Epigenetic regulation of interleukin 6 in age-related cognitive decline and Alzheimer's disease

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Declaration:

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Signed .....

Date .....

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Abbreviations

Abbreviations	Description
AD	Alzheimer's disease
IL-6	Interleukin 6
ACPRC	Age and Cognitive performance research Cohort
RT-PCR	Real time polymerase chain reaction
Aβ	Beta-amyloid plaques
SNP	Single nucleotide polymorphism
NFT	Neurofibrillary tangles
APP	Aβ precursor protein
PSEN1	Presenilin 1
PSEN2	Presenilin 2
MCI	Mild cognitive impairment
TBI	Traumatic brain injury
GWAS	Genetic wide association studies
GxE	Gene environment interaction
ELISA	Enzyme-linked immunosorbent assay
CERAD	Consortium to establish a registry for Alzheimer's disease

Abstract

Introduction: Alzheimer's disease (AD) is a neurodegenerative condition that is becoming a global crisis with no current cure. Neuroinflammation is widely thought to play a role in the pathogenesis of this disorder. Peripheral levels of the cytokine interleukin 6 (IL-6), that has both pro- and anti-inflammatory properties have been linked to dementia and age-related cognitive decline. Polymorphisms in the *IL-6* gene have also been associated with neurodegenerative diseases. However, it remains to be determined how IL-6 levels are regulated in AD within brains and how this relates to neuropathology.

Aim: The aim of this project was to investigate if and how the *IL-6* gene is regulated in the brain during cognitive decline and AD and its relationship to neuropathological markers of AD.

Methods: This study investigated IL-6 within brains from the University of Manchester Age and Cognitive performance research Cohort (ACPRC) and the Dyne – Steele study, that have been longitudinally followed over a 20-year period for cognition and characterised in neuropathology stages. Genotyping for the single nucleotide polymorphism (SNP) rs1800795 in the *IL-6* locus was performed using KASP and *IL-6* gene expression was measured by RT-PCR and normalised using two housekeeping genes (βactin and GAPDH). ELISA was used to determine IL-6 protein levels in brains and bisulphite pyrosequencing was used to quantify methylation levels of CpG sites at the *IL-6* promoter. Finally, immunofluorescence was used to compare control and early AD prefrontal cortex brain samples to enable investigation of the distribution of IL-6 and an important regulator, NFkB.

Results: Between genotypes, no significant difference was observed in age of death, whole brain weight, post mortem delays and neuropathology stages. IL-6 protein levels were analysed at the different neuropathology stages (BRAAK, THAL and CERAD) with no significance determined between each of the stages in the neuropathological hallmarks. Levels of IL-6 protein and *IL-6* mRNA expression were analysed between the different genotypes with no significant differences. *IL-6* gene expression and IL-6 total protein levels showed no correlations, even when further stratified by genotype. Immunofluorescence determined that IL-6 is

present in the prefrontal cortex of both control and early AD samples and NFkB is activated. Distribution of DNA methylation at 4 CpG sites in the IL-6 promoter were determined. CpG site 2 depends on the rs1800795 SNP, which is only present in the G allele carriers. Interestingly, in the G allele carriers, levels of methylation at CpG sites 3 and 4 were significantly lower and higher, respectively. Levels of DNA methylation between control and AD groups were not significantly different at each of the CpG sites and did not correlate with protein levels. Levels of *IL-6* mRNA and DNA methylation stratified by genotype revealed no correlation.

Discussion: This study is the first to investigate IL-6 in AD brains and mechanisms underlying its epigenetic and genetic regulation. IL-6 protein levels and mRNA levels are found not to be impacted by the genotype and in disease state. Interestingly, DNA methylation was shown to be altered in the brains by the presence of a SNP, suggesting gene-environment interactions however, this was not influenced by disease state. In summary, the research has shown that IL-6 is present in control and AD brains and that the SNP has an impact on methylation levels between genotype at different CpG sites. However direct linear correlations between methylation, protein and expression levels did not exist. IL-6 is an important mediator of inflammation and further work is needed to understand how this factor is involved in neuroinflammation and regulated in the pathogenesis of AD.

1.0 Introduction

1.1 Epidemiology of Dementia

Dementia has many forms and the most prevalent form of the disease is Alzheimer's disease (AD) corresponding close to 60% of cases (Rizzi et al., 2014). The analysis of AD brains at post-mortem has led to evidence suggesting the involvement of inflammation in the pathology (Zheng et al., 2016). AD is a neurodegenerative condition, which is progressive over time and results in cognitive decline. Molecular features of AD are beta-amyloid plaques (Aβ) and neurofibrillary tangles (NFT) composed of tau proteins (Jones et al., 2017). There is currently a global crisis surrounding dementia as currently over 40 million people suffer with the disease worldwide (Zheng et al., 2016) and that is set to triple by the year 2050 to 115 million people as life expectancy increases (Liu and Chan, 2014). Women have a higher chance of developing dementia compared to men (Alzheimers, 2015).

1.2 Clinical symptoms of MCI and AD

Mild cognitive impairment is categorised based on thinking skills and memory impairment. An example could be that a person starts to forget important information such as appointments or recent events as well as lacking the ability to make sound decisions (Langa and Levine, 2014). Signs and symptoms of AD can vary depending on the person affected. During the early stages of AD, it is possible for the person to be independent and carry on with day-to-day life. Over time during this stage, it may become noticeable that a person is struggling more to find the correct words or even struggling to remember information they have just read. Eventually, the condition will develop into the moderate AD stage. Once the disease has progressed to this stage, a greater level of care is needed due to the patient becoming less independent in day-to-day life. This stage is usually the longest stage and can last several years. Symptoms become more heightened and more notable. Such symptoms can include forgetfulness and becoming increasingly withdrawn from society, family and friends. The last stage of AD is reached when individuals lose the ability to respond to their environment.

Conversation abilities and controlling movements will become progressively more difficult. As memory and cognitive skills continue to decline, changes in personality may take place. During the last stages of the disease, special care 24 hours a day will be needed. There will be difficulty with physical abilities such as being able to sit or even walk. Due to the deterioration on the individual and their body, they become increasingly open to developing infections such as pneumonia which can be life threatening. Eventually death will occur, as there is no cure for the illness (Alzheimers, 2015).

1.3 Diagnosis of dementia with specific relation to AD

Diagnosis of AD is not definitive until death and a postmortem is completed. During autopsy, the brain may be examined for amyloid plaques and tau proteins which are the classic hallmarks of AD (Kumar and Clark, 2017). However, if some form of dementia is suspected a doctor can carry out a number of tests to aid in trying to diagnose the condition (Neugroschl and Wang, 2011). Recently there have been developments in clinical diagnostic techniques for AD. Blood tests are carried out which would include looking at full blood count, B12, thyroid function, urea, liver function test and glucose levels. Brain imaging can be used for the investigation of AD. Magnetic resonance imaging (MRI) is a useful imaging tool to look at patterns of regional brain atrophy (Kumar and Clark, 2017). More recently, positron emission tomography (PET) is used to indicate characteristic changes in AD brains. Imaging has a key role in the diagnosis of AD, as the brain cannot be assessed until death to perform histopathology for confirmation of AD. Furthermore, imaging is a tool that could have significance in diagnosing AD through diagnostic markers (Johnson et al., 2012). Clinicians often use the mini mental state examination (MMSE) to aid in diagnosing AD and to monitor the progression and severity. This test consists of a scoring system with the score being out of 30. Individuals who score 27 or over are considered as normal. A MMSE score of 20-26 can indicate mild AD in patients. A score between 10 and 20 suggests moderate AD and below 10 would be considered as having severe AD (Kukull et al., 1994). This tool is helping to diagnose AD, however a level of professional judgement is needed as the results can be affected by the cultural

background or level of education of the patient. The neuropathological diagnosis of AD involves looking at 3 different neuropathological staging and they are THAL, CERAD and BRAAK. Description of the 3 different staging can be seen below in table 1

Table 1 - Description of neuropathological staging and brain regions affected

THAL Staging (Serrano-Pozo et al., 2011)	Assess amyloid deposition	Stage 1	Amyloid deposits found in the isocortical region
		Stage 2	Amyloid deposits present in the hippocampal, entorinal cortex, insular and cingulated cortices
		Stage 3	Amyloid deposits present in the thalamus, hypothalamus, white matter and basal forebrain cholinergic nuclei
		Stage 4	Amyloid deposits present in the brainstem structures. e.g. substantia nigra, reticular formation of the medulla oblongata
		Stage 5	Amyloid deposits present in the pons and the molecular layer of the cerebellum
BRAAK Staging (Braak et al., 2006) (Murayama and Saito, 2004)	Assess neurofibrillary tangles	Stage I-II	Classified when neurofibrillary tangles are confined in the transentorhinal region and entorhinal region
		Stage III-IV	Classified when the NFT are involved in the limbicregions. An example of this is in the hippocampus. (Murayama and Saito, 2004)
		Stage V-VI	Significant neocortical involvement, isocortical involvement and substantia nigra. (Murayama and Saito, 2004)
CREAD Stages (Fillenbaum et al., 2008) (Murayama and Saito, 2004)	Assess neuritic plaques	0 - None	No histological evidence of AD.
		1 - Sparse	Sparse formation of neuritic plaques. Uncertain evidence of AD
		2 - Moderate	Moderate formation of neuritic. Suggested diagnosis of AD
		3 - Frequent	Frequent formation of neuritic. Indicate diagnosis of AD

1.4 Risk factors associated with dementia (specifically AD)

There are numerus risk factors that are linked with AD, including lifestyle, vascular and genetic factors (Bekris et al., 2010). The chance of developing dementia varies from person to person. Age is one of the main risk factors for developing dementia. According to Alzheimer's society website, 6 out of a 100 people aged 75-79 will have dementia. This increases with age and 30 out of a 100 people aged 90-94 will suffer from dementia (Alzheimer society, 2018). Making certain changes to the lifestyle can increase or decrease the chances of developing dementia. Maintaining a lifestyle that is healthy could potentially help protect against developing dementia such as regular exercise and mental stimulation (Alzheimers, 2015). Obesity affects many different organ systems and this disease is very damaging. Obesity is associated with higher risk of developing mild cognitive impairment (MCI) and late-life dementia and AD (Nguyen et al., 2014). Recently a number of studies have linked traumatic brain injury (TBI) as a risk factor in developing AD. Results have shown that after TBI, microglia can remain activated months to several years. This is significant as after the injury this function is necessary for phagocytic clearance of debris. The constant cerebral inflammation that occur from this process could intern directly or indirectly cause AD (Donat et al., 2017). There is also a genetic risk that can be associated with dementia which is caused by autosomal dominant mutations. There are a number of genes that can be affected, and these include Aβ precursor protein (*APP*), presenilin 1 (*PSEN1*) and 2 (*PSEN2*), which are linked to AD. There are 3 different alleles associated with *ApoE* gene which include *ε2, ε3* and *ε4* of which *ε4* is seen to increase AD risk in familial and sporadic cases (Liu et al., 2013). It is important to note that just because there is an inheritance of the *ε4* allele it does not guarantee the development of AD. In the brain, the APOE protein binds to Aβ strongly and is present in the plaques found in AD (Jungsu et al., 2009). Genetic factors have been seen to play an important role in AD. Genetic variants in the genome have been analysed through genetic wide association studies (GWAS). A common strategy to assess genetic variants in the genome is through GWAS to observe single nucleotide polymorphism (SNP) arrays to assess the association with AD. Single nucleotide polymorphism of interest during this project is

rs1800795, which resides at -147 base pair upstream from the *IL-6 (*Zhang et al., 2015*)*.

1.5 Neuroinflammation in the brain and in relation to AD

When damage occurs to the human body for example physical injury then inflammation is the response (Weiss, 2008). This inflammatory response is the body's way of controlling and maintaining its homeostasis (Kunnumakkara et al., 2018). There are two types of inflammatory responses and they are acute and chronic inflammation. Acute inflammation usually occurs when the healing process begins and starts to repair the damaged tissues. Whereas chronic inflammation, persists in trying to repair tissue but also damages tissues at the same time (Weiss, 2008). Risk factors that are associated with chronic inflammation include age, obesity, diet, smoking, sleep and stress. Features of chronic inflammation include vasodilation of the blood vessels, blood flow increases and neutrophils move towards the affected area. The continued inflammation presence in the human body can lead to various diseases. Examples can include cancer, arthritis, diabetes and neurological disease. This is through the dysregulation of signalling pathways such as NFκB. This is of importance as targeting the signalling pathways potentially has the effect to eradicate many diseases. There are numerus transcription factors that have been discovered to be linked to inflammation. Some examples of cytokines that are involved include tumor necrosis factor alpha (TNF-α) and interleukins (IL) such as IL-1 and 6. NFκB is an important transcription factor as it is seen to be the key mediator in inflammation. (Kunnumakkara et al., 2018). Neuroinflammation over the past 10 years has developed into a concept that is accepted. However, it is still not presently clear what mechanisms are involved. Neuroinflammation has a role in neurodegenerative diseases (Calsolaro and Edison, 2016).The pathophysiology of neuroinflammation is extremely complex and many different brain cells can drive the activation (Bronzuoli et al., 2016). Neuroinflammation is when there is activation of microglia, astrocytes, macrophages and lymphocytes that result in the release of inflammatory mediators such as cytokines and chemokines (Lee et al., 2010).

1.6 The role of IL-6 in relation to neuroinflammation, MCI and AD

Interleukin 6 (IL- 6) was discovered 30 years ago (María Erta, 2012)and is a proinflammatory cytokine with multiple functions and has neuro-protective properties, but also has degenerative properties (Baune et al., 2012). The molecular weight of IL-6 is 20-30kD. The main source of IL-6 include cells in the immune system and adipocytes. NFkB moderates expression of IL-6 and the protein is upheld in the cytoplasm by the binding of inhibitory proteins (Maggio et al., 2006). IL-6 is shown to be key in chronic inflammation, and levels are elevated in inflammatory diseases (Gabay, 2006). When inflammation occurs which can be acute or chronic, IL-6 proteins are produced at the inflammation site. Microglia and astrocytes are stimulated by the accumulation of Aβ plaques and tau proteins, this leads to the increased production of inflammatory mediators like IL-6 (María Erta, 2012). Many different types of cytokines are secreted by microglia and have been seen to have altered expression in AD patients. As well as several polymorphisms of pro-inflammatory genes are seen to be related to increased risk of AD (Solito and Sastre, 2012). In AD brains, IL-6 accumulates around amyloid plaques as well as other cytokines. This has led to studies looking at pro-inflammatory and anti-inflammatory cytokine levels in cerebral spinal fluid (CSF) and blood serum in patients with AD and MCI (Zheng et al., 2016). Cojocaru et al.(2011) compared IL-6 levels between control and AD patients to determine the correlation of IL-6 levels in the disease pathology of AD. It was discovered that levels of IL-6 were significantly higher in AD patients when compared to the control group. Indicating an increase in production of IL-6 cytokine in AD patients.

1.7 Epigenetic and DNA methylation

Epigenetics is the changes in gene expression which don't change DNA sequence itself. Epigenetic modifications can be altered through life-time and affected by the environment, e.g. in response to nutrients, stress, infections, and toxin exposure (Bayarsaihan, 2016). Epigenetic mechanisms include DNA methylation, changes in histone marking and microRNAs. DNA methylation is one of the most widely studied epigenetic markers. It involves the addition of methyl groups to cytosine residues, usually in a CG sequence, termed CpG. Such methylated CpGs

within promoter regions of genes generally have a repressing activity on gene expression either by blocking transcriptional activators from binding or recruiting repressive histone markers. Patterns of DNA methylation are regulated through the activity of DNA methyltransferases and demethylation enzymes (Moore et al., 2013). Hypermethylation in the promoter region of genes can be associated with transcriptional silencing while hypomethylation induces gene expression (Yasmin et al., 2015). The brain contains some of the highest levels of DNA methylation compared to any other tissue. The epigenetic regulation of genes within the brain is important for neurodevelopment and brain function. Numerous studies have linked changes in the patterns of DNA methylation within the brain to neurological diseases. There is increasing evidence that neurons respond to environmental signals via changes in DNA methylation (Murgatroyd et al., 2009). Epigenetic mechanism mediates the development of chronic inflammation by modulating the expression of pro – inflammatory cytokines such as interleukin's, TNFα and oncogenes of the transcription of NFκB. These molecules are produced by many different cells during chronic inflammation. This can lead to the development of some very serious conditions such as autoimmune disorders, cancers and neurodegenerative conditions (Shanmugam and Sethi, 2013). The inflammatory genes are regulated through DNA methylation, histone modification and transcription factors such as NFκB, FOXP3 and STAT (Bayarsaihan, 2016). DNA methylation levels have been indicated in other genes such as *BDNF* and *OPRK1* promoters in AD patients (Ji et al., 2017). The status of methylation is particularly analysed in the promoter regions of genes, which are linked to AD pathology (Chouliaras et al., 2010a). Promoter methylation is important in the epigenetic mechanism of the regulation of the expression of *IL-6* (Coppieters and Dragunow, 2011). Changes through environmental and functional genes could change hypermethylation and hypomethylation at sites which implicated gene expression (Levine et al., 2015). It is known that the epigenetic process of DNA methylation influences numerous genes that are involved in inflammation. In a recent study Nicolia et al. (2017) focused on late onset AD and neuroinflammation to analyse the possible association with epigenetic modification. Analysis was conducted on human brain tissues from the frontal cortex of healthy middle aged and late onset

AD at different BRAAK stages. In the control group mRNA *IL-6* methylation is seen to decrease as DNA methylation increases. The opposite is noted at BRAAK stages I-II/A, mRNA *IL-6* levels increase as DNA methylation decreases. Whereas at AD BRAAK grouping V-VI/C both IL-6 mRNA levels and DNA methylation decrease. This could be indicating that no relation is present with methylation levels and mRNA expression. Another observation presented was, in the 5-flanking region of the *IL-6* gene high levels of methylation were observed in middle age cases with progressive hypomethylation at stages I-II/A and stages V-VI/C. This is important as hypomethylation is seen to be an epigenetic abnormality. For example hypomethylation is linked to increased inflammatory mediators which could be linked to neuroinflammation and AD. Concluding remarks from this paper is that there is a need to study whole methylation site in DNA promoters and flanking regions, this is to show that expression of cytokines genes is linked with differential DNA methylation in brains from late onset AD patients.

1.8 Regulatory polymorphisms in IL-6

In excess of 3.1 million SNPs are found in the genome of humans if not more. Mental health and its association with polymorphisms has been investigated but there are recently inconsistences between research. Many polymorphisms have the potential to be regulatory. These can be positioned in promoter regions upstream, downstream and in introns. In recent years, the significant development in sequencing has allowed human genome analyses to become cheap and fast. The understanding of the human genome is ever changing and evolving (Paul, 2011). One particular single nucleotide polymorphism is rs1800795 which is found in the promoter region of the *IL-6* gene (Dragašević et al., 2014). The *IL-6* gene is found on the short arm of chromosome 7 (7p21) (Baune et al., 2012) and IL-6 is made of 212 amino acids (Tanaka et al., 2014). This SNP is linked to transcription rates and is linked to various diseases such as diabetes and AD. The IL-6 gene promoter includes numerous regulatory sites to enable the control of gene expression (Popko et al., 2010), such as *NFκB, C/EBP, CREB and AP1* as well as numerous other transcriptional factors regulate *IL-6 mRNA* expression (Wongchana and Palaga, 2012). The C allele in the polymorphism has been seen

to have lower *IL-6* gene expression and IL-6 plasma levels (Capurso et al., 2004). Licastro et al. (2003) reported that in the *IL-6* promoter region the rs1800795 (-174) allele was over represented in AD compared to the control group and seen to increase the risk of AD. *IL-6* polymorphism rs1800795 is located in the promoter region residing at -174 base pair upstream. This polymorphism can affect the transcription of the gene and could alter cytokine production (Liu et al., 2017), suggesting it has an effect upon *IL-6* gene expression and protein levels. The rs1800795 (G) allele has been studied in a number of different conditions and has been found to have higher levels of IL-6. The rs1800795 (C) allele has also been studied in many different conditions and has been associated with lower levels of IL-6 (SNPedia). Noss et al (2015) found an association between the rs1800795 minor allele (C) and the association between increased *IL-6* productions in fibroblasts. On the other hand, in human monocytes and HeLa cell no association was found between increased *IL-6* production and the rs1800795 polymorphism.

2.0 Aims and Objectives

2.1 Aims

To determine epigenetic mechanisms by which the *IL-6* gene is regulated in the brain in neuroinflammation, cognitive decline and AD.

2.2 Objectives

The molecular mechanisms underlying IL-6 regulation in the brain were addressed. A collection of brains assessed longitudinally for cognitive decline (prefrontal cortex, n=67) was measured for protein levels of IL-6 using enzyme-linked immunosorbent assay (ELISA) to test relationship of IL-6 levels in cognitive decline and AD.

Levels of DNA methylation at the IL-6 promoter were determined, using bisulfite pyrosequencing. Gene expression of IL-6 were measured by RT-PCR and genotyping using Kompetitive allele-specific PCR. Finally, the role of genetics and gene-environment interactions in the regulation of IL-6, a polymorphism in the gene was correlated with IL-6 brain expression and DNA methylation. This allowed the determination of whether interactive effects of gene-epigenetic factors could work at the *IL-6* gene.

3.0 Methodology

3.1 Reagents and supplies

All reagents, kits, chemicals and other laboratory equipment used in this project can be found in Appendix 10.1.

3.2 Study population

This project investigated n=67 prefrontal cortex samples acquired from the Manchester Brain Bank (with full ethical approval for all experiments described in section 3). The individual brain samples were from a larger cohort called The University of Manchester Age and Cognitive performance Research Cohort (ACPRC). The pre frontal cortex samples were stored in the -80^{o}C freezer. As seen below in table 2, some of the sample data is missing, which is indicated by the sample size.

Table 2 - Table of characteristics from participants for prefrontal cortex analysis.

Variable	n=67
Age of death	87.51 ± (6.07)
Brain weight (g)[A]	1207.37± (137.37)
Post-mortem delay (hours)[B]	76.14 ± (43.68)
Age at cognitive testing	62.85 ± (5.84)
Females/Males (%)	46 (68.7) / 24 (31.3)

[A] n=43. [B] n=60. All data presented as mean (standard deviation), unless stated otherwise.

3.3 DNA extraction

Frozen prefrontal cortex samples were subjected to DNA extraction using the isolate II genomic DNA kit (Bioline) with a few alterations through previous optimisation. Prefrontal cortex brain samples were placed on dry ice during the cutting stage to stop thawing of the tissues. Brain tissues were weighed in the region of 35 mg, placed in individual eppendorf tubes, and labelled. In each of the tubes 250 µl of lysis buffer and 30 µl proteinase K solution was added and mashed until the samples could pass through the pipette tip. Samples were incubated overnight at 56 ^{o}C on a heat block with a shaking option set to 1200 rpm. After the incubation period, the samples were vortexed and 200 µl of lysis buffer G3 was loaded into each eppendorf, vortexed and incubated for a further 10 minutes at 70^{o}C. In each tube 210 µl of ethanol (96-100%) was added and vortexed vigorously. Samples were then loaded into isolate II genomic DNA spin columns and centrifuged at 11000 x g for 1 minute. To each of the spin columns 500 µl of GW2 wash buffer was added and centrifuged at 11000 x g with flow through discarded. GW2 wash buffer is added for a second time (600 µl) to each spin column and centrifuged at 11000 x g discarding the flow through. Spin columns were centrifuged again and placed in new collection tubes to elute the DNA in 45 µl of elution buffer G at 70^{O}C with an incubation period of 1 minute at room temperature. Spin columns were centrifuged and extracted DNA was collected and purity measured using the Nanodrop 2000c. Purity levels for A260/280 were 2.02 ± 0.08 and A230/260 purity level were 2.22 ± 0.16 indicating DNA purity levels were acceptable.

3.4 Bisulphite conversion

The DNA extracted from the n=67 prefrontal cortex samples were diluted with water to achieve working mix of 20 µl. To the working mix, 35 µl of DNA protect buffer and 85 µl of bisulphite solution was added and vortex to achieve the reaction, indicated when the solution turned blue. The eppendorf's were placed in the eppendorf mastercycler and programmed with the following temperatures, denaturation 95 ^{o} C - 5 minutes, incubation 60 ^{o} C - 10 minutes, denaturation 95 ^{o} C - 5 minutes and incubation 60 ^{o} C – 10 minutes and hold at 18 ^{o} C. When the PCR was completed, 310 µl of BL buffer was added, vortex and centrifuged briefly. A volume of 250 µl of ethanol was added to each eppendorf vortexed for 15 seconds and centrifuged. New collection tubes were placed on each spin column and centrifuged for a further minute to clear any residue left. To each spin column, 500 µl of wash buffer was added and placed in the centrifuge for 1 minute at 15000 – x g. Desulphonation reaction buffer was added to each column and incubate at room temperature for 15 minutes. After the incubation period the samples were centrifuged at 15000x g for 1 minute and flow through discarded. 500 µl of wash buffer was added to each of the columns and placed in the centrifuge and spun at 15000x g for 1 minute. The previous wash step was repeated for a further cycle and centrifuged at 15,000 x g for 1 minute to remove any residual. The spin columns were placed into a sterile 1.5ml micro centrifuge tube and 15 µl of elution buffer was added and incubated for 1 minute, and centrifuge at 15,000 for another minute. An additional 15 µl of elution buffer was added and centrifuged for 30 seconds at 15,000 x g with the flow through collected as the bisulphite converted DNA.

3.5 Designing of IL-6 primers

The genomic browser (https://genome-euro.ucsc.edu/index.html) which is publicly available was used to search for the gene of interest *(IL-6)* with a specific region surrounding the SNP found in the promoter region at -174. The *IL-6* sequence was imported into the primer design software (Pyromark assay design). A graphic view of the assay was shown and with a number of different sets of forward, reverse and sequencing primers with scores out of 100.

Table 3 - Primer information for two sets of IL-6 primers.

	Primer set 1	Primer set 2
Forward primer	AGAAAAAAAGAAAGTAAAGGAA GAGTG	AAAAAGAAAGTAAAGGAAGAGTGG Biotin labelled
Reverse primer	CCTCAAACATCTCCAATCCTATAT TTAT Biotin labelled	CCTCAAACATCTCCAATCCTATATTTA
Sequencing primer	AAGGAAGAGTGGTTT	AAACCTTATTAAAATTATACAATAT
Sequencing to analysis	TGTTTTTTAGYGTTAGTTTTAATG AYGATTTAAGTTGTATTTTTTTTTT TAGTTGTGTTTTGTTATGTTAAAG GAYGTTA TATTGTATAA TTTTAATAAG GTTTTTAATT AGTTTTATTY GTTTTGGTTT TATTTTTATT TTTTAA	AACRTCCTTT AACATAACAA AACACAACTA AAAAAAAAAA T

3.6 Polymerase chain reaction (PCR)

Amplification of DNA through PCR using the eppendorf mastercycler. In a PCR cabinet, the master mix was prepared on ice as followed in table 4 to avoid contamination. The template DNA was added outside the PCR cabinet to also avoid DNA contamination of the cabinet. A period of optimisation was conducted and 2 µl of template DNA was determined to be the ideal amount to add to primer set 1 and 3 µl for primer set 2.

Table 4 - Reagents and volumes for PCR master mix for 1x reaction with a final volume of 25 µl

PCR reagents	Final concentration - 1x reaction
Forward primer	0.5 µl
Reverse primer	0.5 µl
MyTaqHS	12.5 µl
Nuclease free H_20 (NF-H_20)	6.0 µl
Coral dye 10 x	2.0 µl
Template DNA	2.0 µl primer set 1 3.0 µl primer set 2

After a period of optimisation, the PCR conditions that were deemed suitable for the primers are initial denaturation at 95 ^{o} C for 1 minute, denaturation at 95 ^{o} C for 15s, annealing at 52.6 ^{o} C for 15s and extension 72 ^{o} C for 10s. Denaturation to extension was repeated for 49 cycles and was held at 4 ^{o} C. The PCR products were stored at -20 ^{o} C until samples were used for analysis.

3.7 Bisulphite pyrosequencing

DNA extracted from the prefrontal cortex was bisulphite converted and prepared for pyrosequencing. During the set-up period, streptavidin beads were combined with the PCR product and other reagents as followed in the protocol to select out the biotin labeled product. The DNA was denatured to produce single stranded molecules for the sequencing. The heat block was set to 85 ^{o}C with the Q24 plate holder placed on top. The cartridge was cleaned with ddH_2O and checked to ensure that all the channels were clear from blockage. On the workstation, the trays were filled with wash buffer, ddH_20, 70% ethanol and denaturation solution. The pump was switched on and cleaned using ddH_20 in the workstation. The sepharose performance beads (GE Healthcare bioscience) were inverted to mix the solution to avoid damage of the beads via vortexing. Sepharose beads, binding buffer and PCR grade water was added to an eppendorf and mix gently in the volume of 1/40/14 µL. A 96 well plate was cut up to make a 24 well plate and in each well 55 µL of master mix was pipetted and 25 µL of amplified PCR samples. The PCR plate was sealed with a PCR cover film and plate was placed on the shaker (TS-100 thermo-shaker BioSan) for 10 minutes at 1400 rpm. The sequence primer is diluted to 0.3 µM in an eppendorf and vortexed. In the PyroMark Q24 sequencing plate, 25 µL of diluted sequencing primer was added to each well and placed on the workstation. The PCR plate was taken off the shaker and place onto the workstation with the lid removed carefully. The vacuum was turned on and the samples were processed in the following solution. IL-6 PCR samples for 15 seconds, 70 % ethanol for 5 seconds, denaturation buffer for 5 seconds and finally wash buffer for 10 seconds. The vacuum was then inverted 90^{o} for 5 seconds to clear the tubes of any remaining liquid. The vacuum was switched off and placed in the Q24 plate and was shaken from side to side for 10 second to dislodge the beads. The Q24 plate was transferred onto the heat block (DB-2D, Dri-Block®, Techne) left for 2 minutes at 85^{o} C. The PyroMark nucleotides were loaded into the PyroMark cartridge with volumes determined by the PyroMark Advanced software. Once the run was completed then analysis of methylation levels was determined using the PyroMark Q24 analysis feature. When looking at the results generated from pyrosequencing they are often presented as a pyrogram as seen

in figure 16. A pyrogram reports the signal intensity as shown on the y-axis when a base has been added on the x-axis.

3.8 Gel electrophoresis

An agarose gel of 2% was used in gel electrophoresis. To make the 2% agarose gel, 1 g of agarose powder and 50 mls of 1X TBE (Tris, Boric acid and EDTA) buffer is heated at full power in a microwave for 1 minute, once cooled 2.5 µl Midori Green Advance DNA Stain (NIPPON Genetics Europe) was added (0.5 µl/10mls). The gel was poured into a cast and a comb placed to create the wells and allowed to set for around 20/30 minutes. Amplified samples were then prepared with loading dye with the following volumes. Total volume added to each well was 10 µl so 10/5 = 2 µl of loading dye to 8 µl of PCR DNA sample. The gel was placed in the biorad tank with 1X TBE buffer and samples were loaded on to the gel. For sizing of the bands, a 50 bp ladder (Bioline) was ran alongside the DNA on the gel. The tank lid was placed on the tank and attached to the biorad power pack with the voltage set to 90 volts for 70 minutes. The gel was viewed using the licor imaging system and processed on image studio v2.0.

3.9 Genotyping

Kompetitive allele specific PCR assay (KASP) was performed to determine individual genotypes for rs1800795 polymorphism in n=67 prefrontal cortex brain samples. With the probe labelled to detect the alleles 1 and 2 which are labelled FAM and HEX. The sequence is detected through the changes in fluorescence. A 96 well plate was set up for allelic discrimination with diluted DNA extracted from the prefrontal cortex and reaction master mix with three negative controls and three positive controls to aid in analysis. First port of call was to dilute the extracted DNA with an overall concentration of 5 ng/µL in 40 µl final volume with NF-H_20 to dilute the DNA. The KASP master mix was created using 5 µl of KASP master mix solution (KASP^{TM}, LGC), with 3 µl of NF-H_20 and 0.14 µl of rs1800795 primer. In the 96 well skirted plate 8 µl of the master mix and 2µl diluted DNA was allocated to each well taking care to insure template DNA is placed in the correct

well. The plate was covered with a slip and spun to clear any air bubbles that had formed. The AB step one plus real time thermocycler was set up with specific temperatures seen in table 5. The cycle program for this assay included a touchdown step.

Table 5 - KASP assay thermal cycle conditions with touchdown

Protocol stage	Temperature	Duration	Cycles
Step 1 - Hot start Taq activation	94 °C	15 minutes	1
Step 2 Touchdown	94 °C	20 s	10
	68 °C - 0.6 °C decrease to achieve final of 62 °C	60 s	
Step 3 - Amplification	94 °C	20 s	26
	62 °C	60 s	
Step 4 - Read	30 °C	60 s	1

Once the first run had been completed, it was decided that a recycle run was needed to migrate the points of interest further to the clusters. The recycle program steps are indicated in table 6 below.

Table 6 - Recycling conditions for allele discrimination using KASP assay

Temperatures	Duration	Number of cycles
94 ° C	20 seconds	3 cycles
57 ° C	60 seconds	
30 ° C	60 seconds	1 cycle

Once the recycle was completed then the samples were analysed on the Stratagene Mx3000P qPCR system (Agilent) using the Mypro analysis software. Data was exported into Excel and a scatter graph plotted was used to determine the migration and the genotypes of rs1800795 as seen in figure 4.

3.10 Enzyme – Linked immunosorbent assay (ELISA)

The brain tissues were lysed with RIPA buffer (Sigma – Aldrich) which contained protease inhibitors cocktail (Sigma – Aldrich) to extract the protein. Protein concentration of IL-6 was determined using the Abcam high sensitivity human IL-6 ELISA kit. The pre-frontal cortex samples had different total protein concentrations. After ELISA analysis, the results were normalised using BCA total protein results. The control and standards were prepared following a serial dilution in the protocol. In the 96 well plate the blanks, 1X Control solution and standards 2-6 were added in duplicate at 100 µl. IL-6 samples were placed in the appropriate wells at 100 µl and in duplicate when possible. 1X Biotinylated anti-IL-6 was added to each well (50 µl), covered with PCR plate seals and incubated for 3 hours at room temperature (18-25°C). The cover was removed after the incubation period and the liquid aspirated from each well. A volume of 300 µl of 1 X wash buffer was added to each well and the liquid was aspirated. This wash step was repeated three more times. 1X Streptavidin-HRP solution was added at 100 µl into all wells, including the blanks and recovered with PCR plate seals and incubated at room temperature for 30 minutes. The wash step was repeated as above and then 100 µl of Chromogen TMB substrate solution was added into each well and incubate in the dark for 15 minutes at room temperature. Volume of 100 µl of stop reagent was pipetted into each well and results were taken immediately after the stop reagent was added. The absorbance was read on the spectrophotometer using 450 nm as the primary wavelength and 620 nm as the reference wavelength. Three data points were excluded during this process as they fell outside the levels of the kit.

3.11 Real Time polymerase chain reaction (RT-PCR)

Messenger RNA (mRNA) was converted into Complementary DNA (cDNA) using the Tetro cDNA synthesis kit (Bioline, UK) at 500ng of total RNA. This was then diluted 1:10 which equate to 2.5ng/µl cDNA. Primers were predesigned using the NCBI primer-BLAST software. The master mix consisted of 10 µl of 2x QuantiNova Syber green PCR master mix (quigen), 0.8 µl of forward primer, 0.8 µl of reverse primer (table 6 primers for RT-PCR), 6.4 µl RN – H_20 and 2 µl template cDNA with a total volume of 20 µl.

Table 7 - RT-PCR primer information. Forward and reverse primer gene of interest IL-6, 2 housekeeping gene include β actin and GAPDH.

	Forward primer	Reverse primer
IL-6 Gene of interest	5′ - GGTACATCCTCGACGGCATCT- 3′	5′ - GTGCCTCTTTGCTGCTTTCAC-3′
β actin Housekeeping gene	5′- ATCCTCACCCTGAAGTACC - 3′	5′ - ATAGCAACGTACATGGCTGG - 3′
GAPDH Housekeeping gene	5′ CCGCATCTTCCTTTTGCGTCG – 3′	5′ - TGGAATCGCCATGGGTGGA - 3′

Using a 96 well PCR plate, 18 µl of master mix was pipetted into each well. Then 2 µl of cDNA was added with two negative controls and some samples were duplicated. The PCR plate was covered with a clear film and spun with a centrifuged to mix the solution and clear any air bubbles. The biorad RT-PCR machine was set up with the following thermal cycle condition. Denaturation 95 ^{o} C for 2 minutes, denaturation 95 ^{o} C for 5 seconds, annealing at 60 ^{o} C for 5 seconds (read stage) and extension 72 ^{o} C for 10 seconds and this was repeated for 39 cycles. A melt curve analysis was added with the following setting 65 ^{o} C for 5 seconds with increments of 0.5 ^{o} C with a plate read and 95 ^{o} C for 5 seconds.

Using this equation, results were normalised for *IL-6* gene expression against 2 housekeeping genes β actin and GAPDH. These particular genes are important as they act as the internal controls. Primer efficiency was assumed to be 100%.

$$Relative\ gene\ expression = \frac{(E_{\mathrm{GOI}})^{\Delta Ct\ GOI}}{GeoMean[(E_{\mathrm{REF}})^{\Delta Ct\ REF}]}$$

GOI = Gene of interest,

REF = Housekeeping genes,

E= Efficiency (2 = 100%)

3.12 Immunofluorescence

Brain samples were taken from the -80 freezer and placed on dry ice to stop the brain samples from thawing. The samples were mounted onto cork using Neg-50 blue, once dried the samples were mounted on to the cryostat (LEICA CM 3050). The settings for the cryostat included, chamber temperature -16 ^{o} C, chuck temperature -18 ^{o} C and the angle of the cutting blade set to 7 ^{o}. Sections of brain tissue were cut and placed on the charged side of the slide. Using a PAP pen a circle was drawn around the section as this creates a hydrophobic barrier. The brain tissue samples were re hydrated with PBST (1L PBS and 500 µl Tween 20) for 5 minutes. For 30 minutes the slides were blocked in blocking solution which consisted of 4% goat serum and 9.6mls of PBS with 80 µl added per a slide. This is to block unspecific binding of the secondary antibody. Primary antibodies used include anti human IL-6 anti-mouse (1/50) and Anti-NF-kB p65 (phospho S 536) antibody (1/100) which were pipetted into blocking solution and 80 µl added to each slide and incubated in the dark at 4 ^{o} C overnight. Slides were rinsed in PBS for 5 minutes and the secondary antibodies prepared. Alexafluro IgG 568 goat anti mouse was used to detect IL-6 (red) and Alexafluro 488 goat anti-rabbit IgG was used to detect NFκB (green) at 1/200 for 1 hour. The slides were washed in PBS for 5 minutes and mounted with vector shield (h-1200 with DAPI) and covered. Enamel paint was used to fix the coverslips in place before viewing on the Zeiss fluorescence microscope with a 10 x 63 oil magnification.

3.13 Statistical analysis

The SPSS statistics software package (v25.0 for Windows; SPSS, Chicago, IL) was used for statistical analyses and GraphPad v7. Data was tested for normality using the Shapiro–Wilk test. Data which is determined as normally distributed was presented as mean ± standard deviation (SD) or median and 25^{th}-75^{th} percentiles if not normally distributed. Table of characteristic were presented and grouped between genotypes to indicate any significance. IL-6 protein levels and *IL-6* expression levels was analysed using a Mann-Whitney U test. A Kruskal Wallis test was used to analyse the significance of IL-6 protein levels between the 3 neuropathological hallmarks (THAL, CERAD and BRAAK). Methylation percentage among CpG sites were analysed using an independent T-test. Correlations between protein, DNA methylation and gene expression of IL-6 was determined with Spearman's correlation test. Significance threshold of $P=<0.05$.

4.0 Results

4.1 Optimisation of PCR for amplification of IL-6 from bisulphite converted DNA

A period of optimisation was employed to develop the most suitable parameters and conditions for the IL-6 primers. To find the optimal annealing temperature a gradient was performed using different temperatures. These were then viewed on a 2% agarose gel seen below in figure 1.

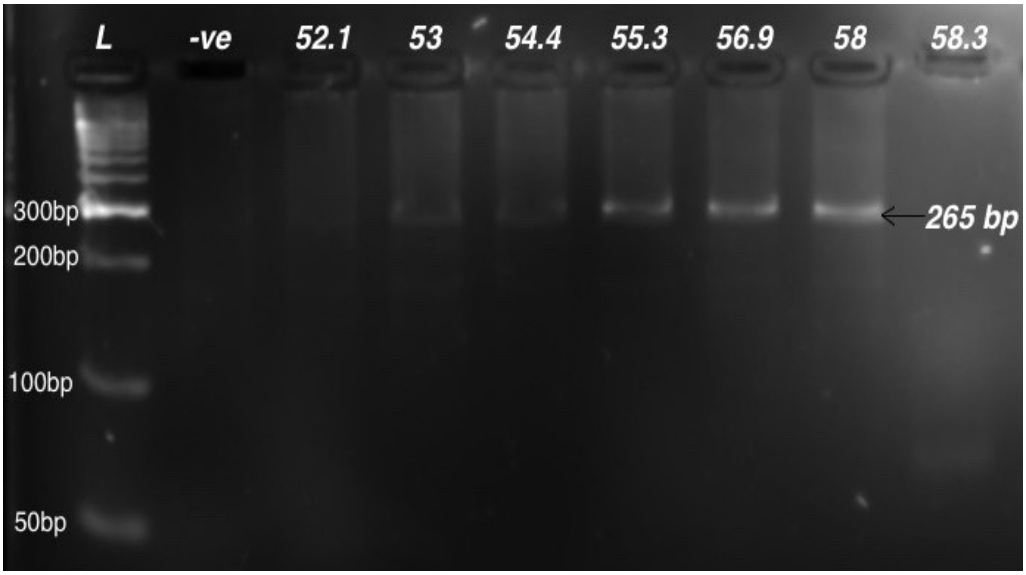

Figure 1 - Gel electrophoresis viewing PCR gradient for optimisation of IL-6 primers using temperatures from 52.1ºC- 58.3ºC. Pooled DNA was amplified using a gradient of temperatures and run on a 2% agarose gel with a DNA ladder and a negative control.

PCR gradient for optimisation purposes for primer set 1 (see table 3) on bisulphite converted DNA extracted from the prefrontal cortex. This revealed that the bands had migrated to the correct region of 265 base pair (bp). Ideally the ladder would have migrated more to allow a clearer picture of the migration and high molecular smears were detected in several wells. Annealing temperature of 58^{o} C was determined the best annealing temperature.

Second gradient was performed to determine if 58^{o} C was the best annealing temperature by employing another gradient to include temperatures greater than 58^{o} C s seen below in figure 2. This was to confirm that 58 ^{o}C is the best working condition for set 1 IL-6 forward and reverse primers.

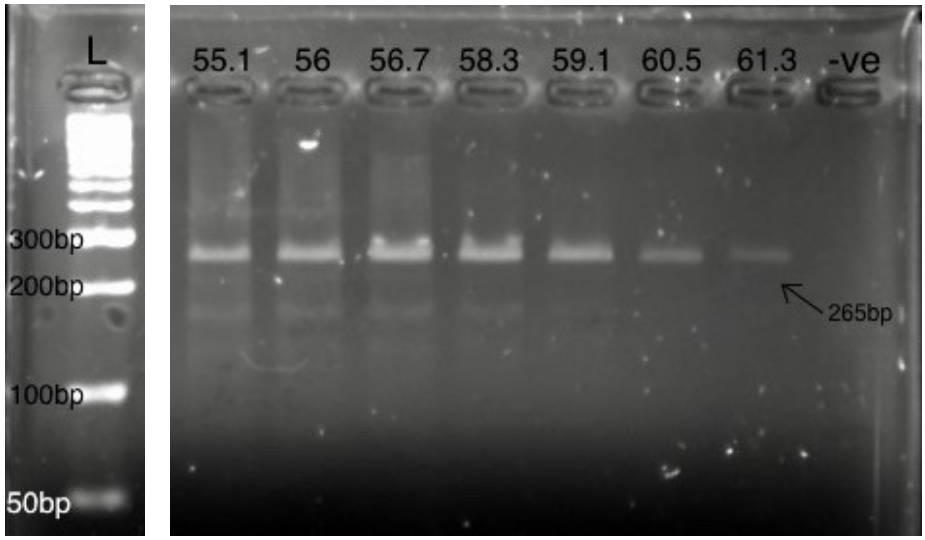

Figure 2 - Gel electrophoresis viewing PCR gradient for optimisation of IL-6 primers using temperatures from 55.1ºC- 61.3ºC. Pooled DNA was amplified using a gradient of temperatures and run on a 2% agarose gel with a DNA ladder and a negative control.

PCR gradient for optimisation purpose for primer set 1 on bisulphite converted DNA revealed the bands had migrated to the correct region of 265 base pair. However high molecular smears were present and the annealing temperature of 58^{o} C was confirmed to be the optimal annealing temperature.

First set of primers were abandoned due to the constant presence of unspecific binding. A period of troubleshooting was employed to determine the cause of the problems with primer set one. The unspecific binding was not resolved by increasing the temperature and altering PCR conditions. The decision was taken to redesign the primers around the same region, as the previous primers would have caused problems with pyrosequencing analysis. Primer set 2 were designed with a base pair length of 261 and the biotin tag was labelled on the forward primer meaning that sequence is now read from right to left. Optimisation to achieve the optimal working conditions was employed and again, a gradient was used with results seen in figure 3.

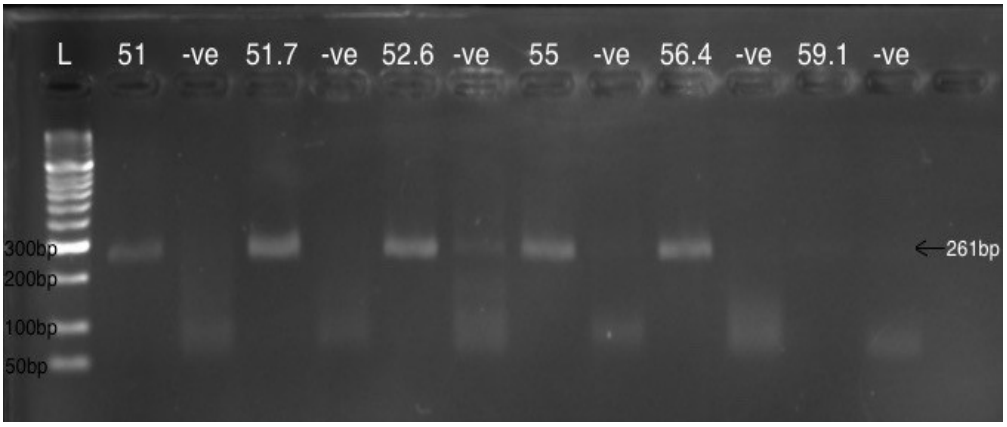

Figure 3 - Gel electrophoresis viewing PCR gradient for optimisation of IL-6 primers using temperatures from 51ºC- 59.1ºC. Pooled DNA was amplified using a gradient of temperatures and run on a 2% agarose gel with a DNA ladder and a negative control for each sample.

Extracted DNA was run on a PCR at different annealing temperatures and viewed on a 2% agarose gel to determine the best annealing temperature for set 2 IL-6 primer forward and reverse. The optimal annealing temperature was determined to be 52.6 ^{o}C which produced the brightest and clearest band. Each negative control produced unspecific bands around 100 base pair.

4.2 Genotyping of IL-6 polymorphism rs1800795

Genotyping for the *IL-6* SNP rs1800795 was preformed using Kompetitive allele-specific PCR (KASP) to determine the genotypes of the SNP in the *IL-6* promoter region. Figure 4 shows an allele discrimination plot with homozygotes GG, homozygote CC and heterozygote CG genotypes.

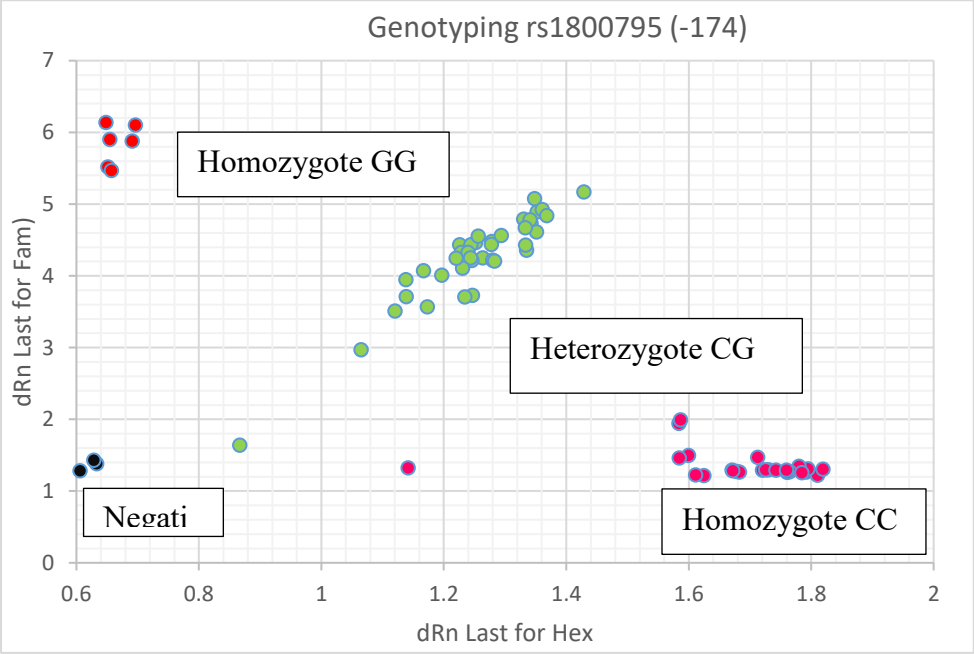

Figure 4 - Allele discrimination of rs1800795 polymorphism. Black circles are 3 negative controls, red circles are homozygote GG genotypes, green circles are heterozygote CG, and pink circles are homozygote CC. Three clusters present represent the n=67 prefrontal.

It is possible to determine the allele frequency and input the results into the Hardy Weinberg Equation. Positive controls were used to help aid in determining the genotype and these samples were from the Myoage study, which had already been genotyped for the rs1800795 SNP.

Allele frequency and Hardy Weinberg equation for genotyping data

- CC genotype 24 samples 35.8%
- CG genotype 38 samples 56.7%
- GG genotype 5 samples 7.5%

Allele frequency

- P = 86/134=0.358
- Q= 48/134=0.642

HWChi-squared test: Chi-square test for Hardy-Weinberg equilibrium (autosomal) p= 0.0559. Regarding the p = 0.056, this just fit within the Hardy Weinberg equilibrium.

4.3 Real time PCR of IL-6 gene expression analysis

Detection of *IL-6* expression levels was detected through RT-PCR and normalised against two housekeeping genes, which included β actin and GAPDH. The SYBR® Green reagent used during this experiment can bind to any double-stranded DNA product. Making it vital to check the melt curve for multiple peak formation as only one peak should be present. Multiple peaks would indicate that there could be primer dimer or nonspecific binding.

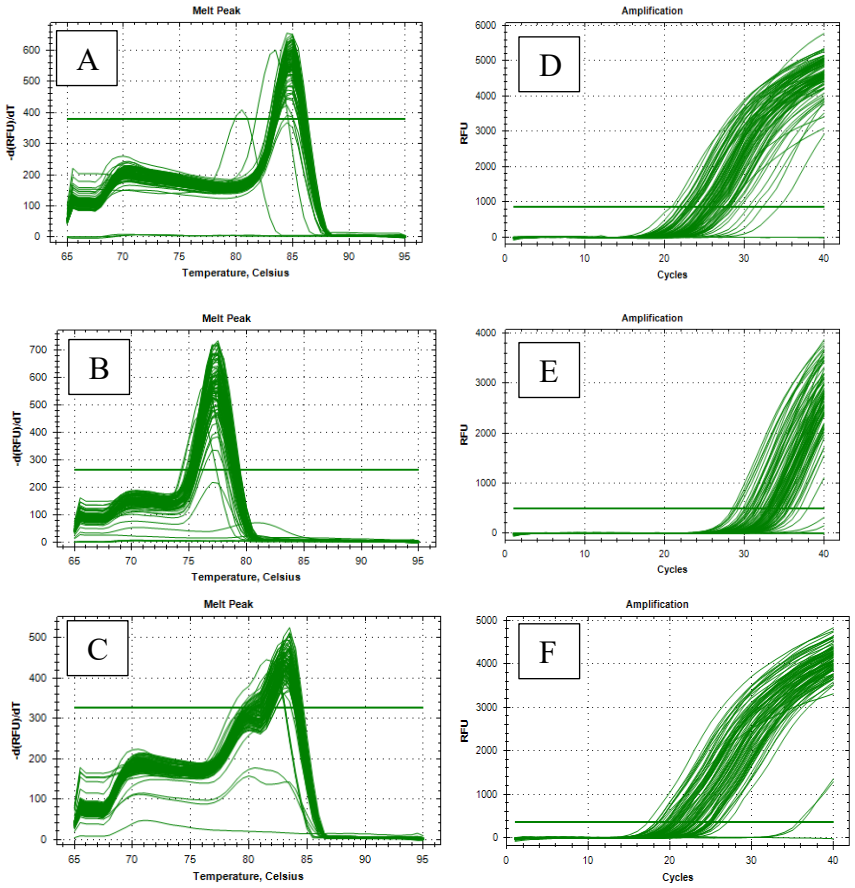

Figure 5 - **Melt peak analysis of *(5A) βactin, (5B) IL-6 and (5C) GAPDH* gene transcripts detected by real-time PCR.** cDNA samples were amplified using RT-PCR with gene of interest and two housekeeping primers. Melting peak analysis was performed to confirm the PCR products. Amplification analysis is shown in figure 5D-F and indicates at which cycle the amplification starts to take place.

Figure 5A, B and C shows melt peak analysis of *β actin*, *IL-6* and *GAPDH*. All three RT-PCR products produce one peak in the melt peak analysis but there is slight wave in the peak shown if figure 5A, B and C. This could indicate primer dimer and unspecific binding.

Further analysis using an electrophoresis gel to determine if the samples have been amplified through RT-PCR and if the deviation is indeed primer dimer or unspecific binding.

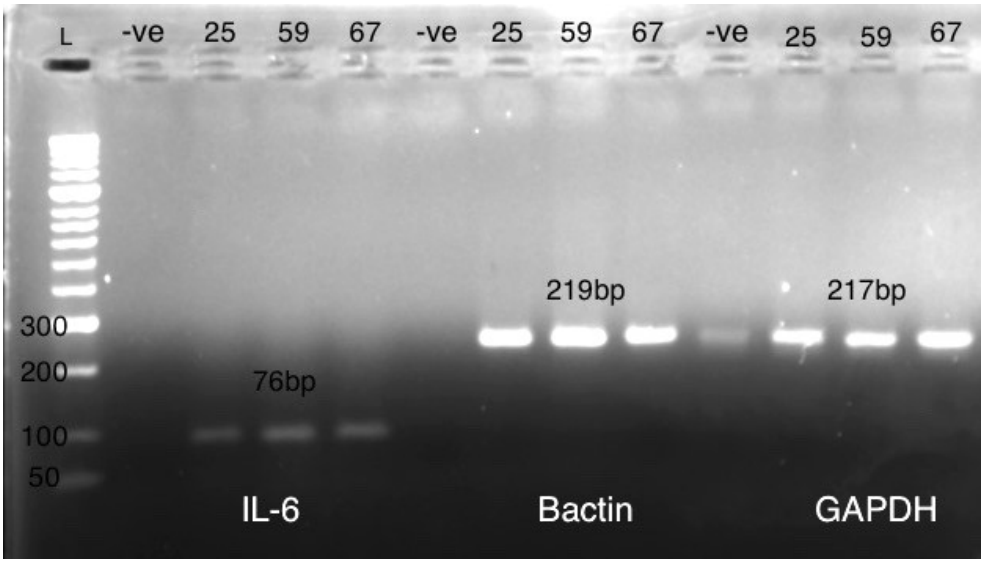

Figure 6 - Gel electrophoresis viewing RT - PCR results for *IL-6* – 76 bp, β actin - 219 bp and GAPDH – 217 bp. A few cDNA samples were run on a 2% agarose gel with a DNA ladder and a negative control for each gene.

Gel electrophoresis was used to determine that the RT-PCR analysis of *IL-6*, β actin and GAPDH had efficiently amplified and no primer dimer is present or unspecific. *IL-6* primers were designed at 76 bp which is consistent to what was seen in the electrophoresis gel and β actin was design at 219 bp and GAPDH was designed at 217 bp and this is seen in the gel electrophoresis in figure 6.

4.4 Brain sample characteristics

4.4.1 Table of characteristic of participants subgroup via genotype

Supplementary information for n=67 prefrontal cortex samples which have been sub grouped by genotype of rs1800795 polymorphism. Genotype CG/GG have been grouped due to the small sample size for GG (n=5) and was continued to be grouped throughout the analysis. Some information is not present for all n=67 prefrontal cortex samples and this is stated in the table below when different.

Table 8 - Table of characteristic of participants subgroup via genotype. Data given in mean ± standard deviation unless stated otherwise. Frequency for CC is n=24 and CG/GG is n=43 unless stated differently.

	CC	CG and GG	p value
Age at death	88.21 ±(4.72)	87.12 ± (6.73)	P = 0.484
Sex (Males/Females)	6/18	15/28	
Whole brain weight (g)	1220.29 ± (114.6)	1201.14 ± (148.62)	P = 0.674
Post mortem, delay (Hours)	77.84± (45.20)	75.16± (43.37)	P = 0.821
THAL stage (amyloid deposition)[b]			P = 0.915
None	6 (25 %)	11 (25.6%)	
1	3(12.5%)	8 (18.6%)	
2	2 (8.3%)	4 (9.3%)	
3	8 (33.3%)	9 (20.9%)	
4	3 (12.5%)	6 (14%)	
5	2 (8.3%)	5 (11.6%)	
CERAD score (neuritic plaques)[b]			P = 0.983
None	7 (29.2%)	11 (25.6%)	
A (Sparse)	7(29.2%)	12 (27.9%)	
B (Moderate)	6 (25%)	12 (27.9%)	
C (frequent)	4 (16.7%)	8 (18.6 %)	
BRAAK stages(neurofibrillary tangles) [ab]			P = 0.424
None	0	4 (9%)	
I – II	12 (52%)	17 (40)	
III – IV	8 (35 %)	17 (40 %)	
V – VI	3 (13 %)	5 (12%)	

Data presented as mean and standard deviation, unless stated otherwise

a- n=23 CC genotype for BRAAK analysis

b- Data presented as n(%)

In the supplementary table 8, key information was analysed to determine if genotype has an effect. Age of death and brain weight are not significantly different upon genotype of rs1800795 polymorphism. Brain tissue determined to have the CC genotype (88.21 ± 4.72) had no significant difference in age of death ($p=0.484$) compared to those with CG/GG genotype. The whole brain weight at post-mortem was not significantly different ($p=0.674$) when compared to the different genotypes, CC (1220.29 ± 114.6). THAL, CERAD and BRAAK scores were determined to have no significance in scoring upon genotype.

4.4.2 IL-6 protein and AD pathology

THAL, BRAAK and CERAD staging are an important classification system when determining AD neuropathology. These scores can be used to determine whether the person has AD or could be classified as a control. IL-6 protein levels were compared with various measures of AD neuropathology to determine if levels are altered at different stages among THAL, BRAAK and CERAD.

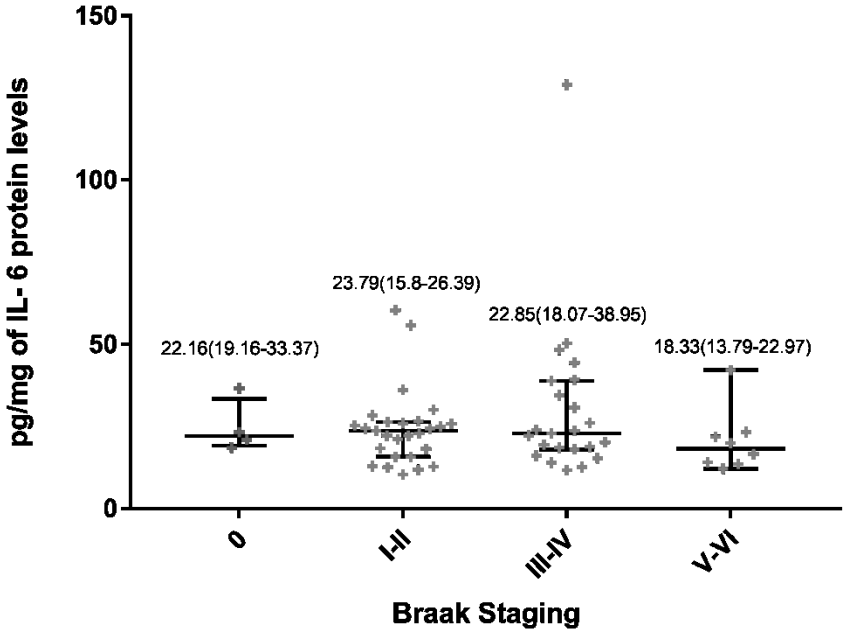

Figure 7 - Relationship of IL-6 protein levels in the different BRAAK stages. Box plot presented as median and 25th-75th percentiles with individual plots of each samples represented. IL-6 protein levels were not found to be significantly different between BRAAK staging (P=0.504) n=62.

Some samples could not be classified into BRAAK staging and so these samples were omitted from the anaysis. Data was deemed to be not normally distributed which resulted in a Kruskal Wallis test being performed to look at potential significance in IL-6 protein levels between BRAAK staging which is scoring neurofibrillary tangle formation. The median and 25th-75th percentiles was reported in each BRAAK group for IL-6 protein levels seen in figure 7. No significance was found in IL-6 protein levels (P=0.504) among the different BRAAK staging.

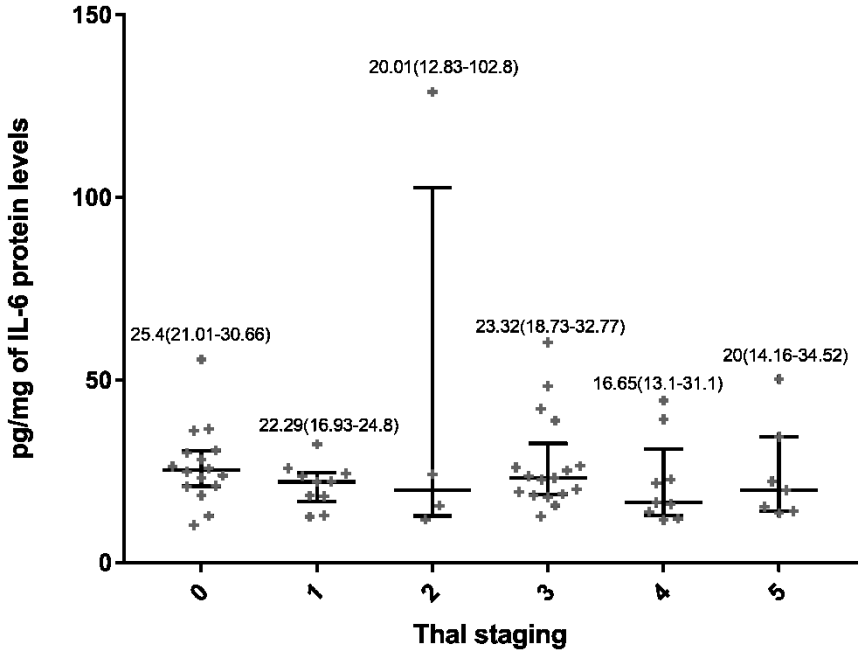

Figure 8 - Relationship of IL-6 protein levels in the different THAL staging. Box plot presented as median and 25th-75th percentiles with individual plots of each samples represented. No significance (p = 0.531) indicated between Thal grouping and IL-6 protein levels with a total sample size of n=63.

Some samples could not be classified into Thal staging and so these samples were omitted from the anaysis. Thal staging data was determined to be not normally distributed. Kruskal Wallis test was performed to look at potential significance in IL-6 protein levels between THAL staging which is looking at amyloid plaque formation. The median and 25th-75th percentiles were reported in each THAL stage for IL-6 protein levels seen in figure 8. No significance was found in IL-6 protein levels (P = 0.531) among the different THAL staging.

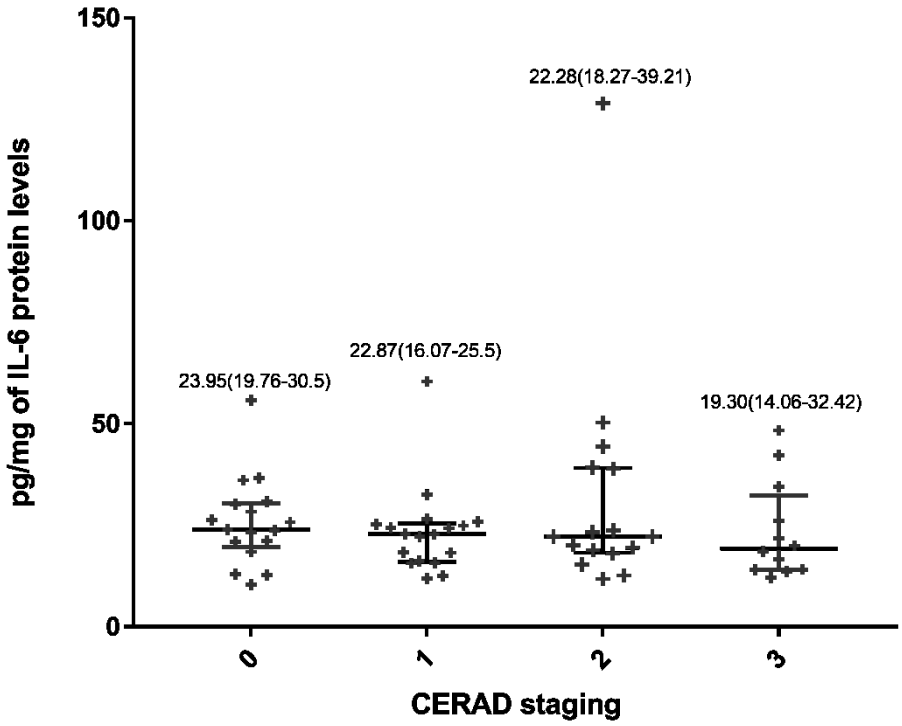

Figure 9 - Relationship of IL-6 protein levels in the different CERAD stages. Box plot presented as median and 25th-75th percentiles with individual plots of each samples represented. No significance (P = 0.718) indicated between stages and IL-6 protein levels with a total sample size of n=63.

Some samples could not be classified into CERAD staging and so these samples were omitted from the anaysis. Data was determined to be not normally distributed and a Kruskal Wallis test was performed to look at potential significance in IL-6 protein levels between CERAD staging which is looking at Amyloid plaque formation. The median and 25th-75th percentiles were reported in each CERAD stage for IL-6 protein levels seen in figure 9. No significance was found in IL-6 protein levels (P = 0.718) among the different CERAD staging.

4.4.3 IL-6 single nucleotide polymorphism, protein levels and gene expression

Levels of IL-6 total protein and relative *IL-6* gene expression were compared between the two different genotypes of the rs1800795 polymorphism to determine if the levels are significantly altered upon which allele is carried. Indicating if particular allele alters protein and expression levels.

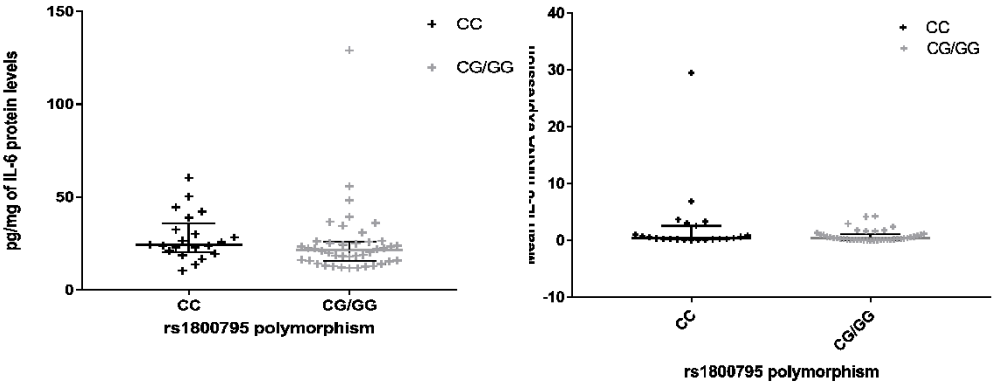

Figure 10 - Gene expression of protein levels in prefrontal brain samples of CC and CG/GG genotype of the rs1800795 polymorphism. Box plot presented as median and 25th-75th percentiles with individual plots of each samples represented. A –IL-6 Protein levels in CC (n21) or CG/GG (n 42) genotype brain samples are not significantly different (p = 0.054) B – mRNA levels of *IL-6* in CC (n23) and CG/GG (n 41) prefrontal cortex samples are not significantly different (p = 0.433).

Interleukin-6 total protein was tested for normal distribution using the Shaprio Wilk test. Results showed that the data is not normally distributed with a p=<0.000. A Mann-Whitney test was therefore performed to determine statistical significance. Interleukin-6 protein levels in carriers of C allele was 24.33 (20.35-35.73) and this was not significantly higher ((309.000 (p value. 0.054)) than G allele carriers. There is no significance in IL-6 protein levels dependent upon genotype and this fits in with the latitude.

Interleukin-6 gene expression was tested for normal distribution using the Shaprio Wilk test which showed a p=<0.000. A Mann-Whitney U test was preformed to determine any significance in expression levels depending on genotype. *IL-6* gene expression in C allele carriers was 0.39 (0.18-2.51) and this was not significantly higher ((415.0 (p value .433)) than G allele carriers.

A correlation determined between gene expression and IL-6 protein levels via genotype was employed to determine if correlation is altered.

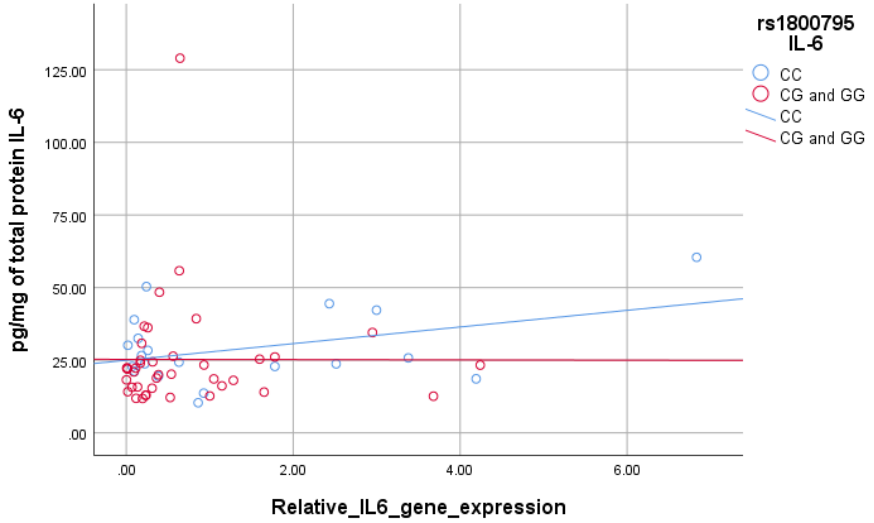

Figure 11 - Correlation of *IL-6* gene expression and protein levels in the pre-frontal cortex determine by genotype. No significant correlation detected in CC (n=20) (p=0.980) and CG/GG (n=40) (p=0.398) of the rs1800795 polymorphism.

Spearman's rho correlation was performed to determine if IL-6 protein levels have a correlation with *IL-6* mRNA. No correlation between *IL-6* mRNA expression and protein levels were observed in either genotype CC (n=20) p=0.980 or CG/GG (n=40) p=0.398.

4.4.4 IL-6 gene expression and protein levels in the control vs early AD group

Levels of IL-6 protein and relative *IL-6* gene expression were compared and related to the control and early AD groups. Sample size was on the smaller side due to not every sample being determined between the control and early AD using the neuropathphysiology staging system. The comparison was determine if there is a higher expression of *IL-6* in prefrontal cortex samples classed as having early AD.

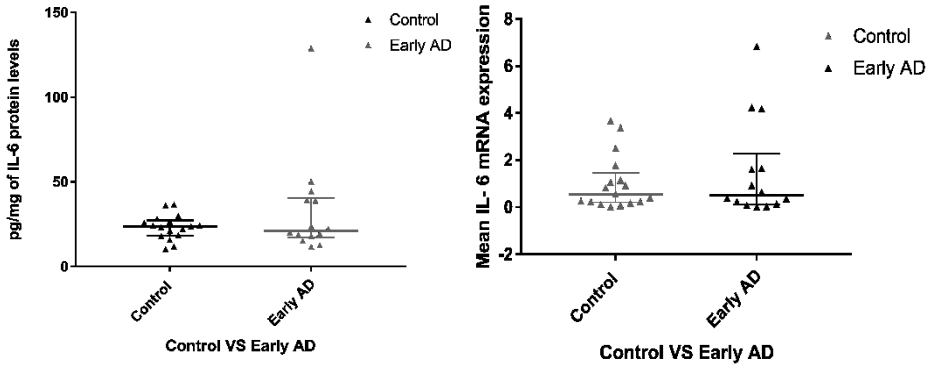

Figure 12 - Gene expression on protein levels in brain of the Control and early AD. Box plot presented as median and 25th-75th percentiles with individual plots of each samples represented. A – IL-6 Protein levels in control group (n=17) and early AD group (n=14) where not significant (p=0.905). B - *IL-6* mRNA levels in control group (n=17) and early AD group (n=14) where not significant (p=0.968). Data presented as median(range) in a box and whisker blot with individual sample marker.

IL-6 protein levels were determined as not normally distributed and data present as median and 25th-75th percentiles. A Mann-Whitney test was performed to determine statistical significance. IL-6 total protein levels in the control group was 23.79 (18.4 – 27.48) and this was not significantly higher (116 (p=0.905)). There is no significant relation between IL-6 protein levels when compared to the control and early AD group.

IL-6 expression levels were determined as not normally distributed and data presented as median and 25th-75th percentiles. A Mann-Whitney test was performed to determine statistical significance. Relative *IL-6* gene expression in the control group was 0.56 (0.21– 1.47) and this was not significantly higher 118(p=0.968) than early AD group. There is no significance in *IL-6* expression levels in the control and early AD groups.

Further step was conducted to assess IL-6 protein levels and *IL-6* mRNA expression levels in control and early AD groups, with further stratification into the two IL-6 genotypes.

Table 9 - IL-6 protein and expression levels in the control and AD stratified by genotype to determine a significance.

	Control	P value	AD	P value
IL-6 protein	N=7 - CC	P=0.626	N=5 - CC	P=0.096
	N=10 - CG/GG		N=9 - CG/GG	
IL-6 expression	N=7 - CC	p=0.874	N=5 - CC	P=0.828
	N=9 - CG/GG		N=9 - CG/GG	

After further analysis of the data with the control and the early AD group being split by genotype, no significance was determined in IL-6 protein level and IL-6 expression levels. Table 9 shows the analysis between the groups and the p values from the analysis which shows no significance.

4.4.5 Immunofluorescence staining

Immunofluorescence staining was used to look at activation of IL-6 and NFκB in the prefrontal cortex in three control and three early AD samples. It is well known that during inflammation NFκB activates IL-6, which accumulates around the nucleus. Control and early AD samples were used to look at IL-6 accumulation and NFκB activation. Due to time constraints only six samples were selected for immunofluorescence staining. No neuronal marker was selected for staining leading to the cell type not being identifiable in the images.

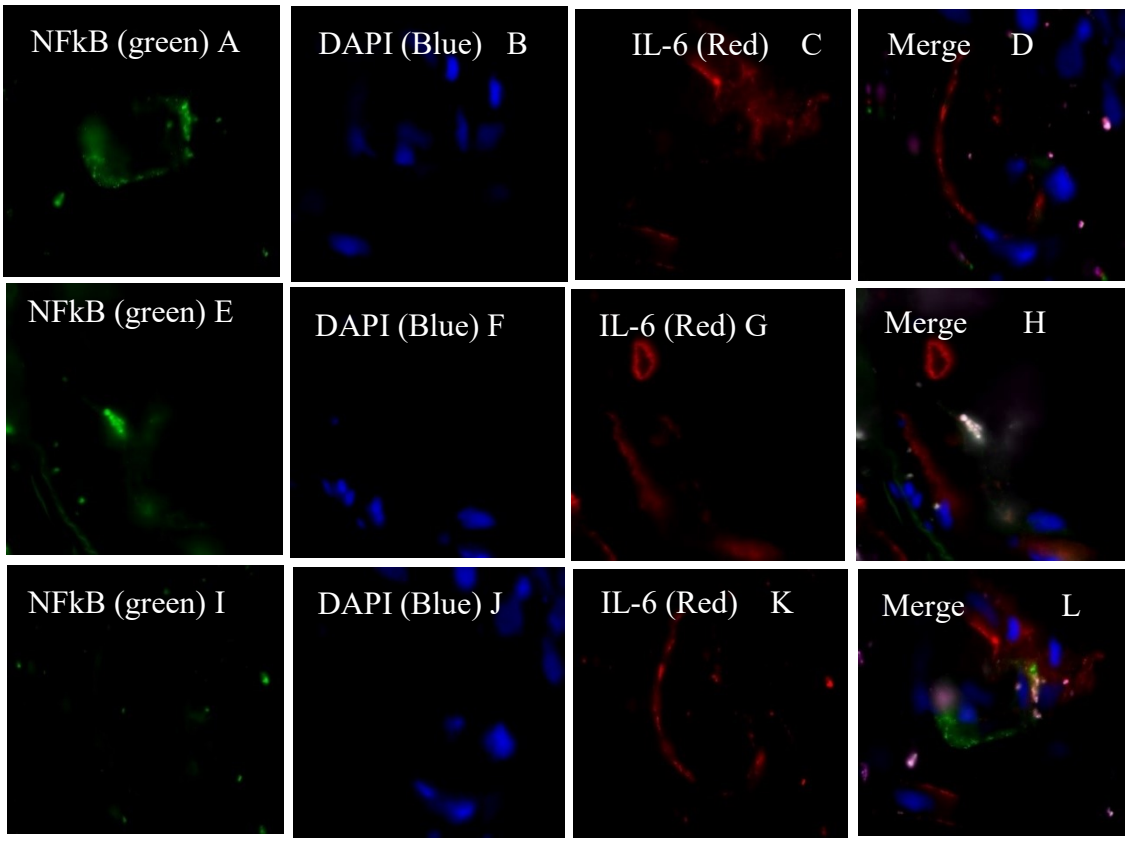

Figure 13 – Immunofluorescent staining results for AD human pre-frontal cortex sample stained for IL-6 and NFκB. A-D sample 13 viewed at 10 x 63 oil magnification stained for IL-6 red, NFκB green and DAPI blue for nucleus. E-H sample 11 viewed at 10 x 63 oil magnification stained for IL-6 red, NFκB green and DAPI blue for nucleus. I-L sample 15 viewed at 10 x 63 oil magnification stained for IL-6 red, NFκB green and DAPI blue for nucleus.

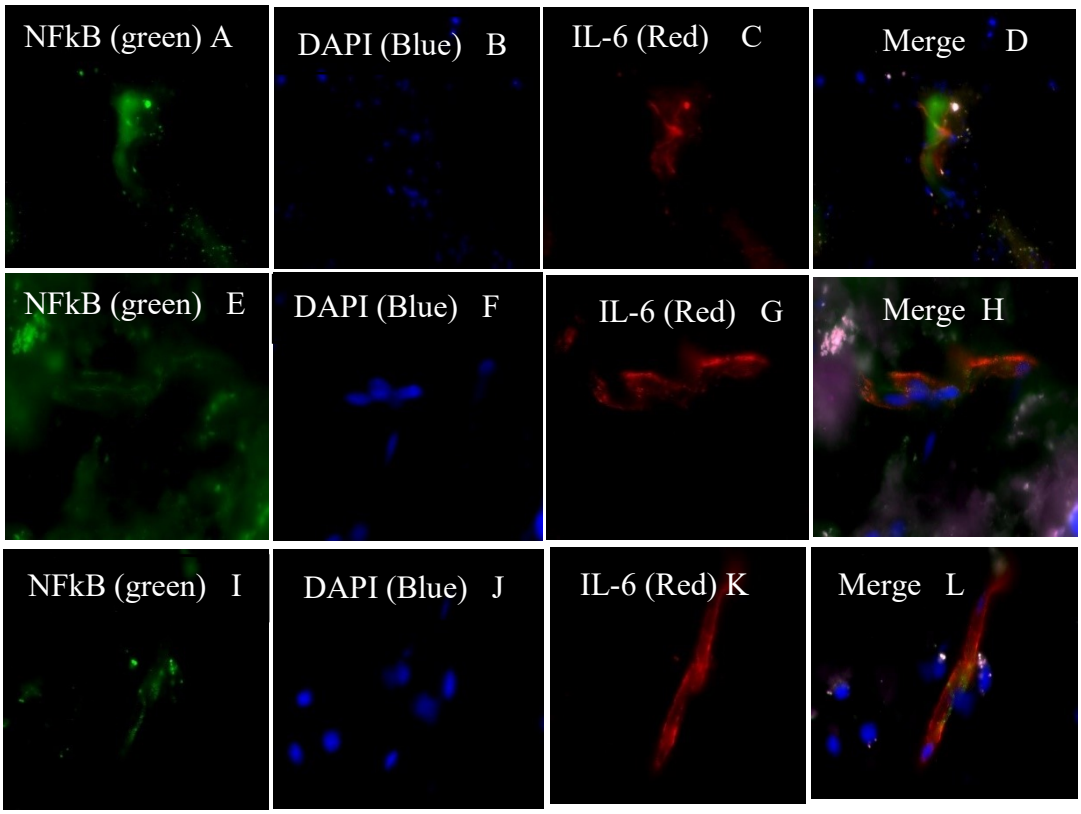

Figure 14 - Immunofluorescent staining of control human pre-frontal cortex sample stained for IL-6 and NFκB. A-D sample 25 viewed at 10 x 63 oil magnification stained for IL-6 red, NFκB green and DAPI blue for nucleus. E-H sample 30 viewed at 10 x 63 oil magnification stained for IL-6 red, NF_kB green and DAPI blue for nucleus. I-L sample 2 viewed at 10x63 oil magnification stained for IL-6 red, NF_kB green and DAPI blue for nucleus.

Immunofluorescent staining was performed to determine *IL-6* localisation in the prefrontal cortex of both early AD and control samples and activation of NF-κB. However, it is not possible to determine which cell type, as no glial cell marker was used to stain for glial cells such as microglia or astrocytes. This was a limitation of the antibodies available. Some degree of auto fluorescence was present in all sample, which is natural fluorescence rather than stained. IL-6 antibody staining was strongly expressed in all samples. Indicating that IL-6 is expressed in early AD and control samples. Ideally, a negative control would have been used during the project however this was not the case. The negative control is important to determine the visualization from the antibody and molecule of interest.

4.4.6 Pyrosequencing pyrogram results from human prefrontal cortex samples

Figure 15 shows a schematic of the *IL*-6 gene and the sequence to analyse for pyrosequencing showing the variant in the polymorphism using primer set 2.

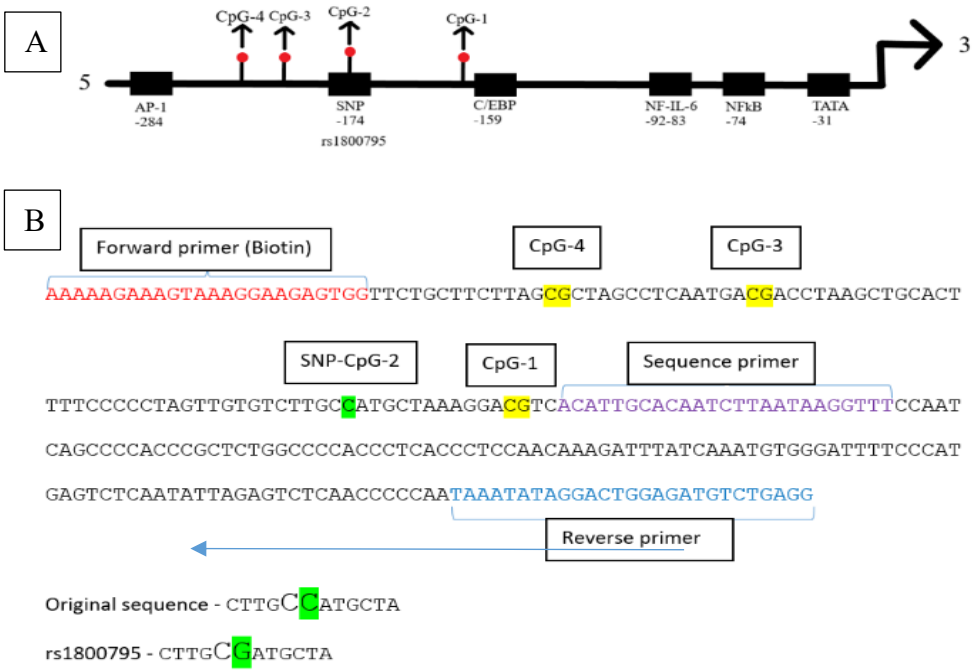

Figure 15 – A – *IL-6* gene promoter region schematic. B - Sequence for analysis highlighted with the forward reverse and sequence primer. A - Red beacon in the IL-6 promoter region schematic indicated 4 CpG sites. B- DNA sequence indicating forward, reverse, sequence primer and CpG site location in the sequence. Highlighted in yellow are the CpG sites for analysis. Highlighted in blue is the position of the rs1800795 polymorphism situated at -174 base pair. Depending on the genotype for this SNP a CpG site is added as seen in the sequence below showing the change at the CpG site.

In figure 15A the position of TATA, NFκB, NF-IL6, C/EBP and AP-1 was determined through reading the literature of another scientist researching in to the *IL-6* promoter region (Poplutz et al., 2014).

Figure 16 shows two different sample results for DNA methylation depending on genotype. The pyrogram was analysed to ensure quality controls have passed and samples have been fully bisulphite converted.

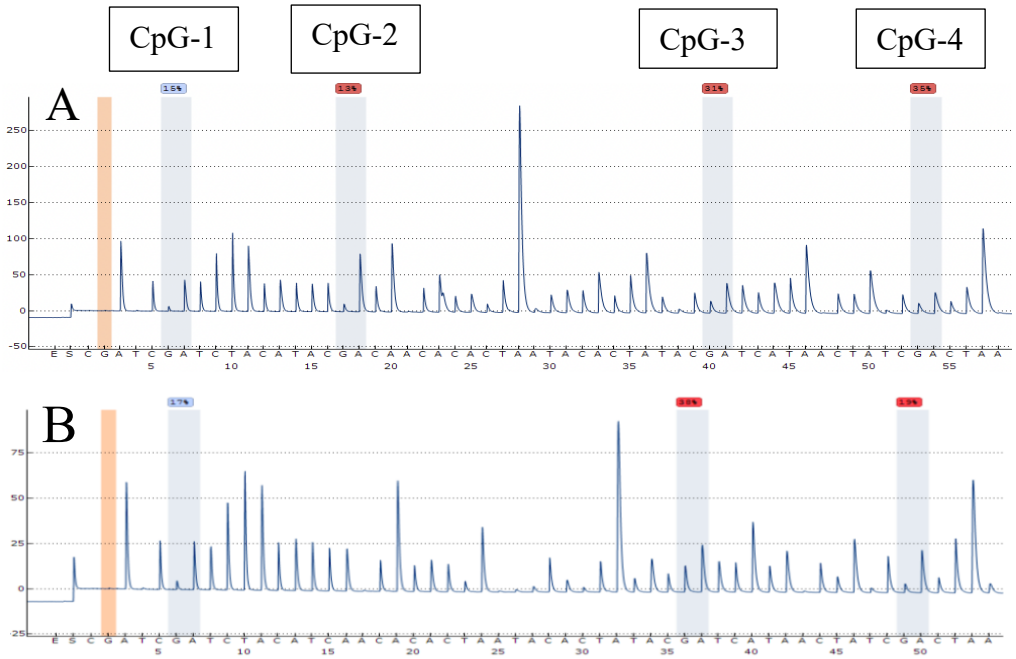

Figure 16 - Pyrograms looking at DNA methylation in IL-6 gene depending on genotype of rs1800795 polymorphism. A - Pyrogram for sample 46 (CG) variant with extra CpG site noted at the single nucleotide polymorphism site. B - pyrograph for sample 9(CC) with 3 CpG sites for anaysis noted extra CpG site is not present.

The control stage passed in both programs indicating that the samples have passed quality control and have been fully bisulphite converted. Figure 16A included 4 CpG sites for DNA methylation analysis. Due to the G allele, being present this generates an extra CpG site, which is rs1800795 SNP. This is indicated at the second CpG site on the pyrograph. The signal generated from the pyrograph on the Y-axis has generated high signal. CpG 1 has passed with a methylation percentage of 16 % whereas the other three CpG site have failed indicated with the methylation percentage being in red. Samples were repeated in duplicate to show if similarity is present upon re-testing of the samples at the failed sites. Figure 16B shows the results from sample 9 (CC) which does not have the extra site at CpG 2, as the C allele does not produce the extra CpG site. Good signal on the Y-axis is indicated and again the first CpG site is highlight in blue indicating a passed result of 17% methylation whereas the other two CpG sites are highlighted in red indicating a failed result. Samples were repeated in

duplicate to indicate if methylation is similar. Ideally, all the samples should have been repeated in duplicate but due to issues that arose with the sequencer it was not possible to gain all the samples in repeats. Some results only had 20 µl of DNA rather than 25 µl, however methylation percentage did not significantly change with the different DNA volumes.

The mean methylation percentages for all samples for each CpG site were presented in a bar grpah to inidicated the percentage methylation at each site.

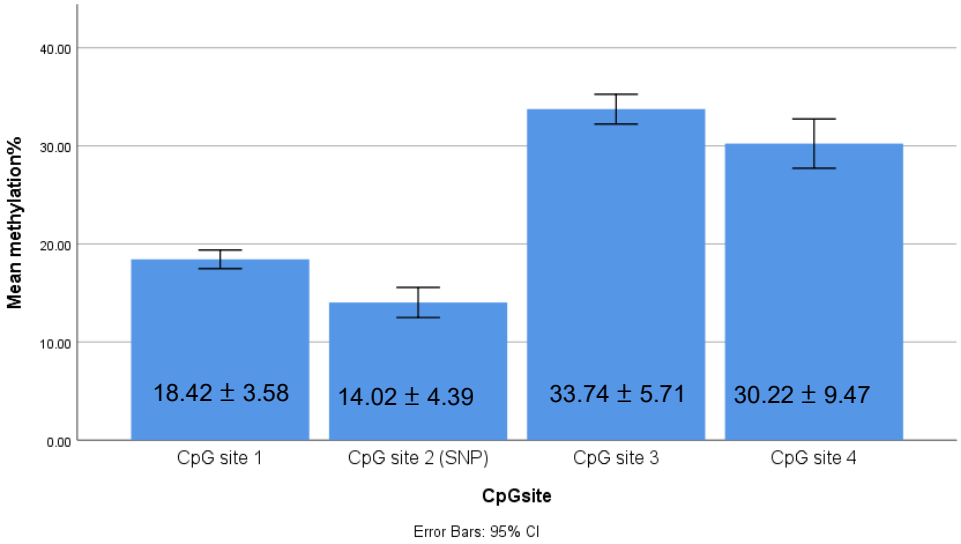

Figure 17 - Mean CpG methylation of *IL-6* in all brain samples at the 4 individual CpG site. CpG 2 methylation is only presented in samples that carry CG/GG genotype (n34) in the rs1800795 polymorphism as the G allele creates an extra CpG site in these samples. In each column, an individual CpG site is represented and the mean ± Standard deviation is presented.

The results presented in figure 17, conclude that CpG site 3 has the highest overall percentage methylation of all the samples compared to the other 3 of which CpG site 2 in only present in the G allele carriers.

4.4.7 Methylation of IL-6 promoter CpG sites

IL-6 percentage methylation in the prefrontal cortex was compared to different genotypes CC and CG/GG. Genotype CG/GG produces an extra CpG site at site 2 whereas CC does not, and this could possibly have an effect on methylation levels.

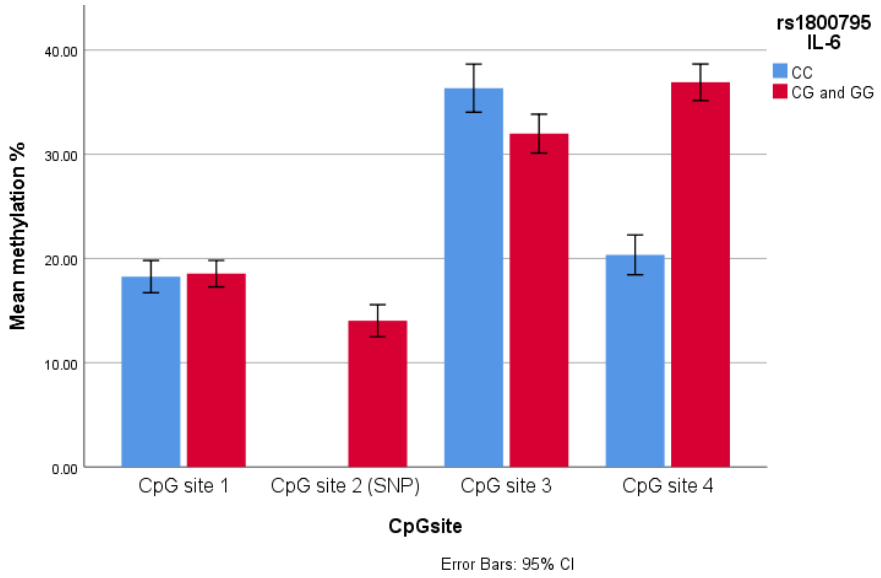

Figure 18 - Methylation percentage at 4 CpG sites of CC (n 23) and CG/GG (n 34) genotype of the rs1800795 polymorphism, data present as mean ± standard deviation. CpG site 1 methylation not significantly different between genotypes (p=0.733). CpG site 2 (SNP) was only present in the G allele carriers. CpG site 3 methylations significantly different between genotypes p=0.004) CpG site 4 methylation significantly different in genotypes (p<0.000).

Data was tested for normal distribution using the Shaprio Wilk test in which the data was determined to be normally distributed. Through the distribution analysis, an independent t-test was selected for analysis to determine if genotype affects the levels of methylation at each CpG site. CpG site 1 percentage methylation in CC genotype were not significantly different (p=0.773) from CG/GG genotype and amounted to 18.26 ± 3.56 and 18.54 ± 3.64 respectively. CpG site 3 percentage methylation in CC genotype group were significantly different (p=0.004) from CG/GG genotype and amounted to 36.37 ± 5.34 and 32 ± 5.34 respectively. CpG site 4 percentage methylation in CC genotype group were significantly different (p<0.000) from CG/GG genotype and amounted to 20.35 ± 4.42 and 36.91 ± 5.03 respectively.

Methylation percentage for all CpG site were tested against the control and early AD groups to determine if there is significant difference in methylation.

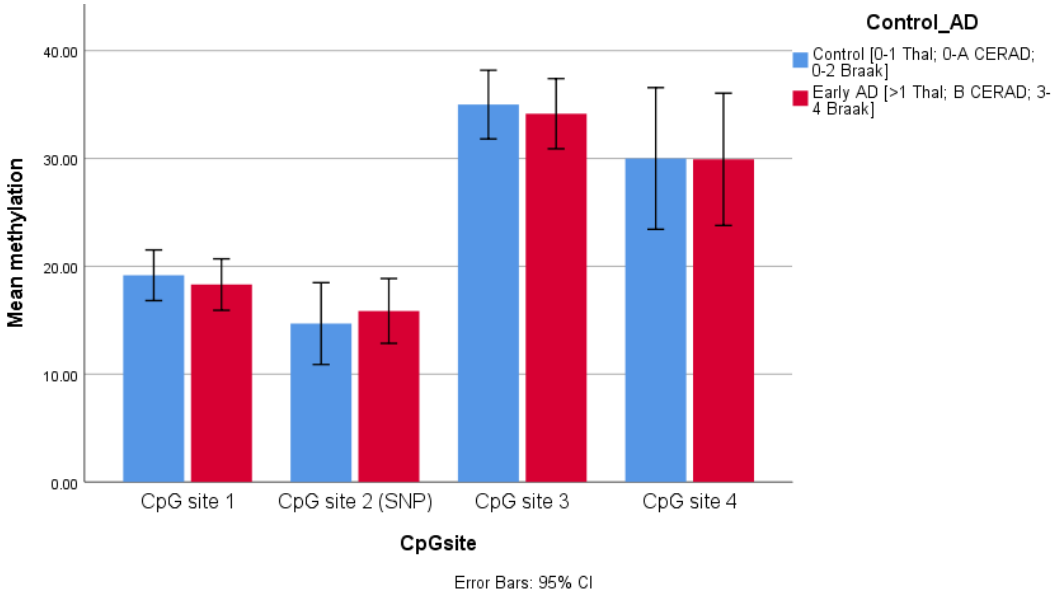

Figure 19 – Mean methylation levels at all CpG 4 sites in control and AD groups were analysed and data presented as mean ± standard deviation. CpG site 1 methylation not significantly different between control and early AD (p=0.586). CpG site 2methylation not significantly different between the control group (p=0.582) and early AD. CpG site 3 methylations was not significantly different between the control group (p=0.692) and early AD. CpG site 4 methylation was not significantly different in the control group (p=0.986).

Data was determined to be normally distributed and an independent t – test was used to analyse if percentage methylation at the 4 CpG sites are different between the control group and early AD group. CpG site 1, percentage methylation 19.17 ± 4.25 in the control group was not significantly different (p=0.586) from the AD group. CpG site 2, percentage methylation 14.69 ± 4.54 in the control group was not significantly different (p=0.582) from the AD group. CpG site 3, percentage methylation 35 ± 5.75 in the control group was not significantly different (P=0.692) from the AD group. CpG site 4, Percentage methylation 30 ± 11.87 in the control group was not significantly different (p=0.986) from the AD group.

4.4.8 Interaction of IL-6 protein and gene expression vs DNA methylation dependent upon genotype

A correlation between IL-6 protein levels to each CpG site was analysed and stratified into genotypes (CC n=20 and CG/GG n=34) to determine if an association or difference occurs depending on the genotype. CpG site 2 methylation is not analysed, as this is only present in the G allele carriers. Looking at figure 20 no correlation was present and further analysis took place.

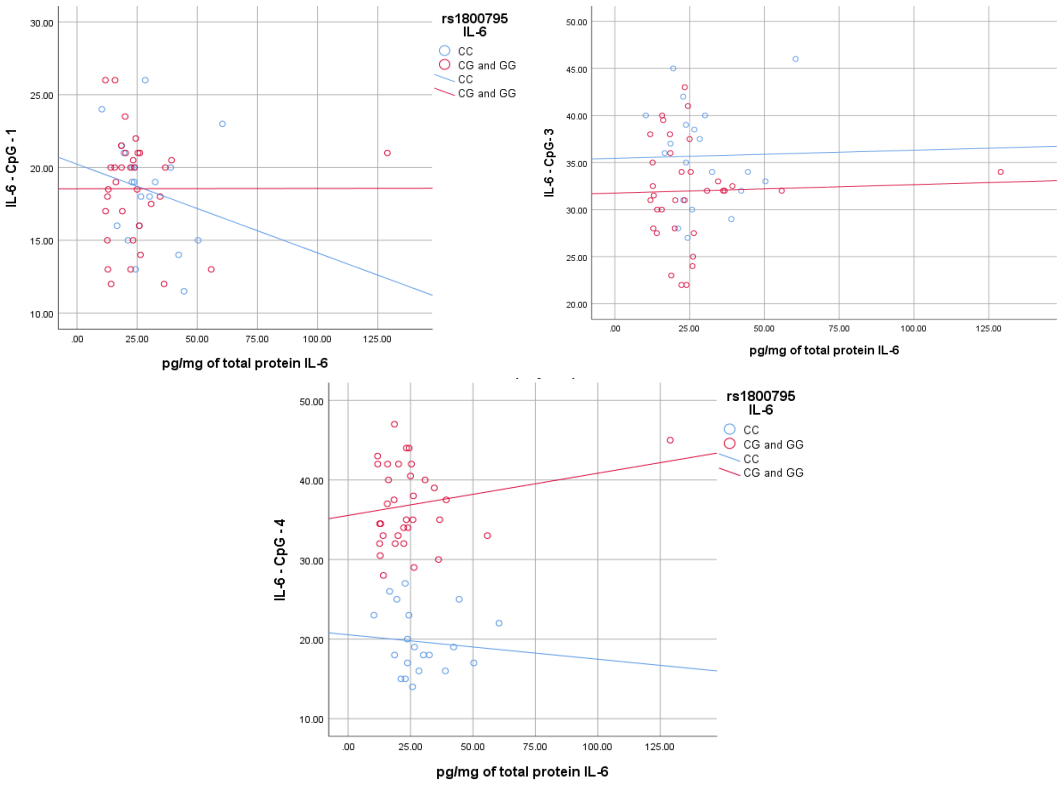

Figure 20 – Correlation of IL-6 total protein and each CpG site methylation depending on the genotype. A– Correlation between levels of IL-6 protein and methylation at CpG site 1 depending upon genotype of rs1800795 polymorphism. B – Correlation between levels of IL-6 protein and methylation at CpG site 3 depending upon genotype of rs1800795 polymorphism. C– Correlation between levels of IL-6 protein and methylation at CpG site 4 depending upon genotype of rs1800795 polymorphism.

A spearman correlation analysis was used to determine the possible relationship between *IL-6* promoter methylation and protein levels. The data was grouped into genotypes to determine significance between CC and CG/GG genotype of rs1800795 polymorphism.

Table 10 - Spearman's correlation analysis on IL-6 proteins levels at each individual CpG compared between genotypes of the rs1800795 polymorphism.

CC	correlation coefficient	p value	CG/GG	correlation coefficient	p value
Total protein IL-6 compared to CpG 1	-.249	P=0.436	Total protein IL-6 compared to CpG 1	.127	P=0.505
Total protein IL-6 compared to CpG 3	-.133	P=0.681	Total protein IL-6 compared to CpG 3	.115	p=0.545
Total protein IL-6 compared to CpG 4	-.277	P=0.383	Total protein IL-6 compared to CpG 4	.198	p=0.294

No correlation was observed in the CC (n=20) genotype and no significances between IL-6 protein levels with CpG site 1 (p=0.436), CpG site 3 (p=0.681) and 4 (p=0.383) were determined. No association was found between IL-6 protein levels in CpG site 1 (p=0.505), 3 (p=0.545) and 4 (p=0.294) in the CG/GG genotype group n=34). This shows that methylation levels are not correlated with protein levels upon genotype.

Further analysis was conducted, and a comparison was determined between each CpG site and *IL-6* gene expression levels stratified into genotype to determine if methylation levels correspond to the levels of expression.

Table 11 - Spearman's correlation analysis of IL-6 gene expression at each individual CpG site compared between the genotypes of the rs1800795 polymorphism.

CC	correlation coefficient	p value	CG and GG	correlation coefficient	p value
IL-6 expression compared to CpG 1	.034	p=0.870	*IL-6* expression compared to CpG 1	.099	p=0.589
IL-6 expression compared to CpG 3	-.049	p=0.828	*IL-6* expression compared to CpG 3	.160	p=0.382
IL-6 expression compared to CpG 4	.030	p=0.894	*IL-6* expression compared to CpG 4	.146	p=0.425

No correlation between *IL-6* gene expression with CpG site 1 (p = 0.870), 3 (p=0.828) and 4 (p=0.894) were found in the CC genotype group n =20. No association was found between IL-6 gene expression levels in CpG site 1 (p=0.589), 3 (p=0.382) and 4 (p=0.425) in the CG/GG genotype group n (34). This shows that IL-6 gene expression is not regulated by DNA methylation and not affected depending on genotype carried in the rs1800795 polymorphism. Overall the result suggests, that protein levels and gene expression levels for the IL-6 gene are not correlated to methylation at each of the 3 CpG sites with CpG site 2 missing as present only in G allele carriers.

4.4.9 Comparison of IL-6 protein levels in the brain in relation to DNA methylation at all CpG sites stratified into early AD and control.

Levels of IL-6 total protein were correlated to each CpG site to indicate if a relationship occurs and if there is any difference between the control and early AD group. This analysis is to indicate if levels of IL-6 protein fluctuate together with each CpG site in control and early AD samples to indicate any correlation.

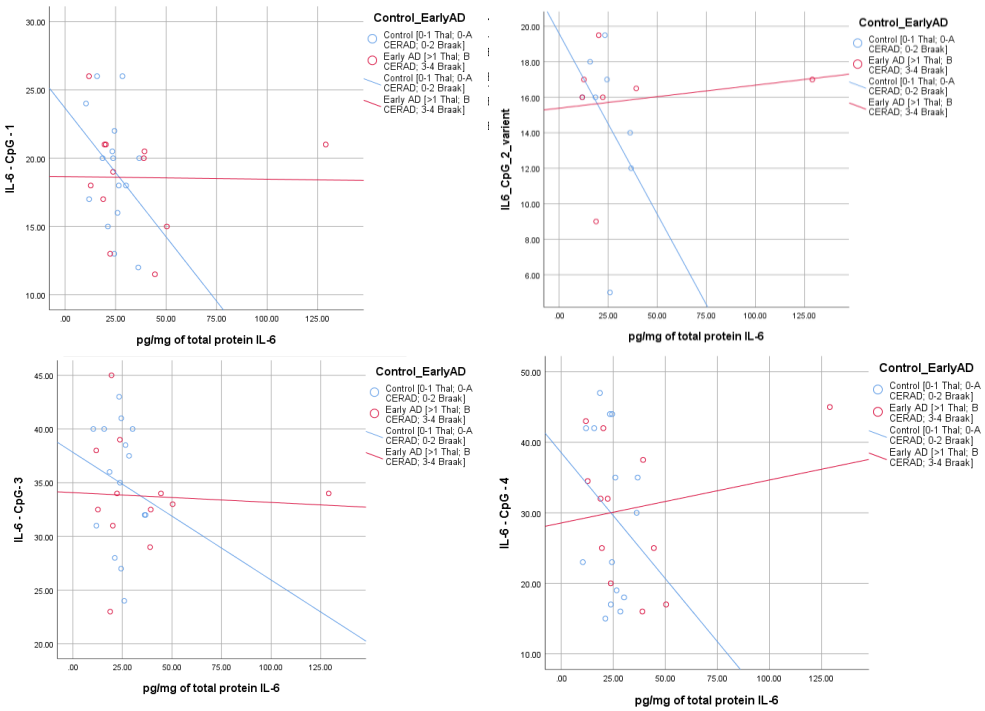

Figure 21 –Correlation of IL-6 protein levels at each CpG site in control and early AD group. A– Correlation between levels of IL-6 protein and methylation at CpG site 1 in control and early AD group. B – Correlation between levels of IL-6 protein and methylation at CpG site 2 in control and early AD group. C – Correlation between levels of IL-6 protein and methylation at CpG site 3 in control and early AD group. D– Correlation between levels of IL-6 protein and methylation at CpG site 4 in control and early AD group.

Looking at the scatter plots, the distribution of samples in the control and early AD group showed no correlation was present. To further enhance analysis a correlation test was performed to confirm the finding in the scatter plot.

Table 12 - Spearman's correlation of each CpG site methylation and IL-6 protein levels in control and AD groups

Control	Correlation coefficient	P value	Early AD	Correlation coefficient	P value
IL-6 total protein compared to CpG 1	-.253	p=0.363	IL-6 total protein compared to CpG 1	-.268	p=0.400
IL-6 total protein compared to CpG 2	-.599	p=0.117	IL-6 total protein compared to CpG 2	.273	p=0.554
IL-6 total protein compared to CpG 3	-.117	p=0.679	Il-6 total protein compared to CpG 3	.011	p=0.974
IL-6 total protein compared to CpG 4	-.269	p=0.333	IL-6 total protein compared to CpG 4	-228	p=0.476

Looking at the results from the control group there was no correlation presented in the individual CpG sites and total protein IL-6 levels. In the early AD group, there was no correlation between IL-6 total protein levels and the individual CpG sites. It has to be noted that the sample size for these groups are small. As not all samples can be grouped into control or early AD using THAL, CERAD and BRAAK scores.

5.0 Discussion

This project investigates the epigenetic regulation of IL-6 within the prefrontal cortex of AD and control brain samples to understand how the *IL-6* gene is regulated during cognitive decline and AD. IL-6, a cytokine with both pro- and anti-inflammatory properties, is seen to have a role in a number of different neurological conditions in which neuroinflammation is a component, such as AD. We found that IL-6 protein levels and *IL-6* mRNA levels were not impacted by genotype and disease state. Interestingly, DNA Methylation was seen to be altered in the presence of a SNP, suggesting GxE. However, this was not influenced by disease state.

5.1 The impact of AD neuropathology and IL-6 protein expression

This is the first study to investigate the levels of IL-6 within the brains of cognitively healthy and AD. Interleukin 6 protein levels in the control and AD samples were collectively analysed to determine if levels are altered at the different stages of AD neuropathology with IL-6 levels compared to BRAAK, THAL and CERAD staging

This study found that protein levels were not significantly altered at the different staging classification among BRAAK ($p = 0.504$), CERAD ($p = 0.718$) and THAL ($p = 0.531$). Overall, the results suggest that protein levels are not significantly altered at the different stages in AD neuropathology classification. This analysis indicates that IL-6 protein expression alone does not correlate with neuropathology. Indicating that other mechanisms could be at work or that IL-6 in other brain regions might have more of a role in AD.

IL-6 is produced by a number of different cell types in the brain which include neurons, microglia and astrocytes (María Erta, 2012). As IL-6 is produced by many different cells this could have an impact as other brain regions may have higher IL-6 levels compared to other areas such as the prefrontal cortex which as of yet is presently uncertain. Further analysis to determine possible brain region and cell-specific variations in IL-6 regulation would be important. As stated above IL-6 is

produced by many different cell types and levels of IL-6 may vary in different brain regions. Such spatial temporal analysis, that may for example reflect particular cell densities of microglia, could shed more light on mechanisms in which IL-6 is controlled and how this might further regulate subsequent neuroinflammatory responses. As well as this, the production of inflammatory factors in the brain from these cells could occur due to the response to injury, inflammation, trauma and neuropathological hallmarks such as amyloid plaques (Harry and Kraft, 2008). This is important to note as during this study, these factors could not be controlled for and this potentially could have an effect. Analysis through blood and CSF have indicated that levels of IL-6 are having an effect on traumatic brain injury (Woodcock and Morganti-Kossmann, 2013). IL-6 is seen to be upregulated in neuroinflammation when infection and injury occurs in the central nervous system (María Erta, 2012). Levels of IL-6 in the CSF have been seen to be significantly increased compared to plasma levels in people who had suffered traumatic brain injury (Woodcock and Morganti-Kossmann, 2013). It must be noted that they are looking at plasma levels whereas this study looked at prefrontal cortex brain tissue. However brain levels of IL-6 may be similarly affected. In conclusion, other factors would have affected the levels of IL-6 and AD neuropathology. During the analysis of the prefrontal cortex it was not known if any had suffered from a TBI or infection which could have in turn affected levels of IL-6 in relation to neuropathology.

5.2 – The impact of -174 polymorphism, IL-6 protein, L-6 mRNA expression levels and difference between control and AD.

In this study, the transcriptional role of the rs1800795 polymorphism in the regulation of protein expression and *IL-6* mRNA in prefrontal cortex brains in relation to cognitive decline and AD pathology was investigated.

Genotyping analysis was first performed to determine the genotype of the n=67 samples. Using the Hardy Weinberg calculation for the SNP analysis, a p value of 0.059 was obtained. The indicates that the genotype fits into the Hardy Weinberg equilibrium validates that the genotypes in this study are correct. However, a p value of 0.059 which suggests the power of the analysis is low and a greater sample size would be needed to increase statistical power for a SNP genotyping analysis.

Interesting, there was a lack of correlation between *IL-6* mRNA levels and IL-6 protein levels. This suggests that many factors may affect the pathway from DNA to RNA to protein. Problems can arise in the translation of mRNA to protein leading to proliferation and death. This is a complex process that does not always lead to a direct correlation between protein and *IL-6* gene expression levels. However, it appears that the SNP has no direct impact in IL-6 protein levels and *IL-6* gene expression levels in control and AD pre-frontal cortex samples collectively.

IL-6 protein levels were analysed between genotypes of the rs1800795 polymorphism (CC and CG/GG) to see if genotype affect IL-6 levels in the prefrontal cortex. The results indicated that IL-6 protein levels boarder on significance (p=0.054) when classed by genotype. This suggests levels of IL-6 in brain tissues may potentially be regulated by the *IL-6* polymorphism rs1800795. Further analysis would be needed with a greater sample size to determine if the SNP indeed influences the protein levels in the brain on replicate studies to understand possible mechanism. Albani et al. (2009) found that there was a correlation between levels of IL-6 plasma and the rs1800795 genotype in aging.

They observed that there are higher circulating levels of IL-6 in the GG genotype compared to the CC genotype. It must be noted that this study was done using peripheral blood rather than human brain tissue. This suggests that protein levels could be dependent on tissue and sample type. Licastro et al. (2003) found that homozygous for the rs1800795 genotype CC had an elevated risk of developing AD. They found that allele variance of the *IL-6* gene does influence brain and blood levels of the IL-6 cytokine in AD patients. Overall, there are still conflicting results and further analysis with a greater sample size would be beneficial, to drawing significant conclusions. Levels of *IL-6 mRNA* were compared to the rs1800795 polymorphism to determine if this particular SNP alters expression levels of *IL-6* found in the prefrontal cortex. Statistical analysis of the results indicated that *IL-6* expression levels were not significantly altered between the genotypes of the *IL-6* polymorphism (p=0.433). This suggests that the G allele does not confer increased transcription in these brain samples of AD and control together.

Levels of IL-6 protein were further analysed to determine if they are altered between the control group and AD group. The prefrontal cortex samples were classified using the BRAAK, THAL and CERAD staging. Statistical analysis of the results found no significance (p=0.905) between the control or early AD groups and the levels of IL-6 protein expressed in the brain tissue. This result suggests that IL-6 protein levels in the prefrontal cortex of the control and early AD groups are not affected by disease state. Further analysis was conducted on RNA expression levels between AD and Control which found no significance in expression levels between the two groups (p=0.097). Indicating that *IL-6* expression levels are no different in the control and AD groups. Further analysis was conducted by further stratifying the samples into the rs1800795 genotypes. No significance was found in the results. The results suggest that levels of IL-6 protein are not altered between genotype and disease state. Studies have been relatively inconsistent when studying *IL-6* polymorphism and interaction to AD risk. One report found that individuals with the CC genotype of rs1800795 have a significantly greater risk of developing Alzheimer's disease (Chen et al., 2016),

whereas there have been several studies conducted by different countries (Italy, Japan and Spain) which have showed a progressive association of the IL-6 G allele in the AD (Mateo et al. 2006, Pola et al. 2002, Shibata et al. 2002). In contrast Depboylu et al. (2004) concluded that *IL-6* (-174) polymorphism does not influence the risk of developing AD in their cohort of German participants. One limitation to the research is the quality of the mRNA in the post-mortem brains and these could potentially effect the analysis and the quality of the results.

IL-6 was seen to be present in the immunofluorescent stained samples of both the control and early AD groups. However due to no neuronal marker being used it was impossible to determine which cells IL-6 accumulated around. The results suggest that IL-6 is present in both healthy controls and AD samples as well as NFkB was activated around the nucleus in both control and AD samples. However, these results cannot be analysed or considered due to sample size and brain tissues not being stained for a neural marker. It can be concluded that IL-6 does accumulate in the brain, however it was not possible to determine if levels were significantly different between the control and AD group. NFkB plays an important role in inflammation as this transcription factor can cause transcription to occur in proinflammatory gene for example IL-6 (Tak and Firestein, 2001). The above result is suggesting the possibility that inflammation indeed could be occurring in not only AD brains but control brains too.

5.3 IL-6 methylation levels

Epigenetic regulation is an important factor in regulating neuroinflammatory systems and has been linked to the pathogenesis of neurodegenerative diseases. One type of epigenetic marker, DNA Methylation, is increasingly implicated in various diseases (Ryan et al., 2017) with strong links between neuroinflammation and epigenetics (Garden, 2013) as well as studies conducted on altering DNA methylation in AD.

Methylation percentages were determined for each CpG site and compared between the rs1800795 (-174 SNP) polymorphisms to assess if the genotype alters *IL-6* methylation levels. The *IL-6* methylation levels were not deemed significant at CpG site 1 between the genotypes (p =0.733). However, CpG site 3 and 4 were deemed significantly different between the two genotype groups (CpG 3 – p=0.004 and CpG 4 -p=<0.000). These results indicate that, within the G allele, methylation levels are increased in neighbouring CpG sites compared to the C allele. Indicating that the rs1800795 SNP has an effect upon methylation levels in the *IL-6* promoter region among the control and early AD prefrontal cortex samples collectively. This is important as this suggests that genotype influences methylation which could in turn affect transcription of the *IL*-6 gene. It is still not clear how methylation at just one CpG site can affect the promoter region. One possible suggestion could be when one CpG site is contained within a transcriptional binding site it could in turn affect the binding. Studies have found that individuals with AD tend to have higher levels of DNA methylation compared to the cognitively normal (Yokoyama et al., 2017). With this in mind, the results would suggests that the C allele may have a protective element through decreasing methylation levels compared to the G allele present at the SNP, which shows an increased risk through increasing methylation levels. This, in turn, could increase the risk of developing AD. It has been suggested, in previous studies, DNA methylation differences at single CpG sites altered *IL-6* expression in Rheumatoid arthritis patient monocytes. However, this association was not deemed to be linked to the rs1800795 genotype (Noss et al., 2015). Methylation levels were compared solely to the control and early AD groups and no significance was

found. This suggests that methylation levels alone are not altered in the control and early AD groups at each of the CpG sites.

IL-6 protein levels were correlated to CpG site 1, 3 and 4 (CpG site 2 SNP was omitted as only present in the CG/GG genotype) to determine if IL-6 levels are correlated with *IL-6* methylation levels depending on genotype present. No correlation was determined in the CC genotype and no significance between IL-6 protein and methylation levels at each CpG site. The CG/GG was determined to have no correlation or significance between IL-6 protein and methylation levels at each CpG site. These results suggest that the SNP does not have a impact on the correlation between levels of IL-6 and methylation, suggesting that other factors could play a role in DNA methylation, e.g. various environmental factors underlying gene by environment (GxE).

IL-6 gene expression levels were compared to each individual CpG site and split into genotype of the rs1800795 SNP. Results indicated no correlation in *IL-6* expression and methylation levels at each CpG site upon genotype. This suggests that the SNP, which produces an extra CpG site in the G allele carriers, does not have a role in altering *IL-6* expression and methylation at each CpG site located in the *IL-6* promoter region. Interestingly, although CpG DNA methylation is generally associated with low gene expression, Noss et al. (2015) found poor correlation between IL-6 expression and promoter CpG methylation.

IL-6 protein levels and gene expression levels were correlated to methylation percentage at each of the CpG sites. Looking at the control group there was no correlation and no significance determined between total protein IL-6 levels and the individual CpG sites, which was noted in the early AD group too. Although a relatively low sample size this result suggests that DNA methylation does not impact on expression levels of *IL-6* and the protein levels in the prefrontal cortex. This implies that other factors could influence DNA methylation and IL-6 levels may well be present in different tissue type. Decreased methylation is generally associated with transcriptional activation. Epigenetic mechanism has been shown

to have a role in the regulation of *IL-6*, allowing regulation independently of genetic sequence. For example, methylation is an important factor in the control of IL-6 and the production of this cytokine may lead to the production being altered as seen in Rheumatoid arthritis (Nile et al., 2008). Furthermore, a twin study looking at DNA methylation, one with AD and other cognitively normal found that levels of methylation were decreased in the prefrontal cortex of the AD sufferers (Mastroeni et al., 2011). In contrast to this a similar study using the same methods found that levels were increased in DNA methylation. An example of this would be a study found that in the middle gyrus levels of methylation had been significantly increased (Coppieters et al., 2014).

In summary, *IL-6* mRNA and IL-6 protein levels are not directly impacted by the rs1800795 polymorphism in the prefrontal cortex samples indicating that other factors could have an impact such chromatin modifications. Methylation levels at CpG sites 3 and 4 were significantly dependent upon genotype. However, when related to IL-6 protein and mRNA expression levels in the brain, no significance was found. This suggests that DNA methylation is not direly regulating brain IL-6 expression and protein levels depend on the genotypes. However, DNA methylation may play a role through gene environment interaction GxE. This is defined as when two different genotypes respond to environment variation in different ways. The environment interaction could include exposure to physical, chemical, biological factors and life events (Tanaka et al., 2014). This leads to the question as to whether such environment interactions could have a potential effect on the transcriptional and expression of IL-6 in this study in response to disease for example. Having different genotypes could influence the response to environmental variation however, this GxE concept still remains largely unclear (Fave et al., 2018).There have been a few studies relating GxE and AD. For example, a study was conducted on AD patients who carry the ApoE4 genotype. They were found to have greater cognitive decline when linked to increased levels of cholesterol compared to normal levels when grouped by the present or absents of the ApoE 4 genotype (Evans et al., 2004). Sporadic cases of AD can be linked to

GxE and, similarly environmental exposure during early stages of life has been seen to impact on complex psychiatric disorders (Chouliaras et al., 2010b)

To summarise, the research has shown that IL-6 is present in control and AD brains and the SNP has an impact on methylation levels between genotype at different CpG sites however direct linear correlations between methylation, protein and expression levels did not exist within this study.

6.0 Limitation

In this particular study using prefrontal cortex samples from the University of Manchester Age and Cognitive performance Research Cohort (ACPRC) it was possible to research the epigenetic regulation of IL-6 in age-related cognitive decline and AD. This study is unique and novel as it included human brain tissue from a well characterised cohort as opposed to previous analysis focused on CSF and blood. However, some limitation exist due to the nature of the research and the tissue type being used, in this case the human brain.

A correlation analysis was conducted on *IL-6* expression levels and RIN values. RIN values were on the lower side (3.69 ± 1.38) indicating that the quality of the RNA is low which is often the case in post-mortem brains. Acceptable RIN values would be over 6. This in turn could affect the results when looking at gene expression. Ideally, better quality RNA would be needed to improve the validity of the results for RNA expression.

The sample size in this study, looking at the effect *IL-6* promoter region SNP, was not adequate with the Hardy Weinberg giving a p value of 0.054, suggesting that a greater sample size is needed for a more robust statistical analysis. When statistical analyses took place on IL-6 protein and expression levels in the control and AD groups stratified by genotype it has to be noted that sample size was further decreased. This was due to some samples not being classified into either being control or early AD. A greater sample size would be better for a more robust statistical analysis.

A limitation of immunofluorescence staining was the brain sample tissue obtained from the brain bank. When sectioning the tissue for IHC the tissues were not cutting smoothly. The temperature in the cryostat was altered +/- several degrees with the starting chamber temperature of -16 to try and improve the sectioning of the samples. It also appeared there was vacuolisation in the brain tissue. As the tissues have come from the brain bank the way the tissue has been frozen could have caused damage. Ideally if more analysis was conducted wax imbedded tissue would be more suitable as the whole brain is dipped in wax and there is less chance of damage.

7.0 Further research

This study has shown that IL-6 is indeed present in the brain and that the SNP has an impact in DNA methylation in the brain. However, linear correlations were not observed between methylation, protein and expression. Further research needs to be conducted on gene regulation in cognitive decline and Alzheimer disease using human brain samples. Targeted approaches such as mapping of methylation are important to allow investigation genetic-epigenetic-protein interactions. However, geneome wide analyses could be important to identify specific immune pathways that perhaps IL-6 might be part of. It is also unclear whether IL-6 might be a cause or consequence of the neuroinflammation in respect to both its pro and anti-inflammatory activities. Further investigation as to these roles and which cells are secreting this cytokine could allow researchers to better determine the pathways by which IL-6 is regulated. Data on activity of microglia, as a marker for neuroinflammation, could be useful to further link this correlates with IL-6 levels. Further research of the polymorphism would be needed with a greater sample size for polymorphism analysis. Though this cohort is well studied with longitudinal and neuropathological data, larger numbers are generally needed for a high-powered association study. A further Genome Wide Association Study (GWAS) analysis would further allow to investigate other SNPs at the IL-6 locus and perform haplotype mapping. GWAS data might also allow the development of polygeneic risk scores for neuroinflammation that IL-6 might form part off.

DNA methylation is an important epigenetic regulator and was measured at the IL-6 promoter. However, other regions of the IL-6 locus might also be important, such as enhancers of which IL-6 has several and more distal promoter regions or the coding region itself. Though targeted DNA methylation by bisulphite pyrosequencing is highly specific and sensitive, geneome wide approaches would allow more coverage of the locus. Another point is whether other epigenetic markers such as various chromatin markers might also have a role in the regulation of IL-6. Histone modification is seen in aging with a decrease in acetylation and increase in phosphorylation Comparing IL-6 expression, protein levels and DNA methylation levels to other parts of the brain, for example the hippocampus would allow comparison to assess to compare if levels are altered in different parts of the brain in relation to AD vs Control.

8.0 Conclusion

By using human brain samples from the prefrontal cortex, we were able to provide an insight into the regulation of the *IL-6* gene during cognitive decline and AD. From this project it was determined that IL-6 does play a role in inflammation with specifics to neuroinflammation. However, it was not determined if the SNP (rs1800795) in the promoter region impacted the regulation of IL-6 cytokine and expression levels.

This study found that DNA methylation at the *IL-6* promoter did not directly associate with *IL-6* expression levels within the brain of individuals with AD. However, the G/C SNP rs1800795 did impact DNA methylation levels. There was obviously no methylation at CpG Site 2 in the CC homozygotes in which this CpG site is no longer present. Methylation at CpG site 3 was significantly decreased in G allele carriers compared to CC homozygotes ($p=0.004$), whereas at CpG Site 4 the G allele carriers showed significantly increased DNA methylation compared to CC homozygotes ($p>0.000$) (figure 18).

This implies that other factors could have an impact such a gene by environment interaction. These results suggest that other factors could influence the

epigenetic regulation of *IL-6* and in a multifactorial mechanism. Biological pathways and gene transcriptional activity can be changed through epigenetics. Factors that could potentially affect this include the environment such as toxins and hazardous exposure which could alter gene expression. Gene environment interaction is an influence that could affect epigenetic regulation through SNP interaction.

Observations during this project indicated that IL-6 is indeed present in both control and early AD but multiple factors could be involved in the regulation of *IL-6* during cognitive decline and early AD. Further analysis would need to be conducted with a greater samples size when determining the SNP significance in relation to expression and protein levels and the impact on AD.

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10.0 Appendix

10.1 table of chemical, reagents and kits

96 well plate	Starline
Q24 sequence plate	Quigen
0.2ml PCR tubes	Starline
Pipette tips 10ul,20ul,200ul,1000ul	
PCR plate cover slips	
Nuclease free water	
PCR grade water	
Isolate 2 genomic DNA extraction kit	Bio line
EpiTec fast Bisulphite kit	Quigen
ELISA anti human IL-6	Abcam
Pyromark q24 regents	Quigen
Agarose powder	
Tris base	
EDTA	
Boric acid	
Syber green dye	Quigen
DNA ladder 100bp	Bioline
MyTaqHS	Bioline
Coral Dye 10x	
70% ethanol	
Denaturing buffer	
Washing buffer	
IL-6 forward primer	Invergion
IL-6 reverse primer	Invergion
IL-6 sequence primer	Invergion
RT PCR IL-6 Forward primer	Invergion
RT PCR IL-6 Reverse primer	Invergion
RT PCR B actin Forward primer	Invergion

RT PCR B actin Reverse primer	Invergion
KASP master mix	
Rs1800795 primer	
IL-6 anti human anti mouse	R&D
Anti-NF-kB p65 (phospho S536) antibody	Abcam (ab28856)
Vector shield DAPI	
Alexfluro IgG 568 goat anti mouse red	
Alexfluro IgG 488 goat anti rabbit green	
StepOne plus	
Fluorescence microscope	
Biorad RT PCR machine	Biorad
Eppendorf master cycler	
Pyro mark sequencer	Quigen
Biorad power pack and tank	Biorad