion Plate were transferred into the EpiTect 96 Plate and mixed with the Buffer BL via pipetting. 250 *µ*l 98% ethanol was added and mixed, then the sampled were processed by turning on the vacuum for 1 min. 500 *µ*l Buffer BW was added and processed. 250 *µ*l Buffer BD was added and then incubated for 15 min at room temperature. After incubating, the samples were vacuum

processed. 500 *µ*l Buffer BW was added and processed and then repeated immediately after. 250 *µ*l 98% ethanol was added and processed. Following this, maximum vacuum was applied for 10 min to remove all residual ethanol. The EpiTect 96 Plate was then removed off the vacuum manifold and tapped vigorously on top of clean absorbent paper until no more drops appeared. The EpiTect 96 Plate was placed on top of an EpiTect Elution Plate and 70 *µ*l Buffer EB was added using a multichannel pipette, followed by centrifuging for 1 min at 5800 x *g.* The elution plate was then sealed for -20°C storage.

PCR Amplification

PCR primers were designed using the PyroMark Assay Design Software 2.0. Target sequences within the promoter regions of the genes were selected using supporting literature of previous DNA methylation studies in other neurological disorders as well as consideration for where CpG sites were located. The primer sequences used for each gene were shown below in Table 3.

Table 3

Gene	FP	RP	SP	Sequence to analyse	Score
NR3C1	GTTGTTATTAGTAGGGGTATTGG	*AAACCACCCAATTTCTCCAATTTCTTT	GAGTTTTAGAGTGGGTTTG	GAGTYGYGGAGTTGGGYGGGGG (22bp)	69
FKBP5	TTTTGGGTTGAGGATAGAAAGG	*AACTTAAAACCACAATACAAACCTCT	GTTGAGGATAGAAAGGT	TTAYGTTTTGTTAAGTGGTTTTTTGGGGGAGTGGGGTGTAGTTTTTTAGAGTTGAAYGG 59bp)	92
NR3C2	*GGTTAGGAGGGTTTTTTATTGGATAATT	CCAAAATCTAAACTACAACTCACC	TTCTTCCCCCTCAACACACTTTTCA	CATCTCTCCAAATATCCTAAAATCRATCAAAAAAAAAACAAAATAAACRTAAACAAATTTAAAACRACC (69 bp)	68

Table3: showing the forward primers (FP), reverse primers (RP) and sequencing primers (SP) for NR3C1, FKBP5, and NR3C2. The sequence to analyse is shown with total number of base pairs (bp), 'Y' indicates a partially methylated site where there is a mixture of cytosine (C) and thymine (T). 'R' is used as the NR3C2 reverse strand is being sequenced, therefore 'R' indicates a mixture of guanine (G) and adenine (A). * indicates biotinylated primer. The score is produced by the pyromark software as an assessment of the likelihood for the analysis to be successful, 60 is used as the minimum threshold for acceptable scores.

The sequence to analyse was selected as they contained multiple CpG sites (referred to as CpG islands) within the promoter regions, this is shown in Figure 2. Previous studies have shown that methylation at these specific promoter CpG sites have been linked to neurological disorders, NR3C1 methylation has found to have links with schizophrenia (Liu et al., 2020), FKBP5 methylation has been found to have a role in post-traumatic stress disorder (Miller et al., 2020) and NR3C2 promoter CpG methylation has been associated with depression and poor stress regulation (Galbally et al., 2020).

Figure 2

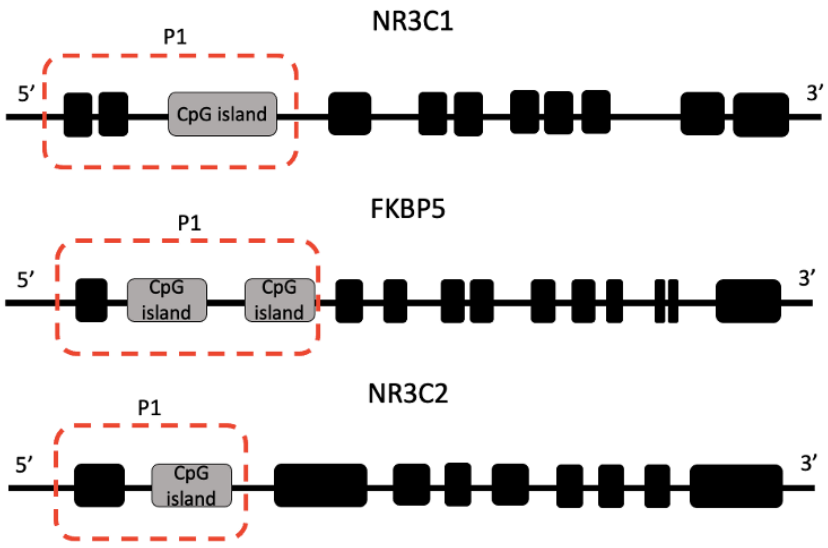

Figure 2: shows a diagram of the NR3C1, FKBP5 and NR3C2 genes. The red area shows the promoter (P1) with the CpG islands labelled. The black boxes represent the exons and the spaces inbetween represent introns.

PCRs were performed in a 96 well PCR plate. Each well contained 10 µl of Meridian Bioscience MyTaq HS mix PCR mastermix, 1 µl of forward primer and 1 µl of reverse primer as well as 6 µl of nuclease free H_2O. Next, 2 µl of sample DNA was added to each well except for the negative where 2 µl of nuclease free H_2O was added to ensure total reaction volume remained the same. The plate was sealed with Thermo Scientific Adhesive PCR plate seals. The plates were then loaded into the Blue-Ray Biotech TurboCycler thermal cycler and was programmed with the following conditions: initial denaturation of one cycle for 3 min at 95°C; 55 cycles of 94°C for 20 sec, 57°C for 20 sec, 72°C for 20s; final extension for one cycle of 72°C for 5 min, then a programmed hold at 4°C indefinitely. Plates were then removed and stored in the fridge.

Gel Electrophoresis

Gel electrophoresis was used to ensure the PCR stage was both successful and contamination free. Initially a 10x TBE solution was made by dissolving 108 g of Fisher Bioreagents Tris base and 800 ml of deionised water, followed by adding 55 g of Fisher Bioreagents boric acid and 9.3 g of Sigma Aldrich EDTA. The solution was stirred until completely dissolved and 200 ml deionised water was added to bring the final volume to 1 L. The stock was diluted 10:1 to make a 1x TBE working solution. A 2% gel was made using 2 g Invitrogen UltraPure Agarose and 100 ml 1x TBE buffer and 10 *µ*l of EMD Millipore GelRed Nucleic Acid Stain, which were mixed and heated together to combine, the gel was then poured into a gel tray with wells and cooled. Once set, the samples were loaded into the gel using 2 *µ*l Meridian Bioscience DNA Loading Buffer Blue and 4 *µ*l of PCR sample, ensuring a negative control PCR was also loaded. Additionally 4 *µ*l of Meridian Bioscience HyperLadder 25bp was added for reference. All 125 PCR products generated the same electrophoretic profile as that illustrated in Figure 3, namely a single, sharp band at the expected amplicon size with no off-target bands or primer-dimer smears, while every negative-control lane remained blank, confirming that all reactions were contamination-free. A representative is shown below in Figure 3.

Figure 3

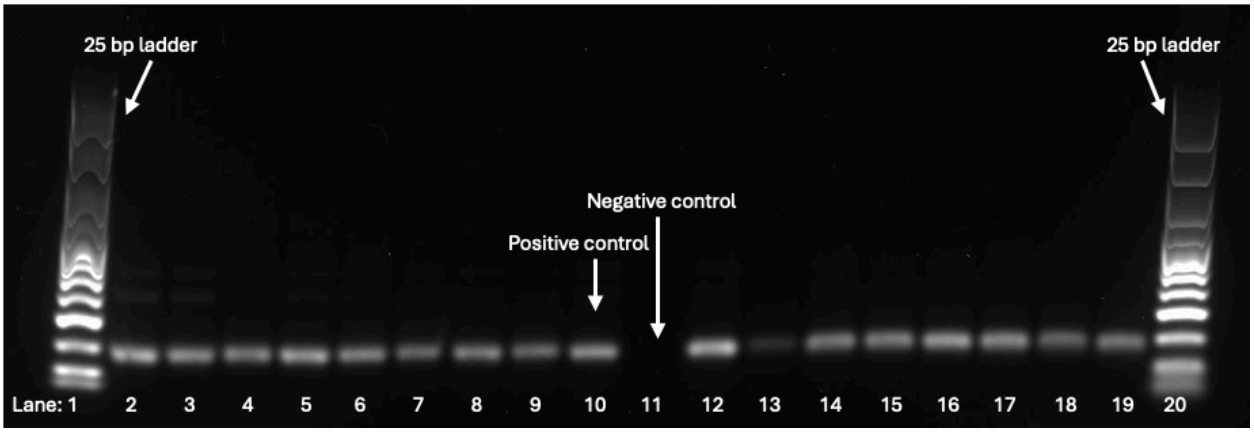

Figure 3: Agarose Gel Electrophoresis of PCR products for NR3C1 gene. The 25 bp DNA ladders (Lane 1 and 20) are used as a molecular size marker. Positive control in lane 10 with a negative control in lane 11. Bands of PCR products for NR3C1 are shown as follows: Lanes 2-5 male dementia samples, lanes 6-9 female dementia samples, lanes 12-15 male control samples, lanes 16-19 female control samples.

DNA Pyrosequencing

The PyroMark Q48 Autoprep Instrument was used for DNA Pyrosequencing. The PyroMark Q48 Autoprep 2.4.2 software was used to create the CpG assay by loading the sequence to analyse for each gene. The sequencing primers designed by the PyroMark Assay Design Software 2.0 software were loaded into the instrument along with the pre-programme volumes of PyroMark Advanced Enzyme Mix, PyroMark Advanced Substrate Mix, Denaturation Solution, Annealing Buffer, Binding Buffer, and Nucleotides. Samples and positive and negative controls were loaded onto the PyroMark Q48 Discs along with 3 *µ*l of magnetic streptavidin-coated sepharose beads. Figure 4:a,b,c and 5:a,b,c are positive and negative representatives for NR3C1, FKBP5 and NR3C2 pyrosequencing traces produced by the Pyromark in this analysis.

Figure 4a

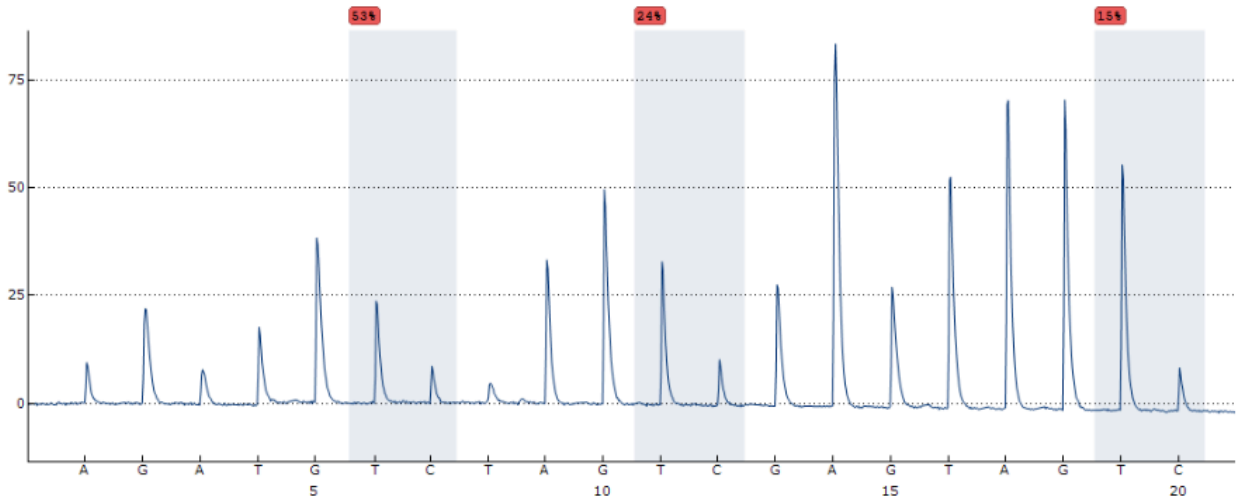

Figure 4a: shows a positive result for NR3C1 methylation, with the y-axis showing the luminescence intensity and the x-axis showing the DNA sequence being analysed and the number of base pairs. The CpG sites are shaded with their percentage methylation above in red.

Figure 4b

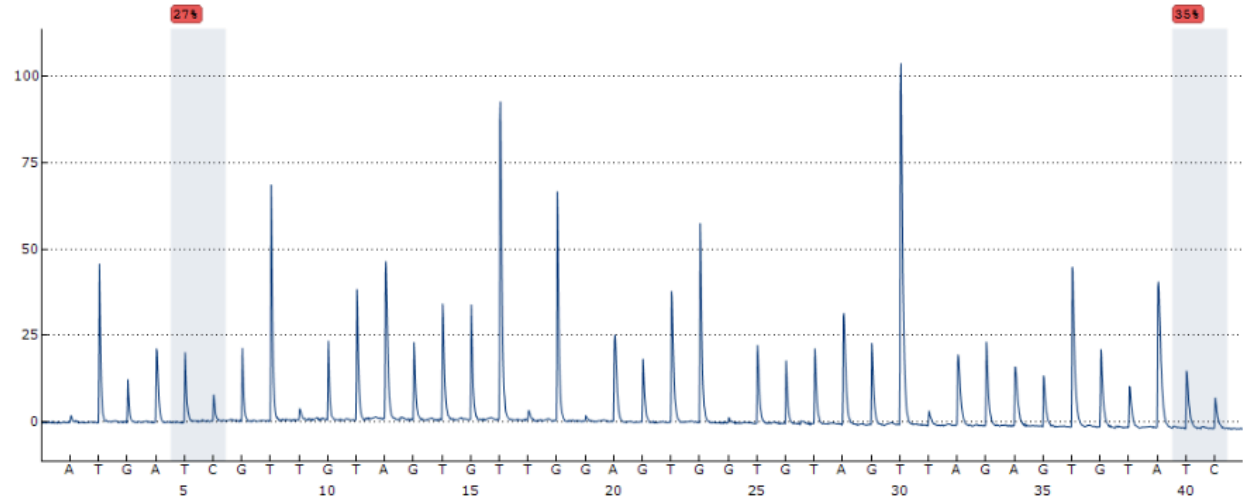

Figure 4b: shows a positive result for FKBP5 methylation, with the y-axis showing the luminescence intensity and the x-axis showing the DNA sequence being analysed and the number of base pairs. The CpG sites are shaded with their percentage methylation above in red.

Figure 4c

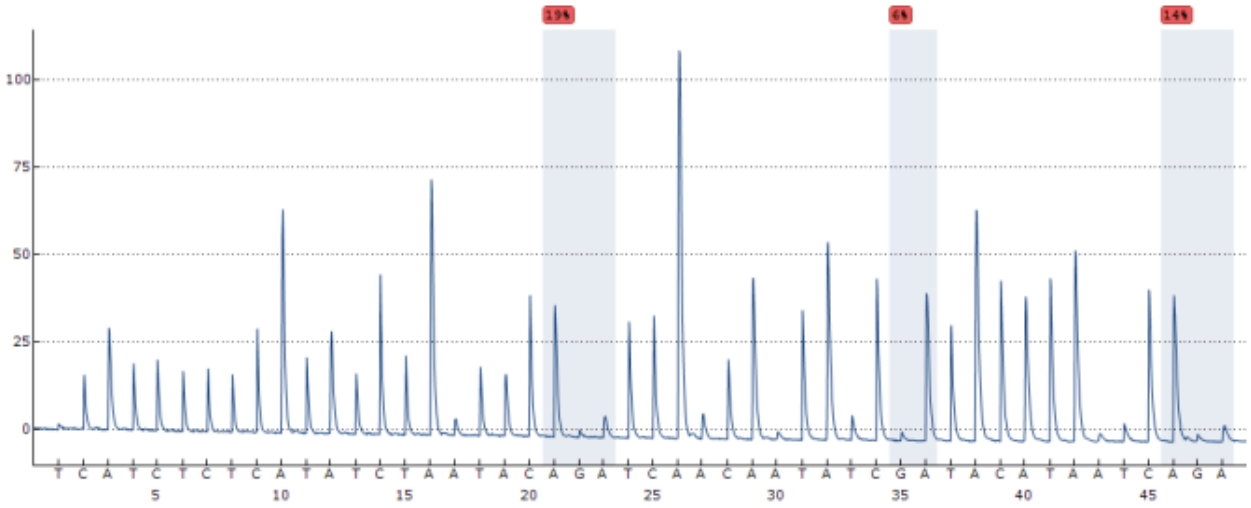

Figure 4c: shows a positive result for NR3C2 methylation, with the y-axis showing the luminescence intensity and the x-axis showing the DNA sequence being analysed and the number of base pairs. The CpG sites are shaded with their percentage methylation above in red.

Figure 5a

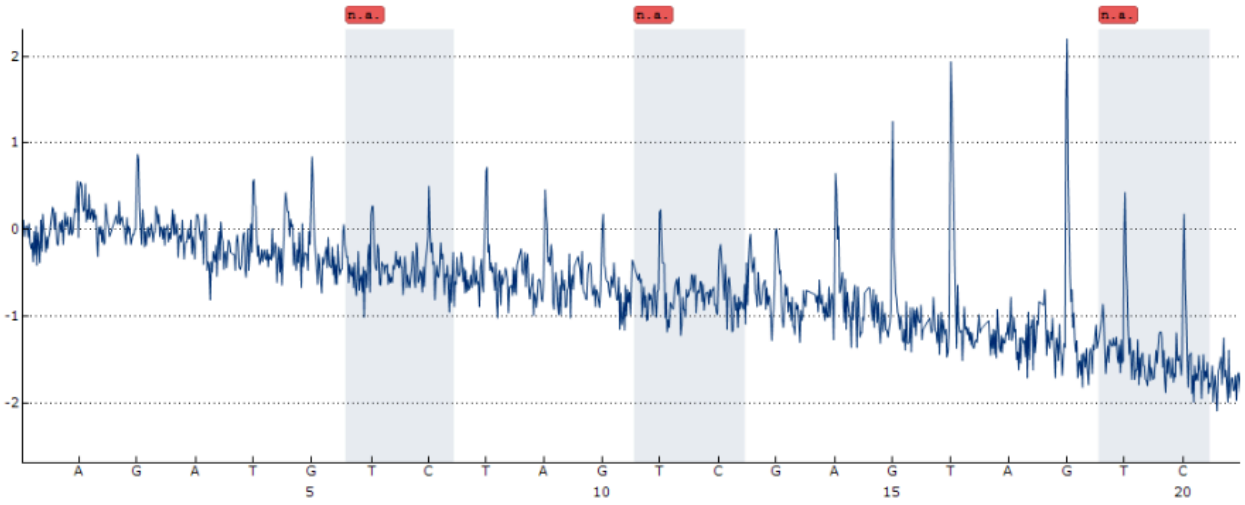

Figure 5a: shows a negative control result for NR3C1 methylation, with the y-axis showing the luminescence intensity and the x-axis showing the DNA sequence being analysed and the number of base pairs.. The CpG sites are shaded with their percentage methylation above in red.

Figure 5b

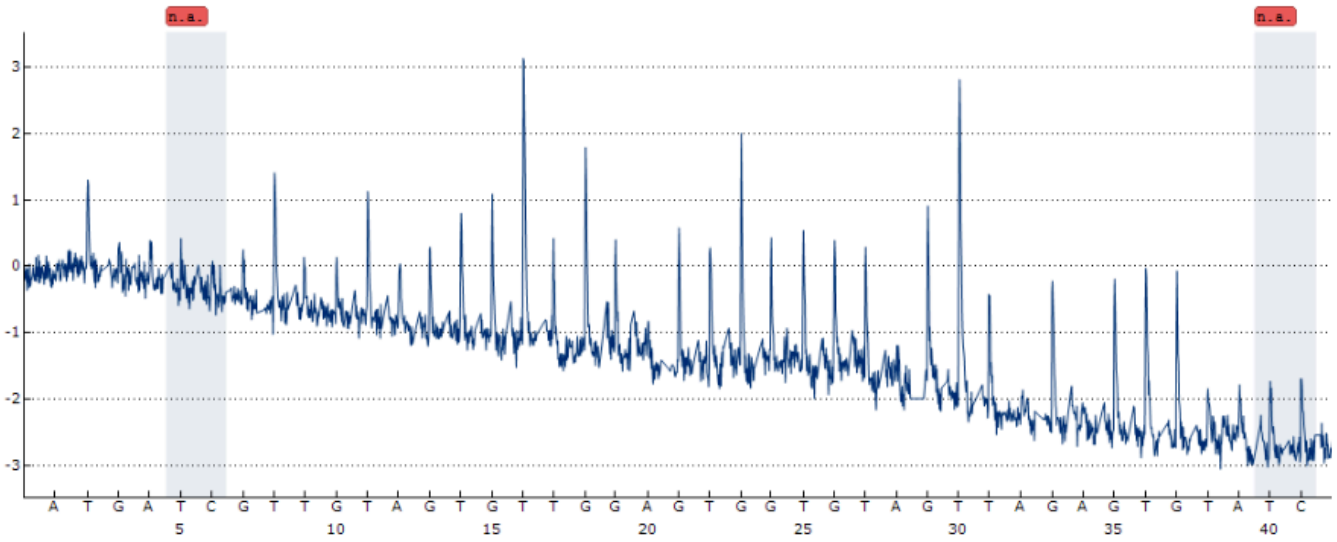

Figure 5b: shows a negative control result for FKBP5 methylation, with the y-axis showing the luminescence intensity and the x-axis showing the DNA sequence being analysed and the number of base pairs.. The CpG sites are shaded with their percentage methylation above in red.

Figure 5c

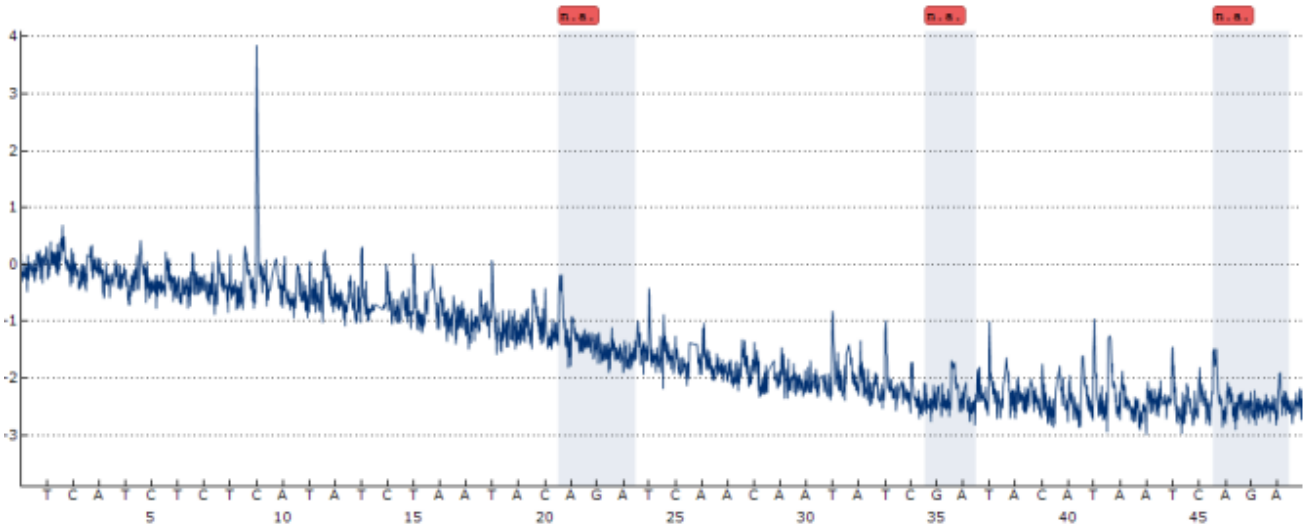

Figure 5c: shows a negative control result for NR3C2 methylation, with the y-axis showing the luminescence intensity and the x-axis showing the DNA sequence being analysed and the number of base pairs.. The CpG sites are shaded with their percentage methylation above in red.

Statistical Analysis

All statistical work was performed in SPSS v.29.0. First, every continuous variable (methylation percentages, cortisol time-points, sleep metrics and demographic factors) was screened with the Shapiro–Wilk test; non-normal variables were natural-log transformed, and where normality could not be achieved non-parametric methods were used. Group differences (dementia vs control; male vs female) were assessed with independent-samples t-tests. Pair-wise associations between methylation at each CpG site and longitudinal phenotypes were explored with Pearson's correlation for normally distributed pairs and Spearman's rank for non-normal pairs. Correlations reaching $p < 0.05$ were entered into simple linear regression models (both "methylation → phenotype" and the reverse direction) to quantify predictive strength (R, R^2, unstandardised B); residual normality was verified with P–P plots. Logistic regression was considered for depression (binary) but abandoned because of severe group-size imbalance. Finally, for the systematic review, study means ± SD were extracted and analysed in RevMan 5 using an inverse-variance random-effects model with 95 % confidence intervals and I^2 to gauge heterogeneity, producing the forest plot in Figure 6.

Initially, A Chi-squared test was explored to compare the full categorical distributions of Thal Phase (0–5), BRAAK stage (0–VI) and CERAD score (0–3) between diagnostic groups, but once the data were stratified by sex more than 40 % of contingency-table cells had an expected count ≤ 5, violating the minimum-cell assumption and yielding unstable statistics. Collapsing adjacent categories to satisfy the assumption would have obscured clinically meaningful gradations in pathology. To preserve that ordinal information while retaining power, we treated the staging scores as continuous severity indices and used independent-samples t-tests (verified with

Mann-Whitney U tests, which gave the same pattern of results). This approach avoids the bias that can arise from arbitrarily merging sparse categories while still testing for group differences in overall pathology burden.

Similarly, a repeated-measures ANOVA was initially planned to examine the joint effects of diagnosis (dementia vs control), sex, and time (five longitudinal waves) on cortisol, sleep, and methylation outcomes. However, the design requires complete data across all factors, and longitudinal attrition plus diary non-return meant that only three donors retained valid observations at every wave for every variable. Restricting the analysis to this tiny, non-representative subset would have produced severely under-powered and potentially biased estimates because the missingness was clearly not completely at random. Rather than impute large blocks of questionnaire-derived data—an approach that can inflate Type I error when sample size is small and the missingness mechanism is uncertain—we adopted a pairwise strategy: (i) correlations were computed with the maximum available cases for each variable pair, and (ii) only those correlations reaching $P < 0.05$ were entered into the regression analysis to quantify effect size while retaining statistical power. This approach preserves information from partial records, reduces bias relative to complete-case ANOVA, and is in line with current recommendations for handling sparsely observed longitudinal neurobiological data (Little and Rubin, 2019).

Results

Meta-Analysis

Table 4 below shows the study characteristics of the studies included in the meta-analysis.

Table 4

	Study Characteristics					Participants		
Study ID	**Authors**	**Year**	**Country of Origin**	**Type of study**	**Aim**	**Control**	**Dementia**	**Outcome**
S1	Sussams R et al.	2020	UK	Longitudinal cohort study	To determine the relationship between psychological stress with cognitive outcomes	n = 68	n =133	Psychological stress, as assessed by the RLCQ or PSS, did not show a link to negative cognitive outcomes in individuals with aMCI. We hypothesise that this lack of association may indicate reduced cortisol production in response to psychological stress as the disease advances.
S2	Popp J et al.	2014	Germany	Longitudinal cohort study	To investigate whether HPA-axis dysregulation occurs at early clinical stages of AD and whether plasma and CSF cortisol levels are associated with clinical disease progression.	n = 37	n = 105	Elevated baseline CSF cortisol levels were linked to more rapid clinical deterioration and cognitive decline in individuals with MCI-AD. These results indicate that HPA-axis dysregulation may emerge during the MCI stage of Alzheimer's disease, potentially accelerating both disease progression and cognitive decline.
S3	Peavy G et al.	2013	USA	Longitudinal cohort study	To investigate associations between chronic stress and diagnostic change.	n = 16	n = 11	Over 2.5 years, 11 individuals progressed from Mild Cognitive Impairment (MCI) to dementia, and 16 from cognitively normal (CN) to MCI. Prolonged stress was linked to MCI to dementia conversion, while cortisol levels did not predict dementia progression.

S4	O'Brian J et al.	1996	UK	Longitudinal cohort study	Examine relationships between the dexamethasone suppression test, cognitive function, depressive symptoms, hippocampal atrophy as assessed via MRI	n = 32	n = 49	The results revealed a connection between ageing and increased dysregulation of the HPA axis in both control and depressed individuals. In Alzheimer's disease, changes in the HPA axis were linked to depressive symptoms and hippocampal atrophy.

Table 4: shows the study characteristics and corresponding study ID for the studies used in this meta-analysis.

Table 5 below shows the extracted data that was entered into the RevMan software for analysis.

Table 5

	Type of Measure		**Control Group**		**Dementia Group**	
Study ID	**Cortisol**	**Cognitive Function**	**Cortisol Levels**	**Cognitive Function**	**Cortisol Levels**	**Cognitive Function**
S1	Awakening cortisol sample (nmol/L)	MOCA, Montreal Cognitive Assessment	11.3 (1.1)	28.0 (0.2)	12.2 (0.6)	22.9 (0.2)
S2	Cerebrospinal fluid cortisol (µg/dL)	MMSE, Mini-Mental State Examination	0.252 (0.251)	28.67 (1.09)	0.555 (0.387)	22.70 (3.06)
S3	Diurnal cortisol (nmol/L)	DRS, Mattis Dementia Rating Score	5.6 (2.3)	139.2 (3.0)	5.8 (1.4)	132.4 (5.7)
S4	Post - Dexamethasone Cortisol (nmol/L)	MMSE, Mini-Mental State Examination	54.8 (48.5)	28.3 (2.1)	80.4 (68.9)	16.6 (5.5)

Table 5: shows the data extracted from the included studies. Values are shown as the mean with the standard deviation in brackets.

Figure 6 below shows the forest plots produced by the RevMan meta-analysis. After all prespecified inclusion and exclusion criteria were applied, only four longitudinal studies met the requirements for quantitative synthesis. The inverse-variance random-effects meta-analysis of these studies (Figure 6) showed slightly higher mean cortisol concentrations in cognitively healthy controls than in dementia cohorts. Although this direction of effect contrasts with the broader literature linking elevated cortisol to neurodegeneration, the finding must be interpreted cautiously: the evidence base is both small and heterogeneous (differences in sampling matrix, collection time points, and assay platform), which limits power and may obscure the true association. To probe the cortisol-dementia relationship more directly, the present work therefore examines promoter methylation in cortisol-binding genes (NR3C1, NR3C2, FKBP5) within post-mortem brain tissue.

Figure 6

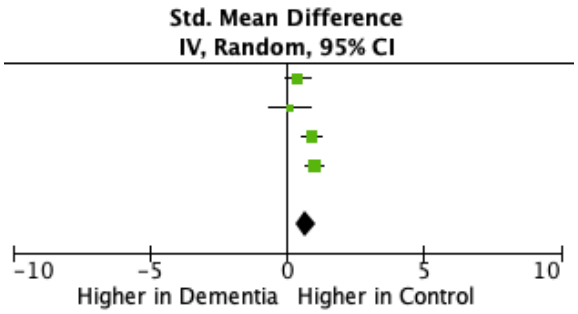

Figure 6: shows the forest plot produced by the meta-analysis for the effect of cortisol levels in dementia vs control. Overall effect is displayed by the black diamond. The green squares show the point estimate of each study. The confidence interval is shown by the lines extending from the green squares. The vertical line represents the line of null effect. Overall, the figure shows that cortisol levels are higher in the control group compared to dementia.

Demographics and Clinical Characteristics

The demographic factors in the dementia and control groups are shown below in Table 6, the post-mortem data was obtained and collated through the Manchester Brain Bank and in-life data was collected by the University of Manchester Longitudinal Ageing Study (Rabbitt et al., 2004).

Table 6

MMU Sample No.	Sex	Age at death	Brain weight (g)	PMD (hrs)	Pathology	Pathological diagnosis	Thal phase	Braak stage	CERAD score
1	F	93	1348	39	Dementia	Mild transitional DLB	3	2 - 3	B
2	M	88		72	Dementia	AD	5	4 - 5	B/C
3	M	85		12	Ctrl	Mild cerebral amyloid	0	1 - 2	0

						angiopathy			
4	M	72	1230		Ctrl	Incipient AD	3	3 - 4	B
5	M	92		37	Ctrl	Age changes only	1	2	A
6	F	91	1520	43.5	Ctrl	Mild SVD	0	1 - 2	0
7	M	96		154	Dementia	Moderate AD pathology	3	4 - 5	B/C
8	M	87	1019	60	Dementia	AD	5	3 - 4	B/C
9	F	91	1157	93	Ctrl	Age changes only	3	1 - 2	A
10	M	89		72	Dementia	AD	4	4 - 5	C
11	F	91			Ctrl	Moderate SVD	3	3	B
12	F	87	1410	80	Dementia	Cerebrovascular disease	4	3 - 4	B
13	M	81	1250		Ctrl	Incipient AD	5	4	A/B
14	M	88		72	Ctrl	Mild cerebrovascular disease	4	1 - 2	A
15	M	78			Ctrl	Probable AD	4	4 - 5	C
16	M	87	1178	87	Ctrl	Mild AD pathology in temporal lobe	1	1 - 2	A
17	M	98	1029	84	Ctrl	Possible AD	3	3	B
18	F	78		144	Ctrl	Age changes only	0	0	0
19	M	82	1020	61	Ctrl	Age changes only	3	0 -1	A
20	M	82	1210	120	Ctrl	Mild AD/parkinson path.	0	1 - 2	A
21	M	82		96	Ctrl	Probable AD	3	4	C
22	M	86	1334	96	Dementia	AD	5	6	C
23	M	76	1359	129.5	Ctrl	Age changes only	1	0 - 1	A
24	M	89	1351	56	Dementia	Moderate AD pathology	5	3 - 4	B
25	F	89	1070	36	Dementia	Age-related tau astrogliopathy with hippocampal sclerosis and secondary TDP-43	2	0 - 1	A
26	F	87		120	Ctrl	Mild AD	3	3 - 4	C
27	M	87	1305	120	Dementia	Parkinson's disease	3	2 - 3	A
28	M	90		6	Dementia	Possible AD	3	3 - 4	B
29	M	94	1150		Ctrl	Age changes only	0	1 - 2	0
30	F	89		128	Ctrl	Incipient AD	5	3	B
31	F	92		24	Ctrl	Moderate cerebrovascular disease	3	0 - 1	A
32	F	92	1270	48	Ctrl	Early/incipient AD	4	4	C
33	M	82	1174	46	Ctrl	Mild DLB	1	0 - 1	0
34	M	91	1216	133	Ctrl	Age changes only	0	2	0
35	F	94	1550	42	Ctrl	Cerebral amyloid	4	2 - 3	A
36	M	89		36	Ctrl	Cerebrovascular disease	0	0	0
37	F	90		156	Ctrl	Age changes only	1	1 - 2	A
38	F	80	1240		Ctrl	Incipient AD	0	2 - 3	0
39	F	90	1134	114.5	Dementia	Corticobasal degeneration	1		A
40	M	79	1290		Ctrl	Incipient AD	3	4 - 5	B
41	M	95	1116	88	Dementia	Possible AD	3	2 - 3	B
42	F	85		12	Ctrl	Age changes only	0	1 - 2	0
43	M	93	1133	70.5	Ctrl	Probable AD	4	4	B

44	M	104	1289	78	Dementia	AD	5	6	C
45	M	100	1058	61.5	Ctrl	Cerebral amyloid angiopathy	1	1	A
46	F	86	1100	26	Ctrl	AD	3	3	B/C
47	M	88	1129	4	Dementia	Early limbic predominant DLB	1	2	A/B
48	F	97	1252	120.5	Ctrl	Argyrophilic Grain Disease with v.mild AD-like tau	0	2	0
49	F	85		187.5	Ctrl	Mild cerebral amyloid angiopathy	0	2 - 3	0
50	M	87	1152	24	Ctrl	Age changes only	2	0 - 1	A
51	M	81	1210	41	Ctrl	Age changes only	0	0	0
52	M	79		116	Ctrl	Argyrophilic Grain Disease	0	2	0
53	M	80	1000	81	Dementia	Probable AD	2	3	B
54	M	76	1204	47	Ctrl	Mild AD changes in temporal lobe	0	1 - 2	0
55	M	89	1450	144	Dementia	Cerebral amyloid angiopathy, moderate SVD	3	1	A
56	M	89		134	Ctrl	Mild AD pathology	2	2 - 3	A/B
57	M	94	1166	86	Ctrl	Early/Incipient AD	3	3 - 4	B
58	M	90	1050	39	Ctrl	Age changes only	1	0 - 1	0/A
59	M	81	1363	44	Ctrl	Early/incipient AD	2	2 - 3	B
60	M	90		41.5	Ctrl	Age changes only	0	0	0
61	M	94	946	111	Ctrl	Age changes only	0	0 - 1	0
62	M	83	1108	94	Dementia	Mild AD changes in temporal lobe	1	0 - 1	A
63	M	90	1217	103	Ctrl	Age changes only	2	2	A
64	M	81	1160	113.5	Dementia	Probable AD	4	3 - 4	B
65	F	86		18	Dementia	DLB	4	4 - 5	C
66	M	87		39	Ctrl	Age changes only	1	1 - 2	0
67	F	89		27	Ctrl	Age changes only	0	2	0
73	M	91	1206 (fixed)	61	Dementia	AD		V	
74	F	88	1160	110	Dementia	Lewy body disease (transitional)		II-III	
75	F	97	1035	26.5	Ctrl	Severe SVD with microinfarction		II	
76	F	90		109.5	Dementia	Incipient AD		III	
77	F	101	1179	135.5	Ctrl	Age changes only		II	
78	F	94	1097	58.5	Ctrl	Moderate SVD		I	
79	F	95	1092	70.5	Ctrl	Age changes only/incipient AD		II-III	
80	F	89	1254	36.5	Ctrl	Age changes only		II-III	
81	M	88		39	Ctrl	Age changes only		0-I	
82	F	88	1128 (fixed)	52.5	Ctrl	Age changes only		I	
83	F	89	1245	171	Ctrl	Age changes only		II	
84	M	90	1309	26	Ctrl	possible AD		III-IV	
85	M	94	1341	59.5	Dementia	Moderate/Incipient AD		IV	
86	F	92	1265	76	Dementia	Probable AD		IV-V	

87	F	90	1333	66	Dementia	Alpha-synucleinopathy neocortical predominant		IV	
88	F	98	1153	37.5	Ctrl	Cerebral amyloid angiopathy		I-II	
89	F	97	1130	121	Dementia	Possible PCA		na	
90	F	92	851	36	Dementia	moderate SVD with ischaemic lesions		II	
91	F	87	1160	170	Ctrl	Moderate AD changes in temporal lobe		III	
92	M	87		92.5	Dementia	Incipient AD		III-IV	
93	M	92	1485	151	Ctrl	DLB		II	
94	F	86	1245	93.5	Dementia	Possible AD		III-IV	
95	M	96	1164	73.5	Dementia	AD		IV-V	
96	F	90	1200 (fixed)	112	Ctrl	Mild tau pathology in temporal lobe		I-II	
97	M	86	1544	33	Dementia	Probable Parkinson's disease		II-III	
98	F	90	1158	143	Ctrl	Age changes only		I-II	
99	M	89	1340	125	Ctrl	Age changes only		I	
100	M	91	1400 (fixed)	133	Dementia	AD		V	
101	M	86	1342 (fixed)	42	Ctrl	Age changes only		I	
102	F	96	1320	68	Ctrl	mild/early DLB (limbic)	3	I-II	A
103	M	91	1220		Dementia	DLB	0	III-IV	0
104	F	88	1230	112	Dementia	Age changes only	2	II	B
105	F	97	1310	44	Ctrl	Age changes only	3	III	B
106	F	90	1090	38	Ctrl	mild cerebrovascular disease	5	II	A
107	F	80	1182	155	Dementia	AD (intermediate probability)	3	V	B
108	M	94	1430	170	Dementia	AD (intermediate probability)	3	V	B
109	M	100	1141	94	Ctrl	Alpha-synucleinopathy brainstem predominant	3	III	B
110	F	103	1043	176	Dementia	AD (intermediate probability)	3	VI	B
111	M	83		69.5	Dementia	AD (intermediate)	3	IV	A
112	F	95	1153	132	Dementia	AD	3	VI	C
113	F	89	1184	80	Dementia	AD (intermediate)	3	III	B
114	M	95	1290	153	Ctrl	Age related changes (mild)	1	II	0
115	F	91		61.5	Dementia	Ageing related changes	3	III	A
116	F	90		141	Dementia	AD	3	VI	B
117	F	88	1143	165.5	Dementia	AD	4	VI	B
118	M	95	1200 fixed	12	Ctrl	Age changes only	0	I-II	0
119	M	89		48	Ctrl	Age changes only	1	II	A
120	F	81	1150 (fixed)	16	Dementia	Limbic Dementia with Lewy Bodies	0	II	0
121	M	90	1284 (fixed)	61	Dementia	multiple cerebral infarctions	0	II-III	0
122	F	86	1166	151.5	Ctrl	Ageing related changes	2	III	B
123	F	85	1275	24	Dementia	AD	3	V-VI	B/C
124	M	89	1272	123	Ctrl	Age changes only	0	II	0

			fixed						
125	M	78	800 fixed	48	Ctrl	DLB (neocortical)	5	V-VI	C
126	M	73		48	Dementia	Corticobasal degeneration	1		C
127	F	84		96	Ctrl	possible AD	3	II-III	B
128	M	88	1027	75	Dementia	AD			
129	F	77	1220	48	Ctrl	Age changes only	0	II	0
130	F	88	1153	96	Dementia	AD	3	VI	C

Table 6: displays the demographic and dementia characteristics for each brain sample. Where data was unavailable the box was left empty. ‘F’ indicates female and ‘M’ indicates a male participant. PMD is the Post Mortem Delay (the time between death and when the samples were harvested). Pathology is recorded as any form of dementia, (AD, Parkinsons and Dementia with Lewy Bodies are all recorded as Dementia) or ‘Ctrl’ indicates Controls where participants were either healthy or had only age changes recorded. Pathological diagnosis is included for more detail on each sample and was used to assign the pathology category, ‘AD’ indicates Alzheimer's Disease and ‘DLB’ indicates Dementia with lewy bodies . Thal phase, Braak stage and CERAD score are used as a measure of dementia severity. Thal phase is a neuroanatomical assessment of Aβ plaques in the brain, 1: Aβ deposits first appear in the temporal lobe. 2: Aβ deposits spread to other neocortical regions and the hippocampus. 3 and 4: Aβ deposits spread to subcortical regions. 5: Aβ deposits spread to the cerebellum and every other region of the brain. Braak staging is used for classifying the progression of Alzheimer's disease and Parkinson's disease pathology based on the distribution of neurofibrillary tangles (NFTs) and Aβ plaques, Stages I-II: Transentorhinal stages, Stages III-IV: Limbic stages, Stages V-VI: Neocortical stages. CERAD (Consortium to Establish a Registry for Alzheimer's Disease) score is used to evaluate the extent and distribution of neuritic plaques in the brain. ‘A’ for amyloid plaques, ‘B’ for NFTs (based on Braak staging), and ‘C’ for neuritic plaques (based on the CERAD score).

The sample population used in this study had female n = 56 and male n = 69, with an average brain weight of 1207 g, and average female age of death = 90 and average male age of death = 88. The dementia group n = 55 with control group n = 70 and a combined group average PMD of 81.5 hours.

IBM SPSS Statistics v 29.0.2.0 was used for all analyses. Dementia and control donors did not differ in age at death (independent-samples t-test, $P = 0.484$) or in sex distribution ($\chi^2 = 0.05, P = 0.826$). To assess whether neuropathology severity varied by diagnosis once sex was taken into account, we performed separate t-tests that compared female dementia cases with female controls and male dementia cases with male controls. In women, dementia was associated with higher Thal Phase ($P = 0.023$) and BRAAK stage ($P < 0.001$) and a trend toward a higher CERAD score ($P = 0.068$); in men, the same contrasts were non-significant (Thal $P = 0.214$, BRAAK $P \approx 0.12$, CERAD $P = 0.139$), mirroring the greater pathology burden typically reported in female dementia, due to women having a 1.9 times higher prevalence of dementia than men (Cao et al., 2020).

Sleep data was also obtained and collected through the University of Manchester Longitudinal Ageing Study. The data was gathered in the form of self-reported sleep diaries. Table 7 shows the group averages across the different sleep variables, the full data table is included in the appendix as Table 20. Data was collected over 5 waves called P1 (conducted 1985), P2 (conducted 1991), P5 (conducted 2001), P6 (conducted 2007) and P7 (conducted 2010). 3 different variables were included, 'hrslp': which is how many hours sleep per night, 'sleff': sleep efficiency calculated by sleep duration divided by duration in bed, and 'wakent': which is how many times participants wake during the night. Data was recorded by the wave ID followed by the variable tested.

Table 7

	Group				
Variable	**All**	**Dementia**	**Control**	**Male**	**Female**
p1hrslp	7.13 ± 0.11	7.15 ± 0.18	7.12 ± 0.13	7.11 ± 0.16	7.15 ± 0.15
p2hrslp	7.03 ± 0.12	6.85 ± 0.24	7.14 ± 0.13	6.99 ± 0.19	7.07 ± 0.16
p5hrslp	6.86 ± 0.18	6.40 ± 0.36	7.14 ± 0.18	6.72 ± 0.28	6.97 ± 0.24
p6hrslp	6.63 ± 0.14	6.37 ± 0.19	6.78 ± 0.19	6.47 ± 0.19	6.78 ± 0.20
p7hrslp	6.92 ± 0.18	6.72 ± 0.27	7.03 ± 0.23	6.94 ± 0.24	6.89 ± 0.27
p1sleff	85.68 ± 1.09	85.79 ± 1.66	85.61 ± 1.46	85.68 ± 1.62	85.68 ± 1.49
p2sleff	84.39 ± 1.27	82.53 ± 2.38	85.49 ± 1.45	83.41 ± 2.01	85.15 ± 1.64
p5sleff	79.23 ± 1.84	74.33 ± 3.34	82.35 ± 1.98	77.97 ± 3.09	80.31 ± 2.18
p6sleff	76.14 ± 1.44	74.40 ± 2.28	77.14 ± 1.86	72.99 ± 2.00	79.10 ± 2.01
p7sleff	77.21 ± 1.67	77.58 ± 2.55	77.01 ± 2.20	78.86 ± 2.22	75.43 ± 2.52
p1wakent	1.21 ± 0.11	1.38 ± 0.21	1.10 ± 0.12	1.52 ± 0.20	0.97 ± 0.11
p2wakent	1.34 ± 0.12	1.64 ± 0.21	1.16 ± 0.14	1.67 ± 0.21	1.08 ± 0.13
p5wakent	1.99 ± 0.14	2.27 ± 0.15	1.80 ± 0.21	2.24 ± 0.21	1.78 ± 0.19
p6wakent	0.50 ± 0.17	1.00 ± 0.37	0.29 ± 0.16	0.00 ± 0.00	0.71 ± 0.22
p7wakent	1.97 ± 0.15	2.10 ± 0.20	1.89 ± 0.21	1.99 ± 0.22	1.95 ± 0.22

Table 7: shows the means for each sleep variable for all participants, as well as the dementia, control, male and female groups and standard error of the mean. The variables measures show 5 waves: P1, P2, P5, P6 and P7 along with the three measures for each wave: 'hrslp': hours sleep per night, 'sleff': sleep efficiency calculated by sleep duration divided by duration in bed, and 'wakent': which is how many times participants wake during the night.

Men scored higher averages when compared to women across all variables on all waves. Independent t-tests were conducted to see if these differences were significant. It was found that men scored significantly higher on p2hrslp (P = 0.047), p6hrslp (P = 0.001), p7hrslp (P = 0.006), p1sleff (P = 0.029), p2sleff (P = 0.0017), p6sleff (P = 0.017) and p7sleff (P = 0.008). The control group typically scored higher mean results compared to the dementia group on the various sleep parameters with the exceptions of p1hrslp, p7hrslp, p1wakent, p2wakent, and p6wakent. However following an independent t-test it was found that the control group was only statistically significantly higher within p5sleff (P = 0.047).

Depression data was obtained and collected through the University of Manchester Longitudinal Ageing Study. Beck and Beamesderfer (1974) recommended that the cut-off scores for the Beck Depression Inventory (BDI) should be determined based on the specific clinical decisions for which the tool is being utilised. The Center for Cognitive Therapy provided the following BDI cut-off score guidelines for patients diagnosed with affective disorders: a score of less than 10 indicates no or minimal depression, a score between 10 and 18 reflects mild to moderate depression, a score from 19 to 29 indicates moderate to severe depression, and a score between 30 and 63 indicates severe depression. In this study, the depression scores were standardised by recording scores below 10 as ‘no depression’ and all scores above 10 as ‘mild to severe depression. Table 8 shows the group averages for percentage depressed across the different depression measures, the full data table is included in the appendix as Table 21 and Table 22. We expected to see a significant difference in percentage depressed between female and male participants due to women having higher prevalence of depression than men with a 1.7 times higher incidence rate compared to men (Albert, 2015). We used a chi-squared test to investigate however found no significant association between depression and gender with the largest significance being P = 0.063 for mood1 vs gender.

Table 8

	Percentage Depressed				
Variable	**All**	**Dementia**	**Control**	**Male**	**Female**
mood1	0.22 ± 0.04	0.29 ± 0.07	0.18 ± 0.05	0.14 ± 0.05	0.28 ± 0.06
mood2	0.17 ± 0.04	0.24 ± 0.07	0.11 ± 0.04	0.16 ± 0.05	0.18 ± 0.05
mood3	0.18 ± 0.04	0.22 ± 0.07	0.14 ± 0.04	0.17 ± 0.06	0.19 ± 0.05
mood4	0.16 ± 0.04	0.20 ± 0.07	0.12 ± 0.05	0.15 ± 0.06	0.17 ± 0.06
mood5	0.26 ± 0.05	0.31 ± 0.09	0.23 ± 0.06	0.17 ± 0.06	0.33 ± 0.07

mood6	0.19 ± 0.06	0.13 ± 0.09	0.19 ± 0.08	0.21 ± 0.10	0.17 ± 0.08
mood7	0.17 ± 0.03	0.17 ± 0.06	0.15 ± 0.04	0.12 ± 0.05	0.20 ± 0.05
mood8	0.16 ± 0.04	0.14 ± 0.06	0.16 ± 0.05	0.11 ± 0.05	0.20 ± 0.05
mood9	0.12 ± 0.04	0.11 ± 0.06	0.13 ± 0.05	0.08 ± 0.04	0.16 ± 0.06
mood10	0.13 ± 0.04	0.11 ± 0.08	0.12 ± 0.05	0.13 ± 0.06	0.13 ± 0.06
mood11	0.36 ± 0.15	0.50 ± 0.29	0.33 ± 0.21	0.17 ± 0.17	0.60 ± 0.24
msev1	0.04 ± 0.02	0.05 ± 0.03	0.04 ± 0.02	0.02 ± 0.02	0.06 ± 0.03
msev2	0.03 ± 0.02	0.05 ± 0.03	0.01 ± 0.01	0.02 ± 0.02	0.03 ± 0.02
msev3	0.03 ± 0.02	0.03 ± 0.03	0.03 ± 0.02	0.02 ± 0.02	0.04 ± 0.03
msev4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
msev5	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.02	0.03 ± 0.03	0.00 ± 0.00
msev6	0.02 ± 0.02	0.00 ± 0.00	0.04 ± 0.04	0.05 ± 0.05	0.00 ± 0.00
msev7	0.02 ± 0.01	0.00 ± 0.00	0.03 ± 0.02	0.02 ± 0.02	0.02 ± 0.02
msev8	0.02 ± 0.01	0.03 ± 0.03	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02
msev9	0.02 ± 0.02	0.00 ± 0.00	0.04 ± 0.03	0.05 ± 0.04	0.00 ± 0.00
msev10	0.02 ± 0.02	0.00 ± 0.00	0.02 ± 0.02	0.03 ± 0.03	0.03 ± 0.00
msev11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 8: shows the percentage depressed for each depression variable for all participants, as well as the dementia, control, male and female groups, and standard error of the mean. Depression variables were measured in 11 waves for two measures; 'mood': participants recorded their mood and then data was standardised to either depression or no depression according to the BDI scale, 'msev': participants recorded their mood severity then data was standardised to either depression or no depression according to the BDI scale.

An independent t-test was conducted to compare the depression means for mood and msev across all waves within the dementia vs control groups and the female vs male groups it was found that no statistically significant differences exist.

Additionally, cortisol data for a subset of brain samples was also obtained and collected through the University of Manchester Longitudinal Ageing Study. Table 9 shows the average cortisol levels for each group across different time points across one day, the full data table is included in the appendix as Table 23.

Table 9

	Average cortisol levels (µg/dL)				
Variable	**All**	**Dementia**	**Control**	**Male**	**Female**
Cort0	14.90 ± 0.82	14.90 ± 1.17	14.90 ± 1.20	14.70 ± 1.08	15.11 ± 1.30

Cort30	23.07 ± 1.71	23.38 ± 2.83	22.77 ± 2.09	21.74 ± 2.31	24.49 ± 2.56
Cort60	20.99 ± 1.34	20.48 ± 2.20	21.42 ± 1.71	22.54 ± 2.12	19.34 ± 1.58
Cort14	10.37 ± 0.85	9.52 ± 0.67	11.07 ± 1.45	10.89 ± 1.37	9.81 ± 1.00
Cort18	6.52 ± 0.44	5.79 ± 0.51	7.12 ± 0.66	6.23 ± 0.60	6.82 ± 0.65
Cort22	4.96 ± 0.65	5.08 ± 0.85	4.85 ± 0.97	4.61 ± 1.03	5.33 ± 0.79

Table 9: shows the average cortisol levels for all participants, as well as the dementia, control, male and female groups and standard error of the mean. 'Cort0' indicates cortisol levels after waking, 'Cort30' indicates cortisol levels after 30 min of being awake, 'Cort60' indicates cortisol levels after 60 min of being awake, 'Cort14' indicates cortisol levels at 14:00, 'Cort18' indicates cortisol levels at 18:00, 'Cort22' indicates cortisol levels at 22:00.

An independent t-test was conducted to compare the mean cortisol level across all timepoints within the dementia vs control groups and the female vs male groups it was found that no statistically significant differences exist.

Methylation Results

Table 10 shows the percentage methylation results obtained from the pyrosequencing analysis.

Table 10

MMU Sample No.	NR3C1 CpG1	NR3C1 CpG2	NR3C1 CpG3	NR3C1 Mean	FKBP5 CpG1	FKBP5 CpG2	FKBP5 CpG Mean	NR3C2 CpG1	NR3C2 CpG2	NR3C2 CpG3	NR3C2 CpG Mean
1	57	32	18	35.67	37	44	40.50	14	10	11	11.67
2	57	32	20	36.33	52	59	55.50	19	12	18	16.33
3	98	49	28	58.33	62	70	66.00	15	12	15	14.00
4	38	15	10	21.00	36	52	44.00	17	12	16	15.00
5	50	25	15	30.00	38	48	43.00	15	9	14	12.67
6	40	26	11	25.67	40	47	43.50	9	9	11	9.67
7	46	24	16	28.67	51	60	55.50	11	9	11	10.33
8	67	30	19	38.67	40	52	46.00	15	12	13	13.33
9	49	23	14	28.67	30	38	34.00	14	11	12	12.33
10	56	23	14	31.00	54	61	57.50	13	9	12	11.33
11	41	18	13	24.00	26	19	22.50	13	9	13	11.67
12	42	19	13	24.67	59	66	62.50	18	12	14	14.67
13	39	25	11	25.00	33	40	36.50	22	13	17	17.33
14	40	20	13	24.33	43	54	48.50	20	12	14	15.33
15	38	17	10	21.67	37	45	41.00	20	13	16	16.33
16	58	28	19	35.00	50	60	55.00	12	10	11	11.00
17	45	24	15	28.00	27	36	31.50	11	9	10	10.00
18	57	27	17	33.67	25	33	29.00	12	11	13	12.00
19	51	32	16	33.00	33	41	37.00	15	7	14	12.00
20	53	24	15	30.67	53	60	56.50	12	10	12	11.33

21	45	21	13	26.33	31	39	35.00	16	9	14	13.00
22	49	26	17	30.67	42	52	47.00	11	9	12	10.67
23	54	25	16	31.67	35	43	39.00	13	9	10	10.67
24	52	21	13	28.67	60	68	64.00	16	12	14	14.00
25	50	20	13	27.67	44	52	48.00	11	6	10	9.00
26	44	19	14	25.67	40	45	42.50	16	13	14	14.33
27	51	21	14	28.67	44	52	48.00	18	12	16	15.33
28	53	25	15	31.00	32	43	37.50	12	11	11	11.33
29	55	25	14	31.33	47	59	53.00	16	12	14	14.00
30	27	18	7	17.33	50	55	52.50	18	10	13	13.67
31	45	27	13	28.33	27	35	31.00	19	16	18	17.67
32	49	20	12	27.00	28	38	33.00	17	11	16	14.67
33	58	22	13	31.00	53	61	57.00	17	12	15	14.67
34	44	21	13	26.00	43	48	45.50	18	11	14	14.33
35	44	21	13	26.00	39	44	41.50	15	11	13	13.00
36	49	23	14	28.67	47	52	49.50	16	11	13	13.33
37	39	20	12	23.67	61	67	64.00	13	10	12	11.67
38	42	27	11	26.67	40	47	43.50	16	11	12	13.00
39	37	9	11	19.00	49	59	54.00	13	10	13	12.00
40	49	23	14	28.67	33	43	38.00	12	5	10	9.00
41	31	20	10	20.33	45	54	49.50	14	9	12	11.67
42	50	30	17	32.33	45	49	47.00	9	8	13	10.00
43	43	26	13	27.33	57	66	61.50	15	10	15	13.33
44	95	48	29	43.00	56	60	58.00	18	10	14	14.00
45	55	26	16	32.33	42	50	46.00	15	10	14	13.00
46	45	21	14	26.67	44	48	46.00	16	11	16	14.33
47	41	18	13	24.00	19	24	21.50	15	11	13	13.00
48	36	17	11	21.33	62	69	65.50	15	10	14	13.00
49	50	27	15	23.00	30	40	35.00	15	10	13	12.67
50	52	23	14	29.67	47	53	50.00	17	11	15	14.33
51	58	28	18	34.67	52	62	57.00	13	10	12	11.67
52	37	24	11	24.00	45	50	47.50	16	10	14	13.33
53	31	19	9	19.67	45	55	50.00	13	10	12	11.67
54	43	24	12	26.33	27	31	29.00	15	10	13	12.67
55	51	25	12	29.33	39	46	42.50	19	6	14	13.00
56	41	21	14	25.33	42	56	49.00	11	10	11	10.67
57	36	16	10	20.67	47	59	53.00	13	11	12	12.00
58	40	17	10	22.33	50	58	54.00	16	13	16	15.00
59	41	19	12	24.00	42	49	45.50	10	11	13	11.33
60	38	18	12	22.67	48	56	52.00	19	12	17	16.00
61	47	22	13	27.33	38	47	42.50	16	10	14	13.33
62	42	22	13	25.67	43	56	49.50	18	14	17	16.33
63	40	15	10	21.67	41	49	45.00	20	9	15	14.67
64	45	21	14	26.67	28	35	31.50	19	14	16	16.33

65	45	23	15	27.67	42	47	44.50	13	9	12	11.33
66	47	21	13	27.00	60	71	65.50	15	10	14	13.00
67	49	25	15	29.67	19	27	23.00	17	9	13	13.00
73	57	29	18	34.67	60	70	65.00	16	12	15	14.33
74	67	33	20	40.00	53	62	57.50	11	9	11	10.33
75	44	21	13	26.00	34	42	38.00	11	10	12	11.00
76	44	20	12	25.33	58	67	62.50	14	11	13	12.67
77	38	19	11	22.67	56	65	60.50	18	12	15	15.00
78	34	18	11	21.00	33	38	35.50	10	7	11	9.33
79	47	22	14	27.67	45	52	48.50	20	12	17	16.33
80	51	22	13	28.67	45	52	48.50	16	11	14	13.67
81	41	21	14	25.33	55	68	61.50	18	13	15	15.33
82	54	22	14	30.00	52	57	54.50	15	9	14	12.67
83	40	19	13	24.00	46	51	48.50	11	10	11	10.67
84	41	20	13	24.67	67	77	72.00	10	9	10	9.67
85	43	19	12	24.67	56	63	59.50	14	10	12	12.00
86	49	22	15	28.67	53	67	60.00	14	10	13	12.33
87	44	20	12	25.33	44	52	48.00	17	12	15	14.67
88	46	20	14	26.67	40	46	43.00	13	7	13	11.00
89	41	19	12	24.00	70	78	74.00	15	10	15	13.33
90	54	26	17	32.33	40	49	44.50	14	7	12	11.00
91	40	21	15	25.33	17	27	22.00	15	10	14	13.00
92	50	21	13	28.00	63	70	66.50	12	9	13	11.33
93	41	19	13	24.33	64	69	66.50	15	9	14	12.67
94	42	20	13	25.00	33	40	36.50	16	12	15	14.33
95	39	18	12	23.00	53	62	57.50	15	10	13	12.67
96	53	24	15	30.67	46	52	49.00	12	10	11	11.00
97	47	22	15	28.00	59	64	61.50	16	9	14	13.00
98	64	31	21	38.67	59	65	62.00	15	10	14	13.00
99	56	27	16	33.00	46	48	47.00	19	11	18	16.00
100	41	26	13	26.67	30	40	35.00	13	6	14	11.00
101	48	28	15	30.33	42	52	47.00	19	13	17	16.33
102	41	24	9	24.67	41	49	45.00	20	11	17	16.00
103	46	28	12	28.67	48	54	51.00	23	14	19	18.67
104	40	25	10	25.00	41	47	44.00	22	14	17	17.67
105	40	25	10	25.00	22	46	34.00	20	13	18	17.00
106	41	26	11	26.00	42	51	46.50	17	12	17	15.33
107	39	25	11	25.00	29	37	33.00	19	12	16	15.67
108	37	25	11	24.33	26	45	35.50	17	11	15	14.33
109	49	30	16	31.67	26	60	43.00	17	12	16	15.00
110	52	32	17	33.67	31	39	35.00	26	15	21	20.67
111	55	34	17	35.33	41	49	45.00	19	14	17	16.67
112	48	29	14	30.33	42	51	46.50	25	16	21	20.67
113	41	28	13	27.33	32	44	38.00	20	12	16	16.00

114	53	26	11	30.00	42	52	47.00	18	13	17	16.00
115	39	24	11	24.67	34	43	38.50	16	10	15	13.67
116	43	25	12	26.67	33	46	39.50	21	13	18	17.33
117	56	34	18	36.00	32	45	38.50	15	7	16	12.67
118	51	30	17	32.67	40	50	45.00	20	13	17	16.67
119	48	27	13	29.33	43	56	49.50	20	14	18	17.33
120	37	24	12	24.33	36	48	42.00	16	12	17	15.00
121	34	22	11	22.33	34	44	39.00	21	9	17	15.67
122	40	26	11	25.67	39	50	44.50	22	13	19	18.00
123	79	43	28	50.00	38	48	43.00	18	13	17	16.00
124	54	32	18	34.67	48	58	53.00	18	12	15	15.00
125	41	26	12	26.33	40	45	42.50	15	12	13	13.33
126	74	38	22	44.67	21	34	27.50	23	13	18	18.00
127	64	35	19	39.33	38	47	42.50	20	13	16	16.33
128	56	34	18	36.00	57	66	61.50	19	13	16	16.00
129	32	14	5	17.00	40	45	42.50	16	8	14	12.67
130	30	15	5	16.67	40	43	41.50	19	12	16	15.67

Table 10: shows the percentage methylation results for NR3C1 CpG1, NR3C1 CpG2, NR3C1 CpG3, NR3C1 Mean, FKBP5 CpG1, FKBP5 CpG2, FKBP5 CpG Mean, NR3C2 CpG1, NR3C2 CpG2, NR3C2 CpG3, NR3C2 CpG Mean for each brain sample identified by the corresponding MMU sample number.

The average percentage methylation results for all samples, the dementia and control group and male and females are shown in Table 11 below.

Table 11

	Average Percentage Methylation										
Group	NR3C1 CpG1	NR3C1 CpG2	NR3C1 CpG3	NR3C1 Mean	FKBP5 CpG1	FKBP5 CpG2	FKBP5 CpG Mean	NR3C2 CpG1	NR3C2 CpG2	NR3C2 CpG3	NR3C2 CpG Mean
All	47.15 ± 0.96	24.06 ± 0.54	13.86 ± 0.33	28.18 ± 0.55	42.45 ± 0.99	51.01 ± 0.99	46.73 ± 0.97	15.96 ± 0.30	10.75 ± 0.18	14.30 ± 0.21	13.67 ± 0.21
Dementia	48.29 ± 1.76	24.98 ± 0.99	14.41 ± 0.62	28.93 ± 0.95	43.08 ± 1.65	51.96 ± 1.57	47.52 ± 1.60	16.47 ± 0.53	10.84 ± 0.33	14.61 ± 0.37	13.97 ± 0.38
Control	46.41 ± 1.09	23.47 ± 0.61	13.51 ± 0.37	27.69 ± 0.65	42.04 ± 1.24	50.39 ± 1.27	46.21 ± 1.23	15.63 ± 0.36	10.69 ± 0.21	14.09 ± 0.25	13.47 ± 0.25
Female	45.82 ± 1.23	23.78 ± 0.76	13.55 ± 0.48	27.58 ± 0.78	40.56 ± 1.51	48.45 ± 1.51	44.51 ± 1.49	15.93 ± 0.50	10.80 ± 0.29	14.42 ± 0.34	13.72 ± 0.36
Male	48.22 ± 1.41	24.29 ± 0.76	14.12 ± 0.45	28.67 ± 0.76	43.96 ± 1.30	53.04 ± 1.26	48.50 ± 1.25	15.99 ± 0.38	10.71 ± 0.24	14.20 ± 0.27	13.63 ± 0.26

Table 11: displays the mean percentage methylation results and standard error of the mean for NR3C1 CpG1, NR3C1 CpG2, NR3C1 CpG3, NR3C1 Mean, FKBP5 CpG1, FKBP5 CpG2, FKBP5 CpG Mean, NR3C2 CpG1,

NR3C2 CpG2, NR3C2 CpG3, NR3C2 CpG Mean for all samples as well as the dementia, control, male and female groups.

Independent t-tests were conducted on SPSS to compare the means in the control vs dementia groups and the female vs male groups and found that men had a significantly higher average methylation for NR3C1 CpG1 (P = 0.05). All other differences in means were not statistically significant. Figure 7 shows a bar chart of the methylation across all samples, dementia and control groups and male and female groups.

Figure 7

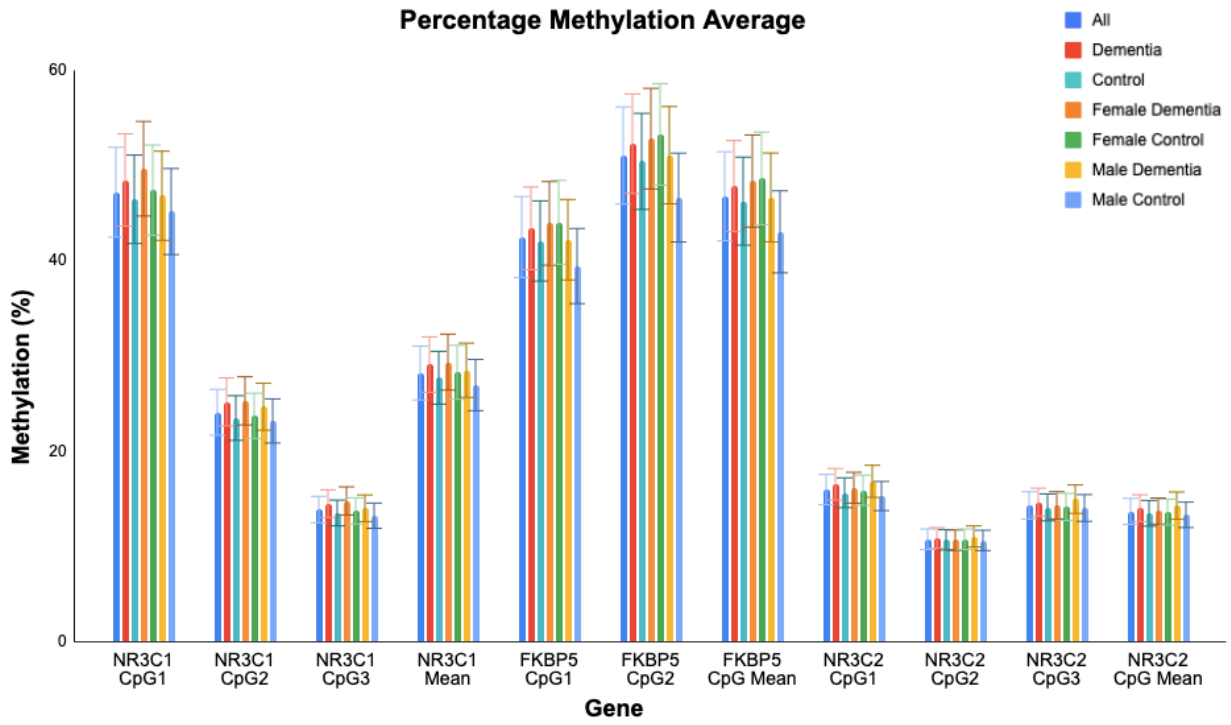

Figure 7: displays a chart showing the percentage methylation for the CpG site on each gene and average percentage methylation for each gene, with error bars showing standard error of the mean.

Normality Testing

A normality test was used to confirm whether the sample data has been drawn from a normally distributed population using SPSS software. This is required as various further statistical tests require a normally distributed sample population. It was expected that the percentage methylation would not be normally distributed.

The variables tested are shown in Table 24 in the appendix and show the distributions based on the Shapiro-Wilk test.

Figure 8 shows representative figures for a normal distribution for the age at death of the samples (P = 0.083) and an non-normal distribution of the NR3C1 CpG1 percentage methylation (P < 0.001).

Figure 8

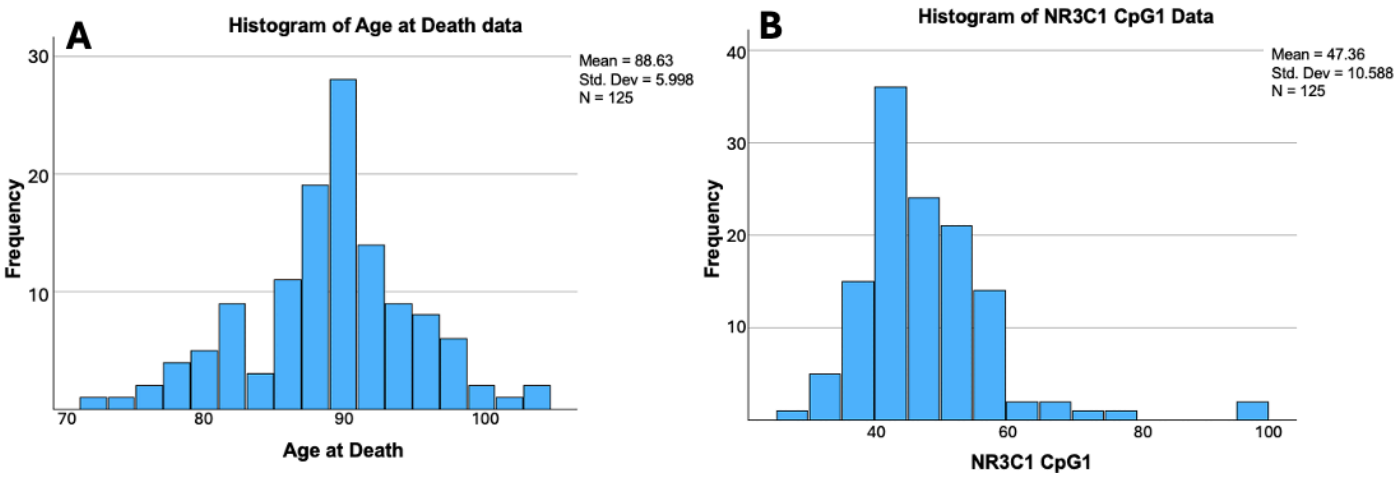

Figure 8: A: shows the histogram produced on SPSS for the normality test of the age at death data, showing a normal distribution (P = 0.083). **B:** shows the histogram produced on SPSS for the normality test of the NR3C1 CpG1 methylation data, showing an abnormal distribution (P < 0.001).

Investigating The Relationship Between Methylation and Cortisol

A correlation analysis was carried out between the cortisol data and percentage methylation data in order to investigate the degree of relationship between them. Pearson's correlation was used where data was normally distributed and Spearman's rank correlation was used where data was not normally distributed. Results are shown in Table 11 and Table 12

Table 11

Spearman's Rank Correlation		
Variables tested	**rho**	**P value**
Cort0 vs NR3C1 CpG1	0.021	0.913
Cort0 vs NR3C1 CpG2	0.234	0.222
Cort0 vs NR3C1 CpG3	0.121	0.533
Cort0 vs NR3C1 CpG Mean	0.044	0.819
Cort0 vs NR3C2 CpG2	0.373	**0.046**

Cort0 vs NR3C2 CpG3	0.269	0.159
Cort30 vs NR3C1 CpG1	-0.005	0.979
Cort30 vs NR3C1 CpG2	0.007	0.972
Cort30 vs NR3C1 CpG3	0.159	0.409
Cort30 vs NR3C1 CpG Mean	0.003	0.989
Cort30 vs NR3C2 CpG2	0.497	**0.006**
Cort30 vs NR3C2 CpG3	0.354	0.059
Cort60 vs NR3C1 CpG1	-0.321	0.079
Cort60 vs NR3C1 CpG2	-0.214	0.284
Cort60 vs NR3C1 CpG3	-0.141	0.45
Cort60 vs NR3C1 CpG Mean	-0.344	0.058
Cort60 vs NR3C2 CpG2	0.185	0.318
Cort60 vs NR3C2 CpG3	0.22	0.234
Cort14 vs NR3C1 CpG1	-0.204	0.272
Cort14 vs NR3C1 CpG2	0.122	0.513
Cort14 vs NR3C1 CpG3	-0.142	0.446
Cort14 vs NR3C1 CpG Mean	-0.148	0.428
Cort14 vs FKBP5 CpG1	0.068	0.716
Cort14 vs FKBP5 CpG2	0.054	0.774
Cort14 vs FKBP5 CpG Mean	0.065	0.729
Cort14 vs NR3C2 CpG1	0.322	0.077
Cort14 vs NR3C2 CpG2	0.152	0.414
Cort14 vs NR3C2 CpG3	0.439	**0.013**
Cort14 vs NR3C2 CpG Mean	0.355	**0.05**
Cort18 vs NR3C1 CpG1	0.401	**0.025**
Cort18 vs NR3C1 CpG2	0.325	0.075
Cort18 vs NR3C1 CpG3	0.392	**0.029**
Cort18 vs NR3C1 CpG Mean	0.401	**0.025**
Cort18 vs NR3C2 CpG2	0.024	0.897
Cort18 vs NR3C2 CpG3	-0.142	0.445
Cort22 vs NR3C1 CpG1	0.171	0.358
Cort22 vs NR3C1 CpG2	0.35	0.054
Cort22 vs NR3C1 CpG3	0.228	0.216
Cort22 vs NR3C1 CpG Mean	0.271	0.141
Cort22 vs FKBP5 CpG1	-0.119	0.525
Cort22 vs FKBP5 CpG2	-0.07	0.709
Cort22 vs FKBP5 CpG Mean	-0.098	0.601
Cort22 vs NR3C2 CpG1	0.024	0.898
Cort22 vs NR3C2 CpG2	0.034	0.858
Cort22 vs NR3C2 CpG3	0.041	0.826
Cort22 vs NR3C2 CpG Mean	0.055	0.77

Table 11: shows the variables for which the Spearman's rank correlation test was conducted. The ‘rho’ indicates the strength of the relationship between two variables, positive values indicate a positive monotonic relationship, negative values indicate a negative monotonic relationship. ‘rho’ values range from -1 to 1 where -1 indicates a perfect

negative linear relationship, 0 indicates no linear relationship and closer to -1 suggests a stronger negative relationship. P value indicates the level of significance, bold values are significant.

Table 12

Pearson's correlation		
Variables tested	**r**	**P value**
Cort0 vs FKBP5 CpG1	0.129	0.506
Cort0 vs FKBP5 CpG2	0.169	0.38
Cort0 vs FKBP5 CpG Mean	0.149	0.439
Cort0 vs NR3C2 CpG1	0.07	0.717
Cort0 vs NR3C2 CpG Mean	0.228	0.235
Cort30 vs FKBP5 CpG1	-0.076	0.697
Cort30 vs FKBP5 CpG2	0.003	0.998
Cort30 vs FKBP5 CpG Mean	-0.037	0.847
Cort30 vs NR3C2 CpG1	0.231	0.227
Cort30 vs NR3C2 CpG Mean	0.347	**0.046**
Cort60 vs FKBP5 CpG1	0.04	0.831
Cort60 vs FKBP5 CpG2	0.084	0.655
Cort60 vs FKBP5 CpG Mean	0.062	0.741
Cort60 vsNR3C2 CpG1	-0.027	0.884
Cort60 vs NR3C2 CpG Mean	0.117	0.531
Cort18 vs FKBP5 CpG1	0.083	0.659
Cort18 vs FKBP5 CpG2	0.038	0.838
Cort18 vs FKBP5 CpG Mean	0.061	0.744
Cort18 vs NR3C2 CpG1	0.001	0.995
Cort18 vs NR3C2 CpG Mean	-0.01	0.959

Table 12: shows the variables for which the Pearson's correlation test was conducted. The ‘r’ indicates the strength of the relationship between two variables, positive values indicate a positive linear relationship, negative values indicate a negative linear relationship. ‘r’ values range from -1 to 1 where -1 indicates a perfect negative linear relationship, 0 indicates no linear relationship and closer to -1 suggests a stronger negative relationship. P value indicates the level of significance, bold values are significant.

Following the correlation analyses it was found that the following correlations were significant with a positive linear relationship: Cort0 vs NR3C2 CpG2 (Correlation coefficient = 0.373, P = 0.046), Cort30 vs NR3C2 CpG2 (Correlation coefficient = 0.497, P = 0.006), Cort14 vs NR3C2 CpG Mean (Correlation coefficient = 0.355, P = 0.05), Cort18 vs NR3C1 CpG1 (Correlation coefficient = 0.401, P = 0.025), Cort18 vs NR3C1 CpG3 (Correlation coefficient = 0.392, P = 0.029), Cort18 vs NR3C1 CpG Mean (Correlation coefficient = 0.401, P = 0.025) and Cort30 vs NR3C2 CpG Mean (Correlation coefficient = 0.347, P = 0.046). Figure 9 shows the scatter plots created for the significant correlations.

Figure 9

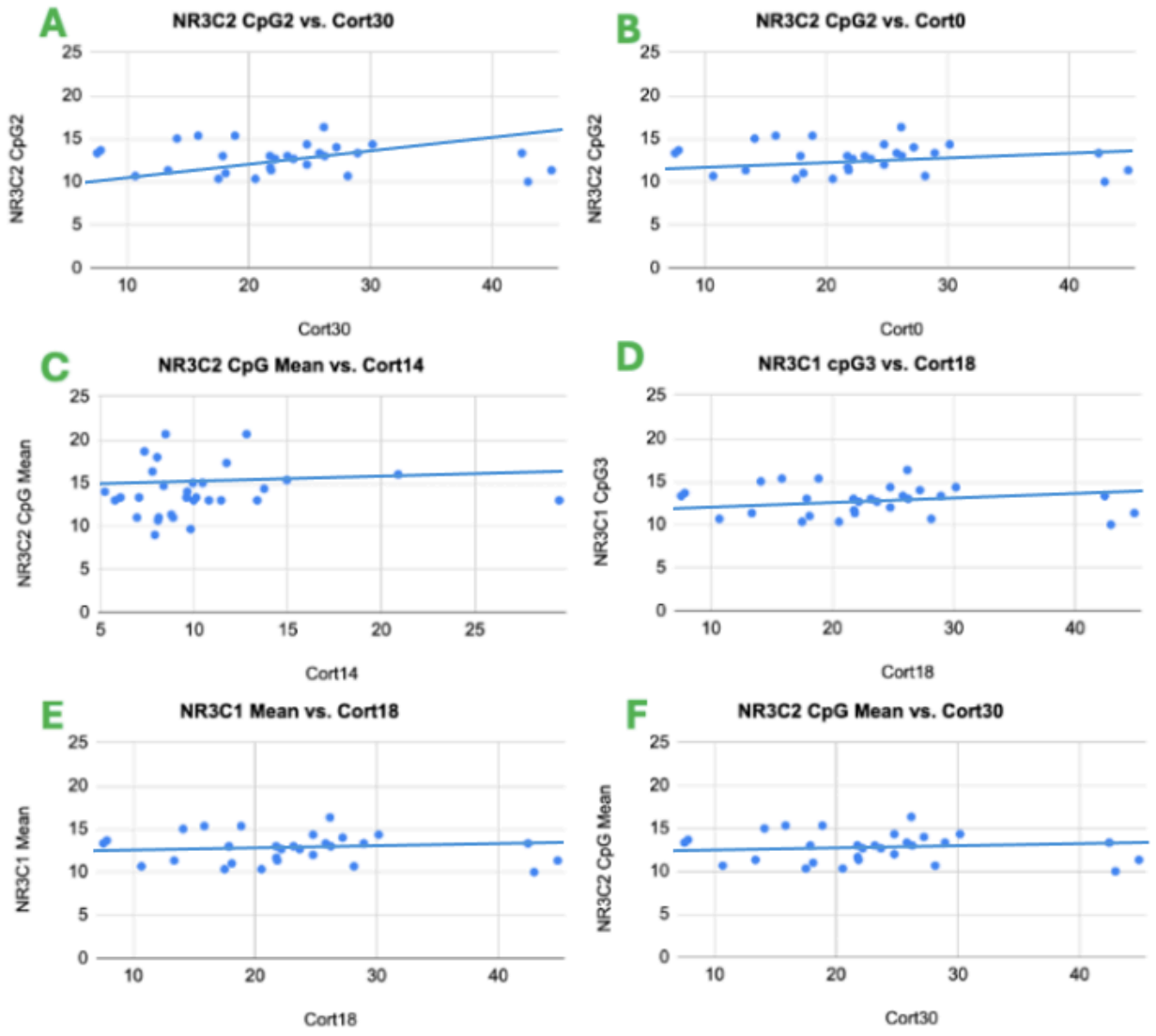

Figure 9: shows scatter plots for all the statistically significant correlations between methylation and cortisol levels with the line of best fit. **A:** shows NR3C2 CpG2 vs Cort30 (Correlation coefficient = 0.497, = P = 0.006), **B:** NR3C2 CpG2 vs Cort0 (Correlation coefficient = 0.373, P = 0.046), **C:** NR3C2 CpG Mean vs Cort14 (Correlation coefficient = 0.355, P = 0.05), **D:** NR3C1 CpG3 vs Cort18 (Correlation coefficient = 0.392, P = 0.029), **E:** NR3C1 CpG1 vs Cort18 (Correlation coefficient = 0.401, P = 0.025), **F:** NR3C2 CpG Mean vs Cort30 (Correlation coefficient = 0.347, P = 0.046).

Investigating The Relationship Between Methylation and Sleep

A correlation analysis was carried out between the sleep data and percentage methylation data in order to investigate the degree of relationship between them. Pearson's correlation was used where data was normally distributed and Spearman's rank correlation was used where data was abnormally distributed. Results are shown in Table 13 and Table 14.

Table 13

Spearman's Rank Correlation		
Variables tested	**rho**	**P value**
p1hrslp vs NR3C1 CpG1	-0.093	0.313
p1hrslp vs NR3C1 CpG2	-0.107	0.245
p1hrslp vs NR3C1 CpG3	-0.114	0.216
p1hrslp vs NR3C1 CpG Mean	-0.082	0.374
p1hrslp vs FKBP5 CpG1	-0.083	0.369
p1hrslp vs FKBP5 CpG2	-0.094	0.307
p1hrslp vs FKBP5 CpG Mean	-0.086	0.351
p1hrslp vs NR3C2 CpG1	0.001	0.994
p1hrslp vs NR3C2 CpG2	-0.118	0.197
p1hrslp vs NR3C2 CpG3	0	0.999
p1hrslp vs NR3C2 CpG Mean	-0.026	0.781
p2hrslp vs NR3C1 CpG1	0.085	0.424
p2hrslp vs NR3C1 CpG2	0.022	0.833
p2hrslp vs NR3C1 CpG3	0.034	0.75
p2hrslp vs NR3C1 CpG Mean	0.068	0.524
p2hrslp vs FKBP5 CpG1	-0.021	0.842
p2hrslp vs FKBP5 CpG2	-0.025	0.815
p2hrslp vs FKBP5 CpG Mean	-0.02	0.849
p2hrslp vs NR3C2 CpG1	-0.149	0.159
p2hrslp vs NR3C2 CpG2	-0.157	0.137
p2hrslp vs NR3C2 CpG3	-0.169	0.11
p2hrslp vs NR3C2 CpG Mean	-0.16	0.129
p5hrslp vs NR3C1 CpG1	-0.195	0.158
p5hrslp vs NR3C1 CpG2	-0.079	0.568
p5hrslp vs NR3C1 CpG3	-0.189	0.172
p5hrslp vs NR3C1 CpG Mean	-0.174	0.209
p5hrslp vs NR3C2 CpG2	-0.107	0.441
p5hrslp vs NR3C2 CpG3	-0.061	0.659
p6hrslp vs NR3C1 CpG1	-0.133	0.201
p6hrslp vs NR3C1 CpG2	-0.065	0.533
p6hrslp vs NR3C1 CpG3	-0.145	0.164
p6hrslp vs NR3C1 CpG Mean	-0.12	0.25
p6hrslp vs FKBP5 CpG1	-0.002	0.988
p6hrslp vs FKBP5 CpG2	-0.053	0.611
p6hrslp vs FKBP5 CpG Mean	-0.025	0.81
p6hrslp vs NR3C2 CpG1	-0.186	0.073

p6hrslp vs NR3C2 CpG2	-0.113	0.279
p6hrslp vs NR3C2 CpG3	-0.134	0.197
p6hrslp vs NR3C2 CpG Mean	-0.147	0.157
p7hrslp vs NR3C1 CpG1	-0.104	0.394
p7hrslp vs NR3C1 CpG2	-0.102	0.405
p7hrslp vs NR3C1 CpG3	-0.058	0.634
p7hrslp vs NR3C1 CpG Mean	-0.113	0.357
p7hrslp vs NR3C2 CpG2	-0.105	0.392
p7hrslp vs NR3C2 CpG3	-0.133	0.275
p1sleff vs NR3C1 CpG1	-0.028	0.765
p1sleff vs NR3C1 CpG2	-0.052	0.575
p1sleff vs NR3C1 CpG3	-0.004	0.966
p1sleff vs NR3C1 CpG Mean	-0.04	0.664
p1sleff vs FKBP5 CpG1	-0.068	0.464
p1sleff vs FKBP5 CpG2	-0.53	0.565
p1sleff vs FKBP5 CpG Mean	-0.061	0.505
p1sleff vs NR3C2 CpG1	-0.123	0.18
p1sleff vs NR3C2 CpG2	-0.19	**0.038**
p1sleff vs NR3C2 CpG3	-0.09	0.328
p1sleff vs NR3C2 CpG Mean	-0.128	0.162
p2sleff vs NR3C1 CpG1	0.115	0.277
p2sleff vs NR3C1 CpG2	0.077	0.47
p2sleff vs NR3C1 CpG3	0.055	0.602
p2sleff vs NR3C1 CpG Mean	0.109	0.303
p2sleff vs FKBP5 CpG1	-0.016	0.882
p2sleff vs FKBP5 CpG2	0.006	0.953
p2sleff vs FKBP5 CpG Mean	-0.01	0.925
p2sleff vs NR3C2 CpG1	-0.126	0.234
p2sleff vs NR3C2 CpG2	-0.117	0.27
p2sleff vs NR3C2 CpG3	-0.155	0.141
p2sleff vs NR3C2 CpG Mean	-0.132	0.213
p5sleff vs NR3C1 CpG1	-0.132	0.342
p5sleff vs NR3C1 CpG2	-0.118	0.396
p5sleff vs NR3C1 CpG3	-0.127	0.362
p5sleff vs NR3C1 CpG Mean	-0.135	0.332
p5sleff vs NR3C2 CpG2	-0.002	0.986
p5sleff vs NR3C2 CpG3	-0.058	0.675
p6sleff vs NR3C1 CpG1	-0.142	0.175
p6sleff vs NR3C1 CpG2	-0.171	0.101
p6sleff vs NR3C1 CpG3	-0.174	0.096
p6sleff vs NR3C1 CpG Mean	-0.175	0.093
p6sleff vs FKBP5 CpG1	0.012	0.908
p6sleff vs FKBP5 CpG2	-0.023	0.828
p6sleff vs FKBP5 CpG Mean	-0.007	0.946
p6sleff vs NR3C2 CpG1	-0.23	**0.027**
p6sleff vs NR3C2 CpG2	-0.117	0.264
p6sleff vs NR3C2 CpG3	-0.198	0.057
p6sleff vs NR3C2 CpG Mean	-0.189	0.07

p7sleff vs NR3C1 CpG1	-0.082	0.501
p7sleff vs NR3C1 CpG2	-0.128	0.294
p7sleff vs NR3C1 CpG3	-0.026	0.83
p7sleff vs NR3C1 CpG Mean	-0.098	0.422
p7sleff vs NR3C2 CpG2	-0.104	0.395
p7sleff vs NR3C2 CpG3	-0.115	0.347
p1wakent vs NR3C1 CpG1	-0.105	0.256
p1wakent vs NR3C1 CpG2	-0.106	0.253
p1wakent vs NR3C1 CpG3	-0.171	0.064
p1wakent vs NR3C1 CpG Mean	-0.153	0.099
p1wakent vs FKBP5 CpG1	0.097	0.297
p1wakent vs FKBP5 CpG2	0.115	0.217
p1wakent vs FKBP5 CpG Mean	0.101	0.277
p1wakent vs NR3C2 CpG1	0.088	0.343
p1wakent vs NR3C2 CpG2	0.157	0.09
p1wakent vs NR3C2 CpG3	0.089	0.335
p1wakent vs NR3C2 CpG Mean	0.102	0.271
p2wakent vs NR3C1 CpG1	-0.132	0.219
p2wakent vs NR3C1 CpG2	-0.11	0.307
p2wakent vs NR3C1 CpG3	-0.237	0.026
p2wakent vs NR3C1 CpG Mean	-0.145	0.178
p2wakent vs FKBP5 CpG1	0.169	0.115
p2wakent vs FKBP5 CpG2	0.19	0.076
p2wakent vs FKBP5 CpG Mean	0.174	0.105
p2wakent vs NR3C2 CpG1	0.182	0.09
p2wakent vs NR3C2 CpG2	0.185	0.084
p2wakent vs NR3C2 CpG3	0.198	0.065
p2wakent vs NR3C2 CpG Mean	0.203	0.058
p5wakent vs NR3C1 CpG1	0.115	0.405
p5wakent vs NR3C1 CpG2	0.035	0.901
p5wakent vs NR3C1 CpG3	0.022	0.873
p5wakent vs NR3C1 CpG Mean	0.106	0.44
p5wakent vs FKBP5 CpG1	0.04	0.774
p5wakent vs FKBP5 CpG2	0.07	0.612
p5wakent vs FKBP5 CpG Mean	0.059	0.67
p5wakent vs NR3C2 CpG1	0	0.998
p5wakent vs NR3C2 CpG2	-0.238	0.08
p5wakent vs NR3C2 CpG3	-0.093	0.501
p5wakent vs NR3C2 CpG Mean	-0.107	0.437
p6wakent vs NR3C1 CpG1	0.355	0.125
p6wakent vs NR3C1 CpG2	0.262	0.264
p6wakent vs NR3C1 CpG3	0.244	0.3
p6wakent vs NR3C1 CpG Mean	0.342	0.14
p6wakent vs FKBP5 CpG1	0.32	0.168
p6wakent vs FKBP5 CpG2	0.42	0.065
p6wakent vs FKBP5 CpG Mean	0.376	0.103
p6wakent vs NR3C2 CpG1	0.009	0.97
p6wakent vs NR3C2 CpG2	0.038	0.873

p6wakent vs NR3C2 CpG3	0.015	0.949
p6wakent vs NR3C2 CpG Mean	0.004	0.985
p7wakent vs NR3C1 CpG1	-0.012	0.926
p7wakent vs NR3C1 CpG2	-0.053	0.675
p7wakent vs NR3C1 CpG3	-0.004	0.975
p7wakent vs NR3C1 CpG Mean	-0.013	0.92
p7wakent vs FKBP5 CpG1	0.021	0.87
p7wakent vs FKBP5 CpG2	0.112	0.37
p7wakent vs FKBP5 CpG Mean	0.062	0.619
p7wakent vs NR3C2 CpG1	0.196	0.114
p7wakent vs NR3C2 CpG2	-0.009	0.94
p7wakent vs NR3C2 CpG3	0.216	0.082
p7wakent vs NR3C2 CpG Mean	0.158	0.204

Table 13: shows the variables for which the Spearman's rank correlation test was conducted. The ‘rho’ indicates the strength of the relationship between two variables, positive values indicate a positive monotonic relationship, negative values indicate a negative monotonic relationship. ‘rho’ values range from -1 to 1 where -1 indicates a perfect negative linear relationship, 0 indicates no linear relationship and closer to -1 suggests a stronger negative relationship. P value indicates the level of significance, bold values are significant.

Table 14

Pearson's Correlation		
Variables tested	**r**	**P value**
p5hrslp vs FKBP5 CpG1	-0.063	0.653
p5hrslp vs FKBP5 CpG2	-0.1	0.472
p5hrslp vs FKBP5 CpG Mean	-0.082	0.557
p5hrslp vs NR3C2 CpG1	-0.103	0.459
p5hrslp vs NR3C2 CpG Mean	-0.09	0.52
p7hrslp vs FKBP5 CpG1	0.151	0.215
p7hrslp vs FKBP5 CpG2	0.104	0.396
p7hrslp vs FKBP5 CpG Mean	0.13	0.288
p7hrslp vs NR3C2 CpG1	-0.172	0.158
p7hrslp vs NR3C2 CpG Mean	-0.161	0.187
p5sleff vs FKBP5 CpG1	0.113	0.416
p5sleff vs FKBP5 CpG2	0.116	0.405
p5sleff vs FKBP5 CpG Mean	0.115	0.408
p5sleff vs NR3C2 CpG1	-0.157	0.256
p5sleff vs NR3C2 CpG Mean	-0.115	0.407
p7sleff vs FKBP5 CpG1	0.161	0.185
p7sleff vs FKBP5 CpG2	0.119	0.329
p7sleff vs FKBP5 CpG Mean	0.143	0.242
p7sleff vs NR3C2 CpG1	-0.149	0.221
p7sleff vs NR3C2 CpG Mean	-0.148	0.224

Table 14: shows the variables for which the Pearson's correlation test was conducted. The ‘r’ indicates the strength of the relationship between two variables, positive values indicate a positive linear relationship, negative values indicate a negative linear relationship. ‘r’ values range from -1 to 1 where -1 indicates a perfect negative linear

relationship, 0 indicates no linear relationship and closer to -1 suggests a stronger negative relationship. P value indicates the level of significance.

Following the correlation analyses it was found that the following correlations were significant with a negative monotonic relationship: p1sleff vs NR3C2 CpG2 (rho = -0.19, P = 0.038), p6sleff vs NR3C2 CpG1 (rho = -0.23, P = 0.027) and p2wakent vs NR3C1 CpG3 (rho = -0.237, P = 0.026). Figure 10 shows the scatter plots created for the significant correlations.

Figure 10

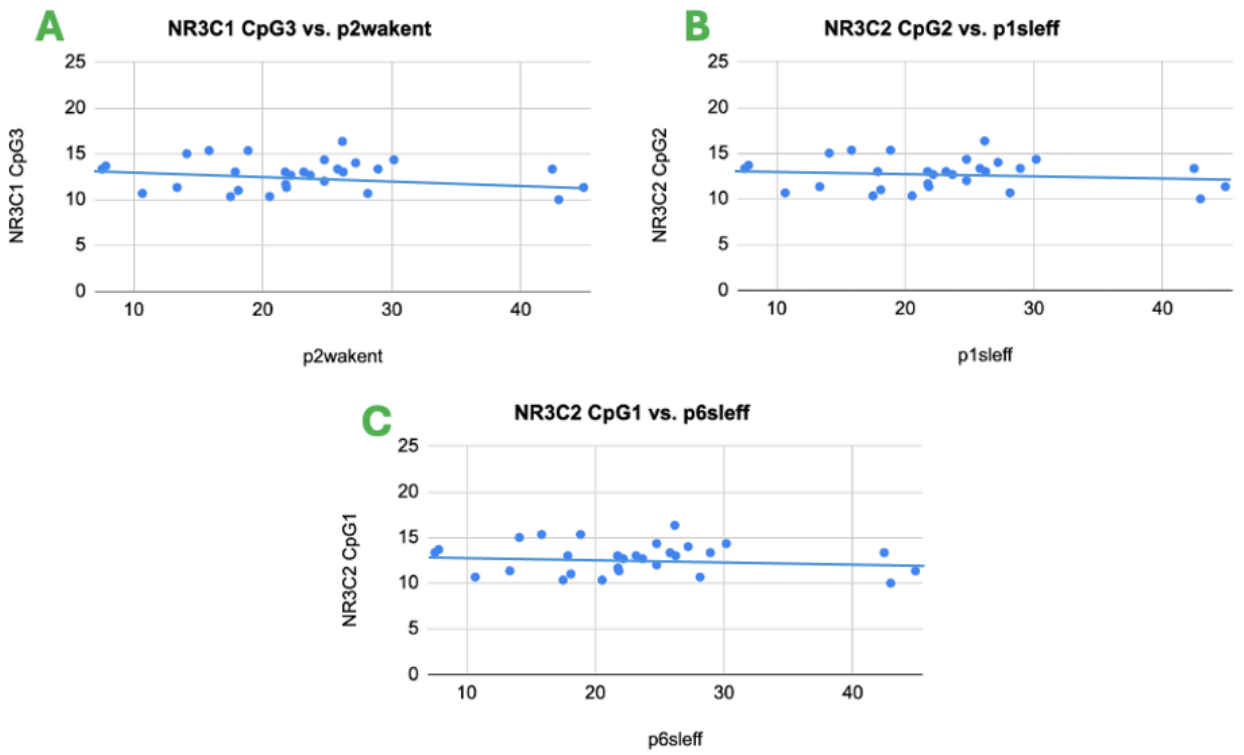

Figure 10: shows scatter plots for all the statistically significant correlations between methylation and sleep data, with the line of best fit. **A:** shows NR3C2 CpG1 vs p6sleff (rho = -0.23, P = 0.027), **B:** NR3C2 CpG2 vs p1sleff (rho = -0.19, P = 0.038), **C:** NR3C1 CpG3 vs p2wakent (rho = -0.237, P = 0.026)

Investigating The Relationship Between Methylation and Depression

As the depression data was standardised into binary format (e.g. coded ‘1’ for depression and ‘0’ for no depression) a point-biserial Pearson’s correlation analysis was used on the depression data and normally distributed percentage methylation and a point-biserial Spearman's

correlation analysis was used on the depression data and abnormally distributed percentage methylation in order to investigate the degree of relationship between them. Results for all depression waves and all gene methylations are shown in Table 15 and Table 16. The depression measures from msev4 and msev11 had zero variance so therefore were excluded from the correlation analysis.

Table 15

Spearman's Rank rho		
Variables tested	**Correlation Coefficient**	**P value**
mood1 vs NR3C1 CpG1	-0.039	0.678
mood2 vs NR3C1 CpG1	-0.005	0.962
mood3 vs NR3C1 CpG1	0.084	0.408
mood4 vs NR3C1 CpG1	-0.098	0.368
mood5 vs NR3C1 CpG1	0.063	0.59
mood6 vs NR3C1 CpG1	0.191	0.337
mood7 vs NR3C1 CpG1	-0.103	0.274
mood8 vs NR3C1 CpG1	-0.044	0.659
mood9 vs NR3C1 CpG1	-0.013	0.91
mood10 vs NR3C1 CpG1	-0.131	0.322
mood11 vs NR3C1 CpG1	-0.072	0.843
msev1 vs NR3C1 CpG1	-0.02	0.836
msev2 vs NR3C1 CpG1	0.019	0.842
msev3 vs NR3C1 CpG1	0.037	0.719
msev5 vs NR3C1 CpG1	-0.148	0.203
msev6 vs NR3C1 CpG1	0.201	0.203
msev7 vs NR3C1 CpG1	-0.007	0.94
msev8 vs NR3C1 CpG1	-0.21	**0.035**
msev9 vs NR3C1 CpG1	0.008	0.941
msev10 vs NR3C1 CpG1	-0.178	0.178
mood1 vs NR3C1 CpG2	-0.157	0.095
mood2 vs NR3C1 CpG2	-0.121	0.207
mood3 vs NR3C1 CpG2	-0.094	0.351
mood4 vs NR3C1 CpG2	-0.185	0.088
mood5 vs NR3C1 CpG2	-0.115	0.324
mood6 vs NR3C1 CpG2	0.032	0.842
mood7 vs NR3C1 CpG2	-0.147	0.12
mood8 vs NR3C1 CpG2	-0.146	0.145
mood9 vs NR3C1 CpG2	-0.044	0.693
mood10 vs NR3C1 CpG2	-0.235	0.074
mood11 vs NR3C1 CpG2	-0.21	0.547
msev1 vs NR3C1 CpG2	-0.124	0.189
msev2 vs NR3C1 CpG2	0.115	0.231
msev3 vs NR3C1 CpG2	0.009	0.928

msev5 vs NR3C1 CpG2	-0.14	0.228
msev6 vs NR3C1 CpG2	0.116	0.463
msev7 vs NR3C1 CpG2	-0.463	0.652
msev8 vs NR3C1 CpG2	-0.161	0.107
msev9 vs NR3C1 CpG2	-0.015	0.893
msev10 vs NR3C1 CpG2	-0.162	0.219
mood1 vs NR3C1 CpG3	-0.09	0.341
mood2 vs NR3C1 CpG3	-0.007	0.943
mood3 vs NR3C1 CpG3	0.026	0.801
mood4 vs NR3C1 CpG3	-0.098	0.368
mood5 vs NR3C1 CpG3	0.028	0.81
mood6 vs NR3C1 CpG3	0.121	0.226
mood7 vs NR3C1 CpG3	-0.181	0.054
mood8 vs NR3C1 CpG3	-0.138	0.168
mood9 vs NR3C1 CpG3	-0.072	0.523
mood10 vs NR3C1 CpG3	-0.225	0.086
mood11 vs NR3C1 CpG3	-0.327	0.357
msev1 vs NR3C1 CpG3	-0.045	0.637
msev2 vs NR3C1 CpG3	0.068	0.478
msev3 vs NR3C1 CpG3	0.056	0.578
msev5 vs NR3C1 CpG3	-0.127	0.274
msev6 vs NR3C1 CpG3	0.169	0.286
msev7 vs NR3C1 CpG3	-0.015	0.871
msev8 vs NR3C1 CpG3	-0.192	0.055
msev9 vs NR3C1 CpG3	0.008	0.94
msev10 vs NR3C1 CpG3	-0.167	0.205
mood1 vs NR3C1 CpG Mean	-0.077	0.415
mood2 vs NR3C1 CpG Mean	-0.031	0.75
mood3 vs NR3C1 CpG Mean	0.034	0.729
mood4 vs NR3C1 CpG Mean	-0.127	0.244
mood5 vs NR3C1 CpG Mean	0.031	0.793
mood6 vs NR3C1 CpG Mean	0.153	0.334
mood7 vs NR3C1 CpG Mean	-0.126	0.184
mood8 vs NR3C1 CpG Mean	-0.08	0.428
mood9 vs NR3C1 CpG Mean	-0.021	0.85
mood10 vs NR3C1 CpG Mean	-0.163	0.217
mood11 vs NR3C1 CpG Mean	-0.071	0.845
msev1 vs NR3C1 CpG Mean	-0.047	0.62
msev2 vs NR3C1 CpG Mean	0.061	0.527
msev3 vs NR3C1 CpG Mean	0.04	0.696
msev5 vs NR3C1 CpG Mean	-0.142	0.221
msev6 vs NR3C1 CpG Mean	1.74	0.27
msev7 vs NR3C1 CpG Mean	-0.018	0.847
msev8 vs NR3C1 CpG Mean	-0.211	**0.034**
msev9 vs NR3C1 CpG Mean	0.002	0.988
msev10 vs NR3C1 CpG Mean	-0.177	0.174
mood1 vs NR3C2 CpG2	0.004	0.964
mood2 vs NR3C2 CpG2	0.183	0.055

mood3 vs NR3C2 CpG2	0.06	0.551
mood4 vs NR3C2 CpG2	-0.012	0.913
mood5 vs NR3C2 CpG2	-0.055	0.639
mood6 vs NR3C2 CpG2	0.084	0.597
mood7 vs NR3C2 CpG2	0.118	0.213
mood8 vs NR3C2 CpG2	-0.033	0.74
mood9 vs NR3C2 CpG2	-0.003	0.977
mood10 vs NR3C2 CpG2	0.117	0.376
mood11 vs NR3C2 CpG2	-0.29	0.417
msev1 vs NR3C2 CpG2	0.116	0.218
msev2 vs NR3C2 CpG2	-0.051	0.595
msev3 vs NR3C2 CpG2	0.01	0.919
msev5 vs NR3C2 CpG2	0.091	0.434
msev6 vs NR3C2 CpG2	0.013	0.934
msev7 vs NR3C2 CpG2	0.021	0.828
msev8 vs NR3C2 CpG2	-0.027	0.787
msev9 vs NR3C2 CpG2	0.097	0.387
msev10 vs NR3C2 CpG2	0.126	0.344
mood1 vs NR3C2 CpG3	0.004	0.964
mood2 vs NR3C2 CpG3	0.183	0.055
mood3 vs NR3C2 CpG3	0.06	0.551
mood4 vs NR3C2 CpG3	-0.012	0.913
mood5 vs NR3C2 CpG3	-0.055	0.639
mood6 vs NR3C2 CpG3	0.084	0.597
mood7 vs NR3C2 CpG3	0.118	0.213
mood8 vs NR3C2 CpG3	-0.033	0.74
mood9 vs NR3C2 CpG3	-0.003	0.977
mood10 vs NR3C2 CpG3	0.117	0.376
mood11 vs NR3C2 CpG3	-0.29	0.417
msev1 vs NR3C2 CpG3	0.116	0.218
msev2 vs NR3C2 CpG3	-0.051	0.595
msev3 vs NR3C2 CpG3	0.01	0.919
msev5 vs NR3C2 CpG3	0.091	0.434
msev6 vs NR3C2 CpG3	0.013	0.934
msev7 vs NR3C2 CpG3	0.021	0.828
msev8 vs NR3C2 CpG3	-0.027	0.787
msev9 vs NR3C2 CpG3	0.097	0.387
msev10 vs NR3C2 CpG3	0.126	0.344

Table 15: shows the Spearman's rank conducted. The ‘rho’ of the relationship variables for which the correlation test was indicates the strength between two variables, positive values indicate a positive monotonic relationship, negative values indicate a negative monotonic relationship. ‘rho’ values range from -1 to 1 where -1 indicates a perfect negative linear relationship, 0 indicates no linear relationship and closer to -1 suggests a stronger negative relationship. P value indicates the level of significance, bold values are significant.

Table 16

Pearson's Correlation		
Variables tested	**Correlation Coefficient**	**P value**
mood1 vs FKBP5 CpG1	0.049	0.604
mood2 vs FKBP5 CpG1	0.117	0.219
mood3 vs FKBP5 CpG1	0.231	0.021
mood4 vs FKBP5 CpG1	0.092	0.399
mood5 vs FKBP5 CpG1	0.182	0.789
mood6 vs FKBP5 CpG1	0.043	0.831
mood7 vs FKBP5 CpG1	0.02	0.22
mood8 vs FKBP5 CpG1	0.123	0.592
mood9 vs FKBP5 CpG1	0.06	0.592
mood10 vs FKBP5 CpG1	0.256	**0.05**
mood11 vs FKBP5 CpG1	-0.356	0.312
msev1 vs FKBP5 CpG1	0.131	0.164
msev2 vs FKBP5 CpG1	-0.182	0.055
msev3 vs FKBP5 CpG1	-0.061	0.546
msev4 vs FKBP5 CpG1	N/A	N/A
msev5 vs FKBP5 CpG1	0.154	0.185
msev6 vs FKBP5 CpG1	-0.281	0.072
msev7 vs FKBP5 CpG1	-0.079	0.402
msev8 vs FKBP5 CpG1	0.105	0.298
msev9 vs FKBP5 CpG1	-0.022	0.843
msev10 vs FKBP5 CpG1	0.157	0.235
msev11 vs FKBP5 CpG1	N/A	N/A
mood1 vs FKBP5 CpG2	0.074	0.434
mood2 vs FKBP5 CpG2	0.107	0.264
mood3 vs FKBP5 CpG2	0.267	**0.007**
mood4 vs FKBP5 CpG2	0.108	0.324
mood5 vs FKBP5 CpG2	0.225	0.051
mood6 vs FKBP5 CpG2	0.11	0.488
mood7 vs FKBP5 CpG2	0.082	0.387
mood8 vs FKBP5 CpG2	0.139	0.164
mood9 vs FKBP5 CpG2	0.098	0.383
mood10 vs FKBP5 CpG2	0.261	**0.046**
mood11 vs FKBP5 CpG2	-0.37	0.293
msev1 vs FKBP5 CpG2	0.145	0.125
msev2 vs FKBP5 CpG2	-0.121	0.207
msev3 vs FKBP5 CpG2	-0.049	0.627
msev4 vs FKBP5 CpG2	N/A	N/A
msev5 vs FKBP5 CpG2	0.158	0.172
msev6 vs FKBP5 CpG2	-0.279	0.073

msev7 vs FKBP5 CpG2	-0.061	0.516
msev8 vs FKBP5 CpG2	0.109	0.276
msev9 vs FKBP5 CpG2	-0.02	0.857
msev10 vs FKBP5 CpG2	0.166	0.209
msev11 vs FKBP5 CpG2	N/A	N/A
mood1 vs FKBP5 CpG Mean	0.063	0.507
mood2 vs FKBP5 CpG Mean	0.114	0.231
mood3 vs FKBP5 CpG Mean	0.252	**0.011**
mood4 vs FKBP5 CpG Mean	0.101	0.355
mood5 vs FKBP5 CpG Mean	0.206	0.074
mood6 vs FKBP5 CpG Mean	0.078	0.625
mood7 vs FKBP5 CpG Mean	0.052	0.583
mood8 vs FKBP5 CpG Mean	0.134	0.182
mood9 vs FKBP5 CpG Mean	0.081	0.472
mood10 vs FKBP5 CpG Mean	0.263	**0.044**
mood11 vs FKBP5 CpG Mean	-0.365	0.3
msev1 vs FKBP5 CpG Mean	0.141	0.136
msev2 vs FKBP5 CpG Mean	-0.155	0.105
msev3 vs FKBP5 CpG Mean	-0.056	0.583
msev4 vs FKBP5 CpG Mean	N/A	N/A
msev5 vs FKBP5 CpG Mean	0.158	0.174
msev6 vs FKBP5 CpG Mean	-0.283	0.069
msev7 vs FKBP5 CpG Mean	-0.072	0.449
msev8 vs FKBP5 CpG Mean	0.109	0.277
msev9 vs FKBP5 CpG Mean	-0.022	0.847
msev10 vs FKBP5 CpG Mean	0.164	0.213
msev11 vs FKBP5 CpG Mean	N/A	N/A
mood1 vs NR3C2 CpG1	-0.189	**0.043**
mood2 vs NR3C2 CpG1	-0.004	0.971
mood3 vs NR3C2 CpG1	-0.111	0.272
mood4 vs NR3C2 CpG1	-0.098	0.37
mood5 vs NR3C2 CpG1	-0.132	0.255
mood6 vs NR3C2 CpG1	-0.033	0.834
mood7 vs NR3C2 CpG1	-0.061	0.521
mood8 vs NR3C2 CpG1	-0.147	0.142
mood9 vs NR3C2 CpG1	-0.182	0.102
mood10 vs NR3C2 CpG1	-0.003	0.979
mood11 vs NR3C2 CpG1	-0.045	0.902
msev1 vs NR3C2 CpG1	-0.1	0.92
msev2 vs NR3C2 CpG1	-0.133	0.163
msev3 vs NR3C2 CpG1	-0.157	0.119
msev4 vs NR3C2 CpG1	N/A	N/A
msev5 vs NR3C2 CpG1	0.068	0.561
msev6 vs NR3C2 CpG1	-0.169	0.285

msev7 vs NR3C2 CpG1	-0.137	0.145
msev8 vs NR3C2 CpG1	0	0.997
msev9 vs NR3C2 CpG1	-0.045	0.687
msev10 vs NR3C2 CpG1	0.071	0.592
msev11 vs NR3C2 CpG1	N/A	N/A
mood1 vs NR3C2 CpG Mean	-0.142	0.133
mood2 vs NR3C2 CpG Mean	0.043	0.656
mood3 vs NR3C2 CpG Mean	-0.06	0.554
mood4 vs NR3C2 CpG Mean	-0.11	0.314
mood5 vs NR3C2 CpG Mean	-0.142	0.22
mood6 vs NR3C2 CpG Mean	-0.004	0.979
mood7 vs NR3C2 CpG Mean	-0.009	0.927
mood8 vs NR3C2 CpG Mean	-0.11	0.274
mood9 vs NR3C2 CpG Mean	-0.145	0.195
mood10 vs NR3C2 CpG Mean	0.033	0.806
mood11 vs NR3C2 CpG Mean	-0.134	0.806
msev1 vs NR3C2 CpG Mean	0.013	0.889
msev2 vs NR3C2 CpG Mean	-0.097	0.309
msev3 vs NR3C2 CpG Mean	-0.086	0.396
msev4 vs NR3C2 CpG Mean	N/A	N/A
msev5 vs NR3C2 CpG Mean	0.059	0.612
msev6 vs NR3C2 CpG Mean	-0.114	0.47
msev7 vs NR3C2 CpG Mean	-0.096	0.311
msev8 vs NR3C2 CpG Mean	-0.023	0.821
msev9 vs NR3C2 CpG Mean	-0.011	0.919
msev10 vs NR3C2 CpG Mean	0.71	0.592

Table 16: shows the Pearson's correlation variables for which the test was conducted. The ‘r’ indicates the strength of the relationship between two variables, positive values indicate a positive linear relationship, negative values indicate a negative linear relationship. ‘r’ values range from -1 to 1 where -1 indicates a perfect negative linear relationship, 0 indicates no linear relationship and closer to -1 suggests a stronger negative relationship. P value indicates the level of significance, bold values are significant.

Box plots shown in Figure 11 were created for the statistically significant correlations between the following: msev8 vs NR3C1 CpG1 (correlation coefficient = -0.2, P = 0.035), msev8 vs NR3C1 CpG Mean (correlation coefficient = -0.211, P = 0.034), mood3 vs FKBP5 CpG1 (correlation coefficient = 0.231, P = 0.021), mood10 vs FKBP5 CpG1 (correlation coefficient = 0.256, P = 0.05), mood3 vs FKBP5 CpG2 (correlation coefficient = 0.267, P = 0.007), mood10 vs FKBP5 CpG2 (correlation coefficient = 0.261, P = 0.046), mood3 vs FKBP5 CpG Mean (correlation coefficient = 0.252, P = 0.011), mood10 vs FKBP5 CpG Mean (correlation coefficient = 0.263, P =0.044) and mood1 vs NR3C2 CpG1 (correlation coefficient = -0.189, P = 0.043).

Figure 11

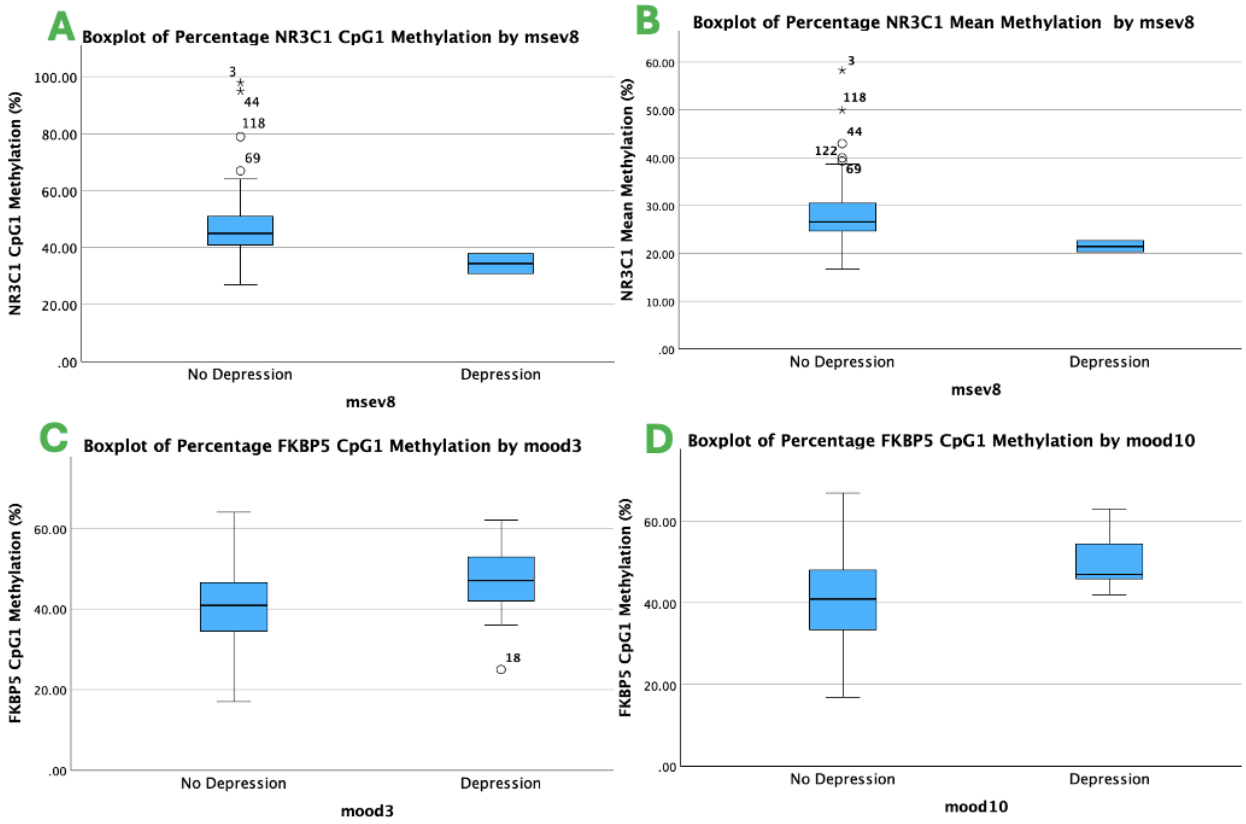

Figure 11: shows various box plots displaying the distribution of methylation levels for participants classified as either "depressed" or "not depressed". A: msev8 vs NR3C1 CpG1 (correlation coefficient = -0.2, P = 0.035), B: msev8 vs NR3C1 CpG Mean (correlation coefficient = -0.211, P = 0.034), C: mood3 vs FKBP5 CpG1 (correlation coefficient = 0.231, P = 0.021), D: mood10 vs FKBP5 CpG1 (correlation coefficient = 0.256, P = 0.05). The central box represents the interquartile range (IQR), with the lower edge indicating the 25th percentile and the upper edge indicating the 75th percentile. The horizontal line within the box denotes the median methylation level for each group. Whiskers extend to 1.5 times the IQR from the box. Outliers (data points falling between 1.5 and 3 times the IQR) are represented by circles (○), while asterisks (*) denote extreme outliers (data points more than 3 times the IQR from the box). These points highlight participants with methylation levels that significantly deviate from the typical range observed in their respective groups, along with the MMU sample number.

Figure 12

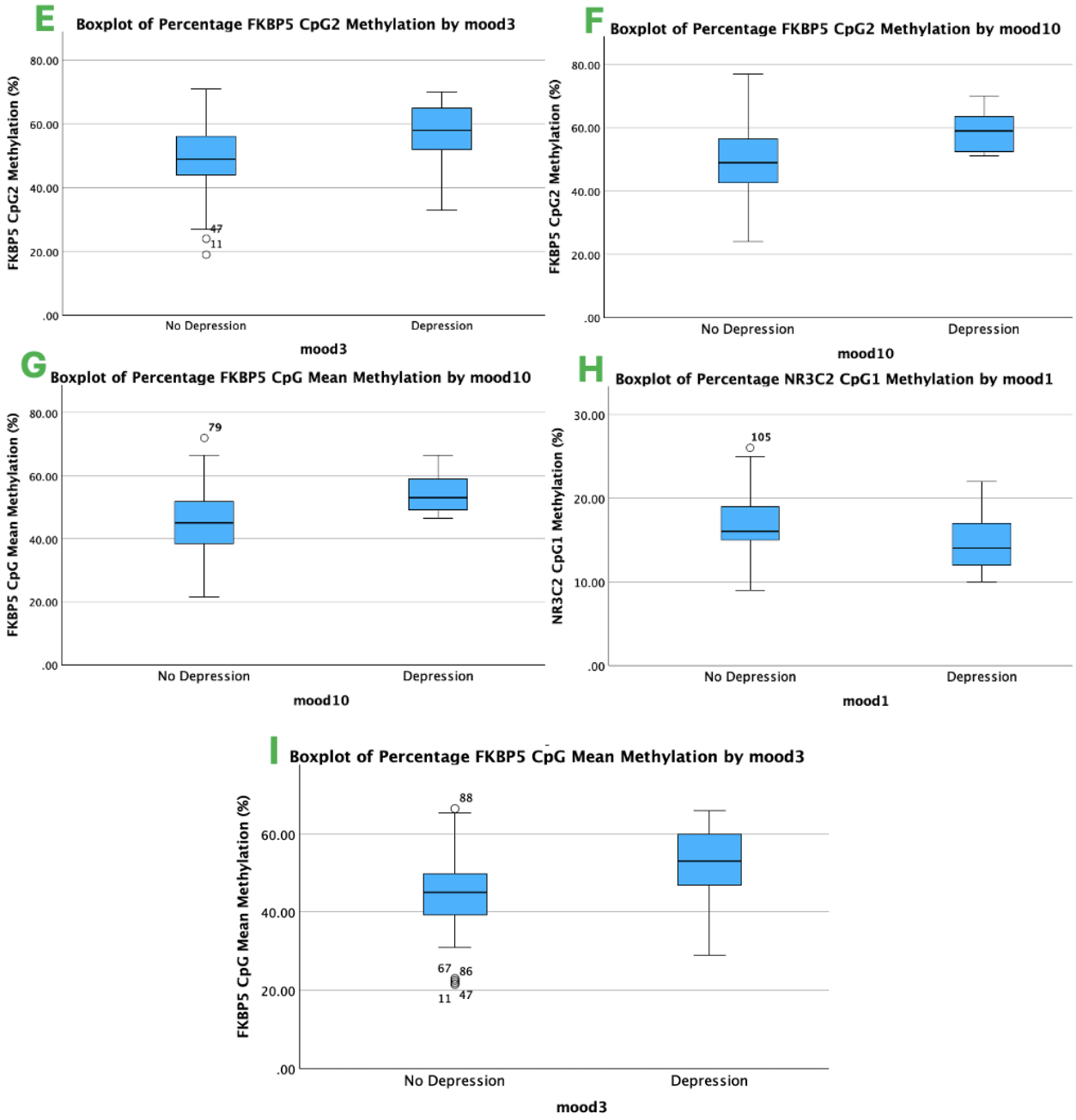

Figure 12: shows a continuation of **Figure 11** Including the box plots: E: mood3 vs FKBP5 CpG2 (correlation coefficient = 0.267, P = 0.007), F: mood10 vs FKBP5 CpG2 (correlation coefficient = 0.261, P = 0.046), G: mood10 vs FKBP5 CpG Mean (correlation coefficient = 0.263, P =0.044), H: mood1 vs NR3C2 CpG1 (correlation coefficient = -0.189, P = 0.043), I: mood3 vs FKBP5 CpG Mean (correlation coefficient = 0.252, P = 0.011). The central box represents the interquartile range (IQR), with the lower edge indicating the 25th percentile and the upper edge indicating the 75th percentile. The horizontal line within the box denotes the median methylation level for each group. Whiskers extend to 1.5 times the IQR from the box. Outliers (data points falling between 1.5 and 3 times the IQR) are represented by circles (○), while asterisks (*) denote extreme outliers (data points more than 3 times the IQR from the box). These points highlight participants with methylation levels that significantly deviate from the typical range observed in their respective groups, along with the MMU sample number.

Regression Analysis

Correlation analyses were initially conducted to examine the relationships between methylation, depression, sleep measures, and cortisol levels, with several significant associations identified. While these correlations provided valuable insights into the strength and direction of the relationships, they did not provide information about the predictive power or the influence of one variable over another. Regression analysis was performed on the significant correlations to gain a deeper understanding of the nature of these relationships allowing us to determine the extent to which methylation levels can predict sleep and cortisol outcomes, and vice versa. As well as understanding how much variance in the dependent variable is explained by the predictor (as indicated by the R-squared value), and the strength of the predictive relationship (through the regression coefficients). Logistic regression was initially considered as a potential method to explore the relationship between methylation and depression, as depression was a binary variable. However, after conducting preliminary analyses and reviewing the assumptions and requirements of logistic regression, it became evident that this approach was not suitable for our data. This is due to logistic regression assuming an adequate sample size and balanced distribution between the two categories of the binary outcome variable. In our dataset, there was a substantial imbalance within the distribution and an inadequate sample size between the depression and non-depression groups, which would have affected the reliability and accuracy of the model's estimates, leading to skewed predictions, which may not reflect the true relationship between the variables. This is likely due to the data being collected over 11 waves and many patients having missing data points. Therefore, we did not run a regression analysis on methylation vs depression. Prior to running the regression analysis all data that was not normally distributed was log transformed using the natural log transformation feature on SPSS. The following variables were transformed according to the results from the Shapiro-Wilk normality tests: Cort14, p1sleff, p6sleff, p2wakent, NR3C1 CpG1, NR3C1 CpG3, and NR3C2 CpG2. First the regression was run as percentage methylation as the dependent variable and the cortisol levels and sleep measure as the predictor variables. Then the regression was run in reverse direction as at this stage, it remains uncertain whether elevated cortisol levels/sleep quality influence methylation or if changes in methylation contribute to increased cortisol levels and poor sleep. Table 17 shows the results from the regression analysis.

Table 17

Regression Analysis						
Dependent Variable	**Predictor Variable**	**R**	**R^2**	**Adjusted R^2**	**ANOVA P value**	**Unstandardised B**
LOG_NR3C2_CpG2	cort30	0.509	0.259	0.232	0.005	0.014
LOG_NR3C2_CpG2	cort0	0.407	1.66	0.135	0.028	0.024
NR3C2_CpG_Mean	LOG_cort14	0.165	0.027	-0.006	0.375	1.332
LOG_NR3C1_CpG3	cort18	0.219	0.084	0.053	0.113	0.028
LOG_NR3C1_CpG_Mean	cort18	0.294	0.086	0.055	0.109	0.026
NR3C2_CpG_Mean	cort30	0.374	0.14	0.108	0.046	0.118
cort30	LOG_NR3C2_CpG2	0.509	0.259	0.232	0.005	18.123
cort0	LOG_NR3C2_CpG2	0.407	1.66	0.135	0.028	6.986
LOG_cort14	NR3C2_CpG_Mean	0.165	0.027	-0.006	0.375	0.02
cort18	NR3C2_CpG_Mean	0.219	0.084	0.053	0.113	3.06
cort18	LOG_NR3C1_CpG3	0.294	0.086	0.055	0.109	3.378
cort30	LOG_NR3C1_CpG_Mean	0.374	0.14	0.108	0.046	1.181
LOG_NR3C1_CpG3	LOG_p2wakent	0.303	0.092	0.077	0.015	-0.201
LOG_NR3C2_CpG2	LOG_p1sleff	1.65	0.027	0.019	0.071	-0.216
NR3C2_CpG1	LOG_p6sleff	0.277	0.077	0.067	0.007	-4.93
LOG_p2wakent	LOG_NR3C1_CpG3	0.303	0.092	0.077	0.015	-0.457
LOG_p1sleff	LOG_NR3C2_CpG2	1.65	0.027	0.019	0.071	-0.126
LOG_p6sleff	NR3C2_CpG1	0.277	0.077	0.067	0.007	-0.016

Table 17: shows the data obtained from SPSS. **Dependent Variable**: the variable that the model is trying to predict. **Predictor Variable**: The independent variable used in the regression model to explain changes in the dependent variable. **R**: is the correlation coefficient, indicating the strength and direction of the linear relationship between the dependent and predictor variables, ranging from -1 to 1 where 0 is no relationship. **R^2**: The coefficient of determination, representing the proportion of the variance in the dependent variable that can be explained by the predictor variables. It ranges from 0 to 1, with higher values indicating a better fit. **Adjusted R^2**: A version of R^2 that accounts for the number of predictor variables in the model, providing a more accurate measure of model fit. **ANOVA P value**: The p-value from the analysis of variance (ANOVA) test, indicating whether the overall regression model is statistically significant. **Unstandardised B**: The unstandardized regression coefficient, representing the amount by which the dependent variable is expected to change for a one-unit increase in the predictor variable (In the units of the original variables).

The regression analysis revealed significant relationships between LOG_NR3C2_CpG2 vs cort30 (P = 0.005), revealing a moderate positive correlation with an R value of 0.509, the R^2 value of 0.259 suggests that approximately 25.9% of the variability in NR3C2 CpG2 methylation levels can be explained by Cort30 with a similar adjusted R^2 value of 0.232. The unstandardised B coefficient for Cort30 was 0.014, indicating that for each unit increase in Cort30, the

percentage NR3C2 CpG2 methylation levels are predicted to increase by 0.014. To further explore the relationship, the regression was run in the opposite direction, where LOG_NR3C2_CpG2 predicts Cort30, the unstandardised B coefficient was 18.123. This means that for each unit increase in LOG_NR3C2_CpG2, Cort30 levels are expected to rise by 18.123 units, suggesting a strong positive relationship in the opposite direction. The regression model of LOG_NR3C2_CpG2 vs cort0 (P = 0.028) revealed a moderate positive correlation with an R value of 0.407. The R^2 value of 0.166 suggests that an estimated 16.6% of the variability in NR3C2 CpG2 percentage methylation levels is explained by Cort0. The adjusted R^2 value of 0.135 reflects a slight adjustment for the number of predictors in the model, showing that a slightly lower percentage of variance is explained when accounting for this. The unstandardised B coefficient for Cort0 was 0.024, predicting that for each unit increase in Cort0, the NR3C2 CpG2 percentage methylation will increase by 0.024. In the reverse regression, where LOG_NR3C2_CpG2 predicts Cort0, the unstandardised B coefficient was 6.986. This indicates that for each unit rise in LOG_NR3C2_CpG2, Cort0 levels are expected to increase by 6.986 units, highlighting a strong positive relationship in the opposite direction. The regression analysis of NR3C2_CpG_Mean vs cort30 was also significant (P = 0.046) with a slightly weaker moderate positive correlation with an R value of 0.374. The R^2 value of 0.14 indicates that 14% of the variance in NR3C2 CpG mean percentage methylation levels is explained by Cort30. The adjusted R^2 value of 0.108 predicts a slightly lower percentage of variance. The unstandardised B coefficient for Cort30 was 0.118, indicating that for each unit increase in Cort30, the mean NR3C2 CpG percentage methylation levels are expected to rise by 0.118. In the reverse regression, where NR3C2_CpG_Mean is used to predict Cort30, the unstandardised B coefficient was 1.181. This suggests that for each unit increase in NR3C2_CpG_Mean, Cort30 levels are expected to increase by 1.181 units, indicating a positive relationship in this direction as well.

The regression analysis of methylation and sleep revealed that LOG_NR3C1_CpG3 vs LOG_p2wakent had a significant relationship (P = 0.015). The R value of 0.303 indicates a weak positive correlation. The R^2 value of 0.092 suggests that 9.2% of the variability in LOG_NR3C1 CpG3 methylation levels is explained by LOG_p2wakent, with the adjusted R^2 value being slightly lower at 0.077. The unstandardised B coefficient for LOG_p2wakent was -0.201, indicating that for each unit increase of p2wakent, the NR3C1 CpG3 percentage methylation levels are predicted to decrease by 0.201 units, demonstrating a negative relationship. In the reverse regression, with LOG_NR3C1_CpG3 as the predictor and

LOG_p2wakent as the outcome, the unstandardised B coefficient was -0.457, suggesting that for each unit increase in LOG_NR3C1_CpG3, LOG_p2wakent decreases by 0.457 units, indicating a stronger negative association in this direction. The regression analysis of NR3C2_CpG1 vs LOG_p6sleff was also significant (P = 0.007). The model revealed a weak positive correlation with an R value of 0.277. The R^2 value of 0.077 suggests that 7.7% of the variability in NR3C2 CpG1 percentage methylation levels is explained by LOG_p6sleff, with a slightly lower adjusted R^2 value of 0.067. The unstandardised B coefficient for LOG_p6sleff was -4.93, indicating that for each unit increase in log-transformed p6sleff, the NR3C2 CpG1 methylation levels are predicted to decrease by 4.93 units, showing a negative relationship between these variables. In the reverse regression analysis we examined NR3C2 CpG1 methylation levels as the predictor variable and LOG_p6sleff as the outcome variable. In this analysis, the unstandardised B coefficient was -0.016, meaning that for each unit increase in NR3C2 CpG1 percentage methylation, the LOG_p6sleff is predicted to decrease by 0.016 units, also reflecting a weak negative relationship. Figure 13 below shows the P-P plots generated on SPSS for the regressions.

Figure 13

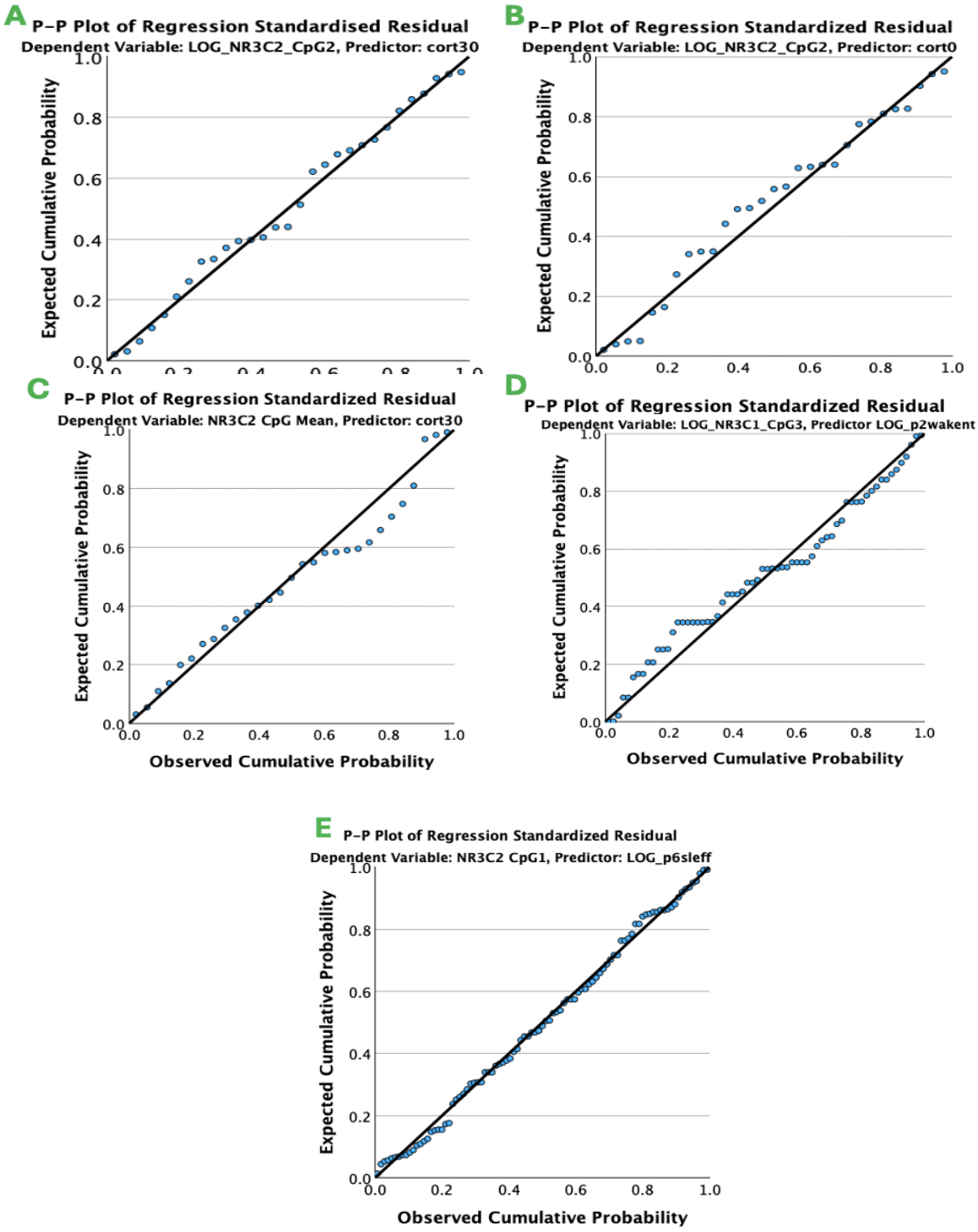

Figure 13: shows P-P plots of the cumulative probability of the standardised residuals against a normal distribution. The diagonal line reflects the expected cumulative probability for a normal distribution. Data points that align closely with this line suggest normality, while deviations indicate potential departures from normality. **A:** LOG_NR3C2_CpG2 vs cort30 (P = 0.005) **B:** LOG_NR3C2_CpG2 vs cort0 (P = 0.028) **C:** NR3C2_CpG_Mean vs cort30 (P = 0.046) **D:** LOG_NR3C1_CpG3 vs LOG_p2wakent (P = 0.015) **E:** NR3C2_CpG1 vs LOG_p6sleff (P = 0.007)

Discussion

Main Findings

To the extent of our knowledge, this is first study to quantitatively analyse DNA methylation in human frontal cortex brain samples to identify the percentage methylation in the promoter region of the 3 stress related genes: NR3C1, FKBP5 and NR3C2 along with over 20 years of longitudinal data on the participants, including cortisol levels, sleep measures and depression scores.

The Relationship Between Sleep and Methylation

This study explored the relationships between DNA methylation in stress-related genes and sleep patterns in healthy individuals and individuals with dementia. The findings revealed significant correlations and regression outcomes, suggesting an intricate interplay between sleep quality and the methylation status of these genes, potentially influencing cognitive decline. The correlation analysis demonstrated significant negative monotonic correlations between sleep efficiency metrics and methylation levels at specific CpG sites. Specifically, sleep efficiency (p1sleff) showed a significant negative correlation with NR3C2 CpG2 methylation (rho = -0.19, P = 0.038), and sleep efficiency (p6sleff) also correlated negatively with NR3C2 CpG1 (rho = -0.23, P = 0.027). Additionally, p2wakent exhibited a negative relationship with NR3C1 CpG3 (rho = -0.237, P = 0.026). The regression analysis of the variables that revealed significant correlations provided more in depth information regarding the relationships between methylation of NR3C1 and NR3C2 and sleep quality. The regression analysis between LOG_NR3C1_CpG3 and LOG_p2wakent revealed a significant relationship (P = 0.015), characterised by a weak positive correlation (R = 0.303). The R^2 value of 0.092 indicates that 9.2% of the variability in NR3C1 CpG3 methylation can be explained by sleep disturbance as

measured by p2wakent. The unstandardised B coefficient of -0.201 indicates a negative relationship, meaning that for each unit increase in p2wakent, NR3C1 CpG3 methylation levels are predicted to decrease by 0.201 units. Comparable findings have been reported in a prospective mother-infant cohort, where chronic maternal sleep disturbance predicted lower NR3C2-promoter methylation in both mothers and neonates, pointing to a bidirectional sleep-mineralocorticoid-receptor epigenetic pathway (Lin et al., 2022). These findings align with the previous studies which have suggested that hypomethylation in the NR3C1 promoter region could result in increased glucocorticoid receptor expression resulting in a sensitisation of the HPA axis (Palma-Gudiel et al., 2015) which can lead to the response system becoming more reactive. Although this does not align with what we were expecting to find, there is still evidence that this overactive stress response could lead to prolonged inflammation which could contribute to cognitive decline (Dobernecker et al., 2023). In the reverse regression, where LOG_NR3C1_CpG3 serves as the predictor for LOG_p2wakent, the unstandardised B coefficient was -0.457, indicating that each unit increase in NR3C1 CpG3 methylation corresponds to a decrease of 0.457 units in sleep disturbance. This stronger negative association suggests that as methylation levels rise, the severity of sleep disturbances may lessen. Thus indicating that epigenetic modifications potentially influence sleep quality or vice versa.

Similarly, the analysis of NR3C2_CpG1 and sleep efficiency yielded significant results (P = 0.007), revealing a weak positive correlation (R = 0.277). The R^2 value of 0.077 indicates that 7.7% of the variability in NR3C2 CpG1 methylation is explained by sleep efficiency (p6sleff). This further emphasises the multifaceted relationship between sleep and methylation patterns in stress-response genes. The negative unstandardised B coefficient of -4.93 suggests a substantial negative relationship, where each unit increase in log-transformed p6sleff predicts a decrease of 4.93 units in NR3C2 CpG1 methylation levels. This could suggest that improved sleep quality could result in increased NR3C2 expression in order to improve stress response which could be dysregulated due to irregularities in cortisol as a result of a disrupted circadian rhythm. In the reverse regression analysis, the unstandardised B coefficient was -0.016, indicating that each unit increase in NR3C2 CpG1 methylation predicts a decrease of 0.016 units in sleep efficiency. Although this relationship is weaker, it reinforces the prior notion, creating a potential feedback loop where sleep disturbances exacerbate epigenetic changes, which in turn affect sleep (Anderson et al., 2021).

These analyses indicate that while there is a measurable relationship, they are relatively modest. This could be due to the fact that the sleep data was collected via a self-reported sleep diary which introduces inaccuracies as well as a decreased sample size as some individuals did not complete the diaries. The smaller sample size may have not adequately captured the full range of variability in both sleep quality and methylation patterns, resulting in weaker correlations. Objective measures, for example polysomnography, could provide more consistent and reliable data, however can be less practical in large studies such as the University of Manchester Longitudinal Ageing Study. Additionally, DNA methylation is influenced by various biological factors, including genetic predispositions, environmental stressors, and lifestyle choices. The combination of these factors could have diluted the strength of the correlation with sleep disturbances.

Overall the findings from these analyses highlight the complex interplay between sleep quality and stress regulation through the epigenetic mechanisms governing the HPA axis involved NR3C1 and NR3C2 genes. As methylation in the promoter regions of these genes can cause transcriptional repression, the results suggest that altered sleep patterns may exacerbate the dysregulation of stress-related pathways associated with dementia (Giallongo et al., 2022). Given that dysregulation of the HPA axis is associated with both impaired sleep and cognitive decline (Jones and Gwenin, 2021), understanding these relationships is crucial. Our findings indicate that interventions aimed at improving sleep quality may help modulate the epigenetic landscape of these genes, potentially offering a preventive strategy against stress-related cognitive decline.

The Relationship Between Cortisol and Methylation

The findings from this study have identified a series of significant positive correlations between cortisol levels and the methylation status of specific CpG sites within NR3C1 and NR3C2 genes. These results provide new insights which may imply that stress regulation pathways influence epigenetic modifications in genes related to the HPA axis and hence may relate to cognitive decline, as both cortisol dysregulation and altered methylation patterns are implicated in dementia progression (Poon et al., 2020).

The significant positive correlations between methylation at NR3C1 and NR3C2 CpG sites and various cortisol timepoints (Cort0, Cort30, etc.) suggest that increased cortisol levels may be linked with increased methylation at these genes involved with stress-responsive pathways. Specifically, NR3C2 CpG2 methylation was modest to moderately positively correlated with cortisol at both baseline and 30 minutes post-awakening (correlation coefficient = 0.373 and correlation coefficient = 0.497, respectively). Similarly, methylation of NR3C1 CpG mean (Correlation coefficient = 0.401) showed significant moderate positive correlations with cortisol at Cort18, further reinforcing the role of these genes in stress response. This pattern mirrors previous work that demonstrated that adults with an exaggerated cortisol-awakening response also exhibited site-specific NR3C1 hypermethylation, suggesting a conserved link between morning cortisol peaks and GR-promoter methylation (Labonté et al., 2014).

The regression analysis revealed significant relationships between NR3C2 CpG2 and cort30 (P = 0.005), revealing a moderate positive correlation with an (R = 0.509), with approximately 23-25.9% of the variability in NR3C2 CpG2 methylation levels explained by Cort30. We found that for each unit increase in Cort30, the percentage NR3C2 CpG2 methylation levels are predicted to increase by 0.014. Whilst for each unit increase in NR3C2 CpG2 methylation, Cort30 levels are expected to rise by 18.123 units, suggesting a strong positive relationship in the opposite direction. The results for NR3C2 CpG2 vs cort0 and NR3C2 CpG mean vs cort30 were similar (R = 0.407 and R = 0.374, respectively). 13.5-16.6% of the variability in NR3C2 CpG2 percentage methylation levels is explained by Cort0, whilst 10.8-14% of the variance in NR3C2 CpG mean percentage methylation levels is explained by Cort30. We found that each unit increase in Cort0, the NR3C2 CpG2 percentage methylation will increase by 0.024 and for each unit rise in NR3C2 CpG2, Cort0 levels are expected to increase by 6.986 units, highlighting a strong positive relationship in the opposite direction. Similarly, we found that for each unit increase in Cort30, the mean NR3C2 CpG percentage methylation levels are expected to rise by 0.118 and each unit increase in NR3C2 CpG mean, Cort30 levels are expected to increase by 1.181 units, indicating a positive relationship in this direction as well.

The regression analysis also revealed a significant relationship between NR3C1 and p2wakent (R = 0.303) the R^2 value of 0.092 suggests that 9.2% of the variability in LOG_NR3C1 CpG3 methylation levels is explained by LOG_p2wakent, with the adjusted R^2 value being slightly lower at 0.077. The unstandardised B coefficient for LOG_p2wakent was -0.201, indicating that

for each unit increase of p2wakent, the NR3C1 CpG3 percentage methylation levels are expected to decrease by 0.201 units, demonstrating a negative relationship. In the reverse regression, analysis suggested that for each unit increase in LOG_NR3C1_CpG3, LOG_p2wakent decreases by 0.457 units, indicating a stronger negative association in this direction. Interestingly, a recent study discovered that lower methylation levels found in the promoter region of the NR3C1 gene were associated with a higher perception of stress and a decrease in perceived control and performance within the mouse population (Dee et al., 2023).

The percentage of variance explained by cortisol ranged from moderate to modest indicate that cortisol levels contribute to, but do not fully account for, the observed methylation changes and vice versa. Additionally, both the correlations (Spearman's and Pearson's) regression correlations are statistically significant but moderate in strength which could largely be due to the small availability of salivary cortisol levels for the individuals in this study as well as the complex variety of influential factors affecting methylation.

Overall the findings support the literature that NR3C1 and NR3C2 are key regulators of the HPA axis (Qing et al., 2021) and methylation of these genes could result in decreased expression and therefore result in an impaired stress response which can result in elevated cortisol levels and increased risk of neurodegeneration (Russell and Lightman, 2019). These links between chronic stress, elevated cortisol levels, and cognitive decline are well-documented, and this study provides further evidence that epigenetics of stress-related genes play a role in elevated cortisol levels which could potentially play a critical role in the pathophysiology of dementia. Additionally, the strong bidirectional relationship between methylation and cortisol. This indicates that not only do elevated cortisol levels potentially influence methylation patterns, but altered methylation may also affect cortisol regulation, potentially exacerbating the dysregulation of the HPA axis observed in dementia patients. Hence, highlighting the need for new therapeutic strategies that modulate methylation at these loci could potentially restore proper stress response function and alleviate the damaging effects of chronic cortisol exposure.

The Relationship Between Depression and Methylation

The results from Spearman's and Pearson's correlation analysis have provided intriguing insights into the relationship between DNA methylation at key stress-related genes and depression in individuals with dementia. Statistically significant correlations were observed between methylation at specific CpG sites within NR3C1, FKBP5, and NR3C2, and measures of depression (MSEV and mood scores).

Specifically, msev8 was negatively correlated with NR3C1 CpG1 (correlation coefficient = -0.2, P = 0.035) and the NR3C1 CpG Mean (correlation coefficient = -0.211, P = 0.034). These findings suggest that higher levels of depression (as indicated by MSEV scores) are associated with lower methylation levels at the NR3C1 promoter, which may result in increased expression of the glucocorticoid receptor and hypersensitivity of the HPA axis.

Given that chronic stress and elevated cortisol levels are associated with depression, cognitive decline and dementia progression, this reduction in NR3C1 methylation may play a key role in the stress-related neurodegeneration observed in these individuals. However, the modest correlation coefficients indicate that additional factors, such as genetic predispositions or environmental influences, likely contribute to this relationship as well as the limitations introduced by the small sample size.

The FKBP5 gene, which acts as a co-chaperone for the glucocorticoid receptor and modulates its sensitivity to cortisol, also demonstrated significant correlations with depression scores. Notably, mood3 and mood10 scores showed positive correlations with FKBP5 CpG1 and CpG2 methylation. For instance, mood3 was positively correlated with FKBP5 CpG1 (correlation coefficient = 0.231, P = 0.021) and CpG2 (correlation coefficient = 0.267, P = 0.007), while mood10 also correlated positively with both CpG sites (CpG1, correlation coefficient = 0.256, P = 0.05; CpG2, correlation coefficient = 0.261, P = 0.046). The FKBP5 CpG Mean values further confirmed this trend, showing significant correlations with both mood3 and mood10 scores.

These results suggest that higher depressive symptoms are linked to increased methylation of FKBP5, which may reduce the gene's ability to regulate NR3C1 sensitivity. Increased FKBP5 methylation could impair the negative feedback loop of the HPA axis, leading to prolonged

cortisol exposure, which is known to contribute to both depression and neurodegeneration. This finding is consistent with the hypothesis that FKBP5 plays a crucial role in the stress response, particularly in how chronic stress may influence the development of depression and cognitive decline, which may contribute to the broader pathophysiological landscape of dementia. These patterns are consistent with large population studies: Großmann et al. (2024) showed that higher FKBP5 CpG methylation was associated with greater depressive-symptom burden, while Li et al. (2023) demonstrated sex-specific links between FKBP5 methylation and adolescent depression scores, reinforcing the relevance of our FKBP5 findings.

The NR3C2 gene exhibited a significant negative correlation between mood1 and NR3C2 CpG1 methylation (correlation coefficient = -0.189, P = 0.043). This suggests that lower methylation at this site is associated with higher depressive symptoms. The NR3C2 gene plays a key role in regulating the body's response to cortisol, particularly in modulating the stress response.

The negative correlation here might imply that reduced methylation at NR3C2 increases receptor expression, possibly altering the balance between NR3C1 and NR3C2 activity in the brain. This imbalance could lead to dysregulation of the HPA axis and exacerbate depressive symptoms in individuals with dementia. These findings further underscore the importance of understanding how epigenetic modifications in stress-related genes contribute to the pathophysiology of both depression and dementia (Dafsari and Jessen, 2020).

Overall, the significant correlations between depression measures and methylation at specific CpG sites within NR3C1, FKBP5, and NR3C2 reinforce the role of stress-related epigenetic mechanisms in the comorbidity of depression and dementia. These findings are particularly relevant given the well-established link between chronic stress, depression, and neurodegeneration. However, once again the modest correlation coefficients suggest that other factors, such as those previously referenced may also be influencing these relationships. Future research should aim to explore the broader methylation landscape, incorporating larger sample sizes and examining additional epigenetic mechanisms, such as histone modifications or non-coding RNA activity, to gain a more comprehensive understanding of how stress, depression, and neurodegeneration interact on a molecular level. Given that depression is widely considered a modifiable risk factor for dementia, early interventions targeting stress-related pathways, such as through antidepressant treatment or lifestyle changes, could offer promising strategies to mitigate the risk of dementia onset and progression (Fernández et

al., 2024). This is of particular importance as studies have found that changes in mood have been found up to 20 years prior to the onset of the typically clinical symptoms of cognitive decline (Caselli et al., 2020). Overall, these findings offer valuable insights into the potential mechanisms linking depression and cognitive decline, paving the way for future research aimed at identifying new therapeutic targets for dementia prevention and treatment.

Conclusions and Future Directions

This study contributes to the growing body of evidence that links cortisol dysregulation with epigenetic modifications to the stress-related NR3C1, FKBP5 and NR3C2. Although there were no significant findings between methylation levels vs dementia and control groups (likely due to limited sample population), there were many other statistically significant relationships revealed. The significant positive correlations observed between cortisol levels and methylation suggest that chronic stress could alter gene expression through methylation and therefore promote dementia progression. Along with relationships between methylation and sleep quality and depression which are also closely linked to chronic stress. Thus, highlighting the value of implementing therapeutic and lifestyle changes to help manage chronic stress and the targeting of epigenetic pathways to aid in prevention and mitigation of neurodegeneration. Additional research is needed to investigate the therapeutic possibilities of modulating DNA methylation in pathways associated with stress. However, the various relatively modest correlation coefficients imply that other factors such as genetic predispositions or other environmental influences, are likely involved in the observed epigenetic changes. Future studies should investigate the broader methylation landscape, utilising larger sample sizes and assessing other epigenetic modifications (such as histone modifications or microRNA expression) to enhance our understanding of the mechanisms driving cognitive decline in the context of stress-related neurodegeneration.

Ultimately, our study has identified multiple statistically significant relationships between stress-related genes promoter methylation and sleep, cortisol and depression which aligns with the supporting literature, emphasising the importance of targeting sleep health, chronic stress and depression as part of a comprehensive approach to reduce the risk of cognitive impairment

and dementia. Stress plays a significant role in dementia and a comprehensive understanding of its molecular mechanisms are crucial for disease management through lifestyle modifications, stress management, or pharmacological interventions. The study population has benefitted from the novel insights produced from this study gaining deeper knowledge into the role of epigenetic mechanisms in dementia patients. We have shown how methylation of stress-related genes can potentially contribute to dementia pathogenesis through its role in various risk factors. These findings can guide the development of further research and targeted therapeutic approaches and personalised healthcare aimed at mitigating stress-related cognitive decline in people with dementia. Further longitudinal studies are needed to elucidate these complex relationships. This bidirectional relationship shown by the regression analysis highlights the complexity of the interactions between these potential risk factors and DNA methylation. Indicating that both factors may influence one another and warrant further investigation. Moreover, as methylation is influenced by both genetic and environmental factors, interventions that target modifiable risk factors, such as reducing chronic stress, improving sleep quality, and promoting healthy lifestyle choices, may help prevent or delay the onset of dementia (Milligan Armstrong et al., 2021; Yang et al., 2022). The future identification of specific CpG sites that correlate with cortisol levels provides a potential biomarker for stress-related cognitive decline, which could be monitored and targeted in future therapeutic approaches. Peripheral blood, saliva, or buccal cells could be taken or swabbed from patients to analyse their methylation profile, although direct samples from the brain exist as a post-mortem option only, some HPA-axis sites (e.g. NR3C1, FKBP5) show moderate blood-brain correlation that could still be utilised as potential biomarkers of brain methylation. Given the role of NR3C1, FKBP5 and NR3C2 in the HPA axis, these therapeutic strategies that modulate methylation at these loci could potentially restore proper stress response function and alleviate the damaging effects of chronic cortisol exposure.

However, our analysis does not determine the relationships directions so we remain unsure whether methylation affects sleep, cortisol and depression levels or whether it is in fact the other way round or both directions simultaneously. This study has benefited by being able to use human brain samples by providing findings with high biological relevance, however it is also limited by being a post mortem study which prevents us to be certain at what point the epigenetic changes were introduced. Therefore, for the future directions of this study it would be beneficial to use a dementia animal model such as the APP23 Alzheimer's mouse model. This would allow use to conduct the study in multiple waves, enabling us to assess methylation

levels at multiple time points as well as implement different test groups such as a chronic stress group (such as exposing them to chronic variable stress), a reduced sleep quality groups (e.g. by introducing sleep deprivation techniques), and a depression group (e.g. by implementing neurotransmitter manipulation) and control group. This experimental design would allow us to build on our findings and elucidate the nature and direction of the relationship between DNA methylation and sleep, cortisol and depression in a mouse model, with the specific aim of determining whether changes in DNA methylation are a causal factor in these variables or if the variables themselves initiate alterations in methylation patterns. Through tracking cortisol levels, methylation patterns, and cognitive decline over time we could potentially establish causal relationships and identify the most critical windows for intervention.

Limitations

Although all the frozen brain samples were obtained from the frontal cortex, their precise position and orientation within this region are unknown. This introduces issues when making direct comparisons between samples, as slight differences in location within the frontal cortex may affect the molecular data. Additionally, the presence of blood vessels and other non-neuronal tissue components means that when sectioning the ~25 mg samples for DNA extraction, there is a risk of variability in tissue composition, which could impact the consistency of the epigenetic analysis. This could be mitigated by implementing alternative techniques such as precise dissection through stereotactic methods or laser capture microdissection (LCM) which would enable us to target more specific and consistent regions within the frontal cortex. Thus, reducing the variability introduced through unknown positioning by obtaining samples from comparable areas.

Another key limitation in this study is introduced through bisulfite pyrosequencing, as it is unable to distinguish between methylated cytosine and hydroxymethylated cytosine, leading to potentially inaccurate readings of methylation levels in samples. It is also possible that some cytosines were not efficiently converted during the bisulfite treatment which could also cause inaccuracies in methylation results. Additionally, the analysis could be subject to PCR bias which could skew methylation quantification. This could be avoided by utilising new technologies for DNA sequencing such as Nanopore technology which can read DNA in its pure form free of PCR and bisulfite bias.

Furthermore, this study did have a moderately large amount of missing data from the longitudinal factors as well as using some objective measure such as self-reported sleep diaries. These factors could have impacted the statistical analysis through introducing inaccuracies in the relationships. To prevent this a larger sample population could be obtained as well as ensuring non-objective measures were used in data collection such as utilising polysomnography.

Perhaps the largest limitation of this study is the sample size. Although 125 brains with over 20 years of longitudinal data is a large population for a study with this design, it is still relatively low when conducting statistical analysis and could be a major contributing factor to the lack of significance found between methylation levels vs dementia and control. It must also be considered that there was a large amount of statistical tests conducted across methylation, cortisol, sleep and depression variables, whilst only a small subset achieved nominal significance, it is statistically plausible that some of these findings represent type I errors that arose by chance. Therefore it is possible to also interpret all $P < 0.05$ associations as exploratory signals rather than definitive effects. Replication in an independent, better-powered cohort, ideally with more complete longitudinal records, will be essential to confirm which of the reported associations are robust. Future studies should aim to use a larger study population such as $n = 1000$, therefore enabling the study to conduct more accurate statistical analysis and uncover the precise role of methylation in stress-related genes.

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Appendix

Nanodrop DNA Quantification

Table 18

MMU Sample no.	Nucleic Acid (ng/uL)	A260/A280	A260/A230	A260	A280	Baseline Absorbance
1	50.6	1.823	1.722	1.121	0.556	-0.078
2	30.2	1.791	0.843	0.571	0.338	0.098
3	30.377	1.809	1.816	0.608	0.336	-0.02
4	40.002	1.902	1.892	0.8	0.421	-0.034
5	20.624	1.828	1.77	0.412	0.226	-0.048
6	22.845	1.799	1.614	0.457	0.254	-0.039
7	73.624	1.846	2.142	1.472	0.798	-0.059
8	25.582	1.881	1.841	0.512	0.272	0.008
9	54.86	1.998	2.008	1.097	0.549	0.03
10	44.832	1.913	1.91	0.897	0.469	0.004
11	24.02	1.843	1.664	0.48	0.261	-0.06
12	61.257	1.891	2.084	1.225	0.648	-0.031
13	43.856	1.936	1.809	0.877	0.453	-0.029
14	27.95	1.876	1.73	0.559	0.298	-0.06
15	88.177	1.891	2.042	1.764	0.933	-0.01
16	37.962	1.919	1.829	0.759	0.396	-0.03
17	18.86	1.783	1.681	0.377	0.212	-0.043
18	44.448	2.002	1.934	0.889	0.444	-0.049
19	51.693	1.912	1.889	1.034	0.541	-0.074
20	18.119	1.698	1.367	0.362	0.213	-0.019
21	27.588	2.027	1.802	0.552	0.272	-0.041
22	29.461	1.805	1.399	0.589	0.326	0.037
23	151.302	2.016	2.129	3.026	1.501	-0.058
24	48.406	1.914	2.003	0.968	0.506	-0.101
25	59.702	1.935	1.884	1.194	0.617	0.042
26	53.698	1.916	1.819	1.074	0.561	0.09
27	75.931	1.891	1.586	1.519	0.803	0.486
28	31.006	1.864	1.842	0.62	0.333	0.07
29	104.103	1.801	1.451	2.082	1.156	0.642

30	56.107	1.957	1.957	1.122	0.573	-0.032
31	91.76	1.961	1.793	1.835	0.936	0.43
32	96.756	1.985	1.807	1.935	0.975	0.379
33	56.272	1.89	1.503	1.125	0.595	0.299
34	86.146	1.763	1.229	1.723	0.977	1.286
35	73.102	1.917	1.568	1.462	0.763	0.244
36	56.774	1.9	1.61	1.135	0.598	0.296
37	65.78	1.886	1.592	1.316	0.698	0.325
38	108.133	1.957	1.822	2.163	1.105	0.434
39	81.999	1.719	1.127	1.64	0.954	1.242
40	59.039	1.702	1.147	1.181	0.694	1.069
41	70.916	1.754	1.223	1.418	0.809	0.945
42	45.169	1.844	1.399	0.903	0.49	0.412
43	34.228	1.915	1.904	0.685	0.357	0.106
44	69.751	1.883	1.623	1.395	0.741	0.495
45	88.497	1.856	1.416	1.77	0.953	0.904
46	68.724	1.838	1.377	1.374	0.748	0.674
47	63.304	1.883	1.518	1.266	0.672	0.404
48	61.44	1.709	1.175	1.229	0.719	0.861
49	98.512	1.855	1.421	1.97	1.062	0.882
50	76.481	1.961	1.944	1.53	0.78	0.098
51	62.769	1.961	1.928	1.255	0.64	0.2
52	58.906	1.917	2.136	1.178	0.615	0.088
53	49.186	1.956	2.121	0.984	0.503	0.143
54	108.634	1.737	1.429	2.173	1.251	1.581
55	109.388	2.022	2.066	2.188	1.082	0.203
56	67.244	1.863	1.478	1.345	0.722	0.65
57	48.933	1.901	1.654	0.979	0.515	0.358
58	45.265	2.051	2.185	0.905	0.441	0.101
59	72.846	1.985	1.961	1.457	0.734	0.239
60	91.677	1.853	1.736	1.834	0.989	0.56
61	45.523	2.031	2.233	0.91	0.448	0.136
62	57.522	1.851	1.599	1.15	0.621	0.451
63	77.835	1.888	1.922	1.557	0.824	0.349
64	84.821	1.826	1.385	1.696	0.929	0.978
65	75.614	1.884	1.835	1.512	0.803	0.392

66	46.101	2.001	2.196	0.922	0.461	0.151
67	54.76	1.989	2.054	1.095	0.551	0.158
73	58.898	1.911	1.593	1.178	0.616	0.504
74	61.537	1.834	1.437	1.231	0.671	0.609
75	60.327	1.698	1.09	1.207	0.711	1.422
76	79.027	1.802	1.339	1.581	0.877	1.095
77	55.376	1.93	1.733	1.108	0.574	0.484
78	58.447	2.021	2.162	1.169	0.578	0.053
79	80.721	1.932	2.12	1.614	0.836	0.049
80	56.305	2.023	2.152	1.126	0.557	0.152
81	64.542	1.986	2.155	1.291	0.65	0.052
82	51.192	2.018	2.149	1.024	0.507	0.052
83	64.302	2.04	2.076	1.286	0.63	0.107
84	64.199	2.022	2.214	1.284	0.635	0.094
85	49.991	2.015	2.046	1	0.496	0.026
86	32.874	2.083	2.16	0.657	0.316	0.097
87	59.922	2.091	2.128	1.198	0.573	0.144
88	43.984	2.069	2.219	0.88	0.425	0.102
89	36.976	1.957	1.633	0.74	0.378	0.26
90	66.037	2.042	2.176	1.321	0.647	0.093
91	63.977	1.958	2.218	1.28	0.653	0.062
92	64.934	1.993	2.13	1.299	0.651	0.071
93	67.756	2.014	1.982	1.355	0.673	0.107
94	38.212	2.004	2.156	0.764	0.381	0.05
95	85.334	2.058	2.203	1.707	0.829	0.113
96	54.923	1.973	2.156	1.098	0.557	0.185
97	43.711	1.971	2.15	0.874	0.443	0.012
98	51.472	1.997	2.043	1.029	0.516	0.078
99	81.244	2.062	2.145	1.625	0.788	0.049
100	47.962	2.061	2.253	0.959	0.465	0.166
101	30.16	2.054	2.076	0.603	0.294	0.143
102	50.645	1.979	2.125	1.013	0.512	0.138
103	50.393	1.967	2.016	1.008	0.512	0.052
104	119.072	1.966	2.181	2.381	1.211	0.191
105	54.541	2.004	2.01	1.091	0.544	0.091
106	59.525	2.035	2.054	1.191	0.585	0.186

107	65.873	1.843	1.429	1.317	0.715	0.359
108	78.837	1.764	1.357	1.577	0.894	1.374
109	58.456	1.913	1.632	1.169	0.611	0.231
110	48.267	1.873	1.575	0.965	0.515	0.23
111	57.619	1.742	1.188	1.152	0.662	0.75
112	88.241	1.759	1.282	1.765	1.003	0.974
113	74.337	1.831	1.424	1.487	0.812	0.609
114	50.49	1.88	1.9	1.01	0.537	0.075
115	84.021	1.881	1.645	1.68	0.893	0.581
116	77.354	1.884	1.53	1.547	0.821	0.541
117	69.398	1.844	1.645	1.388	0.753	0.378
118	24.408	1.817	1.456	0.488	0.269	0.137
119	91.909	1.838	1.689	1.838	1	0.649
120	74.544	1.915	1.703	1.491	0.779	0.535
121	92.822	1.84	1.618	1.856	1.009	0.889
122	56.589	1.89	1.722	1.132	0.599	0.241
123	53.46	1.953	1.966	1.069	0.547	-0.046
124	48.077	1.964	1.938	0.962	0.489	0.073
125	71.237	1.896	1.817	1.425	0.752	0.2
126	126.749	1.787	1.456	2.535	1.419	1.321
127	94.558	1.942	2.081	1.891	0.974	0.114
128	64.349	1.903	1.898	1.287	0.676	0.157
129	70.091	1.88	2	1.402	0.746	0.202
130	88.003	1.82	1.45	1.76	0.967	0.883

Table 18: shows the results from the DNA Quantification Using Nanodrop along with the corresponding MMU sample number. Nucleic Acid (ng/μL) is the concentration of nucleic acids in the sample. A260/A280 is the ratio of absorbance at 260 nm to 280 nm, which assesses purity of the nucleic acid. A ratio around 1.8 approximately indicates pure DNA. A260/A230 is the ratio of absorbance at 260 nm to 230 nm, which also assesses purity. Ratios between 2.0 and 2.2 are considered free from contamination. A260 represents the absorbance at 260 nm (nucleic acids absorption wavelength), used to calculate concentration. A280 is the absorbance at 280 nm, used to assess protein contamination. Baseline Absorbance is the absorbance value measured at the baseline correction wavelength which is subtracted from the sample readings to maintain accurate measurements.

Qubit DNA Quantification

Table 19

MMU Sample no.	Qubit tube conc. (ng/mL)	Original sample conc. (ng/mL)	Sample Volume (µL)
1	227	45.4	1
2	63.1	12.6	1
3	82.9	16.6	1
4	307	61.4	1
5	78.6	15.7	1
6	73.3	14.7	1
7	409	81.8	1
8	144	28.8	1
9	67.3	13.5	1
10	221	44.2	1
11	58.8	11.8	1
12	79	15.8	1
13	56.2	11.2	1
14	52.2	10.4	1
15	105	21	1
16	95.5	19.1	1
17	70.7	14.1	1
18	83	16.6	1
19	98	19.6	1
20	237	47.4	1
21	85.4	17.1	1
22	132	26.4	1
23	58.6	11.7	1
24	126	25.2	1
25	93.9	18.8	1
26	99.6	19.9	1
27	118	23.6	1
28	82.5	16.5	1
29	88.6	17.7	1
30	104	20.8	1
31	109	21.8	1

32	97.5	19.5	1
33	261	52.2	1
34	87.7	17.5	1
35	72.6	14.5	1
36	124	24.8	1
37	84	16.8	1
38	114	22.8	1
39	121	24.2	1
40	19.2	13.84	1
41	79.9	16	1
42	105	21	1
43	98.9	19.8	1
44	103	20.6	1
45	89.5	17.9	1
46	103	20.6	1
47	113	22.6	1
48	120	24	1
49	105	21	1
50	125	25	1
51	278	55.6	1
52	122	24.4	1
53	176	35.2	1
54	136	27.2	1
55	120	24	1
56	91.5	18.3	1
57	74.5	14.9	1
58	118	23.6	1
59	90.9	18.2	1
60	187	37.4	1
61	89.5	17.9	1
62	63.5	12.7	1
63	268	53.6	1
64	75	15	1
65	103	20.6	1
66	93.5	18.7	1
67	188	37.6	1

73	89.3	17.9	1
74	73.4	14.7	1
75	114	22.8	1
76	77.9	15.6	1
77	119	23.8	1
78	190	38	1
79	142	28.4	1
80	280	56	1
81	100	20	1
82	84.8	17	1
83	77	15.4	1
84	138	27.6	1
85	141	28.2	1
86	81.3	16.3	1
87	107	21.4	1
88	133	26.6	1
89	135	27	1
90	182	36.4	1
91	121	24.2	1
92	87.1	17.4	1
93	103	20.6	1
94	77	15.4	1
95	98.9	19.8	1
96	127	25.4	1
97	131	26.2	1
98	153	30.6	1
99	131	26.2	1
100	130	26	1
101	75.7	15.1	1
102	116	23.2	1
103	71.4	14.3	1
104	268	53.6	1
105	84.2	16.8	1
106	57.5	11.5	1
107	114	22.8	1
108	226	45.2	1

109	110	22	1
110	124	24.8	1
111	118	23.6	1
112	187	37.4	1
113	133	26.6	1
114	202	40.4	1
115	153	30.6	1
116	118	23.6	1
117	204	40.8	1
118	78.4	15.7	1
119	291	58.2	1
120	130	26	1
121	237	47.4	1
122	169	33.8	1
123	121	24.2	1
124	215	43	1
125	346	69.2	1
126	97	19.4	1
127	263	52.6	1
128	168	33.6	1
129	261	52.2	1
130	225	45	1

Table19: shows the results from the Qubit DNA Quantification. Qubit tube concentration (ng/mL) shows the DNA concentration measured in the diluted sample prepared in the Qubit assay tube. Original sample concentration (ng/mL) represents the estimated DNA concentration in the original sample calculated by the Qubit reading. Sample volume (µL) indicates the amount of the original sample used for the Qubit assay.

Sleep Data

Table 20

MMU Sample no.	p1hrslp	p2hrslp	p5hrslp	p6hrslp	p7hrslp	p1sleff	p2sleff	p5sleff	p6sleff	p7sleff	p1wakent	p2wakent	p5wakent	p6wakent	p7wakent
1	7	6.5		6		84.85	82.98		76.6		1	1			
2	7	9	10			93.33	92.31	95.24				1	3		
3	8	7.5	7			94.12	93.75	87.5				1.5	1.5		
4	8	8	7.5			91.43	100	83.33				2	2.5		
5	7	6.5		6		77.78	70.27		66.67						
6	6.5	7	7	8	7	100	82.35	87.5	94.12	82.35	1.5	2.5	2.5		2.5
7	6.5		7	8		74.29		71.79	80		1		2.5	2	
8	8	8				100	94.12					1			
9	7	7		7	7	90.32	90.32		84.85	86.1		1			4
10	10	9				100	92.31				1	1			
11	7	7		7		90.32	83.5		93.33						
12	8		7.5			94.12		93.75			2.5		2		
13	8.5	8	8			92.73	96.97	88.89							
14	6			7		63.16			82.35						
15	6	7				70.59	82.35				3				
16	8	8	7	8		88.89	94.12	87.5	80				1		
17	7	8		6	6	100	100		70.59	60	1				1
18	7.75	8	6			88.57	91.43	68.57				3	5		
19	6	5.5		5	4.5	100	91.67		66.67	56.25					1
20	6.5	6.5	6			78.79	83.87	75			1	2.5	2		
21	7	8				82.35	94.12				1	1			
22	6	6.5		8		75	92.86		91.43					1	
23	8	8		7	7	91.43	91.43		87.5	73.68				1	1
24	6.5		5.5	6		81.25		84.62	92.31		1		2.5		
25	9	10	9	8		90	100	100	86.49			2	4		
26	8.5	7.5				89.47	85.71				1.5	1.5			
27	9	10				97.3	90.91								
28	6	6.5				70.59	86.67				1				
29	8	7.5	8.5			91.43	85.71	85			1.5	1.5	2.5		
30	8	8		8	7	88.89	94.12		94.12	93.33		2			
31	7	8		8		82.35	94.12		80		2.5	2			
32	8	7				94.12	87.5				2.5	3			
33	7	7.5		6		87.5	100		77.42		1	1			
34	6.5			6	6	74.29			68.57	66.67	1.5				
35	10	9	9	8	10	100	92.31	94.74	84.21	98.33	1		1.5		
36	8	7	7	6		77.42	77.78	66.67	57.14		1	1.5	2		
37	5.5		5	5	5.5	55		52.63	45.45	61.11	1		2.5		
38	5		8			71.43		88.89			4		3.5		
39	8	8		6	7	100	100		66.67	85.68	4.5	3			3.5

40	9					100									
41	8.5	7.5				89.47	75				2	2			
42	8	8		7		86.49	80		66.67			1			
43	6			8.5		88.89			100		1				
44	6.5	6	6	6	8	74.29	66.67	60	64.86	84.21	1	1	2.5		2.5
45	7	6.5	6	6.5		91.9	88.04	72.73	72.22				1		
46	6.5	6		6		76.47	75		66.67		3	2			
47	6	6		7	7	100	85.71		100	73.68					1.5
48	7	7.45	8	8	9	100	90.3	100	88.89	85.71	2	1	3		3
49	6.5					92.86					3.5				
50	8	7.5		7	8	96.97	93.75		73.68	80	1				2
51	7	8	7.75	8	9	100	100	100	94.12	100					
52		8	8	8	8		100	96.97	88.89	76.19					
53	7	6.5	6.5	6	5	80	76.47	74.29	70.59	55.56	1.5	1.5	2		2
54	6.5				7	76.47				66.67	2				3
55	7	7.5	5	6	6.5	84	85.71	50	66.67	76.47	2.5	3	2.5		2.5
56	6	7		7	9	55.81	70		84.85	83.72	2	1	2.5		1
57	6	5.5		4		75	75.86		66.67		2.5	2.5			
58	8	6		8		100	80		100			1.5			
59	6.75	7	7.5	8	7.5	79.41	82.35	88.24	91.43	90.91	1	1	2		2.5
60	5	5		6.5		68.97	58.82		83.87		2.5	2.5			
61	3	3.5		3	3.5	44.44	50			56					
62	7.5				8	93.75				80					
63	8.5		8	5	5	97.14		91.43	52.63	58.82			2		4
64	6.5	6.5	5.5	6	4.5	92.86	79.59	78.57	80	81.82	1.5	1.5	1		
65	7	7				96.55	100								
66	7		7	8	8	93.33		82.35	94.12	94.12	3		3		4
67	7.75	8	7.5	8	10	100	94.12	93.75	86.49	95.24	1	1.5	3.5		5.5
73	7.75	8		7		100	100		84.85		1	1		1	
74	6.5			5	6	83.87			66.67	77.42	2.5				1.5
75	7.5	7	6.5	7	6	90.91	84.85	72.22	71.79	61.54	1	1	1		1
76	4.5	5		5		48.65	54.05		54.05		3.5	4			
77	6	5	5.5	6	7.5	75	62.5	68.75	70.59	93.75	1	4	2		2.5
78	9			8	8	100			91.43	100					1
79	7	7	7	6	6.5	82.35	82.35	80	66.67	72.22					4
80	7.5	7.5		7	7	90.91	88.24		62.22	66.67	1				
81	7.5	7.5	6	5	5	100	93.75	92.31	45.45	47.62					3
82		7.5		6	7		93.75		70.59	82.35					1.5
83	6	7	7	6	6.5	75	77.78	77.78	77.42	78.79	1.5	1	1.5		1.5
84	8			8	8	84.21			88.89	76.19	1.5				2
85	6.5	7		7		92.86	93.33		87.5		1	1.5			
86	7	6	7	6	6.5	87.5	66.67	87.5	66.67	68.42	1	3	2.5		2.5
87	6.5	6		5	5	76.47	68.57		55.56	51.28	1.5	1.5			2.5
88	8	8		8	7	94.12	88.89		84.21	77.78					2.5

89	4.5	3.5	4	5		69.23	46.67	61.54	57.14		2.5	3.5	2		
90	6.5	7	4	5	5	100	96.55	57.14	76.92	66.67	1	2	2		1.5
91	8	8	8.5	6	6.5	82.05	80	80.95	60	61.9	1	1	1		3.5
92															
93	7.5	7.5		7	7	93.75	93.75		93.33	90.32	2.5	1.5			2
94	7		7		8	84.85		77.78		88.89	2		1		1
95	8			8.5	8	96.97			100	100	2			2	1.5
96	5			6	6.5	61.48			72.73	83.87	2				1.5
97	8			7	10	84.21			75.68	100					4
98	8		6	6	8	100		72.73	75	91.43			2.5		2.5
99	7	7	8	8	8	73.68	73.68	80	94.12	88.89	2	2	1.5		1.5
100	7.5	7	7.5	7	6.5	83.33	75.13	78.95	70	65	1	1	2		2
101	8.5	8.5			8	100	100			80	1	1			2
102	7	6.5	6	5	5.5	77.78	66.67	66.67	58.82	61.11	1	1.5	2.5		2
103	5.5	7	7			68.75	87.5	77.78			2.5	2.5	2		
104	8			8	8	84.21			84.21	84.21					
105	7			8		82.35			88.89		1.5				
106	8	8	8.5	10	11	94.12	96.97	100	95.24	100					
107	8	6		7	8	96.97	82.76		86.6	100					3
108	11			6		100			70.59						
109	7			6	7	87.5			70.59	88.83	2.5				3.5
110	7.5	6	5.5	4	4.5	78.95	70.59	55	47.06	64.29	1.5	2	2.5		2
111	5	5		7		66.67	66.67		82.35		1	2.5			
112	6.5	6.75	7	5	6	83.87	96.43	87.5	58.82	70.59	4.5	2.5	2.5		3.5
113	7					80					1				
114	7	7.5		11	6	80	90.91		100	54.55	2	2		2	3.5
115	7.5	7.25		6	6	95.74	95.6		80	77.42	1	1			2
116	6.5		5.5	6		72.22		48.89	52.17		1		1.5		
117				6	7				66.67	82.35					1.5
118	7			8		90.32			86.49						
119	7.5	7		6	6	83.33	80		64.29	66.67	1	1		1	1.5
120	6	5	6	6	6	80	68.97	75	70.59	70.59	7	4.5	2		2.5
121	9		8.5	8	8	94.74		87.18	82.05	80			3.5		3
122	8.5			5	5	100			64.52	62.5					1.5
123	7	7.5		7	6	82.35	88.24		77.78	66.67	2.5				2
124	5.5	6	5.5	4	4.5	68.75	75	73.33	48.48	60	1	1	3		
125	8	8	8	8	8	88.89	88.89	72.73	80	76.19	1	1	1		1
126	6.5	5.5	3.5			76.47	64.71	58.33			1	1	2.5		
127	5	5.5		5		66.67	73.33		58.82		2.5	3.5			
129	6.5	6	7.5	6		89.66	80	88.24	66.67		1.5	2.5			
130	8	7			7.5	91.43	82.35			88.24	2.5	2.5	2		2.5

Table 20: shows the sleep measures used in this study. The variables measures show 5 waves: P1, P2, P5, P6 and P7 along with the three measures for each wave: 'hrslp': hours sleep per night, 'sleff': sleep efficiency calculated by sleep duration divided by duration in bed, and 'wakent': which is how many times participants wake during the night.

Depression Data 1

Table 21

MMU Sample no.	mood1	mood2	mood3	mood4	mood5	mood6	mood7	mood8	mood9	mood10	mood11
1	No	No	No				No	No			
2	No	Dprs	Dprs	No	Dprs						
3	Dprs	No	Dprs	No	Dprs	No	No	No			
4	Dprs	No	Dprs	No	Dprs		Dprs				
5	No	No	No	No	No		No	No	No		
6	No	No	No	No	No	No	No	No	No	No	
7	No	No	No	No	No		No	No			
8	No	No	No	No		No					
9	No	No	No				No	No	No	No	
10	No	No	No	No	No		No				
11	No	No	No	No	No		No	No	No		
12	No	Dprs	Dprs	Dprs			No				
13	No	No	No	Dprs		Dprs	No				
14	No	No					No	No			
15	Dprs	No	No	No	Dprs		Dprs				
16	No	No	No	No	Dprs	Dprs	Dprs	No	Dprs		
17	No	No	No	No			Dprs	Dprs	Dprs	No	Dprs
18	Dprs	Dprs	Dprs	Dprs	Dprs	Dprs	Dprs		Dprs		
19	Dprs	No	No				No	No	No	No	
20	Dprs		No		Dprs	No	No				
21	Dprs	No	No	No	No		No	Dprs			
22	Dprs	Dprs	Dprs	No	Dprs		No	No			
23	No	No	No				No	No	No	No	
24	Dprs	Dprs	No	No	Dprs		No	No	No		
25	Dprs		Dprs	Dprs	Dprs						
26	No	Dprs	No				No				
27	Dprs	Dprs	Dprs	Dprs			No	Dprs			
28	Dprs	No	No		No		Dprs				
29	No	No	No	No	No	No	No	No			
30	No	No	No				No	No	No	No	

31	No	No	No	No	No		No	No	No		
32	No	No	No	No							
33	No	No	No				Dprs	Dprs	No		
34	No	No					No	No	No	No	
35	No	No	No	No	No	No	No	No	No	No	
36	No	Dprs	No	No	No	No	No				
37	No	No	No	No	No		No	No	No	No	
38	No	No	No	No	No						
39											
40	No	No					No				
41	Dprs	No	No	Dprs	Dprs		Dprs	Dprs			
42	No	No	No				No	Dprs			
43		No					No	No			
44	No	No	No	No	No	No	No	No	No	No	
45	No	No	No	No	No	No	No	No	No	No	
46	No	No	No	No	No		No	No			
47	No	No	No				No	No	No	No	
48	No	No	No	No	No	No	No	No	No	No	
49	No	No	No	No	No		No	No	No	No	
50	No	Dprs	Dprs	Dprs	No		Dprs	Dprs	Dprs	Dprs	
51	Dprs	Dprs	Dprs	Dprs	No	Dprs	No	Dprs	No		
52	No	No	No	No	No	No	No	No	No		
53	Dprs	No	No	Dprs	No	No	No	No	No		
54	No	No					No	No	No	No	
55	No	No	No	No	Dprs	No	No	No	No	No	
56	No	No	No	No		No	No	No	No	No	
57	Dprs	Dprs	Dprs	Dprs	Dprs		Dprs	Dprs	Dprs	Dprs	
58	Dprs	Dprs	Dprs	No	Dprs		Dprs	Dprs			
59	Dprs	No	No	No	No	No	No	No	No		
60	No	No	No	No	No		No	No	No		
61	No	No	No	No	No	No	No	No	No	No	
62	No		No	Dprs		Dprs	Dprs	No	No		
63	No	No	No	No	No		No	No	No	No	
64	Dprs	Dprs	No	No	Dprs		No	No	No	No	Dprs
65	Dprs	Dprs	Dprs	Dprs							
66	No	No	No				No	No	No		

67	No	No	No	No	No	No	No	No	No		
73	No	No	No	No	No		No				
74		No					No	No	No	No	
75	No	No	No	No	No	No	No	No	No	No	
76	Dprs	Dprs	Dprs				No	Dprs			
77	Dprs	Dprs	Dprs	Dprs	Dprs		Dprs	Dprs	Dprs	Dprs	
78	No	No					No	No	No	No	
79	No	No	No	No	No	Dprs	No	No	No	Dprs	
80	No	No	No	No	No		No	No	No	No	
81	No	No	No	No	No	No	No	No	No	No	No
82	No	No	No	No	No		No	No	No	No	
83	No	No	No	No	No	No	No	No	No	No	
84	No	No					No	No	No	No	
85	No	No	No	No	No		No	No	Dprs		
86	No	No	Dprs	No	Dprs	Dprs	Dprs	No	No	No	
87	No	No	No	No	No		No	No	No	No	
88	No	No	No				No	No	No	No	
89											
90	No	No	No	No	No	No	No	No	No		
91	No		No	No	No	No	No	No	No	No	No
92							Dprs	Dprs	Dprs	Dprs	
93	No	No	No	No			No	No	No	No	
94	No	No		No			No	No	No		
95	No	No					No	No	No	Dprs	
96	Dprs	No	No	No	Dprs		No	No	No	No	
97	No	No					No	No	No	No	
98	No	No	No	No	Dprs		No	No	No	No	No
99	No	No	No	No	No	No	No	No	No	No	
100	No		No	No	No	No	No	No	No	No	
101	No	No	No				No	No	No	No	
102	No	No	No	No	No	No	No	No	No	No	
103	No	No		No	No	No	No	No	No	No	
104	Dprs	Dprs	No				Dprs	No	No		
105	No	No					No	No			
106	No		No	No	No	No	Dprs	Dprs	Dprs	Dprs	Dprs
107	No	No	No	No	No		No	No	No	No	

108	Dprs	Dprs					Dprs	Dprs	Dprs		
109	No	No					No	No	No	No	
110	No	No	No	No	No	No	No	No	No	No	
111		No	No	No	No	No	No	No			
112	No	No	No	No	No	No	No	No	No	No	No
113	No		No	No		No	No				
114	No	No	Dprs	No	No		No	No			
115	No	No		No	No		No	No	No		
116	No	No	No	No	No		No	No	No		
117		No					No	No			
118	No	No					No	No	No		
119	No	No	No	No	No		No	No	No	No	
120	No	No	No	No	No	No	No	No	No	No	No
121	No	No	No	No	Dprs		No	No	No	No	Dprs
122		No					No	No	No	No	
123	No	No	No	No	No		No	No	No	No	
124	No	No	No	No	No	No	No	No	No	No	
125	No		No	No	No	No	No	No	No	No	No
126											
127	No	No	No				No	No			
128											
129	No	No	No	No	No	No	No	No			
130	No	Dprs	Dprs	Dprs	No	Dprs	Dprs	Dprs	No	Dprs	No

Table 21: Depression variables were measured in 11 waves for the measure ‘mood’: participants recorded their mood and then data was standardised to either depression or no depression according to the BDI scale.

Depression Data 2

Table 22

MMU Sample no.	msev1	msev2	msev3	msev4	msev5	msev6	msev7	msev8	msev9	msev10	msev11
1	No	No	No				No	No			
2	No	No	No	No	No						
3	No	No	No	No	No	No	No	No			
4	No	No	No	No	No		No				
5	No	No	No	No	No		No	No	No		
6	No	No	No	No	No	No	No	No	No	No	
7	No	No	No	No	No		No	No			
8	No	No	No	No		No					

9	No	No	No				No	No	No	No	
10	No	No	No	No	No		No				
11	No	No	No	No	No		No	No	No		
12	No	No	No	No			No				
13	No	No	No	No		No	No				
14	No	No					No	No			
15	No	No	No	No	No		No				
16	No	No	No	No	No	No	No	No	No		
17	No	No	No	No			No	No	No	No	No
18	No	Dprs	Dprs	No	No	Dprs	Dprs		Dprs		
19	No	No	No				No	No	No	No	
20	No		No		No	No	No				
21	No	No	No	No	No		No	No			
22	No	Dprs	Dprs	No	No		No	No			
23	No	No	No				No	No	No	No	
24	Dprs	No	No	No	No		No	No	No		
25	No		No	No	No						
26	No	No	No				No				
27	No	No	No	No			No	No			
28	No	No	No		No		No				
29	No	No	No	No	No	No	No	No			
30	No	No	No				No	No	No	No	
31	No	No	No	No	No		No	No	No		
32	No	No	No	No							
33	No	No	No				No	No	No		
34	No	No					No	No	No	No	
35	No	No	No	No	No	No	No	No	No	No	
36	No	No	No	No	No	No	No				
37	No	No	No	No	No		No	No	No	No	
38	No	No	No	No	No						
39											
40	No	No					No				
41	No	No	No	No	No		No	Dprs			
42	No	No	No				No	No			
43		No					No	No			
44	No	No	No	No	No	No	No	No	No	No	
45	No	No	No	No	No	No	No	No	No	No	
46	No	No	No	No	No		No	No			
47	No	No	No				No	No	No	No	
48	No	No	No	No	No	No	No	No	No	No	
49	No	No	No	No	No		No	No	No	No	
50	No	No	No	No	No		No	No	No	No	
51	Dprs	No	No	No	No	No	No	No	No		
52	No	No	No	No	No	No	No	No	No		

53	No	No	No	No	No	No	No	No	No		
54	No	No					No	No	No	No	
55	No	No	No	No	No	No	No	No	No	No	
56	No	No	No	No		No	No	No	No	No	
57	Dprs	No	No	No	No		Dprs	No	No	No	
58	No	No	Dprs	No	No		No	No			
59	No	No	No	No	No	No	No	No	No		
60	No	No	No	No	No		No	No	No		
61	No	No	No	No	No	No	No	No	No	No	
62	No		No	No		No	No	No	No		
63	No	No	No	No	No		No	No	No	No	
64	Dprs	No	No	No	No		No	No	No	No	No
65	No	No	No	No							
66	No	No	No				No	No	No		
67	No	No	No	No	No	No	No	No	No		
73	No	No	No	No	No		No				
74		No					No	No	No	No	
75	No	No	No	No	No	No	No	No	No	No	
76	No	No	No				No	No			
77	Dprs	No	No	No	Dprs		No	Dprs	Dprs	Dprs	
78	No	No					No	No	No	No	
79	No	No	No	No	No	No	No	No	No	No	
80	No	No	No	No	No		No	No	No	No	
81	No	No	No	No	No	No	No	No	No	No	No
82	No	No	No	No	No		No	No	No	No	
83	No	No	No	No	No	No	No	No	No	No	
84	No	No					No	No	No	No	
85	No	No	No	No	No		No	No	No		
86	No	No	No	No	No	No	No	No	No	No	
87	No	No	No	No	No		No	No	No	No	
88	No	No	No				No	No	No	No	
89											
90	No	No	No	No	No	No	No	No	No		
91	No		No	No	No	No	No	No	No	No	No
92							No	No	No	No	
93	No	No	No	No			No	No	No	No	
94	No	No		No			No	No	No		
95	No	No					No	No	No	No	
96	No	No	No	No	No		No	No	No	No	
97	No	No					No	No	No	No	
98	No	No	No	No	No		No	No	No	No	No
99	No	No	No	No	No	No	No	No	No	No	
100	No		No	No	No	No	No	No	No	No	
101	No	No	No				No	No	No	No	

102	No	No	No	No	No	No	No	No	No	No	
103	No	No		No	No	No	No	No	No	No	
104	No	No	No				No	No	No		
105	No	No					No	No			
106	No		No	No	No	No	No	No	No	No	No
107	No	No	No	No	No		No	No	No	No	
108	No	Dprs					No	No	No		
109	No	No					No	No	No	No	
110	No	No	No	No	No	No	No	No	No	No	
111		No	No	No	No	No	No	No			
112	No	No	No	No	No	No	No	No	No	No	No
113	No		No	No		No	No				
114	No	No	No	No	No		No	No			
115	No	No		No	No		No	No	No		
116	No	No	No	No	No		No	No	No		
117		No					No	No			
118	No	No					No	No	No		
119	No	No	No	No	No		No	No	No	No	
120	No	No	No	No	No	No	No	No	No	No	No
121	No	No	No	No	No		No	No	No	No	No
122		No					No	No	No	No	
123	No	No	No	No	No		No	No	No	No	
124	No	No	No	No	No	No	No	No	No	No	
125	No		No	No	No	No	No	No	No	No	No
126											
127	No	No	No				No	No			
128											
129	No	No	No	No	No	No	No	No			
130	No	No	No	No	No	No	No	No	No	No	No

Table 22: Depression variables were measured in 11 waves for the measure ‘msev’: participants recorded their mood severity then data was standardised to either depression or no depression according to the BDI scale.

Cortisol Data

Table 23

MMU Sample No.	cort0	cort30	cort60	cort18	cort22
3	20.5	24.7667	20.9333	7.65333	1.55667
6	18.9333	22.1667	28.2333	5.99333	5.86667
16			12.0333	5.62	4.76333
24	12.43	26.2333	20.6667	5.40333	2.53333
25	7.79667	7.83	6.62333	7.94667	4.18667
35			19.3833	8.07	3.65333
36	13.08	30.1667	22.3667	6.55333	6.89

38	7.17	7.54	7.71	4.92	3.08667
48	12.91	23.6667	27.2333	4.17667	1.96333
52	18.8	21.7667	18.1333	13.7667	5.44
55	8.95	13.3567	14.1333	4.91	1.97
59	20.6	42.4333	35.3	6.59667	4.56333
61	9.08	10.6733	12.7733	10.1067	5.5
63	12.17	24.7667	21.9	5.00333	3.33333
64	13.7067	25.8	13.51	5.34333	6.29667
77	16.9333	20.5333	23.8	3.88	2.96667
81	16.0833	28.1333	21.6667	8.51333	4.90667
83	12.2167	18.8567	19.1333	7.54333	2.45
89	16.8333	23.1667	25.9333	2.80667	4.43667
90	14.3867	14.1	14.8	9.15333	4.19667
91	11.2067	21.8333	25	3.64667	2.51333
94	18.6667	17.5	33.15	3.49333	1.57333
98	22.59	26.1667	31.7333	11.2167	19.3133
99	11.25	18.1	16.7333	7.7	3.75667
100	11.7867	15.8333	18.1333	3.87667	12.5333
103	17.9667	17.8667	14.9667	4.86667	4.75667
110	13	21.7333	16.3	7.91667	3.34667
112	16.5333	28.9333	22.0667	8.24333	6.33667
116	14.2667	44.8667	37.1667	5.84333	3.68667
120	17.0333	27.2	22.4333	4.86	4.19333
126	25.2	42.9333	26.8667	6.40333	11.14

Table 23: shows the cortisol data used in this study along with the MMU sample number. ‘Cort0’ indicates cortisol levels after waking, ‘Cort30’ indicates cortisol levels after 30 min of being awake, ‘Cort60’ indicates cortisol levels after 60 min of being awake, ‘Cort14’ indicates cortisol levels at 14:00, ‘Cort18’ indicates cortisol levels at 18:00, ‘Cort22’ indicates cortisol levels at 22:00.

Normality Tests

Table 24

Shapiro-Wilk normality test		
Variables tested	**w value**	**p value**
p1hrslp	0.964	0.003
p2hrslp	0.951	0.002
p5hrslp	0.973	0.271
p6hrslp	0.933	< 0.001
p7hrslp	0.968	0.074
p1sleff	0.924	< 0.001
p2sleff	0.93	< 0.001

p5sleff	0.96	0.068
p6sleff	0.972	0.044
p7sleff	0.969	0.087
p1wakent	0.842	< 0.001
p2wakent	0.909	< 0.001
p5wakent	0.94	0.008
p6wakent	0.669	< 0.001
p7wakent	0.955	0.017
Cort0	0.979	0.803
Cort30	0.932	0.062
Cort60	0.983	0.881
Cort14	0.737	< 0.001
Cort18	0.939	0.076
Cort22	0.719	< 0.001
NR3C1 CpG1	0.857	< 0.001
NR3C1 CpG2	0.913	< 0.001
NR3C1 CpG3	0.877	< 0.001
NR3C1 CpG Mean	0.891	< 0.001
FKBP5 CpG1	0.991	0.58
FKBP5 CpG2	0.99	0.502
FKBP5 CpG Mean	0.989	0.426
NR3C2 CpG1	0.982	0.096
NR3C2 CpG2	0.968	0.005
NR3C2 CpG3	0.969	0.006
NR3C2 CpG Mean	0.982	0.088

Table 24: shows the results from the Shapiro-Wilk Test for Normality of the variables used in this study. The W value measures how well the data fit a normal distribution, ranging from 0 to 1, where 1 indicates a data distribution close to normality. The p-value indicates whether the deviation from normality is statistically significant.