THE GENETICS AND PATHOPHYSIOLOGY OF CLUSTER HEADACHE AND ASSOCIATED DISORDERS

THESIS DISSERTATION
by
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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Clinical Neurology

University College London
April 2021

Declaration

I, Emer O'Connor, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, it has been declared in a statement of contribution at the beginning of each chapter. Where appropriate, collaborators have also been acknowledged. Some chapters are slightly modified versions of the work published by myself that has been reproduced with the permission of the copyright holder.

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Abstract

Cluster headache (CH) is described as one of the most painful conditions known to humans. It effects approximately 60,000 individuals in the UK and carries significant morbidity. It exhibits hereditability evident by reports of familial aggregation and is categorised as a trigeminal autonomic cephalalgia (TAC). Despite this, the exact pathophysiological and genetic drivers of this condition remain elusive. The purpose of this thesis is to examine the clinical and genetic determinants of CH, and thus gain insights into the underlying neurobiological mechanisms.

This work consists of two components. In the first section, I conduct clinical observational studies to further delineate the CH phenotype. I address the postulated association between pituitary adenomas and CH and question the utility of dedicated pituitary imagining in this patient group. I also describe the largest series of Post-Traumatic Headache of Cluster Headache (PTH-CH) and demonstrate its distinct features and increased intractability to treatment. Finally, through meta-analysis, I estimate the prevalence of familial CH to be 6.27% and demonstrate an overlap with concurrent short-Lasting unilateral neuralgiform headache attacks with conjunctival injection and tearing (SUNCT) in familial cases.

The second section explores the genetics architecture underlying CH. I perform a Genome-Wide Association Study (GWAS) to identify replicable susceptibility loci and conduct a downstream analysis. Subsequent genetic correlation analysis showed an overlap with migraine, depression, bipolar and sleep disturbance implying the possibility of a common genetic driver for these conditions, which frequently present concurrently. I then carry out linkage analysis in CH families and replicate a linked

region suggestive of significance on chromosome 2 that also overlaps a genome wide significant locus. Finally, I execute whole exome sequencing and utilise rare variant association tests and segregation analysis to identify causal variants for familial CH.

Acknowledgements

Firstly, a huge thanks to the patients who kindly participated in this research. Your generosity and enthusiasm is greatly appreciated. I hope, with your help, research will advance our understanding of CH and contribute to improved outcomes for future patients. I would also like to extend huge gratitude to Brain Research UK who provided the funding for this research and work tirelessly to advance neuroscience to improve the lives of those suffering with neurological disorders.

I am forever grateful to my supervisor Professor Henry Houlden, for welcoming me into his team and for his unwavering encouragement, support and patience over the last four years. His door is always open for his students. He was invariably on hand to help tackle seemingly insurmountable problems, whether they were real or imagined. Despite my greatest attempts, he never let me give up, even when he probably should have.

I would like to extend a huge thanks to Dr. Jana Vandrovcova, whose supervision was fundamental to the work presented in this thesis, and for her boundless kindness and generosity. I am also very grateful to Professor Nicholas Wood for his expertise and valuable advice in navigating the challenges of this PhD. I would also like to express huge gratitude to Dr Kamal Ahmed for his foresight in supporting and advocating for the careers of trainees and quietly encouraging us to reach our potential. His mentorship and advice has been critical to the completion of this PhD.

Thanks also to my collaborators and those that contributed directly to the work in this thesis. Specifically, I am very grateful to Prasanth Sivakumar, Janice Yip, Ben Simpson, Lou Grangeon, Laura Southgate, Andrea Belin and everyone on the Headache Team at the National Hospital of Neurology and Neurosurgery.

I have met so many wonderful people at the Institute of Neurology (ION) who will always remain close friends. It's impossible to mention everyone, but essential to thank the GEL team including Ambreen Tariq, Flouey Urban, Ifrah Iidow and Katrina Newland who treated me like family when I first moved to London. I owe so much to the members of Office 708 (past, present and honorary) including Monika Madej, Isabel Hostettler, Wayne Yau, Thomas Bourinaris, Nourelhoda Haridy, Ben O'Callaghan, Caroline Neuray ,Michael Chou, and, my partner in crime, Roisin Sullivan. I am confident our strong bond, rooted in our collective efforts to thermoregulate the office, renders our friendship unbreakable. I would also like to thank Stephanie Efthymiou for always being on hand to help, Reza Maroofian for his advice, and David Lynch for his guidance and friendship.

Importantly, I would like to thank the security guards at the ION who cheerfully let me out after late nights in the lab, and the cleaners / maintenance staff who work very hard and do an exceptional job in providing a safe and comfortable environment for students. Their work is absolutely critical and sadly often goes unrecognised.

Huge thanks to the nurses and my colleagues on H2 ward at Guys Cancer Centre where I have worked for the past four years. The endless cups of tea and sympathy offset the inevitable stress that comes with writing your thesis whilst working in a global pandemic.

On a more personal note, I owe so much to my wonderful friends, especially Layan Akijian, Eavan McGovern, Emer Kilbride, James Burns, Justin Penner and Claire Edbrooke. In particular, I would like to thank James who is always there for me and patiently proofread this thesis (including these acknowledgements!). I also want to thank Claire who stayed up through the night helping with formatting and whose friendship has carried me through the most difficult times. I am extraordinarily lucky to have you all in my life.

Finally, my family are the only reason I have made it this far. To my siblings and best friends, Claire and Michael, thank you for always having my back. I will always be grateful to my wonderful parents, Paula and Jack, who sacrificed a lot to ensure I was able to take advantage of every opportunity that presented itself. Your unrelenting support, reassurance and love is fundamental to anything I achieve.

List of Tables

List of Figures

Web resources

Resource	URL
1000 Genomes Project	www.1000genomes.org
ANNOVAR	www.openbioinformatics.org/annovar
bcftools	http://samtools.github.io/bcftools/bcftools.html
Brain RNA-Seq	https://www.brainrnaseq.org
CADD	http://cadd/gs/washington.edu/home
CPDB web tool	http://cpdb.molgen.mpg.de/
dbSNP	http://www.ncbi.nlm.nih.gov/projects/SNP
Ensembl	http://www.ensembl.org/
Exome Aggregation Consortium Database	http://exac.broadinstitute.org/
Fast QC	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
GAS power calculation	http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/index.html.
GATK	https://www.broadinstitute.org/gatk/guide/best-practices.php
Genomics England Panel APP	https://www.panelapp.genomicsengland.co.uk
gnomAD	http://www.gnomad.broadinstitute.org
GTEx Portal	http://www.gtexportal.org/home/
Homozygosity Mapper	http://www.homozygositymapper.org/
Human Brain Transcriptome	https://www.hbatlas.org
Human Genome Variation Society	http://www.hgvs.org
Merlin	http://csg.sph.umich.edu/abecasis/merlin/download/
Michigan imputation server	https://imputationserver.sph.umich.edu/index.html#!
Mutation Taster ver 2	http://www.mutationtaster.org/
National Centre for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov
Online Mendelian Inheritance in Man (OMIM):	http://www.omim.org/
Peddy	https://github.com/brentp/peddy
Picard	http://broadinstitute.github.io/picard/
PLINK	http://pngu.mgh.harvard.edu/purcell/plink/
PolyPhen 2	http://genetics.bwh.harvard.edu/pph2/
Primer 3	http://primer3.ut.ee/
RVTest	http://zhanxw.github.io/rvtests/
SAIGE	https://github.com/weizhouUMICH/SAIGE/
Sequence Ontolology	https://m.ensembl.org/info/genome/variation/prediction/predicted_data.html
SIFT	http://sift.jcvi.org/
UCSC Genome Browser	https://genome.ucsc.edu/

Publications

Publications arising from this thesis:

- Grangeon L, **O'Connor E**, Danno D, Ngoc TMP, Cheema S, Tronvik E, Davagnanam I, Matharu M. Is pituitary MRI screening necessary in cluster headache? Cephalalgia. 2021 Jan 6:333102420983303. doi: 10.1177/0333102420983303. PMID: 33406848.

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Genome Wide Association Study Identifies Risk Loci For Cluster Headache (Annals of Neurology- Accepted / In press)

Manuscripts under review

- **O'Connor E,** Nikram E, Grangeon L, Daisukech D, Matharu M. The Clinical Characteristics Of Familial Cluster Headache (Cephalalgia – in submission)

Other publications (co)authored during this thesis:

- Sullivan R, Yau WY, Chelban V, Rossi S, Dominik N, **O'Connor E**, Hardy J, Wood N, Cortese A, Houlden H. *RFC1*-related ataxia is a mimic of early multiple system atrophy. J Neurol Neurosurg Psychiatry. 2021 Feb 9:jnnp-2020-325092. doi: 10.1136/jnnp-2020-325092. Epub ahead of print. PMID: 33563805.
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Abbreviations

AA	Amino Acid
AC	Allele Count
ACTH	Adrenocorticophic Hormone
AD	Autosomal Dominant
AR	Autosomal Recessive
ASD	Autism Spectrum Disorders
BAM	Binary Alignment Map
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Sub-Cortical Infarcts And Leukoencephalopathy
CADD	Combined Annotation-Dependent Depletion
CAS9	CRISPR Associated Protein 9
CAST	Cohort Allelic Sums Test
CCH	Chronic Cluster Headache
CEX	Coding Exome Oligos
CGRP	Calcitonin Gene-Related Peptide
CH	Cluster Headache
cm	Centimorgan
CMA	Chromosomal Microarray
CMC	Combined Multivariate And Collapsing Test
CNS	Central Nervous System
CNV	Copy Number Variant
CREB	C-AMP-Responsive Element Binding Protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSD	Cortical Spreading Depression
CSF	Cerebrospinal Fluid
DBS	Deep Brain Stimulation
DFFITS	Difference In fits Values
DNA	Deoxyribonucleic Acid
DP	Depth
EA	Episodic Ataxia
ECH	Episodic Cluster Headache
EDTA	Ethylenediaminetetraacetic Acid
eQTL	Expression Quantitative Trait Loci
EREC	Estimated Regression Coefficient Test
ERP3	End Repair Mix
FDA	Food And Drug Administration
FDR	False Discovery Rate
FEPS	Familial Episodic Extreme Pain Syndrome
FHM	Familial Hemiplegic Migraine

fmri	Functional Magnetic Resonance Imaging
FOV	Field Of View
FSH	Follicle- Stimulating Hormone
FSHD	Facioscapulohumeral Muscular Dystrophy
GABA	Gamma Aminobutyric Acid
GATK	Genome Analysis Toolket
GCP	Good Clinical Practise
gDNA	Genomic Deoxyribonucleic Acid
GDPR	General Data Protection Regulation
GERP	Genomic Evolutionary Rate Profiling
GH	Growth Hormone
GMC	General Medical Council
GSA	Global Screening Array
GSEA	Gene Set Enrichment Analysis
GTEX	Genotype Tissue Expression Consortium
GWAS	Genome Wide Association Studies
HGNC	Hugo Gene Nomenclature Committee
HIS	International Headache Society
HLOD	Heterogeneity Logarithm Of Odds
HRA	Health Research Authority
HRC	Haplotype Reference Consortium
HuGe-F	Human Genomics Facility
HWE	Hardy–Weinberg Equilibrium
IBD	Identity By Descent
ICHD	International Classification Of Headache Disorders
IGF-1	Insulin-Like Growth Factor I
ION	Institute Of Neurology
IQR	Interquartile Range
IRAS	Integrated Research Application System
KBAC	Kernal-Based Adaptive Cluster
KO	Knock-Out
LD	Linkage Disequilibrium
LDSC	Ld Score Regression
LH	Luteinizing Hormone
LOD	Logarithm Of The Odds Score
LOO	Leave One Out Analysis
LSD	Lysergic Acid Diethlamide
MABS	Monoclonal Antibodies
MAF	Minor Allele Frequency
Melas	Mitochondrial Encephalopathy, Myopathy, Lactic Acidosis And Stroke-Like Episodes

MIM	Omim Number (Phenotype)
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MRS	Magnetic Resonance Spectroscopy
MS	Multiple Sclerosis
NBS	National Blood Survey Cohort
NGS	Next Generation Sequencing
NHNN	National Hospital Of Neurology And Neurosurgery
NFE	Non-Finnish European
NOS	Newcastle – Ottawa Scale
NPL	Non-Parametric Linkage
nVNS	Non-Invasivse Vagus Nerve Stimulation
OMIM	Online Mendelian Inheritance In Man
ONS	Occipital Nerve Stimulation
PACAP	Pituitary Adenylate Cyclase-Activating Peptide
PEPD	Paroxysmal Extreme Pain Disorder
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PI	Principal Investigator
Polyphen	Polymorphism Phenotyping
PPC	Polymerase Chain Reaction Primer Cocktail
PRISMA-P	Preferred Reporting Items For Systematic Reviews And Meta-Analyses Protocols
PRL	Prolactin
PTH	Post Traumatic Headache
PTH-CH	Post Traumatic Headache - Cluster Headache Variant
PVN	Paraventricular Nucleus
QC	Quality Control
QD	Quality By Depth
Q-Q plot	Quantile-Quantile Plot
R&D	Research And Development
RCT	Randomised Control Trial
REC	Research Ethics Committee
REM	Rapid Eye Movement
RNA	Ribonucleic Acid
RVAS	Rare Variant Association Studies
SAIGE	Scalable And Accurate Implementation Of Generalized Mixed Model
SCA	Spinocerebellar Ataxia
SCAR	Autosomal Recessive Spinocerebellar Ataxia
SD	Standard DEVIATION

SHM	Sporadic Hemiplegic Migraine
SIFT	Sorting Intolerant From Tolerant
SIGN	Scottish Intercollegiate Guidelines Network
SKAT	Sequence Kernal Association Test
SNP	Single Nucleotide Polymorphism
SOA	Statements Of Activities
SOE	Schedule Of Events
SOP	Standard Operation Procedures
SPECT	Single-Photon Emission Computerized Tomography
SPG	Sphenopalatine Ganglion
SSN	Superior Salivatory Nucleus
SSU	Sum Of Squared Score
SUNCT	Short-Lasting Unilateral Neuralgiform Headache Attacks With Conjunctival Injection And Tearing
SVM	Support Vector Machine
SWI	Susceptibility Weighted Imaging
TAC	Trigeminal Autonomic Cephalalgia
TCC	Trigeminocervical Complex
TF	Transcription Factor
TG	Trigeminal Ganglion
Ti/Tv	Transition (Ti) To Transversion (Tv)
TNC	Trigeminal Nucleus Caudalis
TSH	Thyroid Stimulating Hormone
UK	United Kingdom
UTR	Untranslated Region
VBM	Voxel-Based Morphometry
VCF	Virtual Card File
VIP	Vasoactive Intestinal Polypeptide
VI	Variable Importance
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WSC	Weighted Sum Test
WTCCC	Wellcome Trust Case Control Consortium

Contents

Impact Statement

The work presented in this thesis will primarily impact the progression of scientific advancements in the field of cluster headache (CH) through the identification of genetic and clinical factors influencing its development and clinical course. Furthermore, through the creation of a collaborative framework, this initiative provides a foundation for future large-scale consortia studies that will pursue unprecedented insights into this poorly understood condition.

Information derived from the clinical section of this thesis has the potential to inform clinical guidelines surrounding the assessment and management of CH patients. Specifically, the finding of similar rates of pituitary adenomas in this population of patients as compared with the general population has implications for streamlining imaging guidelines and improving cost efficiency. Similarly, the results from the post-traumatic cluster headache (PTH-CH) study indicate the specific traits of this subtype of CH and the requirement for early specialist intervention and tailored management plans in these patients.

As part of this thesis, through a genome-wide association study (GWAS), I detected the first replicable susceptibility loci for CH, and identified an overlap with an established migraine locus. Downstream analysis of these results signals the involvement of microglia and cytokine pathways in the aetiology of cluster. These findings will help to focus future mechanistic and functional studies, potentially leading to identification of druggable targets for novel therapies.

Excerpts from my thesis have already been disseminated through publications in peer-reviewed journals and presentations at both national and international conferences including the Association of British Neurologists (ABN)/ Society of British Neurological Surgeons (SBNS) joint annual meeting and the International Headache Congress (IHC). A network of CH sites within the UK and an international consortium of cluster headache genetics (CCG) was created as part of my project. Enhanced collaboration within this framework will enable long-term recruitment, follow-up, and data collection for large, multi-centre studies both within and outside the field of genetics.

Currently, a large meta-analysis involving the GWAS data generated in this thesis is underway. A portion of my funding was used to genotype smaller cohorts (Greek, Belgian and Spanish CH patients) to enable their involvement in this initiative. The preliminary data generated in this thesis also formed the proposal for a PhD fellowship (£120,000) from Brain Research UK which has been awarded to an incoming UCL student who will attempt to functionally validate the potentially causal variants identified through whole exome sequencing in section IV. This will involve the modelling of *CACNA1G* mutations, identified in families with CH, in xenopus oocytes to demonstrate pathogenicity. This has the potential to reveal an underlying disease-mechanism and provide a therapeutic target for disease management.

I have also engaged in Public and Patient Involvement (PPI) as part of this work. I presented at events organised by the CH support group 'Ouch' and developed a website (www.neurogenetics.co.uk). This is a patient-focused platform designed to improve research accessibility for CH patients. It benefited the recruitment of my study and will have ongoing upkeep so that this interface with the patient population can be maintained. It will also be a prime mechanism to disseminate research findings,

including those presented in my thesis, which have undergone peer-review, and all patients to be involved in curating the content which they would find valuable.

In conclusion, I present my findings with the aim that they may provide benefit to other researchers in the neurogenetic and headache fields during their endeavours to decipher disease-mechanisms and address the existing gaps in translational research in CH. Ultimately, I also hope that this yields downstream outputs to directly benefit affected patients.

Section I: Introduction

1.1 Introduction to Neurogenetics

The field of neurogenetics has advanced exponentially over the last two decades. Next-generation sequencing has facilitated the discovery of several genetic variants as the aetiological drivers of a myriad of neurological diseases, providing fundamental insights into their underlying pathophysiology. In some cases, these developments have translated into the identification of druggable targets and genetic therapy, a process that is likely to accelerate with future technological advances. Considering the potential role of genetics in the generation of neurological treatments, the purpose of this section is to introduce basic genetic principles and establish a framework for the genetic investigation of headache, with an emphasis on Cluster Headache (CH).

The human genome consists of over three billion nucleotides constructed in sequences (genes) that are organised across 23 chromosome pairs. Genes are transcribed into RNA and translated into proteins in the ribosomes. 1-2% of the genome is comprised of ‘protein coding’ exomes; the remaining non-coding DNA, previously referred to as ‘Junk DNA’, is now thought to contribute considerably to the regulation of biological functions with the ability to impact phenotype [1]. These regions transcribe functional RNA molecules, regulatory regions, and repeat sequences which have a structural impact on chromosomes[1]. The expression of genes varies based on cell type, temporal, and physiological factors. This is influenced by short non-coding regions called enhancers that engage with transcription factors necessary for the activation of genes.

Individuals inherit two copies of every gene, one per parent. Rare genetic conditions usually exhibit mendelian models of inheritance, reflecting a single genetic mutation with a large biological effect. In autosomal dominant conditions, only one mutant allele is required to manifest the phenotype, which, if fully penetrant, affects individuals across all generations of a pedigree. Two mutant alleles are needed to manifest autosomal recessive conditions, thus carriers are often asymptomatic or only mildly affected. X-linked diseases are observed in males given they only have one X chromosome, whilst female carriers remain asymptomatic or have mild symptoms. Genetic variation in germline cells is inherited or can occur de-novo during meiosis. Somatic mutations and mosaicism occur later in development, affecting specific cell lineages while others remain unaltered. Rarely, neurological disease can also arise from maternally transmitted pathogenic mitochondrial mutations.

The functional impact of a mutation is dependent on the how it changes the DNA sequence (**Figure 1 A&B**). Single nucleotide polymorphisms (SNPs) are the most frequent type of genetic mutations, subdivided into synonymous or non-synonymous. Synonymous variants are silent mutations that do not impact the amino acid sequence of a protein. Non-synonymous mutations, including missense variants, result in an amino acid change with potential to alter protein function. Nonsense mutations involve the introduction of a premature stop codon, terminating protein translation. Depending on where this occurs in the sequence, it can give rise to a dysfunctional truncated protein. An insertion or deletion of base pairs can cause a frameshift mutation, totally disrupting the reading frame, producing a severely impaired protein. Mutations at the boundary of the intron and exon, or those affecting regulatory sequences used by splicing machinery, result in aberrant mRNA splicing. Trinucleotide repeat expansions

can also have a high impact causing a toxic gain of function. Non-coding mutations and those at 3' and 5', can have a modifying impact on functionality. SNPs can be categorised as rare, low frequency, or common based on their minor allele frequency (MAF) (<1%, 1-5%, >5% respectively).

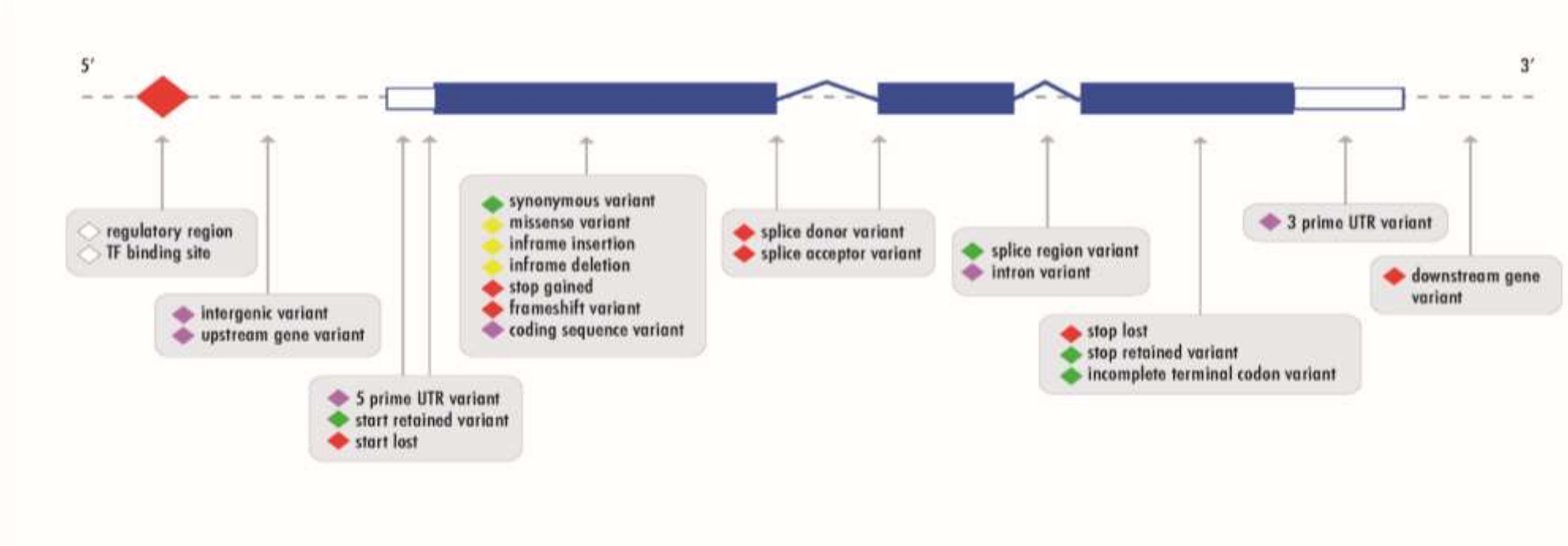


Figure 1 (A) *illustration showing the locations of possible genetic mutations and their associated impact. Exons are represented by solid blue boxes and the grey dashed line represents intronic regions. Regulatory regions are indicated in red. Coloured diamonds adjacent to mutations represent impact of mutations. Red indicates a high impact mutation, yellow indicates a moderate impact, green are low impact mutations and purple are modifiers. B: Information on each mutation as described by sequence ontology (Figures adapted from www.ensembl.org[2]).*

IMPACT	DISPLAY TERM	SEQUENCE ONTOLOGY DESCRIPTION
HIGH	Splice acceptor variant	A splice variant that changes the 2 base region at the 3' end of an intron
HIGH	Splice donor variant	A splice variant that changes the 2 base region at the 5' end of an intron
HIGH	Stop gained	A sequence variant whereby at least one base of a codon is changed, resulting in a premature stop codon, leading to a shortened transcript
HIGH	Frameshift variant	A sequence variant which causes a disruption of the translational reading frame, because the number of nucleotides inserted or deleted is not a multiple of three
HIGH	Stop lost	A sequence variant where at least one base of the terminator codon (stop) is changed, resulting in an elongated transcript
HIGH	Start lost	A codon variant that changes at least one base of the canonical start codon
MODERATE	Inframe insertion	An inframe non synonymous variant that inserts bases into in the coding sequence
MODERATE	Inframe deletion	An inframe non synonymous variant that deletes bases from the coding sequence
MODERATE	Missense variant	A sequence variant, that changes one or more bases, resulting in a different amino acid sequence but where the length is preserved
LOW	Splice region variant	A sequence variant in which a change has occurred within the region of the splice site, either within 1-3 bases of the exon or 3-8 bases of the intron
LOW	Incomplete terminal codon variant	A sequence variant where at least one base of the final codon of an incompletely annotated transcript is changed
LOW	Start retained variant	A sequence variant where at least one base in the start codon is changed, but the start remains
LOW	Stop retained variant	A sequence variant where at least one base in the terminator codon is changed, but the terminator remains
LOW	Synonymous variant	A sequence variant where there is no resulting change to the encoded amino acid
MODIFIER	Coding sequence variant	A sequence variant that changes the coding sequence
MODIFIER	5 prime UTR variant	A UTR variant of the 5' UTR
MODIFIER	3 prime UTR variant	A UTR variant of the 3' UTR
MODIFIER	Intron variant	A transcript variant occurring within an intron
MODIFIER	Upstream gene variant	A sequence variant located 5' of a gene
MODIFIER	Downstream gene variant	A sequence variant located 3' of a gene
MODIFIER	TF binding site variant	A sequence variant located within a transcription factor binding site
MODIFIER	Regulatory region variant	A sequence variant located within a regulatory region
MODIFIER	Intergenic variant	A sequence variant located in the intergenic region, between genes

Figure 1 (B)

1.1.1 Mendelian and Non-Mendelian Disorders

Mendelian conditions are usually monogenic, caused by rare variants with a high effect in causal genes, transmitted from parents to offspring. Although individually rare, these conditions affect millions of families, with more than 8,000 distinct conditions documented to date[3-5]. The study of these diseases has expanded our knowledge of genomic aetiologies and, in turn, provided mechanistic insights into more common conditions[6]. Familial studies involving linkage and segregation analysis are the most useful approach but can be impeded by incomplete penetrance, digenic inheritance, or the presence of a multilocus mutational burden[7]. Approaches such as karyotyping, chromosomal microarray (CMA), whole exome sequencing (WES), and more recently, whole genome sequencing (WGS), have facilitated the discovery of an unprecedented number of disease-causing mutations. Each modality has limitations, for example CMA alone is insufficient to identify copy-neutral, structural mutations and WES cannot effectively detect triploidy or copy number variants (CNV). RNA sequencing and long read sequencing improves the sensitivity of WGS and will likely contribute substantially to future discoveries[8, 9]. A number of mendelian inherited paroxysmal conditions have been attributed to mutations in genes involved in ion channels[10]. These channelopathies encompass a diverse spectrum of phenotypic manifestations and include familial hemiplegic migraine[11-13].

Non-mendelian, or complex neurogenetic disorders arise from interactions between genetic and non-genetic factors. These conditions typically aggregate in families in various inheritance patterns reflecting genetic susceptibility, though they can also occur sporadically. Considering the frequency of these common disorders, it is proposed that a proportion of them are caused by common variation (MAF >5%)[14, 15]. Unfortunately, the identification of the underlying genetic architecture contributing to

these phenotypes is difficult to decipher. Genome wide association studies (GWAS) attempt to identify common genetic markers correlating with, and therefore predisposing to, common disorders[16]. This approach has successfully identified susceptibility loci for migraine[17]. However, associated markers are merely representative of regions that are in linkage disequilibrium (LD) with causal genes, therefore, interpretation of these findings can be challenging. This is further complicated by the need for very large cohorts to sufficiently power GWAS to detect the small effect size of such variants. While GWAS have uncovered the genetic variants associated with a number of complex neurological conditions, the majority of these mutations confer relatively modest increments in risk and account for only a small fraction of heritability[18]. This presents the question as to why such a large proportion of heritability remains unexplained[19]. There is a lack of consensus regarding "missing heritability", however, it has been speculated that a proportion is attributable to low frequency variants (0.5%< MAF <5%) with an intermediate effect that are not captured on genotyping platforms. Furthermore, rare variants (MAF <0.5%) are difficult to detect if they do not have a high effect and therefore do not follow mendelian patterns of inheritance[19]. **(Figure 2)**

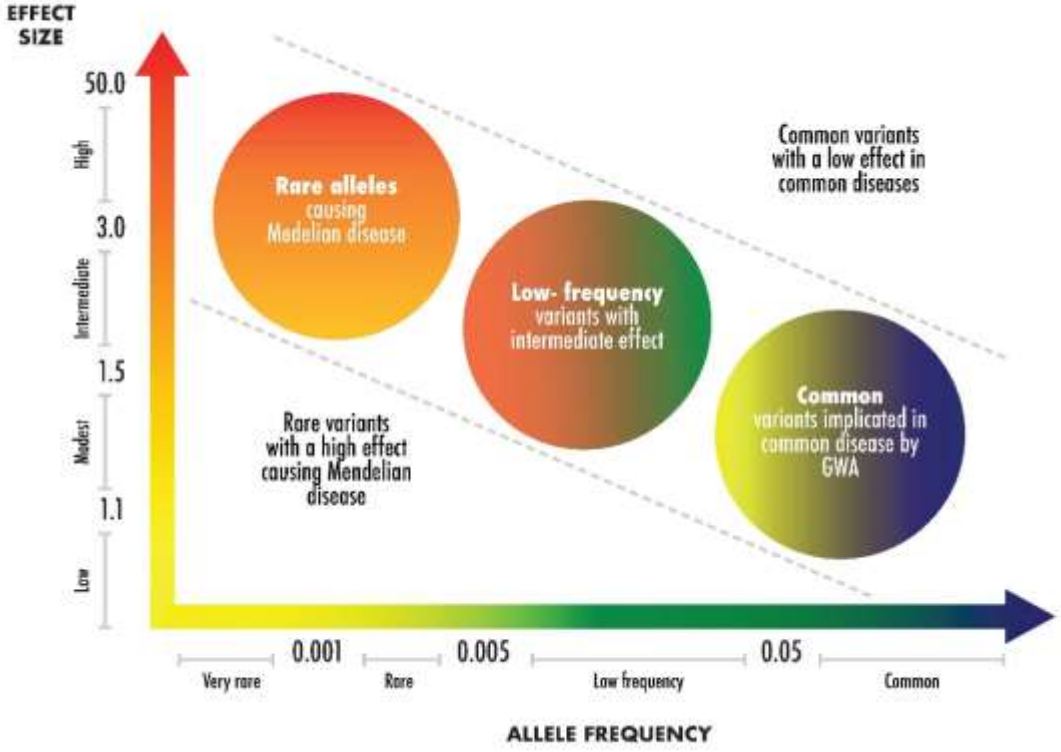


Figure 2 *Feasibility of uncovering causal genetic variants based on their frequency and effect size (odds ratio). Current emphasis lies in identifying variants within the diagonal dotted lines. Rare variants with a large effect size inherited in mendelian patterns are identified through familial studies whilst common variants contributing to common disease are investigated using genome wide association (GWA) studies. (Adapted from Manolio et al[19]).*

1.1.2 The Future of Neurogenetics

The field of neurogenetics is evolving rapidly. Genomic sequencing at a population scale, such as the NHS 100,000 genome project together with advances and improved affordability of sequencing technologies, has contributed considerably to these developments [20]. Genetic testing has become readily available for many monogenic disorders, facilitating the delivery of essential genetic counselling and prognostic information to patients. Similarly, the development of interventions targeting genetic mutations is changing the landscape of how these devastating mendelian conditions are managed. Examples include the use of antisense oligonucleotides for infantile-

onset Spinal Muscular Atrophy, Huntington's disease, and exon-skipping agents in Duchenne muscular dystrophy[21-23]. The clinical benefits in more complex, polygenic neurologic disorders is less clear, however, experimental attempts to use GWAS epigenetic data in combination with clustered regularly-interspaced short palindromic repeats (CRISPR)/CAS9 is promising[24]. These developments illustrate the ability for genetic research to provide unprecedented insights into both the disease mechanisms underlying neurological conditions and offer potential treatment targets. This aligns with the aims of this thesis: to develop an improved understanding of the genetic and pathophysiological factors underlying CH, thus providing a foundation for future mechanistic and clinical studies.

1.2 The Genetics of Headache Disorders

1.2.1 Familial Hemiplegic Migraine

As the archetype of inherited headache disorders, familial hemiplegic migraine (FHM) provides a potential model for the genetic and biological architecture of headache. It is a mendelian inherited condition, but genetically heterogeneous, caused by a single gene mutation with almost complete penetrance. A genetic diagnosis is made in approximately 25% of patients[25]. It is largely inherited in an autosomal dominant pattern and is categorised as FHM1 (MIM 141500), FHM2 (MIM 602481), and FHM3 (MIM 609634). Categorisation is determined according to pathogenic variants in the calcium channel voltage-dependent P/Q type α-1a subunit (*CACNA1A* [OMIM 601011]); ATPase Na+/K+ transporting, α-2 polypeptide (*ATP1A2* [OMIM 182340]); or sodium voltage-gated channel α-1 subunit (*SCN1A* [OMIM 182389]) respectively. All of these genes have a role in ion channels or transmission, suggesting that FHM is a

channelopathy. There is no apparent correlation between genotype and phenotype, often with a wide spectrum of symptoms even within families. This implies additional factors such as sex hormones may modify presentation[11,26].

Over 30 mutations have been reported in *CACNA1A* (FHM1)[27-29]. These are predominantly missense mutations though deletions have been detected[30]. These genetic changes predispose to cortical spreading depression (CSD) through enhanced calcium influx into the presynaptic terminal, triggering glutamate secretion and subsequent neuronal hyper-excitability[29, 31]. Evidence suggests that the severity of symptoms correlates with the functional consequence of the causal variant [32, 33]. FHM1 is allelic with Episodic Ataxia 2 (EA2 [MIM 08500]) and Spinocerebellar ataxia 6 (SCA6 [MIM 183086]), often presenting with overlapping clinical symptoms [34]. Approximately half of patients with EA2 experience concomitant migraine and patients with SCA6 can experience headaches and nausea[35, 36]. In contrast to FHM1 which is caused by gain-of-function mutations, EA2 generally arises from loss-of-function variants, and SCA6 occurs due to a toxic gain-of-function caused by a polyglutamine repeat expansion leading to an aggregation of mutant CaV2.1 channels[37, 38].

FHM2 is caused by mutations in *ATP1A2* which encodes the α-2 isoform of the catalytic subunit of Na+/K+ -ATPase, an ion transport pump[39]. It is highly expressed at the tripartite synapses of astrocytes and modulates glutamate reuptake[13, 40]. Compared to wide type, *ATP1A2* knock-out (KO) mice display neurological deficits and show a reduced threshold for the initiation of cortical spreading depression (CSD), a rapid propagation rate, and slow recovery following depolarization[41, 42]. Dysfunction of the Na+/K+ -ATPase pump is hypothesised to increase susceptibility for CSD by increasing synaptic potassium and glutamate[43, 44]. *ATP1A2* exhibits pleiotropy; mutations cause a spectrum of phenotypes including alternating hemiplegia of

childhood, seizures, intellectual disability, and periodic paralysis [45-49]. *ATP1A2* variants are the most common mutations detected in sporadic hemiplegic migraine (SHM), indicating a higher frequency of de-novo variants in this gene[13].

FHM3 occurs due to mutations in *SCN1A* [12, 50]. It is rare, accounting for only 10% of cases with a molecular diagnosis and encodes the α-1 subunit of the neuronal voltage-gated sodium channel Nav1.1 [50]. Pathogenic variation in this gene leads to impairment of the channels at gamma-aminobutyric acid (GABA)-ergic interneurons, increasing glutamate release and triggering CSD[25, 51]. Mutations in *SCN1A* are frequently associated with epilepsy[51]. Variants can have both a gain- or loss-of-function effect[52, 53]. Concurrent neurological disorders including elicited repetitive transient daily blindness and seizures can occur in some patients with FHM3[54, 55].

1.2.2 Other monogenic disorders associated with migraine

Genes associated with paroxysmal conditions including: Proline-Rich Transmembrane Protein 2 (*PRRT2*, [OMIM 614386]), PNKD metallo-β-lactamase domain-containing protein (*PNKD*, [OMIM 609023]) and Solute carrier family 2 member 1 (*SLC2A1*, [OMIM 138140]) have been proposed as candidates for HM[56-60]. However, due to the clinical heterogeneity of cases and the pleiotropic effects of these genes, causality has yet to be confirmed[60-62]. A frameshift mutation in potassium channel, subfamily k, member 18 (*KCNK18* [OMIM 613655]) which encodes the TWIK-related spinal cord potassium channel (TRESK), segregated in a large pedigree with migraine with aura[63]. TRESK modulates the excitability of neurons of the trigeminal and dorsal root ganglia, impacting the transmission of painful stimuli[64]. More recently, a mutation in ATPase, Na+/K+ transporting, alpha-4 polypeptide (*ATP1A4* [OMIM 607321]) co-segregated in an Italian family with carbamazepine-responsive migraine [65]. Although they provide

plausible candidates, evidence is lacking to definitively attribute causality to mutations in these genes.

Familial migraine has previously been associated with other monogenic conditions. Two pedigrees of patients with familial advanced sleep phase (FASPS) caused by mutations in casein kinase 1 delta (*CK1D* [OMIM 600864]) also had concurrent migraine with aura[66, 67]. *CK1D* is involved in the phosphorylation of PER2 and has a central role in the synchronization of the circadian clock[68]. This would support the proposed role of the hypothalamus in migraine and is further evidenced by the sensitization to pain demonstrated by transgenic CK1D mice[67, 69]. Migraine with aura occurs in cerebral autosomal dominant arterioropathy with subcortical infarcts and leukoencephalopathy (CADASIL), caused by mutations in the Notch receptor 3 gene (*NOTCH3* [OMIM 600276]) and often pre-empts other symptoms by several years[70].

Other mendelian disorders associated with migraine include ROSAH (retinal dystrophy, optic nerve oedema, splenomegaly, anhidrosis and migraine) due to pathogenic variants in the Alpha kinase 1 gene (*ALPK1* [OMIM 607347]) and RVCL (retinal vasculopathy with cerebral leukodystrophy) caused by the 3-prime repair exonuclease 1 (*TREX1* [OMIM 606609]) gene[71, 72].

1.2.3 Migraine and Migraine with Aura

Migraine and Migraine with aura exhibit substantial heritability[73]. The diversity in phenotypes and inconsistent inheritance patterns reflect the genetic complexity of these conditions[74-76]. Several GWAS have been conducted in migraine[17, 77, 78]. Initial studies detected susceptibility loci near genes postulated to have neuronal functions such as the transient receptor potential cation channel, subfamily m, member 8 gene (*TRPM8* [OMIM 606678]), the low density lipoprotein receptor-related protein 1 gene

(*LRP1* [OMIM 107770]), the PR domain-containing protein 16 gene (*PRDM16* [OMIM 605557]) and the Metadherin gene (*MTDH* [OMIM 610323])[78, 79]. A subsequent GWAS revealed additional significant associations in regions near genes involved in synaptic Mads box transcription enhancer factor 2, polypeptide D (MEF2D [OMIM 600663]), Astrotactin 2 (*ASTN2* [OMIM 612856]) and vascular (Transforming growth factor-beta receptor 2 (*TGFBR2* [OMIM 190182]), Phosphatase and actin regulator 1 (*PHACTR1* [OMIM 608723]) function[80].

The largest, a meta-analysis involving 56,674 migraine cases identified 38 independent susceptibility loci, 28 of them novel. This study reflected previous findings with prioritised genes predominantly showing enrichment for vascular functions but also neuronal mechanisms and ion transport[17]. Attributing causality to these signals is difficult as many of the disease-associated SNPs lie in intronic and intergenic regions and are in LD with the causal variant. Fine mapping attempts to overcome this challenge successfully identified a causal susceptibility SNP in PHACTR1 which regulates the expression of the endothelian 1 gene (*EDN1* [OMIM 131240]).

EDN1 promotes vasoconstriction and is highly expressed in vascular smooth muscle, further supporting the aetiological role of vascular mechanisms in migraine. Surprisingly, a subgroup analysis comparing migraine with aura and migraine without aura showed a similar allele frequency across both groups, indicating that the two entities are not genetically distinct[81, 82]. Further subtype analysis also found menstrual migraine to be more highly associated with the Neuropilin 1 gene (*NRP1* [OMIM 602069]) signal[83]. Genetic correlation studies reiterated vascular involvement in migraine, identifying an overlap between migraine with ischaemic stroke and coronary artery disease[84, 85]. Although well replicated, the migraine GWAS have yet to produce

any clinical utility, though they provide clues for future genetic studies in headache disorders [86].

1.3 Phenotype of Cluster Headache

CH is a trigeminal autonomic cephalalgia (TAC) characterised by severe, strictly unilateral pain in the distribution of the first branch of the trigeminal nerve. When untreated, the pain characteristically lasts from 15 minutes to three hours with observable cranial autonomic features such as ipsilateral lacrimation, conjunctival injection, rhinorrhoea, nasal congestion, facial flushing, facial sweating, ptosis, miosis and eyelid oedema **(Figure 3)**[87].

Figure 3 *Photograph showing cranial autonomic features during a CH attack. The image shows left periorbital oedema, partial ptosis, conjunctival injection and tearing. (Reproduced from Nesbitt et al [87] with permissions from BMJ Publishing Group Ltd)*

Unlike migraineurs, who avoid movement during an attack, individuals with CH become restless and agitated. Often referred to as the “suicide headache”, it has been

described as one of the most painful conditions a person can experience; female sufferers report the pain as more severe than childbirth[88]. Attacks occur with variable frequency, ranging from one on alternate days up to eight attacks per day during cluster periods. Cluster periods can persist between weeks and months.

Patients are categorised as having either episodic or chronic cluster headache. A diagnosis of episodic cluster headache (ECH) is assigned when at least two cluster periods lasting from seven days to one year, separated by a pain-free period of ≥3 months is experienced. In contrast, chronic cluster headache (CCH) refers to patients with a remission period lasting <3 months for at least one year[89]. Sufferers can fluctuate between ECH and CCH[90]. Approximately 45-50% of patients develop CCH following ECH (secondary CCH), the remainder are chronic from symptom onset (primary CCH)[91, 92]. It has been suggested that smoking status may influence the switch from the episodic to chronic phenotype[93]. A number of triggers for attacks have been identified including alcohol, strong smells, weather changes, sleep disturbance, and nitro-glycerine medications[94-98].

Characteristically, CH recurs with a circadian pattern, occurring at predictable times each day during cluster periods. These bouts often follow a circannual form, arising at the same time of the year[91, 95, 99]. The high prevalence of nocturnal attacks has resulted in the inclusion of CH as a sleep-related headache in the International Classification of Sleep Disorders[100]. Disordered sleep is frequently associated with CH and up to 80% of patients report nocturnal attacks[101]. Obstructive sleep apnoea and sleep-disordered breathing are also more common amongst CH patients[102-104]. The relationship between CH and the rapid eye movement (REM) to non-rapid eye movement (NREM) sleep stages is heterogeneous and, despite earlier assertions to the contrary, CH does not appear to be a REM locked disorder[105, 106]. Nevertheless,

impaired sleep–wake patterns, decreased REM- sleep and longer REM latencies have been shown during, but not outside, cluster headache bouts[107, 108].

CH is fundamentally a clinical diagnosis. Fulfilment of clinical criteria A to E as outlined in the International Classification of Headache Disorders, 3rd edition code 3.1 (ICHD-3) is required to establish a diagnosis of CH (**Table 1**)[89].

Table 1: ICHD-3 Diagnostic Criteria:

A	At least five attacks fulfilling criteria B-D
B	Severe or very severe unilateral orbital, supraorbital and/or temporal pain lasting 15-180 min (when untreated)
C	Either or both of the following: 1) At least one of the following symptoms or signs, ipsilateral to the headache: a) Conjunctival injection and/or lacrimation b) Nasal congestion and/or rhinorrhoea c) Eyelid oedema d) Forehead and facial sweating e) Forehead and facial flushing f) Sensation of fullness in the ear g) Miosis and/or ptosis 2) A sense of restlessness or agitation
D	Attacks have a frequency between one every other day and 8 per day for more than half of the time when the disorder is active.
E	Not better accounted for by another ICHD-3 diagnosis.

1.4 Epidemiology of Cluster Headache

CH is rare with an estimated prevalence of approximately 1 in 1,000, however, its occurrence varies across geographical regions and has been reported to be as high as 1 in 500[109-114]. These estimations are limited by a frequent delay in diagnosis or misdiagnosis. A delay to diagnosis of up to 2.6 years has been reported in the UK[115]. An earlier age of onset appears to prolong this delay. Extended periods of remission in patients with ECH also impedes a prompt diagnosis[116, 117]. Furthermore, CH is often misdiagnosed. In Eastern Europe and Belgium, misdiagnosis rates have been reported to be as high as 77% and 65% respectively[118, 119]. Notably, a high proportion of CH patients are initially misdiagnosed with migraine, trigeminal neuralgia, sinusitis, or a dental condition[118-121]. Factors contributing to diagnostic uncertainty include the presence of migrainous symptoms, the absence of craniofacial autonomic symptoms, and pain localised to the jaw[120].

CH can occur at any age but frequently presents in the second or third decade and rarely beyond the fifth decade[95, 111]. Overall, it appears to have a male preponderance[122]. In recent years, there has been an increase in the number of females diagnosed, likely due to greater awareness amongst physicians and improved compliance with diagnostic criteria[123, 124]. With regards to lifestyle factors, cigarette smoking is associated with CH and has been reported in 87.8% of chronic sufferers[125, 126].

Psychiatric comorbidities frequently accompany this highly disabling condition. Sufferers have a three times increased risk of developing depression, with self- harm and suicidal ideation reported in 25% - 55% of patients[88, 127]. Associated lifestyle

restrictions, disturbed sleep, and unemployment exacerbate the impact upon the lives of sufferers[95]. The socioeconomic burden of CH is substantial, with a mean direct and indirect cost of €5963 per sufferer over a six month period[128].

1.5 The Pathophysiology of Cluster Headache

1.5.1 Peripheral mechanistic theories

Despite the advances made by pre-clinical, clinical, and neuroimaging studies, the exact pathophysiology of CH remains unclear. Initial theories implicated peripheral mechanisms. In 1952, CH was described as a “histamine headache” due to the elevated levels of histamine and mast cells in blood and scalp skin during an attack[129, 130]. Subsequently, in the 1960s a vascular cause for CH was suggested due to the efficacy of treatments that act as vasoconstrictors such as ergot derivatives[131, 132]. Sterile inflammation within the cavernous sinus was also proposed as a possible cause, however, a single-photon emission computerized tomography (SPECT) /magnetic resonance imaging (MRI) study failed to show evidence of neurogenic inflammation due to a lack of extravasation of plasma proteins into the cavernous sinus during a CH attack[132, 133]. Structural lesions in the pituitary gland, with or without cavernous sinus invasion, were suggested as a possible source of secondary CH based upon the production of neuropeptides such as calcitonin gene-related peptide (CGRP) or substance P [134]. Ultimately, theories implicating peripheral mechanisms failed to explain several key features of cluster headache such as the circadian and circannual rhythmicity of attacks and the efficacy of drugs such as Lithium which act on the central nervous system (CNS)[135, 136].

1.5.2 The trigemino-vascular theory

The most widely accepted hypothesis is that CH is the result of a neurovascular process involving the activation of the trigeminal autonomic reflex, a pathway linking the trigeminal nerve (cranial nerve V) with parasympathetic outflow from the facial nerve (cranial nerve VII) via the superior salivatory nucleus (SSN) in the brainstem[137] (**Figure 4**). The trigeminovascular system consists of a plexus of nociceptive nerve fibres (including thinly myelinated Aδ-fibers and non-myelinated C-fibers) arising from the trigeminal ganglion (TG) to innervate cranial vessels and dura mater[137]. Stimulation of these structures results in the transmission of pain signals to the TG. Projections from these primary nociceptive afferents synapse at the trigeminocervical complex (TCC) in the brainstem, which includes the trigeminal nucleus caudalis (TNC) and dorsal horns of the spinal cord C1/C2. From here, they form ascending connections with thalamic nuclei and send signals to the frontal cortex, cingulate cortex, insula and somatosensory cortex, culminating in pain perception[138]. Activation of the cell bodies of the trigeminal ganglion triggers the release of several neuropeptides including neurokinin A, substance P, and CGRP which act as vasodilators and modulators of nociceptive trigeminal neurons[139]. This can be demonstrated by a rise in serum CGRP concentrations during a CH attack[140].

Trigeminovascular activation by dura and cranial vasculature also triggers parasympathetic outflow from the SSN in the pons to the sphenopalatine ganglion (SPG) and facial nerve. This results in the cranial autonomic manifestations[141]. Furthermore, parasympathetic nerve fibres arising from the SSN travel via the carotid and otic ganglion to produce vasodilation. The partial Horner's syndrome (ptosis, miosis and facial sweating) which can occur during an attack is caused by sympathetic compromise from carotid swelling[142]. This provocation of the parasympathetic nervous

system by the trigeminal nerve is referred to as the trigeminal autonomic reflex[143]. This reflex can also be reproduced in vivo through stimulation of the ophthalmic branch of the trigeminal nerve with capsaicin injection[144, 145]. Stimulation of the mandibular branch of the trigeminal nerve with capsaicin does not illicit dilation of the ipsilateral carotid artery, indicating that the capacity to activate this reflex appears to be exclusive to the ophthalmic branch[146]. Neuropeptides including vasoactive intestinal polypeptide (VIP) and Pituitary adenylate cyclase-activating peptide (PACAP) are also released from parasympathetic neurons, potentiating vasodilation and nociceptive transmission in trigeminal neurons[147, 148]. In recent years, the parasympathetic actions of the SPG has served as a useful target to treat CH through electrical and chemical manipulation, providing further evidence of its role in generating an attack[149].

However, the trigeminovascular theory also has limitations. For example, the resection of the trigeminal nerve root does not obliterate all attacks of CH[150, 151]. Similarly, the activation of the TCC during a CH episode has not been captured on functional imaging though this is may be due to limitations in imaging protocols[152].

1.5.3 The Hypothalamus

Significant evidence exists to implicate the hypothalamus in the aetiology of CH. The hypothalamus has a critical role in the regulation of the sleep-wake cycle, circadian rhythm, autonomic functions, and endocrine systems, all of which can be abnormal in CH[153]. Sleep is a common trigger for CH[95, 154]. Patients frequently report nocturnal attacks, accounting for half of their overall episodes, that can recur at the same time every night[155, 156]. Impaired sleep-wake cycles, reduced total REM-sleep and prolonged REM latencies have also been observed, during, but not outside, a CH bout[107, 108]. These abnormalities in sleep may be indicative of dysfunctional regulation

of the biological clock by the suprachiasmatic nucleus located in the anterior hypothalamus[157]. The secretion of melatonin by the pineal gland in a circadian pattern is also regulated by the suprachiasmatic nucleus[158]. Peak melatonin levels are reduced in CH and accompanied by an abnormal excretory pattern of its metabolite - 6-sulphatoxymelatonin[159-161]. In a small proportion of patients, melatonin is helpful in limiting attacks[162, 163].

The orexinergic system may also be involved. Orexin A and orexin B are proteins produced in the hypothalamus that help regulate wakefulness and nociceptive pathways[164]. The demonstration of lower levels of orexin A in the CSF of CH patients may imply altered anti-nociceptive processing within the hypothalamus[165]. Neuroendocrine changes possibly indicative of hypothalamic dysfunction have also been observed; testosterone levels were reported to be lower in active CH patients compared to controls or those in remission[166]. However, these finding were not reproducible[167]. Twenty four hour cortisol levels were also higher during a bout than in remission periods[160]. There is a loss of the normal diurnal secretory pattern of prolactin though basal concentrations are equivocal to controls[168, 169]. Irregularities in growth hormone secretion has also been observed, with a delayed evening peak in some individuals with CH[170].

It has been suggested that loss of central inhibition by the hypothalamus leads to dysregulation of nociceptive transmissions to the cortical structures involved in processing pain[171]. The hypothalamus receives projections, directly and indirectly, from structures implicated in spinal and trigeminovascular pain processing[172, 173]. For example, rat models have demonstrated that descending projections extend from the paraventricular nucleus of the hypothalamus (PVN) to the SSN, the area responsible for parasympathetic outflow to facial structures during CH attacks[174]. Dural vascular

excitation can induce activation of the paraventricular (PVN) and posterior hypothalamus[175, 176]. The PVN also sends projections to the superior salivary nucleus which appears to contribute to the cranial autonomic manifestations of CH[174]. Additionally, the A11 hypothalamic nucleus, positioned in the periventricular grey matter, exclusively gives rise to the inhibitory dopaminergic projections to the dorsal horn of the spinal cord[177, 178]. Activation or suppression of this nucleus can inhibit or facilitate nociceptive trigeminovascular neurotransmission arising from dural and cranial vasculature, via D_2 dopamine and 5-$HT_{1B/1D}$ receptors[179, 180].

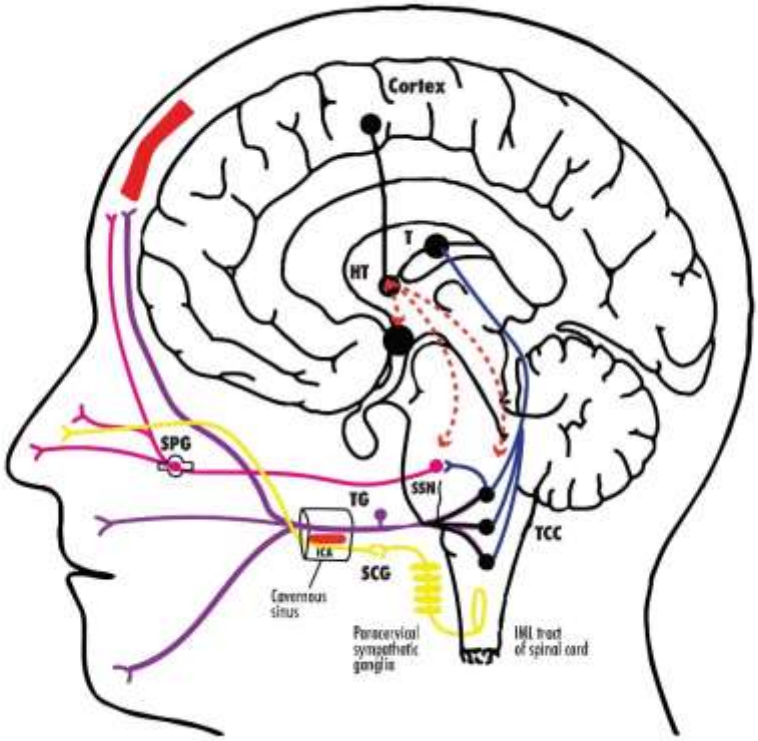


Figure 4 *Pathophysiology of CH: Afferents from cranial vessels and dura (purple) travel via ophthalmic division of the trigeminal nerve. They synapse in the TCC and project to the Thalamus and cortex resulting in pain (blue). Stimulation of the SSN in the pons triggers activation of this parasympathetic reflex via the SPG (pink) and the facial nerve. The HT is connected to the trigeminal system and pain matrix (red dashed lines). A third-order sympathetic nerve lesion causes sympathetic symptoms (yellow). HT = Hypothalamus, ICA = Internal carotid artery, IML = Intermediolateral tract of spinal cord, SCG = Superior cervical ganglion, SN = Suprachiasmatic nucleus, SPG = Sphenopalatine ganglion, SSN = Superior salivatory nucleus, TCC = Trigeminocervical complex. (Adapted from Wei et al.[181])*

1.5.4 Imaging studies

Observations from functional imaging studies further support an aetiological role for the hypothalamus **(Figure 5).** Positron emission tomography (PET) studies have shown activation of the ipsilateral inferior hypothalamic grey matter during a nitroglycerine provoked attack[182]. A subsequent study using PET/magnetic resonance angiography in a larger cohort of 17 individuals with ECH demonstrated activation of areas involved in pain processing and the inferior posterior hypothalamus[183]. These findings were replicated in functional magnetic resonance imaging (fMRI) studies which also revealed activation of the hypothalamus during attacks and atypical functional connectivity in this region[184-186]. Similarly, voxel-based morphometry (VBM) has revealed structural irregularities in the posterior hypothalamus with an increase in grey matter density in cases versus controls[187]. Supporting this, electrode implantation and stimulation of the posterior inferior hypothalamus with a high frequency electrical impulse appears to stop CH attacks[188, 189]. However, tractography studies indicate that the midbrain tegmentum is the ideal anatomical position for deep brain stimulation (DBS)[190]. More recently, VBM imaging showed enlargement of the anterior hypothalamus, the location of suprachiasmatic nucleus, in CH patients[191]. Furthermore, a study using ^{31}P magnetic resonance spectroscopy (MRS) also suggested hypothalamic involvement by demonstrating a reduction in metabolite ratios in patients with CH[192].

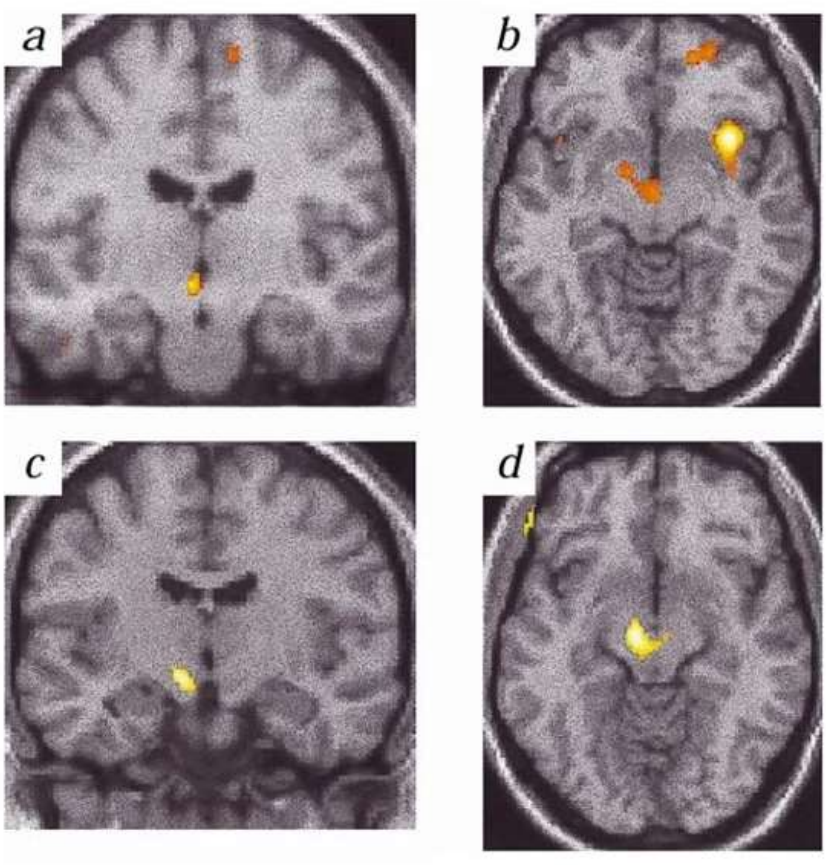


Figure 5 *Positron Emission Tomography (PET) study of cluster headache patients in and out of attack showing ipsilateral activation of the inferior posterior hypothalamus (a and b), with activation of insula and frontal cortex with pain (b). Voxel-based Morphology in cluster headache patients showing structural abnormalities in inferior posterior hypothalamic grey matter (c and d). (Reproduced from May et al[187] with permissions from Nature Medicine)*

Imaging studies also implicate regions involved in the pain matrix in the pathophysiology of CH. An increase in regional blood flow to cortical structures (including the insula cortex, anterior cingulate, and operculum) during CH attacks was first demonstrated back in 1996[193, 194]. Since then, several studies have revealed abnormalities in the pain matrix in CH patients. Metabolic abnormalities in several regions involved in pain, namely the posterior and anterior cingulate cortex, insula, prefrontal cortex, temporal cortex and thalamus, were demonstrated in episodic CH, both in and out of bouts[194]. Reduced functional connectivity has also been shown in the sensorimotor network in patients with ECH[185, 195]. Finally, structural imaging has

shown volumetric changes in CH which appear to differ based on episodic of chronic subtype[196-198].

1.5.4 Neuropeptides

Neuropeptides are released as a consequence of trigeminovascular activation **(Figure 6)**. CGRP, PACAP, neurokinin A, nitric oxide synthase, and substance P are all released from trigeminal sensory fibres. CGRP is a powerful vasodilator and significant evidence exists for its role in CH, triggering interest in its potential as a therapeutic target[140, 199, 200]. PACAP is secreted from both trigeminal sensory and parasympathetic fibres. It also acts as a vasodilator and modulates pain-processing by enhancing nociceptive signalling through neuronal excitation in the CNS[201, 202]. Levels of PACAP-38 are elevated during CH attacks when compared with remission periods in ECH[203]. Another vasodilator, neurokinin A is secreted from the caudal trigeminal nucleus in rat models, initiating cytokine release[204, 205]. Elevated levels of nitric oxide have been observed in the cerebrospinal fluid (CSF) and plasma of CH patients[206]. In addition to its vasodilatory effect, is a marker of active disease in inflammatory conditions such as multiple sclerosis (MS) and lupus[207, 208]. Substance P is immediately released in response to a noxious stimulus and its release has been demonstrated in cats following activation of the trigeminovascular system[209, 210]. It has multiple functions including activation of the inflammatory cascade, vasodilation and nociceptive transmission[211-213].

In addition to sensory fibres, parasympathetic fibres also release neuropeptides due to trigeminovascular activation. Vasoactive Intestinal Peptide (VIP) release reflects parasympathetic activation in both migraine and CH[140, 214]. Although elevated levels have not been demonstrated in serum, it is hypothesised that neuropeptide Y is also

secreted during CH attacks, augmenting trigeminovascular nociceptive transmission[210, 215]. Acetylcholine is a neurotransmitter of the autonomic nervous system. It is released from parasympathetic nerve fibres arising from the SPG. It's release within the meninges appears to mediate lacrimation and intracranial blood flow through the trigemino-parasympathetic reflex in CH[216].

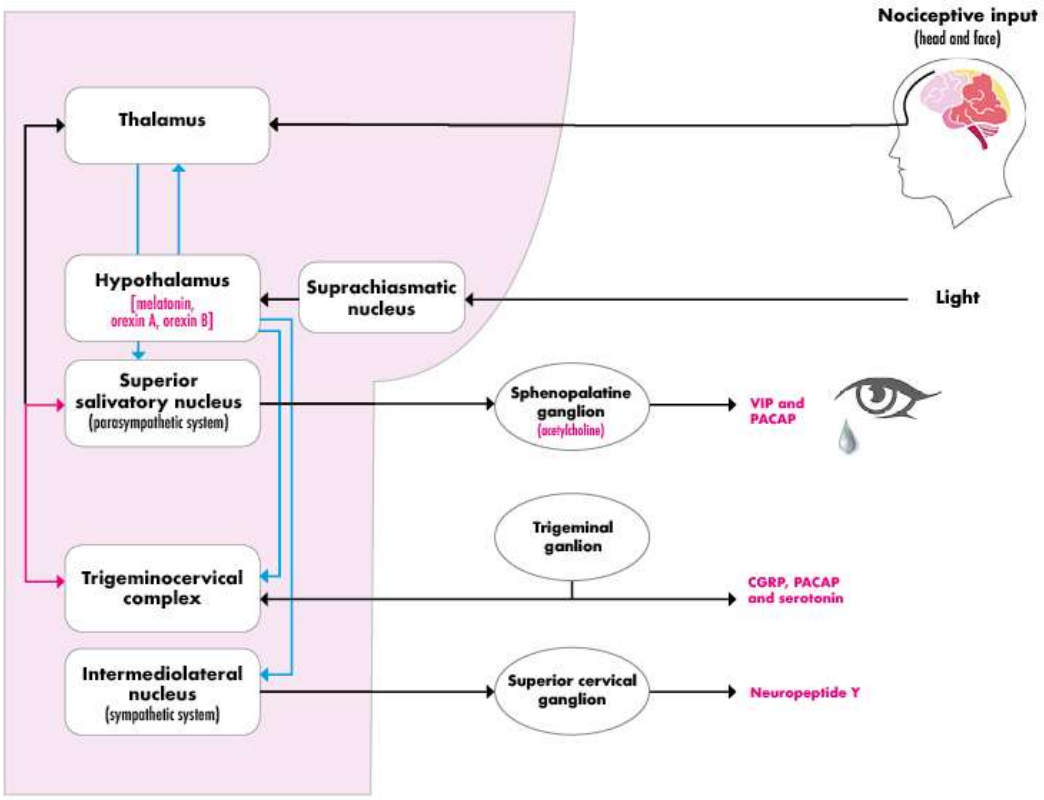


Figure 6 *Illustration of the neuropeptide components of cluster headache. The SSN, TCC and IML are regulated by hypothalamus, which is connected to the suprachiasmatic nucleus. The trigeminal autonomic reflex is triggered by nociceptive inputs from dural and cranial vasculature. Neuropeptides known to be involved in cluster headache are shown in pink (**VIP, PACAP, CGRP, Neuropeptide Y).** (Adapted from Hoffmann et al[217])*

1.6 Treatment of Cluster Headache

1.6.1 Abortive treatments

The treatments of choice in the acute setting include a subcutaneous or intranasal triptan and high flow oxygen. Triptans are selective 5-hydroxytryptamine (5-$HT_{1B/1D}$)

receptor agonists [218] which cause cerebral artery constriction and inhibit neuropeptide release[219]. Evidence exists for two triptans in the treatment of CH. Injectable sumatriptan, 6mg delivered subcutaneously, and intranasal zolmitriptan, 5-10mg, are effective in aborting an attack at 15 and 30 minutes respectively[220-222]. Triptans are usually well tolerated, with side effects such as paraesthesia and chest discomfort reported in less than 10 percent of patients[223]. They are contraindicated in coronary and cerebrovascular disease and guidelines indicate a maximum dose of two injections per day, however, this is based upon limited evidence[224, 225].

Characteristically, high flow oxygen is very effective in relieving attacks, with 75% of sufferers experiencing a relief of symptoms in 70% of attacks if high-flow oxygen is used for 15 minutes[226, 227]. The exact mechanism of effect is not fully understood. Animal models suggest there is direct action on the parasympathetic/facial nerve projections to the cranial vasculature, inhibiting the autonomic pathway and trigeminovascular activation[228]. One drawback to high flow oxygen is an observed rebound headache in some sufferers[229]. This can be ameliorated by extending the period of inhalation and incorporating the use of alternate delivery systems such as a non-rebreather or demand-valve mask[230].

A small amount of evidence exists to support the use of intranasal lidocaine to abort attacks, however this requires larger studies[231, 232]. Therefore, lidocaine should only be considered if triptan and oxygen treatment are contraindicated or ineffective. The use of somatostatin and somatostatin analogues as acute treatments has been suggested based on their inhibition of neuropeptides such as CGRP and VIP, and the observation that CH sufferers have lower levels of plasma somatostatin[233]. Octreotide, a somatostatin analogue, has been shown to be effective in headache relief (52% response in treatment group vs 36% in placebo, $p < 0.01$) [234].

1.6.2 Preventative treatments

Of all the prophylactic options, greater occipital nerve blocks show the greatest evidence in reducing attack frequency with fewer reported adverse effects[235, 236]. The mechanism of action likely relates to the alteration of signalling within the trigeminocervical complex where trigeminal afferents meet the dorsal horn nuclei of the first two cervical segments of the spinal cord[237].

Verapamil, a calcium channel blocker, is often used as the prophylactic agent of choice [238-240]. The exact mode of action of verapamil in CH has yet to be elucidated, however, it is hypothesised that it regulates CGRP-release through the blockade of presynaptic calcium channels[241, 242]. There has only been one double-blind, placebo randomised controlled trial (RCT) of verapamil which demonstrated a significant reduction in attacks (verapamil 0.6 ± 0.88, placebo 1.65 ± 1.01; $p < 0.001$) and analgesia usage (verapamil 0.5 ± 0.87, placebo 1.2 ± 1.03; $p < 0.004$) in the verapamil arm[239]. Common side effects include gingival hyperplasia, leg swelling, and cardiac arrhythmias that can occur in up to 19-38% of patients[241, 243, 244].

Lithium is often used as a second line therapy. To date, all placebo studies involving lithium have had small sample sizes and early endpoints, making them underpowered and unlikely to accurately measure efficacy[245]. A double-blind study (n=24) comparing lithium with verapamil showed both drugs were effective (verapamil 50% and lithium 37% improvement) with a reduction in the use of analgesia (58% in both groups) but considerably more side effects in the lithium group[246].

Other possible alternatives include topiramate which is often used in daily practice despite a lack of evidence to confirm its efficacy. Drug trials have similarly been inadequately powered, with significant variation in the dosage administered[247-249]. The

largest open-label trial involving 33 patients who received up to 250mg per day showed no improvement in symptoms [250]. Melatonin is also an option when other prophylactic agents have failed to resolve symptoms. The distinct circadian rhythmicity and sleep disturbance associated with CH supports its potential benefit. A placebo-controlled RCT consisting of 20 patients demonstrated a reduction in the frequency of attacks in patients taking 10mg of melatonin[162]. In contrast, other studies have shown no therapeutic benefit[251]. An initial open-label trial of sodium valproate 600-2000mg in 15 patients with CH was promising, with complete remission achieved in 9 individuals[252]. A subsequent larger double-blind RCT showed no difference in attack frequency in patients receiving sodium valproate versus controls[253]. Clomiphene was also shown to be useful in two case reports, possibly through hormone manipulation as an inhibitor of gonadatrophin release from the hypothalamus[254, 255].

Given most prophylactic treatments require time to take effect, transitional or bridging therapy is often implemented. This commonly involves a short course of corticosteroids which are thought to lower CGRP levels and improve nocturnal melatonin secretion. There is strong evidence to demonstrate that corticosteroids are very effective in the treatment of CH[256-259]. A recent multicentre, randomised, double-blind, placebo-controlled trial involving 116 participants examining the efficacy of prednisone versus placebo in short-term prevention of ECH showed 2.4 less attacks in the treatment arm difference (95% CI; −4·8 to −0·03, p=0·002). Alternatively, greater occipital nerve blocks can be utilised in the interim, with efficacy that usually exceeds a period of four weeks[235]. Intravenous dihydroergotamine is another option for temporary relief with a success rate of 57-100%[260-263].

1.6.3 Neuromodulation

Non-invasive vagus nerve stimulation (nVNS) is a useful treatment in patients with refractory CH or those intolerant to drug side effects. This can be achieved using GammaCore, a transcutaneous vagal nerve stimulator that recently received FDA clearance[264]. The device is applied to the antero-lateral cervical region and acts through the delivery of high-frequency electrical impulses to the vagus nerve. The exact mechanism of action has yet to be clarified but it appears to have a bilateral inhibitory effect on the trigeminal autonomic reflex and suppresses SSN triggered trigeminocervical neuronal firing[265, 266].

Two prospective, double-blind, sham-controlled RCTs provide compelling evidence for the utility of nVNS in an acute setting, particularly in the episodic subgroup[267-269]. Modulation of the sphenopalatine ganglion (SPG), a key structure in the manifestation of cranial autonomic features, has been suggested as a possible target for intervention[270, 271]. Relief of acute attacks has been illustrated through the electrical modulation of the SPG using an implantable stimulator which is controlled externally using a remote[272-274]. There is also evidence to suggest its utility in the prevention of attacks with significant reduction in attack frequency demonstrated following implantation[274, 275]. Similarly, occipital nerve stimulation (ONS) has been shown to improve refractory CH with one study indicating a 66.7% long-term reduction in pain[276].

1.6.4 Surgery

A number of surgical interventions have been also been proposed but are rarely used in clinical practise. A case series of patients who underwent microvascular decompression of the trigeminal nerve were found to have a favourable response initially, however this decreased over time[277]. The use of stereotactic radiosurgery using Gammaknife to target the SPG or trigeminal nerve root showed a 60% reduction

in pain[278]. However, this can be complicated by dysfunction of the trigeminal nerve in up to half of patients[279]. Central neuromodulation can regulate cortical and subcortical structures involved in pain processing. A subset of patients can qualify for deep brain stimulation (DBS) of the posterior hypothalamus if they have medically-refractory CCH and recurrent episodes >2 years, with strictly unilateral attacks for >1 year[280]. Evidence for this mostly consists of case studies and case series, however targeted neuromodulation near hypothalamic and midbrain structures can illicit short and long-term relief in most patients[239].

1.6.5 Other Therapies

CGRP has an established role in the generation of a CH attack. CGRP has been shown to increase in both nitro-glycerine-induced and spontaneous attacks and normalises with treatment[140, 199]. The CGRP pathway can be manipulated via either the peptide or canonical receptor using monoclonal antibodies (MABs). Two MABs have been investigated for their efficacy in CH. Galcanezumab was approved recently for use in ECH based on a study involving 106 participants, that demonstrated a reduction in the frequency of attacks by 3.5 attacks per week (95% CI; 0.2 to 6.7, p =0.04) when compared with placebo[281]. Recently, a trial of fremanezumab as a preventative treatment in ECH was prematurely terminated due to a lack of observable benefit at 4 weeks. Trials of both galcanezumab and fremanezumab were negative in CCH[282].

Illicit drug use is more prevalent amongst CH sufferers, many of whom report deriving relief from substances such as psilocybin and lysergic acid diethlamide (LSD)[283]. One case series involving 53 CH sufferers showed encouraging results for LSD as a preventative treatment and psilocybin as both a preventative and abortive treatment

[284]. The efficacy of oral psilocybin is currently being investigated in a double-blind randomised study[285].

1.7 The Genetics of Cluster Headache

Evidence suggests that CH has a genetic predisposition, with an estimated risk that is 5-18 times higher in first degree relatives of sufferers, and 1-3 times higher in second degree relatives[286]. Cases of concordance amongst monozygotic twins have been reported[287-290]. This may reflect selection bias as a Swedish twin registry found discordance amongst twin pairs to be more frequent, although both monozygotic and dizygotic twins were included in this cohort[291]. Similarly to migraine, familial aggregation of CH is observed[292-295,296]. Estimations of the rate of family history of CH differs across studies. For example, one study had a familial rate of 2.3% with a low Falconer's heritability index, indicating a high likelihood of additional modifiers in CH aetiology[297]. In contrast, a positive family history in up to 20% of patients, inferring a 39 fold relative risk has also been reported[298]. The largest heritability study to date, involving 370 probands, illustrated familial CH in 7% of cases indicating a multifactorial, as opposed to monogenic, aetiology[299].

Incomplete penetrance and inter-familial phenotype variability has been reported. An earlier age of onset in the offspring of parents with CH may infer anticipation[300], however, large multigenerational pedigrees would be required to demonstrate this. There also appears to be a greater proportion of female sufferers in familial cases[301]. Segregation analysis show an inheritance pattern consistent with AD with reduced

penetrance [302]. AR modes of inheritance have also been demonstrated reflecting the genetic heterogeneity of this condition[303].

To date, the vast majority of genetic studies have consisted of association studies based on the targeted investigation of single nucleotide polymorphisms (SNPs) in candidate genes with a hypothesised role in the development of CH. These studies were underpowered, lacked consistent replicable findings and produced conflicting results (**Table 2**).

The first genetic study of CH involved a case report of an individual from Japan with a missense mutation (3243A>G) in the mitochondrial gene *MT-TL1* (mtRNA$^{Leu(UUR)}$ [OMIM 590050])[304]. This is a known causal variant for mitochondrial encephalopathy, myopathy, lactic acidosis and stroke-like episodes (MELAS), a condition strongly associated with headaches, especially in the initial stages. Attempts to replicate this in other cases of CH have been unsuccessful - only two small Italian (n=47) and German (n=22) cohorts have assessed this association[305, 306].

1.7.1 Candidate Gene Studies

Since then, several candidate association studies have been conducted. (**Table 2**) The circadian periodicity of CH attacks and suggested hypothalamic involvement in its pathophysiology generated interest in genes involved in the circadian clock and biology of sleep. This included the circadian locomotor output cycles protein kaput gene (*CLOCK* [OMIM 601851]) which is highly expressed in the suprachiasmatic nucleus and plays a critical role in the regulation of circadian rhythms and diurnal preference (**Table 2**, row 2) . Analysis of the rs1801260 (3111T>C) 3'UTR SNP in CH failed to observe an association[76, 307-310]. A Swedish group conducted a study of additional SNPs in the *CLOCK* gene and found that the minor allele of the rs12649507

was more frequent in cases versus controls (OR 1.29, 95% CI;1.08-1.54, p= 0.0069).This association was strengthened when patients were stratified for diurnal variation in their headache (OR 1.44, 95% CI;1.15-1.77, p = 0.0009)[311]. Furthermore, they showed that mRNA expression of CLOCK in fibroblasts was higher in cases with rs12649507. The period circadian protein homolog3 gene (*PER3* [MIM 603427]), another core component of the circadian clock which negatively regulates CLOCK, was also proposed as a candidate by a Norweigen group (**Table 2**, row 3)[312]. Variants in this gene have been associated with familial advanced sleep phase syndrome 3 and diurnal preference, however, no association was found with CH[312, 313].

Genes involved in the hypocretin system (orexin-A, orexin-B) and their receptors (orexin receptor type-1, orexin receptor type-2) were also presented as possible candidates (**Table 2**, row 4). These regulate sleep –wakefulness, pain modulation and other complex homeostatic functions [314, 315]. Furthermore, they are highly expressed in the posterolateral hypothalamus which is implicated in CH as demonstrated by imaging studies and a lower level of hypocretin in the CSF of individuals with CH [165, 316, 317]. An Italian group detected an association between CH and rs2653349 (1246A>G [p.I308V]), a SNP in the Hypocretin Receptor 2 (*HCRTR2* [OMIM 602393]), in a study involving 109 cases and 211 controls, (OR 6.79, 95% CI; 2.25-22.99, p = 0.0003)[318]. This was replicated by a German group who compared 226 CH sufferers with 266 controls and similarly demonstrated that individuals homozygous for the G allele were at higher risk of developing CH (OR 1.97, 95% CI; 1.32 to 2.92, p = 0.0007)[319]. A meta-analysis which included these studies alongside 575 Dutch patients replicated, an association with the homozygous genotype, albeit with a significantly lower (OR 0.69, 95% CI; 0.53-0.90, p = 0.006). An analysis of the Dutch

cohort alone however, could not reproduce this association[320]. Similarly, a number of other population based studies also failed to replicate these findings implying that this association may be population dependent[309, 310, 321].

Based on the observation that alcohol acts as an immediate trigger for a CH attacks, variants in the alcohol dehydrogenase 4 (*ADH4* [OMIM 103740]) gene (including rs1800759 and rs1126671) implicated in alcohol dependence were investigated (**Table 2**, row 7). In one Italian study involving 110 CH cases and 203 controls, the non-synonymous variant (I309V) increased the risk of CH (OR 2.33, 95% CI; 1.25-4.37, p = 0.006) [322]. These findings were reproduced in a second Italian study (p=0.03) which also demonstrated an association between CH and rs1800759 (p=0.03) and rs1126671 (p=0.03)[309].However, the association with rs1800759 was contradicted by subsequent studies in a Chinese and larger Swedish population[310, 323]. Given the lack of functional evidence, small sample sizes, and insufficient power of the Italian studies, it is likely that this represents a type 1 error.

Observations regarding response-to-treatment also informed the choice of candidates. As previously discussed, verapamil is frequently used as a prophylactic agent of choice, implying a possible role for calcium activated channels in the aetiology of CH [324]. A Swedish group conducted a study to establish the overrepresentation of SNPs associated with verapamil-responsiveness in a cohort of 628 CH patients and 586 controls. This showed a significant association between rs1531394 in Anoctamin 3 (*ANO3* [OMIM 610110]) and CH that was strongest under a recessive model (OR 1.52, 95% CI;1.11–2.06, p=0.0086), however this association was not verapamil-response dependent and did not impact expression in fibroblasts (**Table 2**, row 9)[325]. Based on

the use of mono-amine oxidase inhibitors in migraine and the identification of abnormalities in tyrosine metabolism in CH, genes for trace amines were examined[326-328]. This involved linkage analysis in two large pedigrees with CH, but did not demonstrate a significant LOD score[328]. The antagonism of 5-hydroxytrptamine (5-HT)$_{1B/D}$ with triptans inspired a candidate study of the guanine nucleotide-binding protein, beta-3 (*GNB3* [OMIM 139130]), involving a cohort of 231 individuals with CH[329]. An alternate splice variant (C825T) is suggested to predict response to treatment for a number of serotonergic drugs[330]. The C825T variant results in alternate splicing with 41 amino acids deleted from GNB3, however, its functional impact has yet to be clarified. The study demonstrated a higher response rate to triptans in individuals heterozygous for the genotype but this was not statistically significant (OR 1.04, 95% CI; 0.40-2.72, p=0.66). Expression and electrophysiological experiments showed that the variant does not impact ion channel function in rat sympathetic neurons[331]. The use of triptans in CH also triggered the investigation of solute carrier 6, member 4 gene (*SLC6A4* [OMIM 182138]) which encodes for a protein that clears 5-HT from synaptic spaces. This involved a cohort of 148 CH patients but failed to identify any correlation with triptan responsiveness[332].

Other hypothesis-driven candidate studies which failed to identify an association with CH. Two common variants in the FHM1 gene, *CACNA1A*, were investigated in a Swedish population of 75 cases and 108 controls but did not show significant enrichment (**Table 2**, row 8)[333]. However, the methodology of this study was flawed as it sought to identify an association with an intragenic CAG repeat in the 3UTR region rather than a causal mutation for FHM. A variant in MTHFR, also linked with migraine, was investigated by a German group but did not produce an association (**Table 2**, row

10)[334]. Genes implicated in respiratory disorders were also examined due to a higher prevalence of sleep apnoea and cigarette smoking amongst CH sufferers. The serpin peptidase inhibitor A, member 1 gene (*SERPINA1* [OMIM 107400]), implicated in Alpha 1 Antitrypsin deficiency related emphysema, was considered and screened in a cohort of 55 cases and 55 controls (**Table 2**, row 13). No correlation was detected, however phenotype analysis found that patients with an S or Z allele had a higher attack frequency (p =0.02)[335]. The high prevalence of smoking dependence amongst CH patients lead to the examination of variants of genes encoding the nicotinic acetylcholine receptor (*CHRNA3* [OMIM 118503], *CHRNA5* [OMIM 118505]) in a cohort involving 65 patients (**Table 2**, row 14)[336, 337]. This did not identify a risk variant but suggested that the A allele at rs578776 in the 3'-UTR may be protective. Variants in the Nitric Oxide Synthase gene (*NOS1* [OMIM 163731]), examined due to the role of nitro-glycerine in triggering attacks, were also uninformative (**Table 2**, row 11).[338] Impaired iron homeostasis in the periaqueductal grey matter in migraine and chronic daily headache lead to the examination of the gene homeostatic iron regulator (*HFE* [OMIM 613609]) as a potential candidate (**Table 2**, row 12). Once again, no association was ascertained, however, individuals with a minor allele of the H63D variant appeared to have a later age of onset[339, 340]. Overall, candidate association studies have been uninformative and have failed to identify reproducible associations. This is consistent with the observation that such studies can produce false positives and require robust replication and validation to accurately attribute association[341].

1.7.2 Family studies

A lack of large multigenerational pedigrees and the reduced penetrance of CH hinders familial studies. A family based candidate approach has been used in attempts to identify segregating variants in *CACNA1A, CLOCK, ADH4, HCRTR2* and trace

amines[309, 328, 342]. The only hypothesis-free familial study to date involved a linkage study of five Danish pedigrees. It did not produce a significant LOD scores but showed a suggestion of linkage at four loci, two on chromosome 2 and one on chromosome 8 and 9, suggesting locus heterogeneity. However, these findings were not reproducible when extended to a further 33 pedigrees[321].

1.7.3 Genome- Wide Association Studies

There has been one GWAS performed on CH to date (**Table 2**, row 16)[343]. Significantly underpowered, involving only 99 patients and 360 controls of Italian ancestry, no variant reached statistical significance. However, they detected seven distinct loci suggestive of association. The most significant SNP (rs1006417) was on chromosome 14 (OR 0.34, 95% CI; 0.21–0.55, $p=1.4x10^{-6}$), upstream to the leucine-rich repeat and fibronectin domain-containing protein 5 gene (*LRFN5* [OMIM 612811]). A second SNP (rs12668955) suggestive of significance (OR 0.48, 95% CI 0.34–0.66, $p=9.1x10^{-6}$) within the adenylate cyclase-activating polypeptide 1 (*ADCYAP1R1* [OMIM 102981]), which encodes for pituitary adenylate cyclase-activating peptide (PACAP) was also detected. This was presented as an interesting candidate as it has been implicated in pain-processing in animal models and observed to induce migraines in health individuals administered PACAP[344, 345]. Gene based association analysis identified the membrane metalloendopeptidase gene (*MME* [OMIM 12050]) as the most significant gene ($p=2.5x10^{-5}$) with lead variant rs147564881 representing a missense mutation)[343]. MME encodes for neprilysin which is involved in the hydrolysation of substance P, neuropepetide Y, bradykinin and brain natriuretic peptides that have a known role in the regulation of trigeminal nociceptive signals [137, 346]. However, these findings were not replicated in a much larger Swedish study of 542 CH patients and 581 controls[347].

1.7.4 Gene Expression Studies

A small number of studies attempted to use expression analysis to decipher the genetics of CH. These studies were limited as they were considerably underpowered and utilised RNA derived from lymphoblasts, which may not be reflective of expression patterns in brain. One study involving only three patients with ECH demonstrated differential expression in over ninety genes[348]. A number of these genes were involved in calcium-binding and two human leukocyte antigen genes (*HLA-DQA1* [OMIM 146880] and *HLA-DQB1* [OMIM 604305])[348].

Another performed whole transcriptome analysis in eight CH cases and ten lithium responsive bipolar patients in an effort to identify a common expression profile between the two phenotypes with a therapeutic response to lithium. They reported altered expression of RNA-binding motif 3 (*RBM3* [OMIM 300027]) and nuclear receptor subfamily 1 group D member 1 (*NR1D1* [OMIM 602408]) between both groups and controls[349]. *RBM3* and *NR1D1* interact with a number of genes involved in circadian pathways including *CLOCK*. Additionally, they demonstrated an upregulation of tryptophan hydroxylase 1 (*TPH1* [MIM 191060]) which has a critical role in serotonin metabolism [350].

A group involving 39 CH patients and 20 controls could not replicate any difference in expression in single gene tests but on examination of functional gene sets found differential expression in GABA receptor function and channel related genes[351]. The methodology of this study was lacking as it failed to control for the dynamic nature of RNA and samples were collected with variable timing, with some cases sampled within 24 hours of an attack and others within 94 days.

In conclusion, expression studies have failed to produce meaningful results. This is likely reflective of the use of non-neuronal tissue samples for RNA extraction and the lack of power due to the small cohort size of these studies.

Table 2: Candidate gene studies in CH

Num	Gene OMIM	Function	Hypothesis	Country	Cases/ Controls	Variant/ Marker	MAF in gnomAD	Results P value	Reference
1	*MT-TL1* (OMIM: 590050)	Mitochondrial tRNA for leucine	Variant found in one case with CH. Variant associated with MELAS which is frequently complicated by migraine	Italy	47	Nt3243A>G	-	No Association	Cortelli et al [305]
				Germany	22	Nt3243A>G	-	No Association	Seibel et al [306]
2	*CLOCK* (OMIM: 601851)	Transcription factor essential to the maintenance of circadian clock.	Circadian / diurnal periodicity of CH attacks Disturbed sleep in CH patients	Italy	107/210	rs1801260	0.26	No Association	Rainero et al[308]
				Italy	101/100	rs1801260	0.26	No Association	Cevoli et al[76]
				Italy	54/200	rs1801260	0.26	No Association	Zarrilli et al[309]
				Sweden	449/677	rs1801260 rs11932595 rs12649507	0.26 0.4 0.3	No Association No Association 0.0069	Fourier et al[311]
				Chinese	112/192	rs1801260	0.26	No Association	Fan et al[310]
3	*PER3* (OMIM: 603427)	Generate circadian rhythms through differential expression to light exposure	Circadian / diurnal periodicity of CH attacks Disturbed sleep in CH patients	Norway	149/432	rs57875989	0.12	No Association	Ofte et al [312]
4	*HCRT* (OMIM: 602358)	Encodes hypocretin with role in regulation of autonomic and endocrine homeostasis	Hypothalamic involvement in CH headache attacks as shown in imaging studies and lower levels of hypocretin in the CSF	Italy	109/ 211	rs4796717 rs8072081	0.74 0.0025	No Association No Association	Rainero et al [318]
5	*HCRT1* (OMIM: 602358)	Encodes for Hypocretin 1 receptor				rs1056526 rs2271933	0.49 0.55	No Association No Association	

6	*HCRT2* (OMIM: 602358)	Encodes for Hypocretin 2 receptor		Italy	109/211	rs2653349 rs1027650	0.84 0.016	0.0003 No Association	
				Germany	226/266	rs2653349	0.84	0.00007	Schurks et al[319]
				Denmark, Sweden, UK	259/267	rs2653349 rs3122169	0.84 0.78	No Association	Baumber et al[321]
				Italy	54/200	rs2653349	0.84	No Association	Zarrilli et al [309]
				Netherlands	575/874	rs2653349	0.84	No Association	Weller et al[320]
				China	112/192	rs2653349	0.84	No Association	Fan et al[310]
7	*ADH4* (OMIM: 103740)	Encodes alcohol dehydrogenase 4, critical enzyme in ethanol oxidation rate.	Alcohol as trigger for CH	Italy	110/203	rs1800759 rs1126671	0.51 0.76	No Association 0.006	Rainero et al[322]
				Italy	54/200	rs1800759 rs1126671	0.51 0.75	0.03 0.03	Zarrilli et al[309]
				Sweden	390/389	rs1800759 rs1126671	0.51 0.75	No Association No Association	Fourier et al[323]
				China	112/192	rs1800759	0.51	No Association	Fan et al [310]
8	*CACNA1A* (OMIM: 601011)	Encodes pore-forming subunit of P/Q type VGCC	Variants in the gene cause FHM1	Sweden	75/108	D19S1150 CAG expansion	-	No Association	Sjostrand et al[352]
9	*ANO3* (OMIM: 610110)	Encodes for anoctamin-3, a calcium activated chloride channel	Calcium-activated ion channel investigated due to effectiveness of Verapamil as a prophylactic agent	Sweden	628/586	rs1531394	0.51	0.0086	Ran et al[325]
10	*MTHFR* (OMIM: 607093)	Encodes methylenetetrahydrofolate reductase, necessary for remethylation of	Previous link between variant in MTHFR and migraine	Germany	147/599	rs1801133	0.31	No Association	Schurks et al[353]

		homocysteine to methionine							
11	*NOS1* (OMIM: 163731)	Encode for nitric oxide which is involved in neurotoxicity associated with stroke and neurodegenerative diseases and neural regulation of smooth muscle.	Due to role of nitric oxide in vasodilation, neurotransmission and inflammation	Sweden	91/111	Dinucleotide repeat (NOS1a) Trinucleotide repeat (NOS1b)	-	No Association	Sjostrand et al[338]
	NOS2A (OMIM: 163730)					Bi-allelic repeat Pentanucleotide repeat	-	No Association	
	NOS3 (OMIM: 163729)					CA repeat in intron 13	-	No Association	
12	*HFE* (OMIM:613609)	Encodes protein which binds to transferrin receptor reducing affinity for transferrin	The observation of higher iron concentrations in pain-mediated structures in the brain	Italy	109/211	rs1800562 rs1799945	0.034 0.11	No Association	Rainero et al [340]
13	*SERPINA1* (OMIM: 107400)	Encodes alpha-1-antitrypsin, a deficiency of which causes emphysema	Higher frequency of smoking / sleep apnoea in CH	Germany	55/55	F, M, S, Z alleles	-	No Association	Summ et al [335]
14	*CHRNA3-CHRNA5* (OMIM: 118503) (OMIM: 118505)	Encodes the nicotinic acetylcholine receptor which mediates fast signal transmission at synapses	High smoking prevalence	Italy	65/263	rs16969968 rs6495306 rs578776	0.26 0.37 0.39	No Association No Association 0.038	Cainazzo et al [337]
15	*TAR1, TAR3, TAR4, TAR5, PNR, GPR58*	G-coupled receptors	Due to treatment response of migraine to mono-amine oxidase inhibitors and	Italy	-	Haplotype / Family based analyses	-	No Association	Aridon et al[328]

	(MIM:609333) (MIM:604485) (MIM: 604849)	activated by trace amines	elevated levels of trace amines in migraine and cluster headache.						
16	*ADCYAP1R1* (MIM:102981) *MME* (MIM:120520) *LRFN5* (MIM:612811)	Secretion of growth hormone, ACTH, cetecholamines. Neurotransmissio n Metalloendopepti dase, known to degrade opioid peptides amongst other substrates Cell adhesion molecule known to promote neurite growth with role in presynaptic differentiation	Genes in LD with signals suggestive of significance in GWAS involving 99 Italian patients and 360 controls	Sweden	542/581	rs12668955 rs147564881 rs1006417	0.42 0.001525 0.18	No Association No Association No Association	Bacchelli et al[343]

1.8 Aims and outline of thesis

The aim of this thesis is to gain insights into the pathophysiological and genetic determinants of CH. To achieve this goal I employed two approaches. Firstly, in section three of this thesis, I conduct clinical observational studies on a large cohort of CH cases derived from an ongoing study at the tertiary referral headache clinic at the National Hospital of Neurology and Neurosurgery (NHNN), Queen Square, London. Secondly, in section four, I explore the underlying genetic architecture of CH through the development of an international consortium of CH centres and the application of a number of established methods including GWAS, family-based analysis and rare variant association analysis.

1.8.1 Aims of clinical section

- To estimate the incidence of pituitary adenomas in CH and the value of dedicated pituitary imaging in the routine assessment of these patients.

- To explore the relationship between head trauma and the development of CH and delineate the clinical features of Post Traumatic Cluster Headache (PTH-CH).

- To estimate the prevalence of a positive family history of CH through the consolidation of results from previously published cohorts through systematic review and meta-analysis.

- To describe the phenotype of and identify clinical features unique of familial CH.

1.8.2 Aims of genetics section

- To conduct a GWAS in order to identify common genetic mutations (MAF >5%) that contribute to the development of CH. To attempt to replicate these findings through international collaboration with other CH centres.

- To perform linkage analysis and whole exome sequencing in a cohort of families with CH.

- To conduct a rare variant burden analysis to identify rare genetic mutations (MAF<0.05%) with an aetiological rule in CH.

Section II: Materials and Methods

2.1 Methods Summary

This thesis is comprised of two key work packages, a clinical component and a predominant genetic component. The cohorts and subsections of each component are outlined in **Figure 7**. These required separate methodologies which are detailed below.

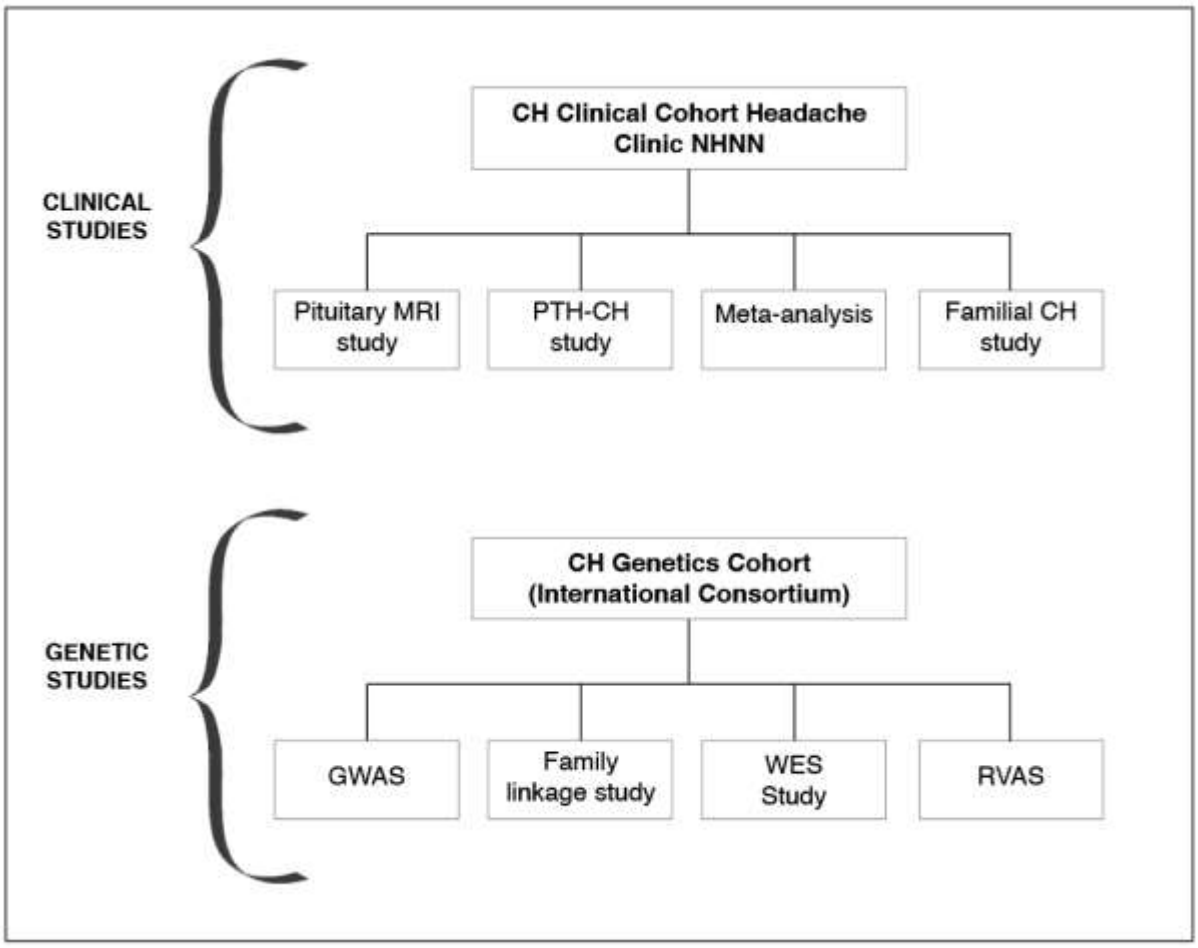


Figure 7. *Breakdown of component cohorts.*

2.2 Clinical Studies

2.2.1 Ethical Approval and Data protection

Ethical approval for the clinical studies was obtained by my collaborator and subsidiary

supervisor, Dr Manjit Matharu Consultant Neurologist, NHNN, from local Research Ethics Committee (REC no:11/LO/1709) as part of an ongoing research project. All clinical data was de-identified and stored on an encrypted database (Microsoft Excel, Microsoft Corporation, Redmond, WA, USA) at NHNN in accordance with general data protection regulation (GDPR).

2.2.2 Recruitment Strategy

All cases included in the clinical studies outlined in section three were recruited as part of an ongoing study at the headache clinic at the NHNN, which was initiated in January 2007. From 2007–2017, CH cases were consented and recruited by clinical fellows and the headache clinical nurse specialists. My role in case collection included follow-up of participants (in clinic or review of clinical notes), data entry for the cohort dataset, and where appropriate, re-phenotyping of cases, collection of additional data, and assessment of possibly affected probands and their affected relatives.

2.2.3 Clinical phenotyping and inclusion criteria

All CH cases attending the headache clinic were diagnosed in accordance with the diagnostic criteria as outlined by the classification committee of The International Headache Society. Depending on the timing of recruitment, the most recent diagnostic criteria was used for the study. Earlier cases were diagnosed using the 2004 International Classification of Headache Disorders, 2nd Edition (ICHD II)[352]. Participants recruited between 2013 and 2018 were diagnosed using the International Classification of Headache Disorders-3beta edition (ICHD-3β)[124]. From 2018 the third edition of the International Classification of Headache Disorders (ICHD-3) was used [353]. Recruited cases were reviewed longitudinally at outpatient follow-up throughout this period, allowing for diagnostic confirmation.

All of the participants I recruited for this study were diagnosed using ICHD-3β or ICHD3. The main difference between these criterion is the required remission period for a diagnosis of CCH which was revised from less than one month (ICHD-3β) to less than three months (ICHD3). Patients are now characterised as having either ECH (attacks separated by a pain-free duration of ≥3 months) or CCH (remission period lasting <3 months). The diagnostic criteria used in each study is outlined in their respective methodology sections. In cases of diagnostic uncertainty, where one of the diagnostic criteria was not met, the consensus of two independent neurologists was required to establish a diagnosis of probable CH. In cases where clinical features overlapped with paroxysmal hemicranias and an optimum indomethacin trial had not been carried out (or was contraindicated), they were defined as probable TAC and excluded from the analysis.

The data collected included demographics, type of CH, laterality, site of pain, features of attacks including frequency, duration, and severity, quality of pain, and associated symptoms such as autonomic and migrainous features.

2.2.4 Concurrent primary headache disorders

The presence of a co-existing headache disorder was diagnosed using the ICHD3β/ ICHD3 criteria (depending of time of recruitment). Of note, patients diagnosed with SUNCT (Short-lasting unilateral neuralgiform headache attacks with conjunctival injection and tearing) in section 3 and 4 of this thesis fulfilled the criteria as outlined below. (**Table 3**)

Table 3: ICHD-3β diagnostic criteria for SUNCT

A	At least 20 attacks fulfilling criteria B–D
B	Moderate or severe unilateral head pain, with orbital, supraorbital, temporal and/or other trigeminal distribution, lasting for 1–600 seconds and occurring as single stabs, series of stabs or in a saw-tooth pattern
C	At least one of the following five cranial autonomic symptoms or signs, ipsilateral to the pain: Conjunctival injection and/or lacrimation Nasal congestion and/or rhinorrhoea Eyelid oedema Forehead and facial sweating Forehead and facial flushing Sensation of fullness in the ear Miosis and/or ptosis
D	Occurring with a frequency of at least one a day.
E	Not better accounted for by another ICHD-3 diagnosis.

2.2.5 Treatment response

To evaluate the response to acute and preventive treatments, we used a modified definition of intractable CH based on the consensus statements and expert opinions published in the literature (see **Table 4**) [354, 355]. A satisfactory response to a trial of treatment was defined as a ≥50% reduction in mean attack frequency for preventive medication and as a ≥50% reduction in pain ≥50% of the time for acute medication [96]. Participants were classified as indeterminate in cases of insufficient data (less than four trials of preventive treatment or lack of either sumatriptan injection or high flow oxygen as acute treatment). Response to treatment was determined based on the last available clinical letter.

Table 4: Definitions of intractable response to treatments (modified from European Headache Federation consensus guideline and Goadsby et al) [354, 355]

Adequate trial performed - Appropriate dose: Decision left to the clinical physician - Appropriate length of time: At least 1 month for melatonin trial At least 3 months for all other preventive therapeutics
Failed trial - Unsatisfactory response: Less than 50% reduction in mean attack frequency for preventive treatment Less than 50% reduction in pain at least 50% of the time for acute treatment - Side-effects requiring cessation of treatment - Contraindications to use
Intractable to acute treatment - Failure to respond within 15 minutes of subcutaneous sumatriptan use and - Failure within 30 minutes of high dose and flow rate oxygen use
Intractable to preventive treatment: - Failure of at least 4 classes among Verapamil Lithium Topiramate Gabapentin Methysergide Melatonin

2.2.6 Confirmation of family history

A family history of CH was initially recorded by proxy from probands. Affected relatives who were available underwent a phone or in-person interview with a neurologist to confirm a diagnosis of CH. Deceased relatives, or those unavailable for interview, were included if they had evidence of a previous CH diagnosis by a neurologist in their clinical notes.

2.2.7 Neuroimaging techniques

MRI brain or MRI with dedicated pituitary views were conducted as part of patient's

routine assessment on either a 1.5 (Siemens Avanto, Ernlagen) or 3 Tesla (Siemens Trio, Ernlagen) MRI scanner. A specified protocol for pituitary imaging involving 3 mm thick T1- and T2-weighted coronal and sagittal sections of the sella region was carried out on all cases included in the study. If the pituitary gland was not clearly visualised on the pre-contrast views, further coronal and sagittal high-resolution 3 mm sequences were utilised [356]. These sequences were performed with a small field of view (FOV) focus on the sella.

The T1 sequence with coronal and sagittal views was useful to show anatomical details, particularly for the posterior pituitary bright spot. T2 coronal or sagittal views enabled visualisation of the diaphragm sella, arterial flow-voids, and where present, identified haemorrhage or the pathognomonic 'dot sign' of rathke cleft cyst. Small T1 dynamic coronal FOV were used at multiple time-points to aid the identification of microadenomas which typically show delayed enhancement. In the presence of a lesion, the tumour was measured and any evidence of cavernous sinus invasion was recorded. Maximum tumour diameter was calculated and used to categorise tumours as either macroadenoma (> 10 millimeters) or microadenoma (<10 millimeters) [357].

In the case of macroadenomas, signal change indicative of haemorrhage, necrosis, cystic transformation or calcification was evaluated and further investigated with susceptibility weighted imaging (SWI) or CT where appropriate. Herniation of the subarachnoid space in the sella turcica was indicative of primary empty sella or an arachnoidocele. This was demonstrated by a sella filled with CSF and transversed by the infundibulum (infundibulum sign). It was categorised as a partial empty sella if less than 50% of the space was filled with CSF [358, 359]. Cystic formations, which also demonstrate the infundibulum sign, were classified as either rathke's cleft cyst, arachnoid cysts, pars intermedia cyst or as anterior pituitary cyst.

2.2.8 Systematic Review

To provide an accurate estimate of the rate of familial CH, I conducted a systematic review of all available publications reporting a family history of CH. At inception, the review was registered with PROSPERO, the International Prospective Register of Systematic Reviews (registration number CRD42019157309) to ensure transparency and avoid duplication. The aim of a systematic review is to provide an accurate and reliable summary of accumulative evidence. Methodological weaknesses and inconsistent reporting can diminish their value, therefore a structured and transparent approach is recommended[360]. With this in mind, I conducted the systematic review in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses protocols (PRISMA-P) 2015 guidelines[361]. This consists of a checklist including 27 items and four-point flow diagram.

A comprehensive search strategy was devised involving the interrogation of the following electronic databases: MEDLINE, PUBMED, EMBASE, CINAHL. A pre-specified search criteria was implemented under the guidance of a professional librarian search specialist. A combination of free text terms and medical subject headings (MeSH) were employed to improve the sensitivity of the search. Search terms for family history were combined with CH search terms using the "AND" function to identify relevant publications. As an extra step, references of all included publications were screened to identify additional articles known to the authors. The details of the search criteria are shown in the appendix (Section III, 3.3.3a). All titles and abstracts were independently extracted and reviewed by two researchers (EOC and BS). The pre-defined eligibility criteria, further elaborated on in section 3.3.3a, was used to select publications for inclusion. There were no additional restrictions in terms of date of publication, study size, geographical location or language. Full texts were

obtained for abstracts meeting inclusion criteria. Again, these were reviewed by two independent researchers (EOC and BS), and a third reviewer (JV) intervened to reach consensus if there was disagreement. The results were reported in accordance with PRISMA guidelines.

Data extracted included: (i) study design (ii) publication date (iii) ethnicity of population studied (iv) protocol for data acquisition (v) diagnostic criteria used (vi) sample size (vii) male to female ratio (viii) proportion of cohort with a positive family history (ix) male to female ratio of patients with familial CH. Data was extracted by two researchers (EOC and BS) independently and identified discrepancies were corrected by a third researcher (JV)

Eligible studies were analysed independently to diminish the risk of bias and to assess their quality. To achieve this we utilised a modified Newcastle – Ottawa scale (NOS) quality assessment tool. The NOS was originally created specifically to interrogate the quality of nonrandomized comparative studies prior to inclusion in meta-analysis [362]. The scale consists of eight items across three major domains including (i) Selection of study groups to ensure appropriate representation (ii) Comparability of the groups (iii) Ascertainment of outcome under examination. Each of the outcome categories were assigned a star rating whereby good quality was assumed when a score of greater than six stars was reached [362]. Any publication not achieving this quality standard was omitted from the meta-analysis.

2.2.9 Meta-analysis

The function of a meta-analysis is to statistically synthesize the quantitative results from several studies investigating a corresponding research question. In this study, this was achieved through the observation of proportions, whereby proportion

represents the amount of cases with a family history of CH divided by sample size [363]. Meta-analysis of these proportions produced a weighted average proportion derived from a one-dimensional binomial measurement using a random-effects model [364, 365]. This method of meta-analysis has shown superior precision over other techniques which concentrate on measurements of effect size such as odds ratio, mean difference or relative risk [366, 367].

This meta-analysis was conducted using the 'meta' and 'metafor' packages in the R statistical environment (version 3.6.1, 2019-07-05) and the R code used is included in the appendix of this thesis (Section III, 3.3.3c)[368]. In order to improve statistical variance and distribution of proportional data, transformations (logit and double-arcsine) were applied in accordance with the protocol outlined by Wang[369].

Overall, data was extracted from seven eligible studies and formatted in Microsoft Excel whereby each column represented a mandatory variable required to calculate effect and formulate plots. The data was then imported to R as a csv. Transformations were applied to raw proportions to create normal distribution across the selected studies to improve the validity of the test. Proportions were performed using a framework with two distinct approaches: the logit and the Freeman-Tukey double arcsine[370, 371]. The raw/direct proportions were calculated and the distribution of untransformed, logit and double-arcsine transformed proportions were analysed. Density plots were used to inspect the distributions of the proportions for normality using and tested using the Shapiro-Wilk test. As the Logit-transformed proportions were more consistent with normal distribution, this transformation was used for the analysis. This allowed for the calculation of a pooled effect size and corresponding sample variance.

To detect between-study variability, resulting from the different populations and protocols used, heterogeneity was quantified using the I^2 statistic. This is the ratio of between-study variance to the observed variance (i.e., the sum of between- and within-study variance). Due to high inter-study variation as denoted by a high I^2, a random-effects model was selected for estimation of family history in CH. Leave-one-out analyses (LOO) was then conducted whereby each study excluded one by one and the summary proportion is re-calculated based on the remaining n-1 studies. This analysis enables an assessment of the influence of each study on summary proportion. Accompanying diagnostic plots used to pinpoint influential studies included: externally studentized residuals, difference in fits values (DFFITS), Cook's distances, covariance ratios, LOO estimates of the amount of heterogeneity, LOO values of the test statistics for heterogeneity, hat values and weights. Studies with a statistically significant influence on the fitted model were identified as outliers and excluded and the model was re-fitted.

A gender-segregated analysis was then conducted on all studies. For this analysis, within-group estimates of τ^2 were not pooled due to the lack of a common between-study variance component across both genders. We initially fitted a mixed-effects model involving a calculation of all summary effect sizes using the τ^2 within each subgroup and then fitted two separate random effects models. Estimated statistics from each model were then combined and fitted with a fixed-effect model.

2.3 Genetic Studies

2.3.1 Ethical Approval and Data protection

Ethical approval was obtained as part of a substantial amendment to the ethical protocol (REC 07/Q0512/26 amendment #9) of an existent study (Investigation of human neurological ion channel or episodic neurological disorders, 07/N018). This included approval for an additional five headache specific recruitment sites and the development of a study website. Statements of Activities (SOA) and a completed Health Research Authority (HRA) Schedule of Events (SOE), accompanied the submission to the Integrated Research Application System (IRAS) for HRA Approval. All samples were collected in accordance with General Medical Council (GMC) guidelines for Good Medical Practise and the Helsinki Declaration[372, 373].

In accordance with GDPR guidelines. All data collected was de-identified and stored on an encrypted database in a clinical environment. Each site was equipped with a site file which included a delegation log with attached qualifications and Good Clinical Practise (GCP) certificates of all individuals involved in recruitment. A screening and enrolment and a sample collection log was established at each individual site. Standard operation procedures (SOPs) were outlined and overseen by local prinicipal investigators (PI).

2.3.2 Recruitment Strategy

In order to conduct a sufficiently powered GWAS, a large homogeneous cohort of CH cases is required. A number of CH features impede this process. Firstly, CH is a relatively rare disorder with a frequency of occurrence in approximately 1 in 1,000 people. Therefore, to recruit the required number of cases for GWAS, collaboration and multi-centre recruitment over a prolonged period is necessary.

Secondly, CH is clinical diagnosis, lacking a diagnostic test or biomarker. While phenotypically distinct, overlapping symptoms with other primary headache disorders can lead to misdiagnosis. The misclassification of cases presents a potential confounder and is entirely dependent on adherence with diagnostic criteria and the skill of phenotyping by the assessing neurologist.

Recruitment for the GWAS occurred from 2007 to 2018. It began at the NHNN as part of a larger study entitled ‘Investigation of human neurological ion channel or episodic neurological disorder’. I developed an additional five sites across the UK through collaboration with local headache specialists to enhance recruitment (**Figure 8**).

Figure 8: *CH recruitment sites across the UK*

Each site was required to provide a research and development (R&D) capacity & capability letter and I liaised with site PIs to oversee the signing of contracts between the sponsor and local committees at individual sites. I obtained an honorary contract at each site and worked in collaboration with consultant neurologists to establish a recruitment team which included myself, headache specialists nurses and clinical fellows.

2.3.3 Patient and Public Involvement (PPI)

To augment recruitment, I developed a departmental website www.neurogenetics.co.uk using wordpress **(Figure 9)**

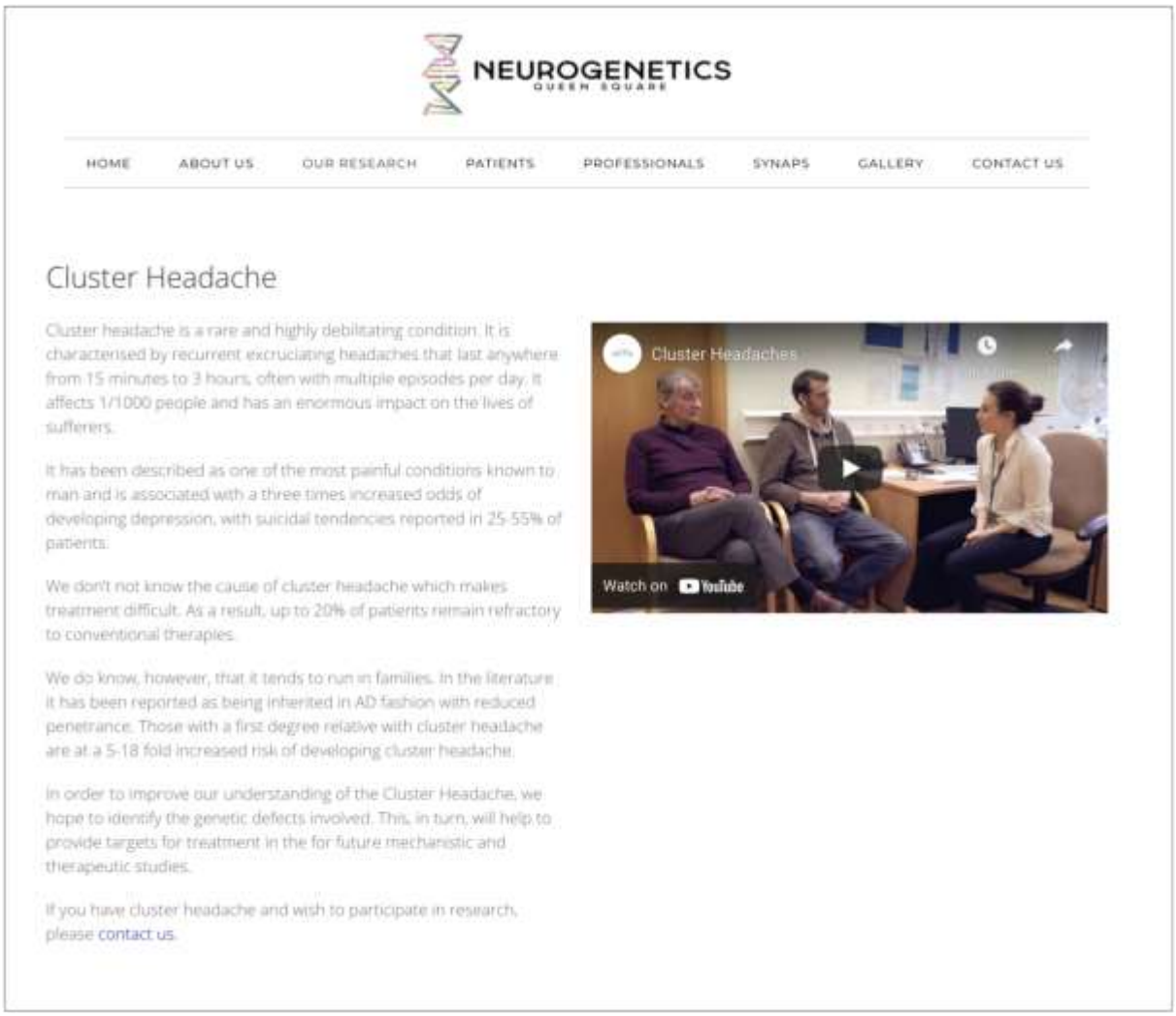


Figure 9 *www.neurogenetics.co.uk*

Through collaboration with colleagues and patients, I curated patient-focused content to promote neurogenetic research and advertise the study. This enabled patients to register interest for the study; these individuals could then be approached for screening. Finally, I advertised the study through OUCH UK a CH support group, via their website and by giving talks at meetings, to facilitate the participation of additional individuals.

2.3.4 Phenotyping of cases

Patients recruited prior to 2016 were phenotyped by the headache team at the NHNN. I subsequently reviewed the clinical notes of these patients to ensure they were strictly adherent to the ICHD3β and collected additional data as detailed below. Where necessary, patients were contacted and interviewed over the phone or reviewed in clinic if there was any ambiguity around their diagnosis or if required information (ethnicity, smoking status etc) was missing.

All external enrolling sites were headache clinics lead by specialists in the field. At these sites, patients were phenotyped by a specialist neurologist before I screened them for suitability prior to inclusion. Only patients who strictly fulfilled the ICHD-3β diagnostic criteria, were included [374]. Two independent neurologists were required to establish a diagnosis where one of the criteria of ICHD-3β were not met, and a consensus was required prior to inclusion. Additional information collected on each participant included ethnicity, smoking status, family history of CH, age of onset and temporality of attacks (ECH or CCH).

2.3.5 Sample Collection

Based on patient preference, either whole blood or saliva were collected from all participants for DNA extraction. All resources, including EDTA bottles and Oragene

saliva kits were provided to the sites and samples were transferred to the Institute of Neurology (ION), Queensquare, for extraction. All participants provided informed consent for analysis of their DNA and access to their clinical records for research purposes. If patients were unable to travel to a recruitment site, they were contacted by phone and received postal consents, that were later returned accompanied by a saliva sample in a pre-posted envelope.

2.3.6 Control Data

Control genotype data was obtained from the Wellcome Trust Case Control Consortium (WTCCC) through a WTCCC Data Access Agreement. Controls consisting of the 1958 birth cohort and NBS National Blood Survey cohort (NBS), the details of which are outlined elsewhere [375]. An additional cohort of UK controls who did not report a history of headache were also included as detailed in Section 4.1.

2.3.7 Familial Cluster Headache Study

All patients with a positive family history of CH were recruited to this arm of the study. Participants were recruited in a similar manner to the GWAS study above. Only participants with an affected relative available for interview and confirmation of diagnosis were included. Blood samples were provided by affected individuals with informed consent. Where available, additional affected individuals within families were consented and provided whole blood for DNA extraction. DNA was also collected from unaffected family members where possible for segregation analysis. Due to the rarity of familial CH, additional families were recruited through international collaboration. The sites involved in this collaboration are listed in **Table 5.**

Table 5: Collaborators on familial CH WES study

Origin	Clinical Centre	Collaborator	Study Group
UK	NHNN	Houlden	Synaptopathies
Leiden, The Netherlands	Leiden University Medical Centre	Prof Arn MJM van den Maagdeberg	LUCA
Stockholm, Sweden	Karolinska University Hospital	Prof Christina Sjöstrand	N/A
Copenhagen, Denmark.	Rigshospitalet-Glostrup, University of Copenhagen	Prof Rigmor Jensen	N/A
UK, London	Kings College London	Dr. Laura Southgate and Prof Richard Trembath	N/A
Belgium, Ghent	Ghent University Hospital	Prof Koen Paemeleire	N/A
Greece	Glyfada Headache Centre	Dr Michael Vikelis	N/A

2.3.8 Laboratory Methods

2.3.8 (a) DNA extraction

DNA was extracted from either saliva or peripheral blood based on the participants sampling preference. Extraction from peripheral blood was conducted by the neurogenetics laboratory at the NHNN. Genomic DNA (gDNA) was extracted from 5-10mls of whole blood samples using the FlexiGene (Qiagen) kit in accordance with the instructions, as provided by the manufacturer. This involved the addition of lysis buffer to each sample, centrifugation to isolate cell nuclei and mitochondria, and then resuspension in a protease enriched denaturation buffer. DNA then underwent precipitation in isopropanol and was washed with 70% ethanol.

Saliva samples were collected using OG-600 Oragene DNA saliva kits. A proportion of samples were sent to LGC bioresearch technologies (Germany, www.bioresearchtech.com) for extraction and I extracted the remaining samples using the sbeadex livestock kit (protocol J). Sbeadex kits use superparamagnetic microparticles in a two-step binding mechanism to bind and purify nucleic acids. Combined with the washing steps, this unique process removes impurities and potential inhibitors of enzymatic reactions very effectively. I used the NanoDrop ND-1000 spectrophotometer to quantify and assess the quality of DNA extracted. The concentration was estimated at 260nm and 260/280 and 260&230 absorbance ratios were used to assess purity. I diluted the samples to the required concentration specific to the technique being employed for analysis using autoclaved distilled H2O. For WES, DNA concentration was measured using the Qubit 2.0 flurorometer (Thermo Fisher Scientific). This technique measures the concentration through the detection of the amount of double stranded DNA bound to a fluorescent dye. This technique provides a more accurate measurement of concentration as the flurorescent dye used does not bind to degraded DNA or other proteins.

2.3.8 (b) Genotyping

DNA from CH cases were genotyped on the Ilumina Infinium Global Screen Array (GSA, http://www.glimdna.org/assets/2017-infinium-global-screening-array-illumina-data-sheet.pdf).

I prepared the samples to provide DNA concentrations of 200ng in 4ul, in 96 well abgene storage plates (thermofischer) and sent them to the Human Genomics Facility (HuGe-F) Erasmus Medical Centre (www.glimdna.org) for genotyping.

Genotyping involves the whole genome amplification of gDNA and enzymatic fragmentation to provide approximately 300bp fragments. DNA fragments then undergo purification and hybridization on Illumina Beadchips, which consist of microbeads with attached SNP-specific oligonucleotide capture probes. Hybridized fragments undergo labelling with allele specific antibodies and are imaged with a two-confocal laser and projected on an Illumina BeadArray reader. Genotypes are then called based on the colour signal emitted. The GSA array contains over 700,000 update SNPs with 50,000 customised SNPs to highlight neurological and pharmacogenetic markers.

2.3.8 (c) Whole exome sequencing (WES)

WES was used in CH familial studies to fine map regions suggestive of linkage and for segregation analysis. I prepared all samples for WES which involved the dilution of DNA to 20ng/µl or 50ng/µl with TE buffer or autoclaved distilled H20 (Dh20). The prepared samples were then transferred to Macrogen (https://www.macrogen.com/) for processing.

In brief, WES was generated using a protocol for 100-bp /150-bp paired-end reads and analysed on one of three platforms; illumine HiSeq 4000, Novaseq or Hiseq X. The human reference genome USC hg19 was used to align sequence reads. Preparation of samples was conducted in accordance with the Agilent SureSelect Target Enrichment system version 6 (**Figure 10**), to pick up coding regions, capturing them from NGS genomic fragment library.

Library preparation involves the shearing of each sample using a covaris sonicator to create fragments of double stranded DNA. Samples are then incubated with AMPure XP beads on a midi plate. Overhangs produced from shearing are converted to blunt

ends with the addition of ERP3 (End Repair Mix). This mix consists of 5’ to 3’ polymerase to fill the 5’ overhangs and 3’ to 5’ exonuclease to eliminate the 3’ overhangs. On completion of end repair AMPPure XP beads are used to select library size. Ligation between blunt fragments at the time of the adapter ligation reaction, is prevented by the addition of an ‘A’ nucleotide to the 3’ end of fragments. A complementary ‘T’ nucleotide attached to the 3’ end of the adapter gives a reciprocal overhang for the ligation of the fragment to the adapter. This method reduces the rate of chimera formation. For this reason, an A-tailing mix and buffer are added to each sample.

In preparation for hybridization on the flow cell, multiple indexing adapters are ligated to DNA fragment ends by ligate adapters. Samples can then be pooled together following the addition of ligation mix, buffer and unique adapter indexes. A stop ligation buffer is used to arrest the reaction following incubation of the samples with the adapter. The coverage of sequencing is proportional to the number of samples pooled together, whereby a higher number of samples will result in lower coverage. DNA fragments, now ligated with adapters at both ends of every molecule, are amplified using PCR in the process of enrichment. Adapters are annealed using PCR primer cocktail (PPC) to perform PCR.

Library quality control (QC) is achieved with a bioanalyszer (Agilent) and Qubit is used for library quantification. DNA libraries are then pooled and hybridized to enable capture probes to bind with exons and flanking regions. Coding exome oligos (CEX) are mixed with the library pool and a capture buffer before being inserted into a thermal cycler. Probes hybridized to exons and flanking regions are captured using streptavidin magnetic beads. Non-specific binding is eliminated with two heated washes. The

eluted exon enriched library then undergoes a second hybridization to enhance specificity of the captured regions prior to sequencing.

Sequencing is carried out on an illumina platform which enables processing of multiple DNA sequences in parallel. This is achieved through the combination of the oligo-primed fragments with nucleotides and DNA polymerase in the flow-cell channels which is then linearized and denatured to create a single strand. Nucleotides are then tagged with a fluorescent label and the 3'-OH is blocked so that each base incorporated is observed. Imaging of the flow cell lane is carried out after the incorporation of each base. The 3' block is then removed to accommodate the incorporation of another nucleotide by DNA polymerase. This cycle is repeated and the same process is conducted on both DNA strand ends to produce paired end reads. Data de-multiplexing is then carried out to produce files for alignment. These processes are summarized in **Figure 10**.

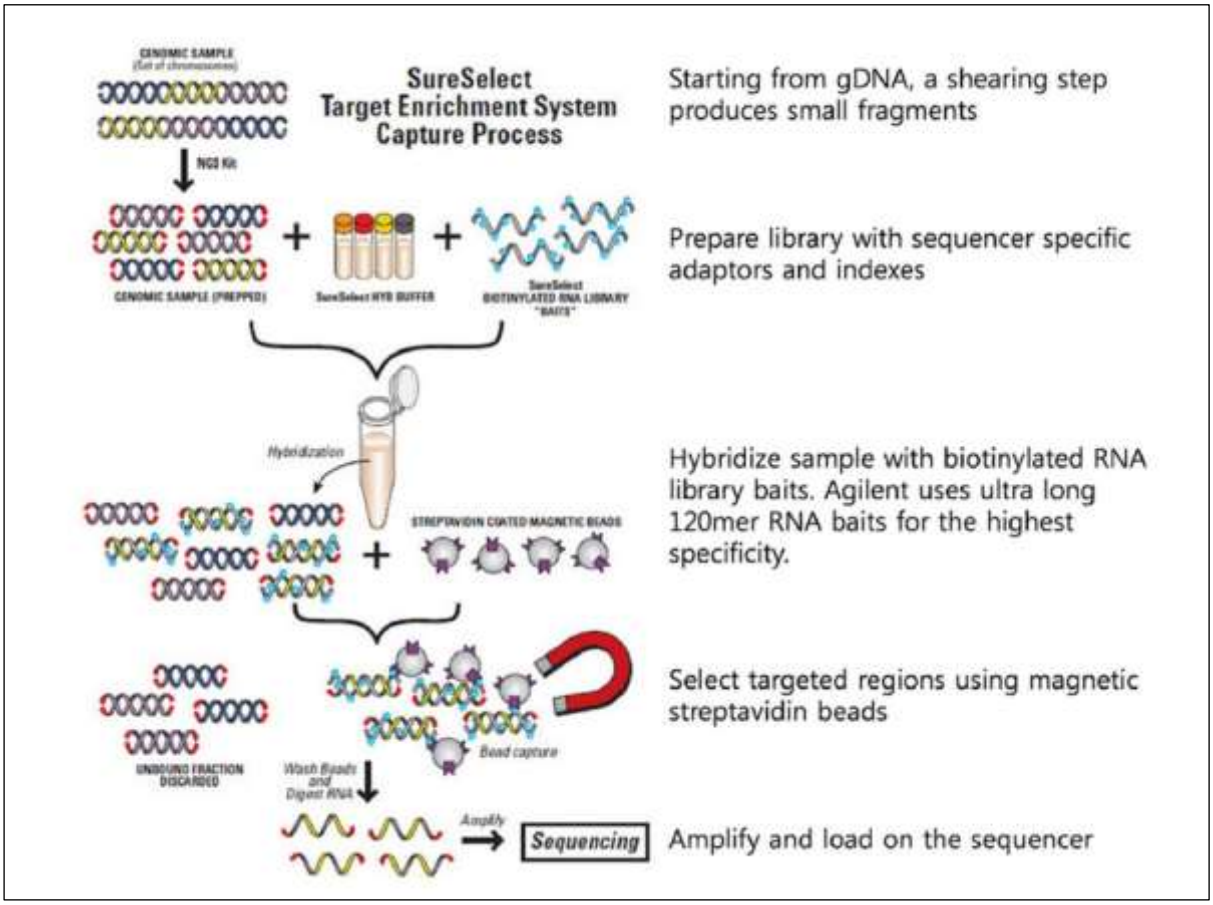


Figure 10: *DNA library preparation and target enrichment. (Figure reproduced from Macrogen; https://dna.macrogen.com)*

2.4 Bioinformatic Processes

2.4.1 Genome Wide Association Study

The protocol and commands used can be found in Appendix section IV , 4.1.5..

2.4.1 (a) Sample QC

The following QC steps were carried out prior to association analysis:

- Missingness : In order to remove variants or samples with excess missingness, variants with a genotype call rate <95% and individuals with a call rate of <98% were excluded using PLINK [376].

- Sex Check : The purpose of this step was to remove samples with ambiguous sex or inconsistent sex information, possibly indicating contamination or sample mishandling. This was demonstrated by calculating the X-chromosome and comparing it to the self-reported sex. This was achieved using the sex check function in PLINK [376] by inspecting the homozygosity of the X chromosome and associated F value whereby a value <0.2 indicates female sex >0.8 is male.
- Relatedness: This is performed to avoid cryptic relatedness and remove duplicated samples. Relatedness is estimated by calculating identity by descent (IBD) of all sample pairs. This is conducted on pruned SNPs of autosomal chromosomes only. Using PLINK [376], relatedness is estimated by calculating the pi-hat ratio (0.5 infers first-degree, a value of 0.25 second-degree etc). A threshold of >0.2 was used for my dataset.
- Heterozygosity: This is the proportion of heterozygous genotypes per sample. This step excludes individuals with outlying heterozygosity rates which may indicate contamination or inbreeding. All samples deviating five SD from the mean were excluded.
- Ethnicity: Population stratification presents a potential confounder which can lead to spurious associations. I conducted this in two steps. Firstly, Use the PCA command in PLINK [376] to run principal components analysis on the pruned dataset from the previous step and used the first 20 principal components to exclude outlying individuals. Secondly, I used Peddy [377] and excluded non-European samples following visual inspection.

2.4.1 (b) Variant QC

- Missingness of SNPs: In this step SNPs missing in a large proportion of participants (low genotype calls) are removed. Missingness was calculated with PLINK [376] and variants with a genotype call rate of <95% were removed.
- Deviation from the Hardy-Weinberg equilibrium: Cases and controls are interrogated separately at this step (10E-8 for cases, 10E-6 for controls). Deviation from HWE may indicate genotyping error or can indicate evolutionary selection.
- Minor allele frequency (MAF): SNPs were then filtered to include only those with a MAF of >0.01.

2.4.1 (c) Imputation

I used the Haplotype Reference Consortium (HRC v1.1) on the Michigan server for imputation [378]. Genotypes were encrypted during upload and download (SSL or SFTP) and the server carries out automatic checks for quality, phasing and imputing.

2.4.1 (d) Post-imputation QC

A list of monomorphic SNPs and pruned Variant calling format (VCF) files were generated using bcftools [379]. Variants with an info score < 0.4 or a MAF <0.01 were removed.

2.4.1 (e) Association testing

Association analysis was conducted using SAIGE, a tool for binary traits particularly robust for unbalanced case-control datasets and relatedness[380]. SAIGE was then ran in two steps. Firstly, it ran a null logistic mixed model to estimate variance component and other model parameters. The second step involved an association test between

each genetic variant and phenotypes by applying SPA to the score test statistics. The first four principal components were used as covariates.

2.4.2 Linkage Analysis

This method localises the area in the genome within which causal mutations are likely to lie and is particularly useful to identify genetic candidates for mendelian disorders. It is a statistical method which correlates phenotype with a chromosomal region based on the principle that variants, or genetic markers, in close proximity on a chromosome are likely to be inherited together during meiosis. The distance between genetic markers will be greater the higher the number of recombination events. In contrast, a lower number of recombinations will reduce the distance between two markers. Thus, the location of a causal variant can be identified by uncovering regions that tend not to recombine in the affected members of a pedigree. Therefore, to achieve meaningful results, a monogenic inheritance pattern and accurate phenotyping of affected and unaffected individuals is essential.

Linkage analysis can be parametric or non-parametric. A parametric approach is used when the pattern of transmission of the disorder is known. Alternatively, a non-parametric (model-free) analysis is conducted, using identity by descent (IBD) if the pattern of inheritance is unknown. The recombination fraction (θ) refers to the number of recombinant offspring divided by the sum of the non-recombinant and recombinant offspring. It can be interpreted as a measure of distance between loci ranging from 0 for loci that are adjacent to 0.5 for distant loci. A Logarithm of Odds (LOD) score is a statistical test which calculates the probability of two loci (or a genetic marker) and a disease marker being linked compared with the probability of it happening by chance. By this calculation, an LOD score of >3 is considered significant evidence for linkage, a score between -2 and 3 is inconclusive and a score below -2 excludes linkage. To

allow for locus heterogeneity, the heterogeneity LOD score (HLOD) is also calculated which is independent of the trait model parameters.

All samples for linkage analysis were genotyped using the Illumina GSA array (see above). Quality control was conducted using PLINK[376]. SNPs with a call rate of <90% and monomorphic SNPS were excluded using the --geno 0.1 and --maf 0.05 commands. After variant pruning, equally distributed SNPs across the genome (1cM intervals) were identified using the mapthin software[381]. MERLIN software was used to perform linkage analysis [382]. All files (.ped .dat .map .model) were created as per instructions. The affected status (0=unknown, 1=unaffected, 2=affected), sex (1=male, 2= female) and pedigree structure of each individual was specified in the .ped file. The .map file outlined the positions of genetic markers measured in centimorgans (cM). The .model file indicates the model being used, penetrance and disease allele frequency. Input files and pedigrees were validated using PEDSTATS[383] and the function:

> pedstats –d file.dat –p file.ped.

The pedwipe tool was used to clean genotyping errors detected in pedstats output using the following commands:

>merlin –d dat.txt –p ped.txt –m map.txt --error

>pedwipe –d dat.txt –p ped.txt

2.4.2 (a) Parametric Linkage analysis

Using MERLIN[382] , I then performed genome-wide parametric linkage analysis using:

>merlin –d file.dat –p file.ped –m file.map –model file.model –markerNames --pdf –tabulate

Haplotype analysis using MERLIN with the command:

>merlin -d haplo.dat -p haplo.ped -m haplo.map –-best –-horizontal

2.4.2 (b) Non-parametric analysis

merlin -d file.dat -p file.ped -m file.map --tabulate --pdf --markerNames --npl —exp

2.4.3 Bioinformatic processing of WES data

Bioinformatic processing of raw data was carried out by the Bioinformatics team at UCL (Dr. Jana Vandrovcova, David Murphy and Hallgeir Jonvik). Sequence reads with respective quality scores were generated, annotated and aligned to the reference genome using software such as bwa-0.7.12,GATKv3.40 or SnpEff as part of a standardised bioinformatics pipeline. Novoalign (Novocraft technologies) was used to align paired-end sequencing reads from FASTQ files. SAMtools[379] was used to create a BAM file. Duplicate reads were eliminated and statistics were generated using picard tools[384]. Variant calling and realignment of indels was achieved using the Genome Analysis Toolkit (GATK)[385]. ANNOVAR[386] was used for variant annotation.

2.4.4 Variant Prioritization

Following bioinformatics processing of WES data, a final list of called variants is generated in a VCF file. Up to 25,000 variants can be generated from WES and in monogenic conditions with autosomal dominant inheritance, as often observed in CH families, the phenotype can be caused by a single variant. For this reason, there is a requirement to develop a filtering method to exclude variants that are unlikely to contribute to the phenotype and identify potentially pathogenic mutations. I followed the following prioritization pipeline to identify variants of interest:

2.4.4 (a) Quality Control (QC)

Initially, false positive/artefactual variants are filtered out based on a number of quality measurements including phred scores (base quality scores), segmental duplication scores (to identify false positive calls), and depth (DP, number of reads in a region).

2.4.5 (b) Frequency

Variants are then interrogated based on their frequency in publically available population databases such as gnomAD, EXAC and 1000 Genome project [387, 388]. Common variants observed frequently in the general population are unlikely to have a large enough effect size to cause mendelian conditions. Considering CH has a frequency of approximately 1 in 1,000, it would be expected that causative variants have a low frequency in population databases. Therefore, variants that were common in the general population were excluded from downstream analyses, retaining only variants with frequency of < 1% on gnomAD[388]. Variants with a frequency >1% in the Institute of Neurology in-house dataset which contains WES of approximately 10,000 individuals with neurological conditions were also excluded.

2.4.5 (c) Segregation

Variants are prioritized based on the mode of inheritance as indicated in pedigrees. In CH, the most frequently postulated pattern of inheritance is autosomal dominant with reduced penetrance. Heterozygous variants were therefore selected out in these families. I then filtered for variants that segregated in families in two or more affected individuals.

2.4.5 (d) Site / Predicted impact of mutation

Variants are also categorised based on their location. It is presumed that the majority of disease-causing variants are located in exons, therefore, I focused on mutations

occurring in coding regions including exons and splice-sites. Mutations were also included based on their estimated impact (**Figure 1 A&B**) prioritising insertions, deletions, non-synonymous missense, stop lost, stop gained, splice donor or acceptor and frame-shift mutations.

2.4.5 (e) Expression

Variants can then be further narrowed down by expression pattern, prioritising only genes that are expressed in brain according to Genotype Tissue Expression Consortium database [389].

2.4.5 (f) Predicted Pathogenecity

The pathogenicity of identified variants was estimated using in-silico pathogenicity software. Examples include; mutation tester [390] which is useful for indels, SIFT [391] and Polyphen-2 [392] which evaluate non-synonymous missense mutations and the CADD score [393] which also covers non-coding regions. Tolerance to variation can also be indicated by probability scores provided by ExAC and accessed through gnomAD. A z-score of >2 indicates intolerance to missense mutations and pLI score >0.8 implies intolerance to loss of function mutations.

2.4.5 (g) Conservation

The Genetic Evolutionary Rate Profiling (GERP^{++} score)[394] uses evolutionary conservation to evaluate variants. It compares conservation of an amino acid across several species to estimates the impact of missense variants. Conservation across species implies the variation in this residue is likely to be pathogenic which is indicated by a GERP^{++} score of >2.

2.4.6 Rare Variant Association Study (RVAS)

In contrast with GWAS, rare-variant studies enable the detection of variants with a greater effect size and impact on phenotype. The use of NGS, in this case WES, in such studies has the potential to provide direct insights into the genes and the underlying pathways and biology of a condition. It is well established that mendelian conditions are often caused by rare, highly penetrant variants that impair reproductive fitness [395]. However, the exact contribution of rare variants to common and complex conditions requires rare-variant analytical approaches that detect genes with an excess of rare variants in cases versus controls. Prior to conducting association testing, quality control steps were carried to avoid systemic biases.

2.4.6 (a) Data Processing and Quality Control

Following initial processing of raw WES data (by UCL bioinformatics team), all samples were processed under a similar workflow (**Figure 11**). A copy of the script used can be found in appendix (Section IV, 4.4.4a). GVCF files for all samples in each cohort were merged using Genomics DBI Import and variants were called using the Genotype GVCF tool (GATKv4.1)[396]. After GATK VSQR variant recalibration merged VCF files were normalised using bcftools v1.10[379]. Finally, multi-allelic variants were removed.

The quality control processes are summarized in **Figure 11**.

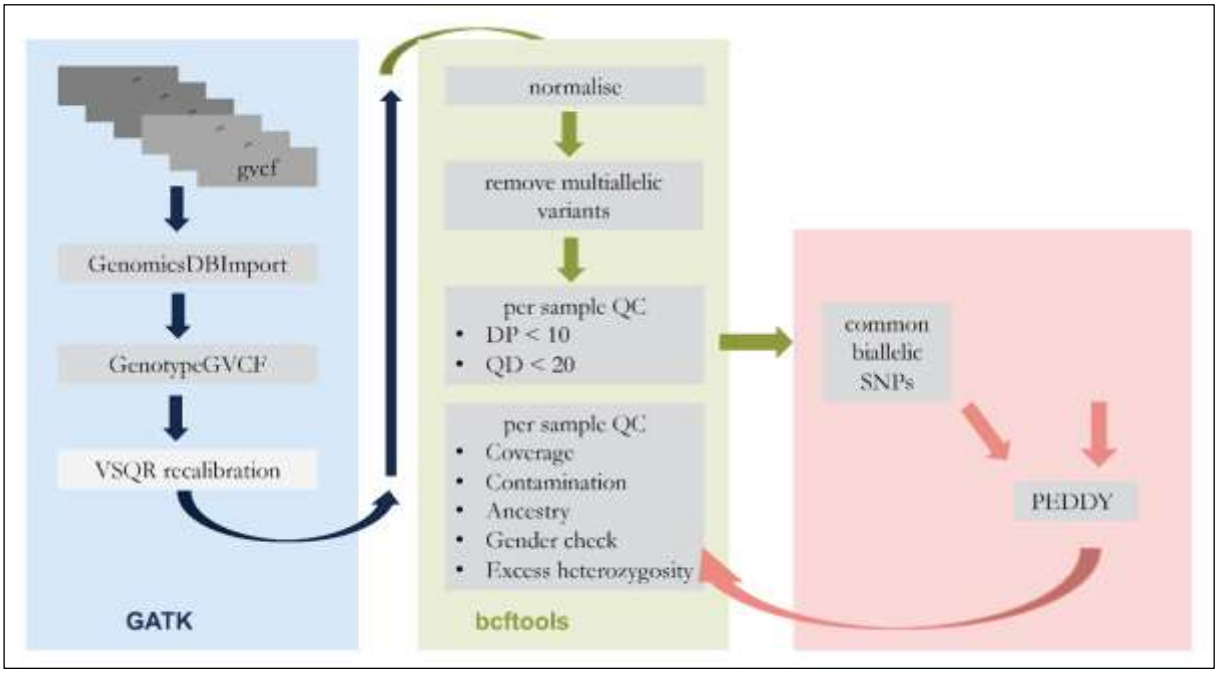


Figure 11 *Schematic outlining data processing and quality control steps including pre-QC filters and annotations, genotype level QC, variant level QC and sample level QC.*

2.4.6.(b) Genotype QC

Genotype-level quality metrics generated by GATK were assessed to remove low quality per sample genotype calls. All genotypes with DP of < 10 and quality by depth (QD) < 20 were set to missing. Samples with low coverage, high contamination, low Transition (Ti) to Transversion (Tv) ratio were then removed.

2.4.6 (c) Variant QC

F-miss was calculated to identify samples with a high degree of missingness. Variants were annotated based on call rate, Hardy–Weinberg equilibrium (HWE), allele frequency, and filter all positions with a genotyping rate < 95% and/or minor allele frequency > 0.01.

2.4.6 (d) Sample QC

In this step, non-european ancestry, incorrect gender, or samples with excess heterozygosity were removed. Per sample quality control was performed using a combination of PLINK/SEQ and Peddy [376, 377] .

2.4.6 (e) Rare –Variant Association Testing

There are several approaches to rare variant association studies. Here, I outline some of the most commonly used methods.

2.4.6 (i) Single – Variant association testing

This involves conducting a univariate test at every rare variant and estimating its significance using an appropriate threshold of significance as defined by p-value, while allowing for multiple testing [397]. In our analysis, we used Fisher's exact test and controlled for multiple testing using the false discovery rate (FDR) which is defined as the expected proportion of incorrect rejections of the null hypothesis [398]. This is the standard approach for GWAS but lacks power to detect rare variants with similar effect sizes in rare variant studies [397]. Single variant tests can however be useful if the sample size is sufficiently large with variants are not very rare but have a large effect size.

2.4.6 (ii) Burden tests

Burden tests collapse information on genetic variants within a defined region to generate a score based on the number of minor alleles of each variant within that group [399, 400]. There are a number of configurations developed to calculate this score. The MZ test calculates burden based on the number of rare variants for which an individual has at least one minor allele [401]. Similarly, the cohort allelic sums test (CAST) uses the assumption that the presence of any rare variant increases disease risk [402]. This score is then compared between cases and controls to examine for an association between

the score and the trait. The combined multivariate and collapsing test (CMC) is an extension of CAST and provides a combination test to include both common variants and sets of rare variants [403]. The weighted-sum test (WSC), also an extension of CAST, assigns weight to rarer variants based on the assumption that they have a larger effect on disease [404].

Burden tests are based on the assumption all rare variants are causal and acting in the same direction. When this assumption is violated there is a signficant reduction in power [405]. Therefore, while useful when a large fraction of variants are acting in the same direction, the utility of burden tests decreases with the presence of trait-increasing and trait-decreasing variants [402, 404-406]. Several adaptive extensions have been proposed to resolve this issue. The data-adaptive sum test (aSum) predicts the effect direction of variants prior to conducting the burden test integrating estimated effect direction [407]. The estimated regression coefficient test (EREC) overcomes this by assigning a weight based on the regression coefficieint of each variant [400]. The Kernal-based adaptive cluster (KBAC) uses adaptive weighting, combining variant calssification of both non risk and risk variants [408]. Whilst these adaptive extensions overcome the limitations of classic butden tests, they often require two step procedures and are computationally intensive [409].

2.4.6 (iii) Variance component tests

This model groups together variants with the same effect according to their allele frequency in cases and controls, thus providing a robust test when both protective and risk variants are present [410]. Included under this model are the C-alpha test, the sequence kernal association test (SKAT), and sum of squared score (SSU) test [405, 411, 412]. This approach is more useful when there are both trait-increasing and trait-decreasing variants present but becomes less effective when a large proportion of

variants act in the same direction (e.g are all risk). Additionally, a small sample size or overall minor allele count can give rise to a type 1 error with this method, prompting additional adjustments and permutations [413].

2.4.6 (iv) Combination Analysis

This combines both burden and variance-component methods to give a robust anaysis in the presence of both trait-increasing and trait-decreasing variants. A number of strategies have been proposed to combine both, including Fisher's method and a modification of the SKAT method called SKATO [413, 414]. Unfortunately, a combined approach can also be slightly less powerful if the assumptions underpinning either component are largely true.

The underlying genetic architecture of CH is largely unknown, therefore, I conducted several premutations of the above techniques as part of my rare-variant association analysis using RVTests [415].

Section III: Clinical Studies

3.1 Pituitary MRI Screening in Cluster Headache

Statement of contribution: Data for this study was collected from the headache clinic at the National Hospital for Neurology and Neurosurgery (Queen Square, London, UK) over ten years by headache clinical nurse specialists and headache clinicians under the supervision of Dr. Manjit Matharu. My contribution included the organisation, analysis and interpretation of data in collaboration with Dr Lou Grangeon (visiting clinical fellow). Statistical analysis was conducted by Dr. Thanh Mai Pham Ngoc of the Mathematics Institute of Orsay, Paris Sud University. A version of this chapter has been published in Cephalalgia.(doi: 10.1177/0333102420983303.)

3.1.1 Introduction

Cases of pituitary disease mimicking CH have previously been reported in the literature [416-419]. Pituitary disease frequently presents with headache in approximately 33-72% of patients with pituitary tumours [420, 421]. A prospective study involving eighty-four individuals with pituitary tumours demonstrated CH occurred in 4%, potentially indicating an over-representation of CH in this patient group [422]. However, as this study was carried out in a tertiary referral neurosurgical centre, there may be a ascertainment bias. Other studies investigating pituitary adenomas in CH were limited by the small sample sizes [416-418]. Therefore, due to a lack of sufficiently powered neuroimaging studies, it remains unclear as to whether a higher prevalence of pituitary tumours occur in patients presenting with CH.

Consequently, the necessity for pituitary imaging in CH patients is a matter of debate. MRI of the pituitary is often recommended in CH with atypical features, symptoms of pituitary disease, abnormalities on neurological examination, or a poor treatment response [418]. Atypical features or so-called “red flags” include an older age of onset, prolonged duration and/or higher frequency of attacks, bilateral pain, and the absence of autonomic symptoms [416, 423, 424]. However, national guidelines such as the French headache society and Scottish Intercollegiate Guidelines Network (SIGN) guidelines advise pituitary imaging with MRI for all patients with CH [425, 426]. Unnecessary imaging can result in the erroneous attribution of CH to an incidental lesion. As incidental pituitary lesions are identified on MRI in up to 10% of the general population, this is a considerable risk [427-429].

3.1.2 Aims and Objectives

1. To examine the incidence of pituitary adenomas in CH patients and thus determine the requirement for routine dedicated pituitary MRI imaging.
2. To assess for clinical features that may be predictive of pituitary adenomas in CH patients.

3.1.3 Methods

3.1.3 (a) Cohort Collection

The recruitment strategy is detailed in the methodology section, subsection 2.2.2. In addition to data collection on demographics and clinical features of CH, each patient underwent a comprehensive endocrine clinical history and examination focusing on pituitary symptoms such as menstruation changes, galactorrhoea, hirsutism, erectile dysfunction, and acromegaly.

3.1.3 (b) Neuroimaging and pituitary assessment

The pituitary MRI protocol is described in methods section 2.2.7. In line with standard clinical practice, routine pituitary function tests were assessed including the following parameters: prolactin (PRL), growth hormone (GH), insulin-like growth factor-type I (IGF-I), cortisol, adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, oestrogen, and progesterone. All analyses were carried out in the laboratory at NHNN, London.

To assess the incidence of pituitary adenomas, we only included patients who underwent MRI with dedicated pituitary views. To identify the clinical predictors of pituitary adenomas, we divided the cohort into two groups, those with (cases) or without (controls) a pituitary adenoma.

3.1.3 (c) Statistics

Descriptive statistics were expressed as mean with standard deviation (SD) and median with interquartile range (IQR). Missing data was not replaced by imputation techniques. The two groups of patients were compared using a Mann-Whitney nonparametric test for quantitative data and a Fisher exact or chi-square test for qualitative data and the threshold for statistical significance was set to $p \leq 0.05$. For multivariate analysis, random forests were used. Random forests are an efficient, general-purpose approach for classification and regression[430]. The principle of random forests combines a multitude of binary randomized decision trees and aggregates their predictions by averaging.

In this study, after having split our genuine dataset into training and test sets, we used the R package VSURF based on random forests to perform variable selection[431].

Variable selection is a crucial issue in classification problems to remove irrelevant explanatory variables. To perform variable selection, VSURF ranked the explanatory variables according to the variable importance (VI) criterion in the random forest framework. At the end of the ranking phase, a threshold was computed. Only variables with VI exceeding this threshold were retained for the interpretation and prediction steps.

Weighted random forests were used to address dataset imbalances. This is a frequently utilised approach to compensate for imbalance and improve the prediction accuracy by adding a larger weight to the minority class. The R software was used to conduct our multivariate analysis [368].

3.1.4 Results

3.1.4 (a) Demographics

A total of 718 patients were included in the study, of whom 504 were male (70.2%). The mean age was 49.9 years (SD 12.3) and the mean age at CH onset, 31.4 years old (SD 13.0). The mean follow-up time in our headache clinic was 7.4 years (SD 3.9).

3.1.4 (b) Imaging Assessment

Routine MRI brain was performed in 267 patients while 376 patients underwent a routine MRI brain along with dedicated pituitary views (**Figure 12**).

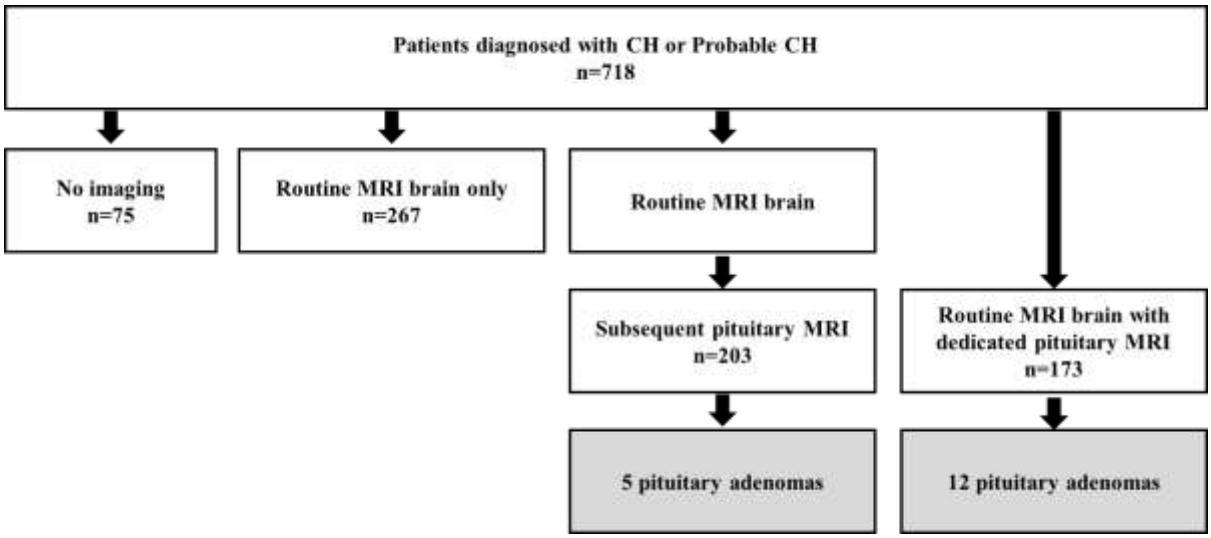


Figure 12: *Flow diagram illustrating imaging results of patients recruited to the study. Seventy-five patients did not undergo any neuro-imaging and were lost to follow-up.*

No significant differences were found between the patients who underwent routine MRI and those who received pituitary MRI in terms of gender, age of onset or diagnosis (**Table 8**). Younger patients (48.2; SD 11.8 vs 51.9; SD 12.5, $p < 0.001$) and those with a shorter history of CH (16.5 SD 10.2 vs 20.7 SD 11.5, $p < 0.001$) were more likely to undergo a dedicated pituitary MRI. A total of 41 (5.7%) patients had already had a pituitary MRI prior to attending our headache clinic.

Table 6: Demographics of patients who underwent routine MRI brain only compared with MRI brain with dedicated pituitary views.

Male	188 (69.6%)	261 (70.0%)	0.93
Female	82 (30.3%)	112 (30.0%)	0.93
	Median [IQR]	Median [IQR]	
Age (yrs.)	51.9 +/- 12.5; 50 [22-89]	48.2 +/- 11.8; 48 [22-78]	<0.001
Age at onset (yrs.)	31.3 +/- 14.4; 29 [5-81]	31.7 +/- 12.2; 30 [10-72]	0.34
CH duration (years)	20.7 +/- 11.5; 18 [0-64]	16.5 +/-10.2; 14 [0-53]	<0.001
	n(%)	n(%)	
ECH	119 (44.1%)	136 (36.5%)	0.07
CCH	123 (45.5%)	198 (53.0%)	0.07
PECH	11 (4.1%)	16 (4.3%)	1.00
PCCH	17 (6.2%)	23 (6.2%)	1.00

Abbreviations:
Mean +/- standard deviation, median [min-max] for quantitative data, n (%) for qualitative data
ECH: Episodic cluster headache;
CCH: Chronic cluster headache;
PECH: Probable episodic cluster headache;
PCCH: Probable chronic cluster headache

3.1.4 (c) Radiological pituitary abnormalities

Of the 376 patients with dedicated pituitary views on MRI, 35 (9.3%) had a pituitary abnormality. Ten abnormalities were diagnosed on pituitary MRI after the routine MRI brain was interpreted as normal.

Details regarding the pituitary abnormalities found are presented in **Table 7**. Non-functioning microadenoma (n=13) were the most common abnormality followed by empty sella syndrome (n= 8), Rathke's cleft cyst (n=5), other cystic formations (n= 4), functioning microadenoma (n=2), spontaneous pituitary haemorrhage (n=1) and macroadenoma (n=1). One patient had a microadenoma but it had not been ascertained whether it was functioning or non-functioning. Cavernous sinus invasion was found in one patient whose headaches began eight years after a non-functioning microadenoma was found on an MRI performed for a minor head trauma.

Of the three functioning adenomas that were found, two were prolactinomas and one was a IGF-1 secreting micro-adenoma. Two patients were symptomatic: one had galactorrhoea secondary to their prolactinoma. This had been diagnosed 23 years after first CH attack. The other patient had menstruation changes and hirsutism with a microadenoma; pituitary blood profile was not available and they were lost to follow up. In terms of treatment, the two prolactinomas only required medical management with dopamine agonists. Treatment did not result in the resolution of their CH. Notably, lesions identified on pituitary MRI performed following a normal brain MRI (n=10), did not require specific treatment.

Table 7: Pituitary lesions identified on dedicated pituitary imaging

Nature of the lesion		n (35)	Detected on routine MRI	Cavernous sinus invasion	Pituitary related symptoms	Medical treatment	Surgical treatment
Micro-adenoma	Functioning	2	1	0	1	1	0
	Non-Functioning	13	2	1	0	0	0
	Data not available	1	0	0	1	DNA	DNA
Macro-adenoma	Functioning	1	0	0	0	1	0
Pituitary haemorrhage		1	1	0	0	0	0
Empty sella	Partially	6	0	0	0	0	0
	Fully	2	2	0	0	0	0
Rathke's cleft cyst		5	0	0	0	0	0
Other cystic formation	Anterior pituitary cyst	1	0	0	0	0	0
	Pars Intermedia cyst	3	0	0	0	0	0

Abbreviations:
DNA: Data not available, MRI: Magnetic resonance imaging

3.1.4 (d) Routine pituitary blood profile

Of the 718 patients, 342 (47.6%) underwent a pituitary function profile. Of the patients with a pituitary abnormality, 28 (80.0%) profiles were available. In addition, the results of 254 (72.3%) patients with normal pituitary imaging were also reviewed. Minor

abnormalities were found in 71 patients with normal pituitary imaging (27.9%) and in 8 patients with a pituitary lesion (28.5%). This was not statistically significant (p = 0.64).

Only three significant blood test abnormalities were found in the cohort. This included an increased prolactin in two patients with prolactinomas (8806 mIU/L and 2000 mIU/L respectively) and an elevated IGF-1 (107 nmol/L) in a patient with a functioning micro-adenoma.

Comparison between patients with pituitary adenomas and patients with normal pituitary imaging

Univariate analyses is summarized in **Table 8**

Table 8: Demographics and Univariate analysis

	No pituitary adenoma (n=359, mean [SD])	Pituitary adenoma (n=17)	*P* value
Demographics and Diagnosis			
Age (years old)	48.05+/-11.85	49.11 +/- 10.67	0.89
Age at onset (years old)	31.64+/- 12.17	28.88 +/- 12.5	0.44
Sex			
Male	252 (70.1%)	10 (58.8%)	0.46
Female	107 (29.8%)	7 (41.2%)	0.46
Diagnosis			
ECH	126 (36.9%)	12 (34.3%)	0.76
CCH	178 (52.1%)	21 (60.0%)	1.00
Probable CH	37 (10.8%)	2 (5.7%)	0.52
Headache Duration	16.27+/-9.84	20.23+/-15.85	0.51
Duration, frequency of attacks			
Duration of attack	103.9 +/-162.39	103.23 +/- 58.33	0.43
Frequency	2.99 +/2.12	2.76 +/1.34	0.86
Laterality			
Strictly unilateral	292 (82.49%)	12 (70.6%)	0.21
Side-variable	58 (17.0%)	4 (23.5%)	0.55
Bilateral	9 (2.5%)	1 (5.9%)	0.37
Site			
Orbital / Retro-orbital	241	26	0.78
Nasal	25	4	0.04*
Frontal	122	6	1.00
Temple	161	15	0.08
Parietal	54	7	1.00
Vertex	42	5	0.24
Occiput	75	7	0.77
Cranial autonomic features and restlessness			
Ptosis	210	17	0.66
Eye oedema	130	13	0.30
Conjonctival Injection	257	11	0.55
Miosis	10	3	0.45
Lacrimation	279	27	0.44
Nasal blockage	203	24	0.23
Rhinorrea	213	20	0.29
Sweating	180	17	0.78
Facial flush	143	16	0.25
Aural fullness	73	5	1.00
Restlessness	283 (82.9 %)	30 (85.7 %)	0.23
Response to medical treatment			
Intractable to acute treatment	14 (4.33%)	0	1.00
Intractable to preventive treatment	84 (32%)	5 (45.4%)	0.47

Abbreviations:

Mean +/- standard deviation for quantitative data, n (%) for qualitative data, ECH: episodic cluster headache, CCH: chronic cluster headache, CH: cluster headache

The only significant item was the nasal location (p= 0.04). Regarding the response to CH medical treatment, no difference was found between the patients with pituitary adenomas and those with normal imaging in terms of acute and or preventive treatment (p = 1.00 and p = 0.47 respectively).

No variable achieved statistical significance on multivariate analysis. **Figure 13(A)** displays the variable importance (VI) mean graphs (over 50 runs of random forests) in descending order for the dataset variables. **Figure 13 (A)** includes all variables whereas **figure 13 (B)** is a zoom of the top one. The red line is the threshold value. The VI mean varied between -0.0025 and 0.0005, indicating that no variables are relevant as clinical predictors of pituitary adenoma.

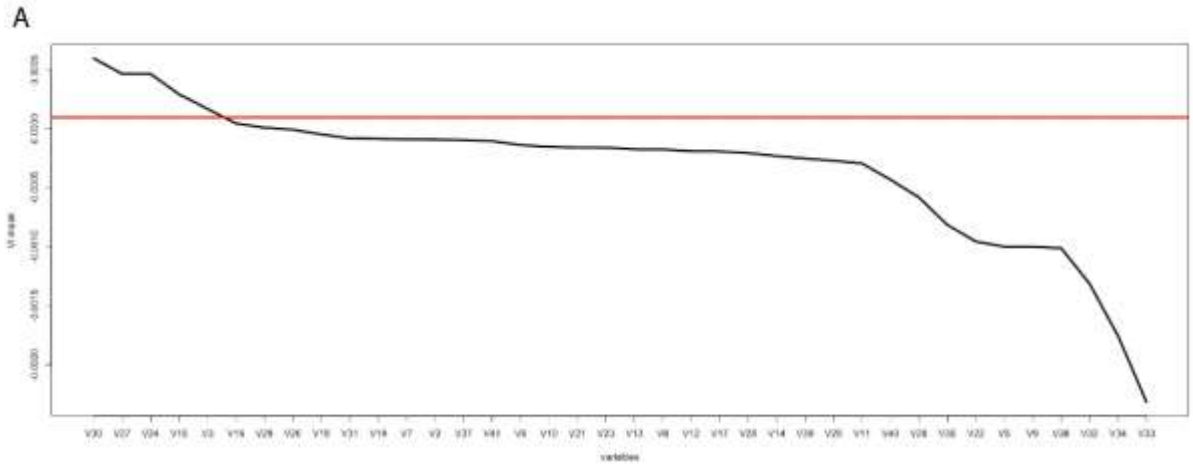


Figure 13 (A)

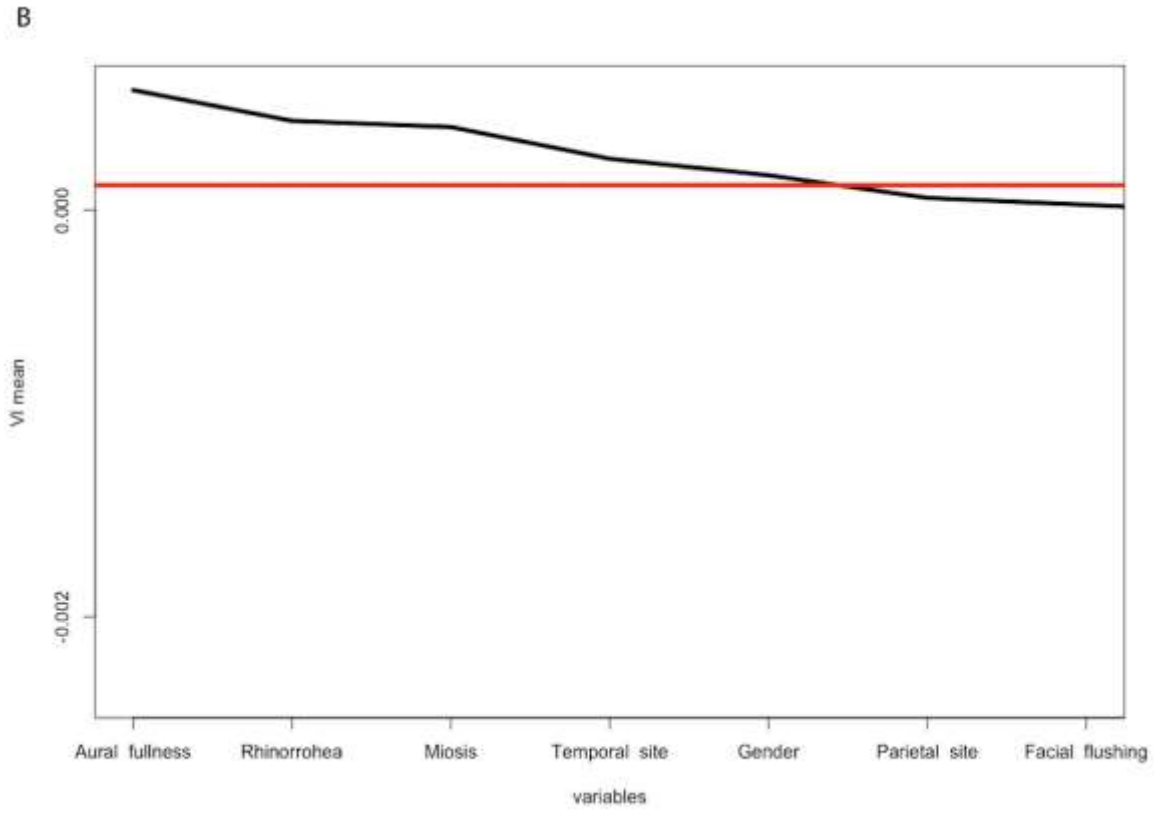


Figure 13 (B)

Figure 13 (A&B): *Multivariate analysis illustrating no variable achieved significance. A: VI (on the X-axis) of all variables recorded , in descending order (on the y-axis). The red line is the threshold value . B: Focus on items above the threshold value (in red): Only five items were above the line but with minor significance, of no clinical relevance (VI mean < 0.002) Variables legend:V2: Current age, V3: Gender, V5: Age of Onset, V6: Episodic CH, V7: Chronic CH, V8: Probable CH , V9: Strictly Unilateral, V10: Side Variable, V11: Bilateral, V12: Site RetroOrbital,V13: Site Orbital, V14: Site Frontal, V15: Site Temporal, V16: Site Parietal, V17: Site Vertex, V18: Site Occiput, V19: Site Nasal, V20: Absence of dysautonomic feature, V21: Ptosis, V22: Eye Oedema, V23: Conjonctival injection, V24: Miosis, V25: Lacrimation, V26: Nasal Blockage, V27: Rhinorrea, V28: Sweating, V29: Facial Flush, V30: Aural Fullness, V31: Restlessness, V32: Average attack frequency per day, V33: Minimum attack frequency per day V34: Maximum attack frequency per day, V35: Average Attack Duration, V36: Minimum Attack Duration, V37: Maximum Attack Duration, V39: Intractable to Acute treatment, V40: Intractable to Preventive treatment, V41: Follow-up duration.*

3.1.5 Discussion

A limitation of this study is the lack of a control cohort of patients without CH undergoing the same imaging protocol to compare the frequency of pituitary adenomas with our cases. However, pituitary adenomas were identified in 4.5% of cases in this large cohort of CH patients. These findings are consistent with previous estimates of the incidence of pituitary adenomas in the general population MRI and CT scans, which

ranges from 1.5% to 31%, with an average rate of 10.6% [432, 433]. As a result of recent developments in imaging protocols, such lesions are now regularly detected [433]. These findings are also reflective of a large autopsy series of 178 pituitary glands that identified incidental lesions in 34% of cases [427]. Similarly, a meta-analysis incorporating autopsy and imaging studies demonstrated pituitary adenomas in approximately 16.7% of the general population [428]. Furthermore, previous evidence of an over representation of CH in endocrine clinics, does not equate to an over-representation of pituitary adenomas in CH clinics.

Macroadenomas and functioning microadenomas have been reported in CH [418, 420, 434]. However, the pathophysiological mechanism underlying this association is unclear. Stretching of the dura mater as a result of a suprasellar extension or invasion of the cavernous sinus, in turn causing direct irritation of the trigeminal nerve was initially postulated[418, 424]. An absence of a correlation between tumour volume and the presence of headache deviates from this assumption [134, 420, 435]. Biochemical abnormalities and the release of "nociceptive peptide" by pituitary adenomas has also been suggested as a potential cause for headache [421, 436].

In this cohort, the diagnosis of a pituitary adenoma required medical treatment in only two cases, one of which lacked the symptoms of pituitary disease and therefore risked remaining undiagnosed. Thus, in the entire cohort, pituitary imaging benefitted only a single patient. This study sought to clarify the utility of MRI with dedicated pituitary views in the assessment of CH patients without excluding the usefulness of a routine brain MRI overall. Pituitary abnormalities in ten individuals had not been diagnosed prior to MRI with dedicated pituitary views but mostly represented adenomas or cysts that were not of clinical significance and did not have therapeutic consequences. Therefore, we challenge the usefulness of performing an additional MRI with dedicated

pituitary views after routine brain MRI, especially in the absence of symptoms of pituitary disease.

Our findings also dispute the need for screening pituitary function tests in these patients. Only two clinically relevant abnormalities (including in one symptomatic patient) were detected out of the 342 sets of tests conducted. Additionally, “minor abnormalities” of no clinical significance were found in equal proportion in individuals with and without pituitary adenomas. This indicates that in the setting of a normal MRI brain, pituitary function tests are unhelpful.

One limitation of this study is an imbalanced dataset, with only 5% of patients with pituitary adenomas. However, we attempted to compensate for this in our statistical analysis. No clinical characteristic or atypical headache feature was isolated as predictive of a pituitary adenoma, contradicting a previous study that suggested patients with pituitary lesions present atypically and respond poorly to treatment [418]. Other limitations of this study include it’s retrospective nature and absence of MRI with dedicated pituitary view in all cases. However, a prospective series is unlikely to impact the incidence of pituitary lesions.

The interpretation of the significance of pituitary lesions detected in CH patients is challenging. As per the ICHD-3 criteria for “headache attributed to pituitary hyper- or hypo-secretion”, evidence supportive of causation includes a temporal relationship between the onset of headache and pituitary dysfunction and/or improvement following treatment of the pituitary lesion. Patients with a pituitary adenoma in this study, including those with cavernous sinus invasion (n=1) or functioning lesions (n=2) did not fulfil these criteria.

Previous evidence suggests that approximately 49% of patients experienced an improvement in their headaches following surgical intervention for a pituitary tumour[422]. In our study, CH continued unchanged in patients with prolactinomas treated with dopamine agonists. While these findings do not exclude the role of pituitary lesions in the aetiology of CH, it signals that many are incidental lesions that do not impact CH management. Therefore, headache as an indication for the treatment of pituitary tumours remains controversial [435, 437]. This conflicts with some clinical guidelines such as the Endocrine Society of Colombia which lists unremitting headache is an indication for surgical intervention [438]. Additionally, the lack of consistent guidelines for the management of non-functioning lesions, which when invasive could present with CH, creates further challenges [439].

The relevance of pituitary lesions in CH is similar to that of vascular lesions. As with pituitary adenomas, evidence suggests that carotid dissection or aneurysms can be associated with CH [419, 440-442]. Therefore, it is possible that MR angiography is of equal diagnostic value as pituitary MRI in this group of patients. The problem with dedicated imaging of all patients for pituitary abnormalities (or vascular lesions), in addition to routine brain MRI, is that the diagnostic yield is very low while the chance of detecting an incidental, clinically irrelevant lesion is relatively high. Therefore, the cost effectiveness of this diagnostic approach is questionable.

This is the largest study of pituitary imaging in a CH cohort. The high proportion of patients with CCH (50%) is reflective of the study being performed in tertiary referral centre [301]. Despite this, only 5.7 % of cases in this study underwent pituitary MRI prior to attending the clinic, precluding referral bias in terms of pituitary screening. However, replication of our findings in a secondary care setting would be helpful, though the aforementioned cost implications may be a barrier.

In conclusion, this study demonstrates that pituitary adenomas do not occur at a higher frequency in CH patients compared to the general population. Furthermore, no clinical predictive factors distinguished CH patients with adenomas from primary CH. These findings suggest that the diagnostic assessment of CH patients should not routinely include pituitary screening with dedicated MRI pituitary scans. It should be performed if there are symptoms or features on standard brain MRI that are indicative of pituitary disease. Considering the low incidence of pituitary lesions in CH patients, this recommendation would improve cost-effectiveness and provide a more targeted diagnostic evaluation.

3.2 A Study of the Phenotype of Post-Traumatic Headache with Cluster Headache

Statement of contribution: Data for this study was collected from the headache clinic at the National Hospital for Neurology and Neurosurgery (Queen Square, London, UK) over ten years by headache clinical nurse specialists and headache clinicians under the supervision of Dr.Manjit Matharu. My contribution included the organisation, analysis and interpretation of data in collaboration with Dr Lou Grangeon (visiting clinical fellow). Advanced statistical analysis was conducted by Dr. Thanh Mai Pham Ngoc of the Mathematics Institute of Orsay, Paris Sud University. A version of this chapter has now been published in the JNNP (doi: 10.1136/jnnp-2019-322725)

3.2.1 Introduction

Post-traumatic headache (PTH) is characterised as headache that develops within seven days of a head trauma [353]. It is difficult to determine the true prevalence of this type of headache, however, it is thought to be more frequently associated with mild rather than severe head injury [443, 444]. Tension-type headache and migraine without aura are the usual associated phenotypes [445]. Trigeminal autonomic cephalalgias (TAC) have been reported following head injury [446-448], however only two cases fulfil the International Classification of Headache Disorders (ICHD3) criteria for post-traumatic headache and cluster headache. The vast majority of cases are described with a prolonged or unknown latency between CH onset and head trauma [449-451].

Recent functional imaging data suggests that ipsilateral hypothalamic activation with subsequent trigemino-vascular and cranial autonomic activation underpin the pathogenesis of CH [452, 453]. Its relationship with head trauma remains unclear.

According to Lambru et al, there are three hypothesis explaining the link between head injury and CH. Firstly, CH may occur as a direct result of head trauma, secondly, head trauma may only increase the risk of developing CH, and finally, that the personality traits associated with CH may predispose to head trauma [449].

Although most PTHs resolve within 12 months of injury, approximately 18–33% of PTHs persist, leading to loss of work capacity and significant fiscal consequences [454, 455]. Post-concussion syndrome with associated depression, cognitive dysfunction, and insomnia also contribute to this economic burden [456]. This highlights the clinical and medico-legal imperative to define post-traumatic headache with a cluster headache phenotype (PTH-CH) accurately.

3.2.2 Aims and objectives

The aim of this study is to define the characteristics of PTH-CH and compare them with primary CH.

3.2.3 Methods

Recruitment strategy, phenotyping, and clinical/treatment data have been discussed in the methodology section 2.2. All consecutive patients with prior head trauma and diagnosed with CH according to ICHD-3β were assessed [353]. Only patients with a maximum latency period between head trauma and the first CH attack of seven days, meeting criteria for both PTH and CH, were included. Concurrent headache conditions were also categorised using ICHD-3β.

Each patient was longitudinally followed and evaluated for the cause and mechanism of head trauma, initial symptoms, such as posttraumatic amnesia or loss of consciousness, and neuroimaging. In case of headache of greater than 3 months' duration, the PTH-CH was called "persistent". According to the International Headache

Society (IHS) classification, head trauma was defined as severe or mild[353]. In order to ascertain specific characteristics in PTH-CH patients, they were compared against a control cohort of all patients fulfilling the primary CH criteria during the same time period.

3.2.3 (a) Controls

In order to ascertain specific characteristics in PTH-CH patients, a comparison with a control cohort was used. This included all patients who attended the headache clinic during the same period and who fulfilled the criteria for primary CH. The same evaluation and data collection conducted in the PTH-CH group was performed in the control cohort. Patients with an antecedent history of head trauma or those with secondary CH were excluded from the final control cohort.

3.2.3 (b) Statistics

Descriptive statistics were expressed as mean with standard deviation (SD). The two cohorts were compared using a Mann-Whitney nonparametric test for continuous data and a Fisher exact and Chi-square test for categorical data. For the multivariate analysis, we adopted a three-step approach. Firstly, we balanced our two groups of PTH-CH and primary CH patients. A common statistical approach to deal with imbalanced learning is to alter the class distribution to get a more balanced sample. Secondly, we performed variable selection to select a subset of relevant covariates for our model construction, and finally, a logistic regression with the previously selected features. Note that we did not split the data into a training and a test set due to the highly imbalanced dataset. Adopting the standard holdout strategy would lead to high variance estimates of the accuracy measure. Instead, a specific holdout version adapted to imbalanced learning was used. More specifically, missing values were

corrected by imputation techniques based on random decision forests. Then the ROSE algorithm was used to balance the data [457].

The ROSE function creates an artificial balanced sample according to a smoothed bootstrap approach. Then, on the ROSE sample, variable selection was performed to get a subset of important covariates to feed into the logistic regression. The LASSO algorithm was used to select relevant explanatory variables [458]. The LASSO performs automatic variable selection and is capable of selecting groups of correlated variables. Logistic regression was conducted with the variables selected by the LASSO. All the numerical results were performed with the software R[368]. The randomForest, ROSE and glmnet packages were used [457, 459]. The threshold for statistical significance was set to $p \leq 0.05$.

3.2.4 Results

We identified 26 patients diagnosed with PTH-CH within the defined study period, of whom 19 (73.0%) were male (**Table 9 & 11**). The control cohort involved 631 individuals with primary CH. Patients with a history of antecedent head trauma (n=52) or secondary CH (n= 26) were excluded from the final control cohort (n=553). No difference between the PTH-CH and primary CH patients was found regarding demographic data such as current age, sex, age of onset, and follow-up duration allowing for an accurate comparison.

PTH-CH was persistent in all cases. The mean age of patients with PTCH at recruitment was 48.4 years (SD 11.2) and the mean age at CH onset, 31.8 years old (SD 13.5). The mean follow-up time in our headache clinic was 6.5 years (SD 13.5). Five PTH-CH patients (19.2%) were diagnosed with ECH or probable ECH phenotype and 21 (80.7%) with CCH or probable CCH phenotype.

Of the 21 patients with CCH phenotype, 15 were chronic from onset. Of the remaining patients, six developed the ECH phenotype immediately after the injury. The mean duration of attacks was 87.3 minutes (SD 55) and the mean frequency was 3.3 daily (SD 1.8). At least one autonomic feature was present during the attacks in the entire case group, with restlessness during an attack reported in all but two patients. At least one migrainous feature was reported in 24 patients (92.3%). There was a distinct circadian periodicity with the attacks occurring predictably during the night in 24 (92.3%) patients. Regarding concomitant headaches, six (23.0%) patients suffered from episodic migraine and five (19.2 %) patients from chronic migraine. One developed chronic migraine at the same time as PTH-CH, whereas three patients also suffered from PTH with a chronic migraine phenotype but related to a separate head injury. All patients could clearly distinguish CH attacks from migraine pain.

Of the 26 PTCH patients, a family history of cluster headache in at least one first-degree relative was confirmed in four individuals (15.3%) from three families. Two patients were brother and sister and their mother also suffered from CH, as confirmed at clinic. The affected relatives of the two remaining PTCH patients with probable family history were deceased. Nevertheless, one (the mother of the PTCH patient) had previously been diagnosed with CH and the other (the uncle of the PTCH patient) had a history that was highly suspicious for CH (extremely severe headaches localised behind the eye and occurring during the night).

Regarding the response to medical treatment, two patients (7.6%) were considered as intractable to acute treatment and 11 (42.3%) as intractable to preventive treatment. Three of them benefited from invasive neuromodulation including occipital nerve stimulation (ONS), sphenopalatine ganglion stimulation (SPG) and ventral tegmental region deep brain stimulation.

The mechanisms of head trauma are summarized in **table 10**. The vast majority of patients (n = 23, 88.4%) sustained mild injury. Three traumas (11.6%) were considered as severe due to the duration of loss of consciousness and/or presence of anterograde amnesia. No intracranial haemorrhage was reported, though two skull fractures were described. The different mechanisms of head injury included road traffic accident (n= 5), mechanical fall (n= 4), collision with an object (n=7), an assault (n= 3) or direct penetration of the head by metal or glass (n=2). In addition, dental extractions (n=5) were assessed as the ICHD defines head injury as “penetration of the head by a foreign body” but were not included for comparison to the control cohort, considering that they were not strictly consistent with other PTH-CH patients. Following the trauma, five patients had cranial bruising and three had deep laceration. The first CH attacks occurred a few hours after the trauma in seven patients and immediately after in five patients. The CH attacks were ipsilateral to the head injury in all patients in whom the trauma was clearly one-sided, except one (patient 2). Due to a wider and often bilateral injury, laterality was indeterminate in 11 patients.

Table 9: Demographics and clinical characteristics in patients with post-traumatic cluster headache

	Sex	Age of onset	Current age	Follow-up duration (years)	Type of CH	Other associated headache	Characteristics of attacks						Response to treatment	
							Laterality	Average duration (min)	Average frequency (per day)	Autonomic feature	Restlessness	Migrainous symptoms	Intractable to acute treatment	Intractable to preventive treatment
1	M	15	34	10	CCH*	-	Side variable	50	3	C, L, N, R, E, FS, P	Yes	N, V	No	No
2	M	32	54	10	CCH	Episodic migraine	L	60	2	C, L, N, R, E, FF, P	Yes	PT, PN, OSM, N	No	No
3	M	18	38	10	ECH	-	R	120	3	C, L, N, E, FS, P	Yes	PT, PN, N, V, OSM	No	No
4	F	36	54	10	CCH*	Episodic migraine	Side variable	90	3	N, E, P	Yes	PT, PN, N, V, OSM	No	Yes
5	M	33	64	8	CCH*	-	R	90	6	L, N, R, FS, FF, P, AF	Yes	PT, PN, OSM	No	Yes
6	M	43	57	8	CCH	Episodic migraine	L	150	1	L, R	Yes	PT, PN	No	No
7	M	20	34	3	CCH*	-	R	40	5	C, L, N, R, E, FS, P	Yes	-	No	No
8	M	10	55	8	PECH	-	R	180	6	C, L, N, R, FS, FF, P, AF	Yes	PT, PN	No	Yes
9	M	22	38	8	CCH	Chronic migraine	L	50	5	C, L, N, R, E, FS, FF, P	Yes	PT, PN, OSM	No	No
10	M	26	52	8	CCH*	-	R	120	2	C, L, N, FF, FS, P	Yes	OSM	Yes	Yes
11	F	45	58	8	CCH	Episodic migraine	L	120	2	FS, AF, P	Yes	PN, N, MS	No	Yes
12	F	29	42	7	CCH	-	L	120	6	C, L, N, R, E, P	Yes	N, OSM	No	Only 3 trials done
13	F	11	23	5.5	CCH	-	Side variable	90	2	C, L, R, E, P	Yes	PT, N	NA	NA

14	M	54	57	5	CCH	-	L	150	1	C, FF, FS	No	-	Yes	Yes
15	M	48	59	5	CCH		L	45	2	C, L, N, R, E, FS, P	Yes	PT, PN	No	Yes
16	F	47	61	4	CCH	Chronic migraine	Side variable	120	3	L, N, FF, FS	Yes	-	No	Yes
17	M	24	33	2.5	CCH	Chronic migraine	L	30	5	C, L, N, R, E, FS, FF, P	Yes	PT, PN, N, V, OSM	No	Only 3 trials done
18	M	45	52	5	PECH	Episodic migraine	L	210	3	C, R, E, FS	Yes	N	No	No
19	M	56	62	2	CCH	Chronic migraine	L	180	2	NA	Yes	PT, PN	No	Yes
20	F	51	64	8	ECH	-	L	30	3	C, L, N, R, FS, P	Yes	PT	No	Only 3 trials done
21	M	28	45	1	CCH*	-	L	120	4	C, L, N, R, E, FS, FF, P	Yes	PT	No	No
22	M	38	48	4.5	CCH	-	R	30	6	C, L, N, R, E, FS, FF, P	Yes	-	No	Yes
23	M	26	39	10.5	CCH	-	L	20	8	C, L, N, R, FS, FF	Yes	PT, PN	No	Yes
24	M	24	36	6.5	CCH	Chronic migraine	R	60	2	C, L, N, P, AF	No	PT, PN, N	No	No
25	M	33	48	7	CCH	-	L	60	4	C, L, N, R, E, P	Yes	PN, N	No	Only 3 trials done
26	F	17	52	5	ECH	Episodic migraine	L	45	3	L, N, R	Yes	PT, PN, N, OSM	No	Only 3 trials done

* Secondary chronic cluster headache phenotype (initially episodic and subsequently transformed to chronic variant)

Abbreviations:

CCH – Chronic Cluster headache, CH – Cluster headache, ECH – Episodic Cluster headache, L = Left, NA = Not available; R = Right,
Autonomic Feature – C (Conjunctival injection), L(Lacrimation), N(Nasal congestion), R(Rhinorrhoea), P(Ptosis), E(Eyelid oedema), FS(Facial sweating), FF(Facial flushing), AF(Aural fullness) Migrainous symptoms – PT (Photophobia), PN (Phonophobia), OSM (Osmophobia), N (Nausea), V (Vomiting), MS (Motion sensitivity)

Table 10: Characteristics of head trauma in patients with post-traumatic cluster headache

	Side of Injury	Association with headache		Details of head trauma	Associated features			Neuroimaging
		Ipsilaterality	Time from injury to CH onset		Loss of consciousness	Amnesia	Skin bruise or laceration	
1	Vertex	Indeterminate	5 days	Head hit by a block of concrete while standing on a riverbank for fishing	2-3 min	-	Deep laceration	Normal
2	Left	Yes	Immediate	Cheek and occiput hit while attacked by a group, then was thrown down flight of stairs	20-25 min	Anterograde	Bruising & laceration	Left zygoma fracture
3	Left	No	24 hours	Hitting the front left aspect of head while walking into a metal pole	-	-	-	Normal
4	Unclear	Indeterminate	24 hours	Concussion to occiput and neck while playing netball	-	-	-	Normal
5	Forehead	Indeterminate	7 days	Banging the head on the beam of a door at home	-	-	-	Normal
6	Left	Yes	7 days	Accidental fall during water skiing, hitting on the left face	2 min	-	-	Normal
7	Unclear	Indeterminate	24 hours	Unknown cause of syncope during driving, then hitting a tree with head concussion	15 min	-	-	Normal
8	Unclear	Indeterminate	Immediate	Involved in road traffic accident but no further details available	-	-	-	Normal
9	Left	Yes	Few days	Attacked by a group. Left side of head was punched and then kicked twice	10 min	Anterograde	Bruising	Normal

10	Unclear	Indeterminate	7 days	Head was hit when he was serving in the army. No further detail available	5 min	-	-	Normal
11	Left	Yes	Immediate	Fell forward and hit on left forehead while having ice skating	4 hours	-	Marked bruising	Basal skull fracture
12	Left	Yes	Few days	Attacked in a pub with the left temple punched by a customer	-	-	-	Normal
13	Unclear	Indeterminate	5 hours	Head was hit when the bus swerved suddenly	-	-	Mild bruising	NA
14	Left	Yes	Few days	A piece of glass fell on the left side of the head when dismantling a conservatory	-	-	-	Incidental aneurysm
15	Left	Yes	Immediate	Assaulted by a prisoner with a metal mop base on the left cheek	-	-	Deep laceration	Normal
16	Left	Yes	Immediate	Left side of head and neck was hit by an object falling from a building	-	-	-	Normal
17	Unclear	Indeterminate	Few hours	Involved in road traffic accident; was run over by a car while riding a motorcycle	5 min	-	-	Normal
18	Left	Yes	3 days	Involved in road traffic accident but no further details available	Duration unknown	-	-	Normal
19	Left	Yes	Few hours	Fell off the bed and hit the corner of left eye on table	-	-	-	Normal
20	Left	Yes	Immediate	Bashed her head against the corner of a desk	-	-	Bruising	Normal
21	Unclear	Indeterminate	3 days	Involved in road traffic accident: collision with a car while riding a motorcycle	-	-	-	Normal

22	Right	Yes	1 day	Root canal treatment of a right upper tooth followed 7 days after by insertion of crown	-	-	-	Normal
23	Left	Yes	3 days	Dental extraction with dislocation of the jaw	-	-	-	Normal
24	Right	Yes	7 days	Lower right dental extraction	-	-	-	Normal
25	Left	Yes	Few days	Upper left dental extraction	-	-	-	Normal
26	Bilateral	Indeterminate	24 hours	Four wisdom teeth extraction	-	-	-	Normal
Abbreviations: RTA – Road Traffic accident, MRI – Magnetic resonance imaging, CT – Computed tomography, NA: Not available								

Univariate analyses are presented in **table 11**. PTH-CH patients were more likely to be diagnosed with the CCH phenotype (80.9% versus 50.4%; p= 0.006) and with associated chronic migraine (19.0% versus 7.6%; p =0.05) than the control cohort. A parietal location of referred pain, which was the most common site of injury, was more common in the PTH-CH group (38.0% versus 16.0%; p = 0.008). They were also at a higher risk of being intractable to preventive treatment than the control cohort (42.8% versus 16.0%; p = 0.002). The results remained unchanged when including the five dental extractions cases.

Table 11: Univariate analysis results of comparison between post-traumatic headache with cluster headache phenotype (PTH-CH) and primary cluster headache (Control cohort)

	PTH-CH (n=21)	**Control cohort (n=553)**	***P value***
Demographics			
Age (years old)	49.3 SD+/- 11.9	48.8 SD +/- 12.4	*0.56*
Gender (M/F)	15/6	379/174	*0.77*
Follow-up duration (years)	6.47 +/- 3.0	6.04 +/- 2.8	*0.43*
Age at onset (years old)	32.9 SD +/- 14.4	31.3 SD +/- 13.6	*0.56*
Family History of CH	4 (19.0%)	50 (9.0%)	*0.12*
ECH and/or PECH CCH and/or PCCH	4 (19.1%) 17 (80.9%)	274 (49.5%) 279 (50.4%)	***0.006***
Episodic migraine Chronic migraine	5 (23.8%) 4(19.1%)	99(17.9%) 42 (7.6%)	0.49 **0.05**
Duration, frequency of attacks			
Usual duration (min)	97.8 +/- 56.0	90.8 +/- 64.0	*0.44*
Average frequency per day	3.0 +/- 1.5	2.8 +/- 2.1	*0.08*
Laterality			
Strictly unilateral	16 (76.1%)	444 (80.2%)	*0.64*
Side-variable	5 (23.8%)	91 (16.4%)	*0.37*
Bilateral	0	18 (3.2%)	*0.40*
Site & referred pain			
Orbital / Retro-orbital	11	398	**0.05**
Frontal	6	176	0.75
Temple	11	267	0.71
Parietal	8	89	**0.008**
Occiput	5	111	0.67
Cranial autonomic features and restlessness			
Ptosis	16	304	**0.05**
Eyelid oedema	12	192	**0.03**
Conjunctival Injection	15	365	0.60
Miosis	1	22	0.85
Lacrimation	16	428	0.89

Nasal blockage	14	320	0.42
Rhinnorhoea	13	330	0.83
Facial sweating	14	265	0.09
Facial Flush	9	208	0.63
Aural Fullness	3	86	0.87
Restlessness	20	428	0.06
Response to medical treatment			
Acute treatment			
Intractable	2	16	0.23
Indeterminate	4	130	
Responsive	16	407	
Preventive treatment			
Intractable	9	89	**0.002**
Indeterminate	8	210	
Responsive	4	254	

Mean +/- standard deviation for quantitative data, n (%) for qualitative data

CCH: Chronic and/or probable chronic cluster headache; CH: Cluster headache; ECH: Episodic and/or probable episodic cluster headache; F: female; M: male; PTH-CH: post-traumatic headache with cluster headache phenotype

For multivariate analysis, we obtained a simulated sample of 270 PTH-CH patients and 304 non-PTH-CH patients. On this balanced dataset, in a binary logistic framework, the LASSO selected 33 variables over the 37 original ones.

We found significant positive association between PTH-CH and family history CH (OR 3.32; 95% CI, 1.31 - 8.63, p = 0.012), chronic cluster headache phenotype (OR 3.29; 95% CI, 1.70 - 6.49, p <0.001), temporal location (OR 2.04; 95% CI, 1.10 - 3.84, p =0.024), parietal location (OR 14.82; 95% CI, 6.32 - 37.39, p <0.001), eye oedema during attacks (OR 5.79; 95% CI, 2.57 - 13.82, p<0.001), miosis during attacks (OR

11.24; 95% CI, 3.21 - 41.34, p <0.001), rhinorrhoea (OR 2.65; 95% CI, 1.26 - 5.86, p =0.013), facial sweating (2.53, 95% CI, 1.33 - 4.93, p= 0.005); and restlessness (OR 4.63; 95% CI, 1.16 - 22.19, p= 0.039). Multivariate analysis confirmed intractability to acute (OR 12.34; 95% CI, 2.51 - 64.73, p=0.002) and preventive treatment (OR 16.98; 95% CI, 6.88 - 45.52, p <0.001) as independent characteristics of PTH-CH patients. Associated chronic migraine had one of the highest OR (10.35; 95% CI, 3.96 - 28.82, p <0.001), (**Table 12**). Conversely, PTH-CH patients were less likely to present with frontal referred pain (OR 0.09; 95% CI, 0.03 - 0.19), absence of cranial autonomic features during attacks (OR 0.09; 95% CI, 0.01 - 0.60), conjunctival injection (OR 0.33; 95% CI, 0.12 - 0.86), lacrimation (OR 0.25; 95% CI, 0.09 - 0.66) or flush (OR 0.28; 95% CI, 0.12 - 0.59).

Table 12: Results of multivariate logistic regression model comparing post-traumatic headache with cluster headache phenotype (PTH-CH; n=21) and primary cluster headache (Control cohort; n=553)

Predictive factor	OR	95% CI	p value
Family History of CH	3.32	1.31 – 8.63	0.012
Chronic cluster headache phenotype	3.29	1.70 – 6.49	<0.001
Temporal location	2.04	1.10-3.84	0.024
Parietal location	14.82	6.32 – 37.39	< 0.001
Presence of eye oedema	5.79	2.57-13.82	< 0.001
Presence of miosis	11.24	3.21-41.34	< 0.001
Presence of rhinorrhoea	2.65	1.26-5.86	0.013
Presence of facial sweating	2.53	1.33-4.93	0.005
Restlessness	4.63	1.16-22.19	0.039
Associated Chronic Migraine	10.35	3.96 – 28.82	< 0.001
Intractable to Acute Treatment	12.34	2.51-64.73	0.002
Intractable to Preventive Treatment	16.98	6.88 – 45.52	< 0.001

Abbreviations: CH: cluster headache; CI: Confidence interval; PTH-CH: post-traumatic headache with cluster headache phenotype; OR: Odds-ratio

3.2.5 Discussion

This is the largest series of PTH-CH reported to date. This study describes the clinical characteristics of twenty-six PTH-CH patients, who developed headaches within seven days of head trauma that strictly fulfil the ICHD-3 criteria for both PTH and CH. Five patients who underwent dental extractions were kept apart, considering that these cases have a post-surgical rather than a typical post-traumatic aetiology. Univariate analysis revealed that PTH-CH patients were more likely to have the chronic variant of CH, parietal site of pain, prominent cranial autonomic features particularly ptosis and eyelid oedema, intractability to preventive treatments and have concurrent chronic migraine. A multivariate logistic regression model demonstrated that PTH-CH patients are also more likely to have a family history of CH, CCH, temporal and parietal site of pain, prominent cranial autonomic features (particularly eyelid oedema, miosis, rhinorrhoea and facial sweating), restlessness, intractability to acute and preventive treatments, and associated chronic migraine. These findings suggest that PTH-CH has a distinct clinical phenotype with more severe cluster headache phenotype that is less responsive to treatments compared to primary CH.

In primary CH, wide activation of ipsilateral trigeminal nociceptive pathways involving the trigeminal-autonomic reflex leads to central activation through the trigeminal nucleus caudalis and the superior salivatory nucleus in the brainstem [138, 449]. In contrast, it is suggested that PTH results from more localized changes in the trigeminal pathway due to direct damage at the site of trauma [460, 461]. Factors including axonal injury, reduced cerebral circulation, and the inappropriate release of local neurotransmitters may play a role in the initial emergence of PTH-CH [445, 449]. Consistent with previous reports, there was a propensity for referred pain in the parietal and temporal regions during PTH-CH attacks in our cohort [462]. This likely

reflects localized changes. Furthermore, the majority of patients with strictly unilateral headache reported pain on the side ipsilateral to the injury. Although the pain distribution may lead to attribution to some event in the past, this is consistent with previous findings and supports the hypothesis of peripheral involvement in PTH-CH pathogenesis via direct damage to local nociceptive structures [463, 464].

Nevertheless, diffuse changes such as excessive neuronal depolarization and the release of excitotoxic neurotransmitters may underpin the pathogenesis of PTH-CH [445]. The sensitization of central trigeminal neurons in PTH as a consequence of the initial trauma-related inflammatory process within the cranial meninges and the calvarial peri-osteum may also be involved[465]. During primary CH attacks, the ipsilateral cranial autonomic features testify of cranial parasympathetic activation and sympathetic hypofunction due to a central disinhibition of the trigeminal autonomic reflex [466]. The more frequent eyelid oedema, miosis, rhinorrhoea and facial sweating during in PTH-CH compared to primary CH in our study may reveal a marked disinhibition of pain pathways after trauma. Such long-term modulation of central pain pathways in CH following trauma would also explain the vast majority of chronic variant found in our study. More than 80% of the PTH-CH patients suffered from the chronic variant and fifteen of them developed the chronic form from onset, without any intermittent pain free period. This further supports a continuous sensitivity to common headache triggering factors [465].

Post traumatic headache with a chronic migraine phenotype co-occurred in 19% (4/21) of our cohort, exceeding the estimated 3% incidence in the general population and reaching the high odds-ratio of 10.35 in multivariate analysis [467]. Although PTH can be heterogeneous, patients could clearly distinguish their CH attacks form their migraine pain, arguing more for true concomitant headaches than one heterogeneous

phenotype of the same type of headache. Similarly, this may result from central sensitization with dysfunction of brainstem antinociceptive centres (implicated in migraine) as well as the hypothalamus (implicated in CH)[182, 468]. Thus, head trauma may lead to global alteration in the “pain neuromatrix”, with concomitant forms of CH and migraine. The marked intractability of PTH-CH to both acute and preventative treatment could be another direct consequence of these modifications.

The rarity of CH following trauma, compared to migraine and tension-type headache, remains unexplained [447]. Nevertheless, in a study of military service members with a history of mild traumatic brain injury (n=95), TAC type (n= 6) was the second most prevalent after migraine type[451]. The emergence of CH phenotype may be due to a genetic susceptibility. We found that four (15.3%) PTH-CH patients had family history of CH in first-degree relatives, exceeding the estimated prevalence of CH of 1.2% in general population. There were three times the odds of having a family history of CH in case of PTH-CH. This implies that genetic risk in combination with an environmental trigger, such as head trauma, may be required to develop the phenotype.

PTH-CH may occur only in cases where there is hypothalamic disruption secondary to trauma, and subsequent reorganisation [469]. Indeed, the somatosensory system is capable of reorganization following peripheral denervation and pain[470, 471]. There is supporting evidence for the involvement of posterior hypothalamus in primary CH attacks[472, 473]. Head trauma can lead to a loss of hypocretin-producing neurons in the posterolateral hypothalamus and CH attacks are known to occur when the orexinergic system is down regulated[474, 475]. Indeed, the circadian periodicity is one of the hallmark of CH attacks and concerns 70% of patients[476]. The unusual high rate of circadian periodicity, documented in 92% of PTH-CH patients in our study, may be suggestive of hypothalamic dysfunction related to trauma.

Weaknesses of this study include that our data was collected at a tertiary headache centre. Comparison with a large control cohort who attended the same clinic during the same period minimizes this referral bias. Retrospective data may involve memory biases, but the rarity of the PTH-CH entity makes a prospective study unlikely to be feasible. Disability from PTH is compounded by coexisting post-traumatic stress disorder[477]. Unfortunately, we did not formally perform psychological assessment or comparison with primary CH in terms of quality of life. Post-traumatic sleep and mood disturbances can plausibly influence the development and perpetuation of headache, but also the response to treatment[456]. In terms of intractability, a large number of patients were classified as indeterminate due to incomplete trials. Finally, rather broad confidence intervals were found after logistic regression owing to small number of PTH-CH patients.

Strengths of the study include the relatively large sample of patients who strictly fulfil the ICHD criteria for both PTH and CH, allowing us to consider our conclusions as strongly reliable. An association between head injury and CH has already been described in the past but most injuries were quite remote from the first CH attack[452]. The definition of PTH implies a close temporal relationship, established as the occurrence of headache within 7 days after head trauma. This stipulation might be somewhat arbitrary but yields a stronger evidence of causation, leading to a higher specificity of ICHD criteria[353]. Indeed, a high proportion of CH patients sustained head injury several years prior to CH onset, suggesting an association between trauma and CH which goes beyond the rare occurrence of PTH-CH cases, thus raising the hypothesis of distinctive lifestyles in CH patients[478]. In conclusion, this series is the first to describe in detail the specific clinical characteristics of PTH-CH. We demonstrated that PTH-CH is more likely to present as chronic form, with marked cranial autonomic

features and temporo-parietal location of attacks in patients with family history of CH. They have a considerably higher risk of intractability to treatment and associated chronic migraine. This unique evolutive profile possibly reflects sensitization of the pain neuromatrix and hypothalamus following trauma.

3.3 A Systematic Review And Meta-Analysis of the Prevalence of Familial Cluster Headache

Statement of contribution: In collaboration with Mr. Ben Simpson, I conducted this systematic review and meta-analysis. My contribution included the collection of data for original cohort, the organisation and analysis of data. Mr Ben Simpson led the meta-analysis which was carried out under his supervision. A version of this chapter has now been published in the Journal of headache and pain (doi: 10.1186/s10194-020-01101-w)

3.3.1 Introduction

Those with a family history of CH appear to have an increased risk of developing the condition [292-295]. Estimations of the presence of a positive family history amongst sufferers varies across studies. For example, in one cohort the familial prevalence was 2.3%, with a low Falconer's heritability index, indicating a high likelihood of an environmental cause [297]. Others, however, estimate a positive family history in up to 20% of patients, inferring a 39 fold relative risk [298]. Inter-familial clinical variability has also been observed, with an earlier age of onset reported in the offspring of parents with CH, inferring the possibility of anticipation [300]. There also appears to be a higher proportion of female sufferers in familial cases [301]. These findings have provided a

basis for familial studies and genetic association studies in genes with a putative role in the pathophysiology of CH [76, 309, 310, 319-321, 479, 480].

3.3.2 Aims and objectives

The purpose of this study was to perform a systematic review and meta-analysis of all familial CH studies in order to derive a prevalence estimate.

3.3.3 Methods

This systematic review was registered with PROSPERO, the International Prospective Register of Systematic Reviews (registration number CRD42019157309) and carried out in accordance with the guidelines for Preferred Reporting Items for Systematic Reviews and Meta-analysis Protocols (PRISMA- P)[481]. All published studies of interest were identified through a search involving the following electronic databases: MEDLINE, PUBMED, EMBASE, CINAHL.

A pre-defined search strategy was formulated which included a combination of relevant medical subject headings (MeSH), associated synonyms and free text [482]. To identify studies reporting a family history the following terms were used; "family" OR "familial" OR "hereditary" OR "heritability" OR "hereditability" OR "inherit" OR "inherited" OR "genetic" OR "genes" OR "gene". These were added to terms for Cluster Headache including "Trigeminal Autonomic Cephalalgia" OR "TACS" OR "Cluster Headache" OR "cluster headaches" combined using the 'AND' operator. To ensure a robust review, references from cited articles were also screened. Finally, experts were also consulted to identify additional missed literature. The details of the search strategy used for individual databases is summarised in the appendix (Section III, 3.3.3a).

3.3.3 (a) Eligibility criteria and data extraction

All studies reporting the prevalence of familial CH within a defined cohort of CH patients were included in the analysis. The inclusion criteria defined a positive family history as a clinical diagnosis of CH, in one or more affected individuals, who were a first or second-degree relative. To avoid an over representation of familial history, only studies that confirmed a diagnosis of CH by a neurologist in an affected relative were included in the systematic review. All abstracts were independently analysed by two authors and those fulfilling the eligibility criteria were included for full-text review. A separate assessment of the included studies was conducted by two authors independently and the following data was extracted for analysis: study design, year of publication, population studied, methodology of data acquisition, diagnostic criteria employed, number of participants, gender ratio, percentage reporting a family history and gender ratio of patients with familial CH.

To minimise the risk of bias, all eligible studies were independently analysed using a modified Newcastle – Ottawa appraisal checklist, a tool designed to appraise cohort studies on three main areas: the selection of the study groups, the comparability of these groups; and the ascertainment outcome for [483]. The total score of the modified version is limited to 7 stars with removal of sections pertaining only to longitudinal studies.

3.3.3 (b) Unpublished cohort

We included an additional unpublished cohort of patients who attended the headache clinic at the National Hospital for Neurology and Neurosurgery (Queen Square, London, UK) between January 2007 and April 2017. Details pertaining to their recruitment and phenotyping are described in methodology. A total of 645 patients were included in the cohort. Of these, 456 (70.69%) were male. A family history of

cluster headache was reported in 66 patients (10.2%).18 cases were excluded as relatives did not fulfil the ICHD3β criteria for cluster headache or were uncontactable. Overall, 48 (7.44%) individuals had a confirmed family history of CH.

3.3.3 (c) Statistical analysis

Further details on meta-analysis can be found in methods section 2.2.9. All scripts and commands used are available in appendix (section III, 3.3.3c).

3.3.3 (d) Estimation of relative proportion of effected probands with positive family history of CH:

For included studies, we extracted the total number of affected probands with a first or second degree relative with a clinical diagnosis of cluster headache and the total number of cases in the study [297, 302, 484-487]. The raw/direct proportions were calculated and the distribution of untransformed, logit and double-arcsine transformed proportions were compared. The distributions of the proportions were assessed for normality using density plots and tested using the Shapiro-Wilk test. Logit-transformed proportions most resembled a normal distribution therefore, this transformation was used for the analysis. To account for potentially high inter-study variation and high I^2, a random-effects model was fitted for estimation of family history in CH.

After fitting a model to all included studies, leave-one-out analyses (LOO) and accompanying diagnostic plots were used to identify influential studies including: externally studentized residuals, difference in fits values (DFFITS), Cook's distances, covariance ratios, LOO estimates of the amount of heterogeneity, LOO values of the test statistics for heterogeneity, hat values and weights. Briefly, each study was removed one at a time, and the summary proportion is re-estimated based on the remaining n-1 studies. Studies with a statistically significant influence on the fitted

model were removed as outliers and the model was re-fitted. All data analysis and visualisation was performed using the R statistical environment (version 3.6.1) using the “metafor" and "meta” packages[368]. The analysis was performed as outlined by Wang [369].

We also performed a gender-segregated analysis to ascertain a family history prevalence for males and females.

3.3.4 Results

3.3.4 (a) Systematic review

Following the removal of duplicates, the search strategy identified 1281 studies, all of which were published between 1994 and 2015 (**Figure 14**). Following a screening process which excluded 1260 unsuitable abstracts, 22 full-text articles were assessed for eligibility and 7 were selected for inclusion. To avoid over estimating family history, 15 of the 22 studies were removed due to inadequate clinical confirmation in affected relatives. The remaining full texts consisted of seven cohort studies with an estimated prevalence of family history of CH ranging from 4.9% to 26.3%. After being supplemented with local cohort data, [O’ Connor (2020)], the included studies consisted of a total of 3,415 CH patients, 238 of which reported a positive family history of cluster headache. **Table 13** summarises the extracted data.

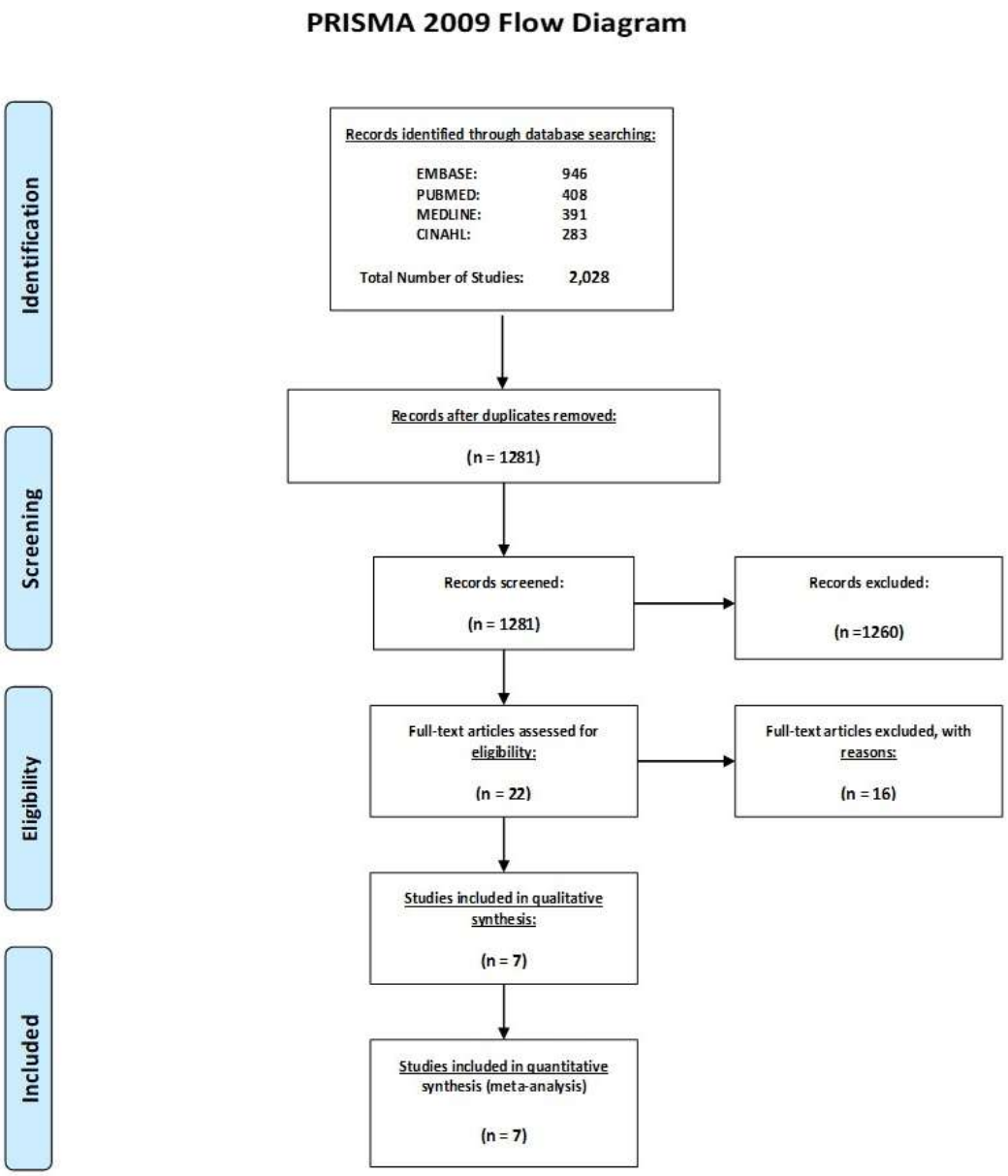


Figure 14: Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. Schematic showing breakdown of screening process. doi:10.1371/journal.pmed1000097

Table 13: Data extracted from identified studies

Country	Authors	Study Design	Diagnostic Criteria	Method of data acquisition	Sample size (n)	Probands with FCH (n, %)	Males with FCH	Females with FCH
USA	Kudrow and Kudrow (1994)	Retrospective Study	Adhoc Committee on classification of headache 1962 ICHD-1	Proband interview Semi-structured phone interview Direct examination	300	26 (8.6%)	7	19
Denmark	Russell *et al* (1996)	Retrospective Study	ICHD-1	Questionnaire Semi-structured phone interview Direct examination	366	25 (6.8%)	17	9
Italian	Montagna *et al* (1997)	Retrospective Study	ICHD-1	Semi-structured phone interview	222	5 (2.25%)	NA	NA
Italian	Leone *et al* (2001)	Retrospective Study	ICHD-1	Semi-structured phone interview Direct examination	785	44 (20%)	29	15
French	El Amrani *et al* (2002)	Consecutive case-series	ICHD-1	Direct examination	220	44 (10.75%)	12	8
Italian	Torelli and Manzoni (2003)	Retrospective Study	ICHD-1	Semi-structured phone interview	186	20 (4.34%)	30	8
Italian	Taga *et al* (2015)	Retrospective Study	ICHD3-beta	Clinical documentation	691	40 (4.92%)	28	12
UK	O'Connor *et al* (2020)*	Retrospective Study	ICHD3-beta	Semi-structured phone interview Direct examination	645	48 (7.44%)	35	13

** O'Connor et al represents unpublished local cohort. CH: cluster headache,, FCH: Familial CHICHD: International Classification of Headache Disorders, , NA: Not available*

All seven studies scored 6 or higher in our risk of bias assessment Newcastle – Ottawa appraisal checklist, demonstrating a low risk of bias, therefore all seven studies were included for meta-analysis (**Table 14**).

Table 14: Newcastle-Ottawa Quality Assessment Scale for cohort studies with awarded stars per category

Study	Selection	Comparability	Outcome	Total number of stars
Kudrow and Kudrow (1994)	★ ★ ★ ★	★ ★	★	7
Russell et al. (1996)	★ ★ ★ ★	★	★	6
Montagna et al. (1997)	★ ★ ★ ★	★	★	6
El Amrani et al. (2002)	★ ★ ★ ★	★	★	6
Torelli and Manzoni (2003)	★ ★ ★ ★	★ ★	★	7
Taga et al. (2015)	★ ★ ★ ★	★	★	6
Leone et al. (2001)	★ ★ ★ ★	★	★	6

A maximum of 7 stars can be awarded in total. Selection category = maximum of 4 stars. Comparability = maximum of 2 stars. Outcome =maximum of 1 star

Estimation of relative proportion of effected probands with positive family history of CH:

The study data was transformed using the logit-transformation and normality was confirmed using density plot (**Figure 15**) and Shapiro-Wilk test (p=0.9889).

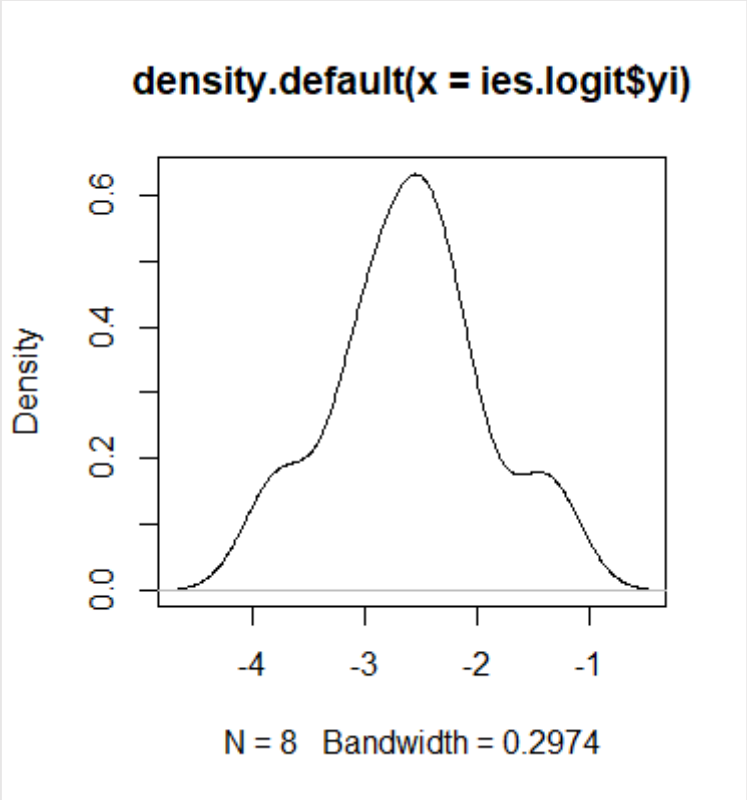


Figure 15: Density plot confirming normality following transformation of data.

The random-effects model identified a high degree of study heterogeneity (I^2 = 90.95%, p < 0.01). It estimated the prevalence of family history in CH patients to be 7.21% (95% CI:4.69–10.92%). Inspection of the externally studentized residuals indicated that the Leone et al study had a high z-value (3.05) and therefore may be an outlier. Diagnostic plots also indicated the presence of an outlier and are shown in **Figure 16**.

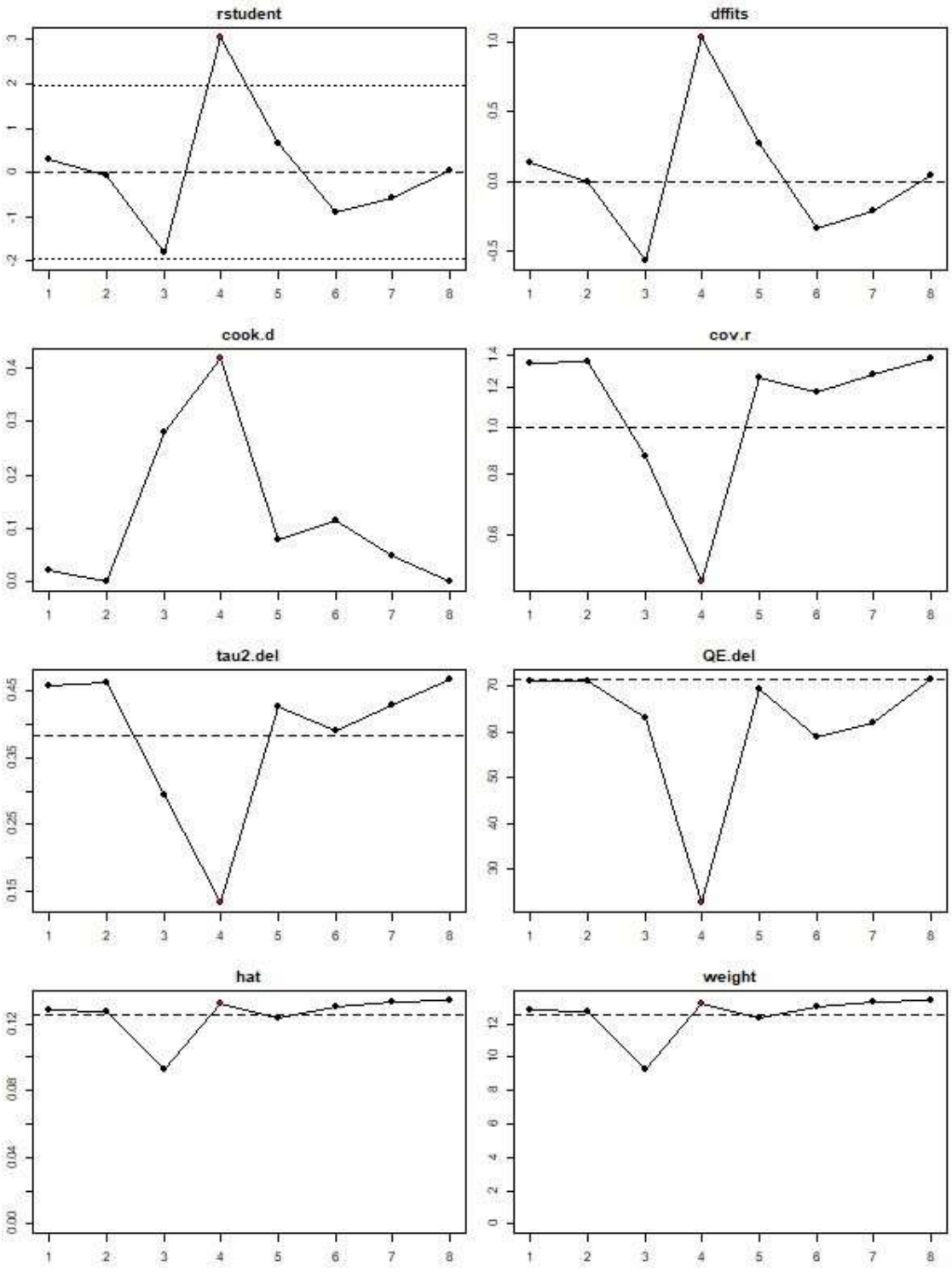


Figure 16: Diagnostic plots indicating outlying study

Leave one out (LOO) analysis revealed that removal of the Leone et al study produced the greatest reduction in the I^2 heterogeneity from 90.95% to 76.75% compared to removal of other studies (**Table 15**). As a result, the Leone et al (2001) study was removed from the final meta-analysis. The remaining seven studies reported the proportion of family history in CH between 2 and 11%. The estimated true proportion of CH patients with a positive family history was 6.27% (95% CI:4.65–8.40%) and overall I^2 of 73% (**Figure 17**).

Table 15 : Leave one out (LOO) analysis : removal of Leone (2001) study reduced the I^2 heterogeneity from 90.95% to 76.75%.

Study	estimate	z-value	p-value	ci.lb	ci.ub	Q	Qp	tau2	I^2	H^2
Kudrow Kudrow	0.068522	-8.03122	9.65E-16	0.037454	0.122093	71.2786	5.55E-14	0.573291	93.12907	14.55407
Russell	0.071372	-7.8481	4.22E-15	0.038919	0.127302	70.89675	6.67E-14	0.5812	93.23762	14.78768
Montagna	0.081983	-9.40515	5.20E-21	0.051217	0.128724	62.86965	3.10E-12	0.357634	90.75166	10.81275
Leone	0.060042	-13.8083	2.27E-43	0.041438	0.086248	20.87984	0.000854	0.181164	76.74742	4.957224
El Amrani	0.066217	-8.41919	3.79E-17	0.036885	0.116062	69.25927	1.46E-13	0.535263	92.97622	14.23735
Torrelli Manzoni	0.0775	-8.10609	5.23E-16	0.044122	0.132626	57.60195	3.80E-11	0.499239	91.92747	12.3877
Taga	0.075286	-7.88475	3.15E-15	0.04182	0.131849	60.86631	8.05E-12	0.544286	92.12084	12.69171

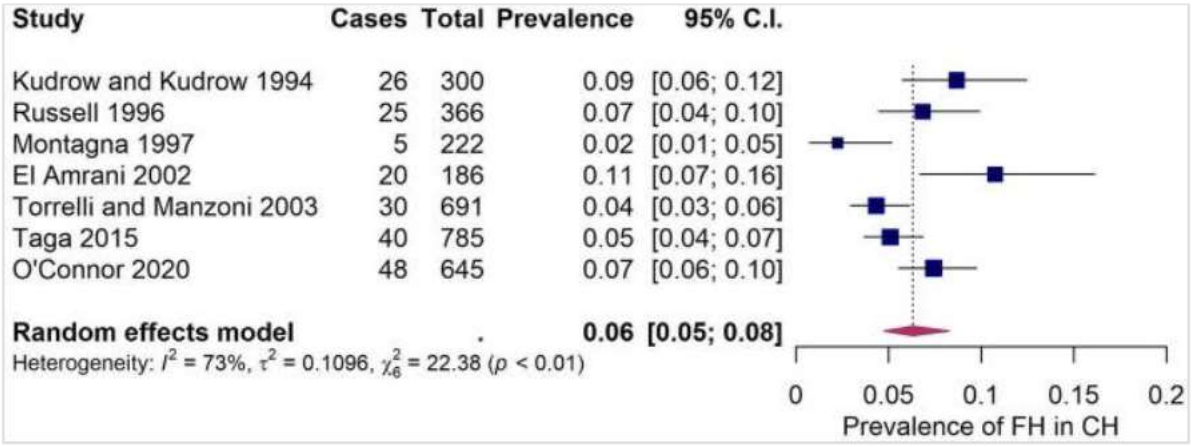


Figure 17: *A random effects model was fitted to estimate the true prevalence of family history in cluster headache patients. The study author and year (study), total number of cases with a positive family history (cases), total number of participants (total), prevalence proportion (prevalence) and 95% confidence intervals (95% C.I) are displayed along with measures of study heterogeneity. All values rounded to one significant figure.*

Moderator analysis was then performed in order to identify any potential confounding variables, in particular: the year of publication, sample size, and study design. Moderator analysis showed both sample size and year of publication did not show evidence of influencing the study outcome ($p > 0.05$).

Finally, in the seven studies we assessed potential publication bias using a funnel plot and Egger regression testing illustrating outliers. Funnel plots were roughly symmetrical (**Figure 18**).

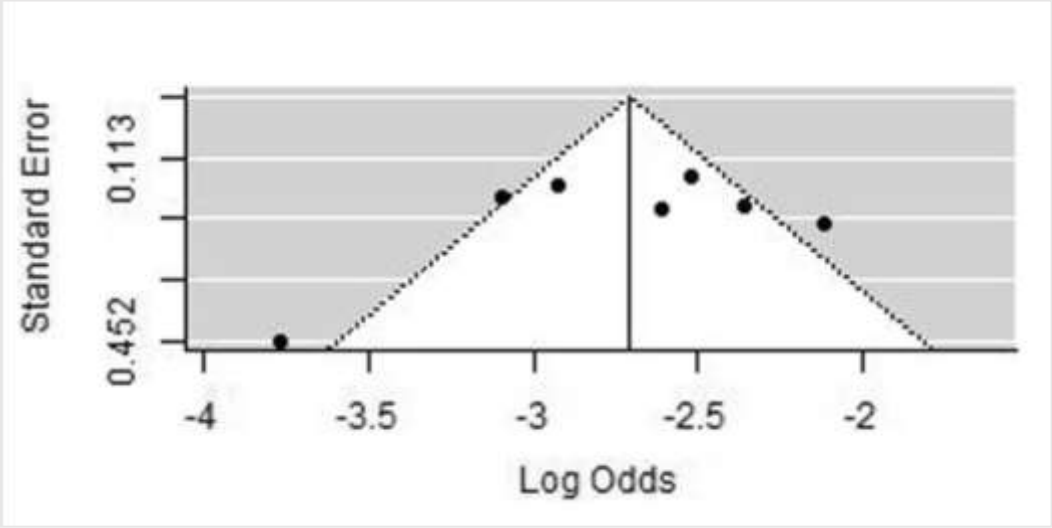


Figure 18: *Funnel plot to assess potential publication bias. The x-axis shows the estimated prevalence (log odds) compared to the y axis which shows study precision in the seven selected studies.*

Eggers test was not significant (p=0.1701) indicating no clear evidence of publication bias. Performing the same analysis with the inclusion of the O'Connor cohort did not affect this result (p=0.1127).

3.3.4 (b) Estimation of family history prevalence in male and female patients

In total, studies that had gender segregated numbers: Kudrow and Kudrow (1994), Russell (1996), Leone (2001), El Amrani (2002) Torelli and Manzoni (2003), Taga (2015) and our unpublished cohort: O'Connor (2020) were included. We represented each study with a male and female estimate of family history prevalence. Leone (2001) was identified as an outlier in our initial analysis. We continued to exclude this study for two reasons: our method of analysis results in two separate entries per study (one male, one female), causing influential studies to be over-represented which may skew outlier analysis, and secondly, these estimates are not truly independent. Based on the identified literature, we chose not to assume a common between-study variance component across males and females. Therefore, we did not pool within-group estimates of τ^2. We used a mixed-effects model whereby all summary effect sizes were calculated using separate τ^2 within each subgroup (males and females), then two separate random effects models were fitted. We combined the estimated statistics from each model and fitted a fixed-effect model as outlined by Wang [369].

Overall, Leone et al and Torelli and Manzoni et al were excluded as outliers. The fitted models for the subgroups estimated the prevalence of familial CH at 6.47% (95% CI: 5.27-7.92%) and 9.26% (95% CI: 6.29-13.43%) for males and females respectively **(Figure 19).**

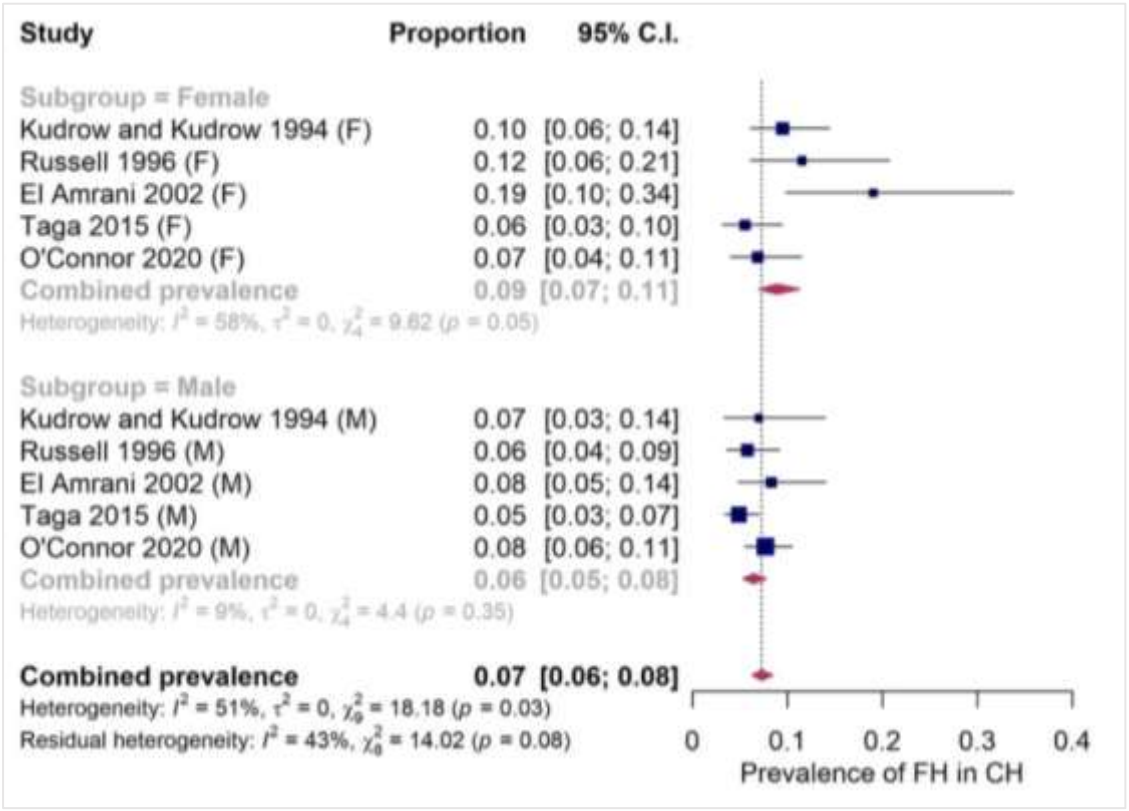


Figure 19: *A random effects model was fitted to each subgroup which estimates the true prevalence of family history in female and male cluster headache patients. The study author and year (study), total number of cases with a positive family history (cases), total number of participants (total), prevalence proportion (prevalence) and 95% confidence intervals (95% C.I) are displayed along with measures of study heterogeneity. All values rounded to one significant figure.*

The overall I^2 for the male only model was just 9.14% and heterogeneity was no longer statistically significant (p= 0.354), while the I^2 for the female model was 58.42% and remained significant (p=0.047). While the summary estimate was larger for females than males, the results of the test of moderators revealed the subgroup summary estimates were not significant (p= 0.106). Therefore, we combined the estimates, producing a similar, albeit slightly higher estimate as our initial analysis of 6.98% (95% CI:5.83- 8.35).

Moderator analysis revealed an association between the model estimates and study size (p= 0.0176) (**Figure 20**). The R^2 indicated that approximately 64.2% of the true

heterogeneity in the observed effect sizes are accounted for by sample size. This may potentially explain the heterogeneity seen in the female-only estimates as overall there were fewer females across studies. The year of publication did not significantly influence the estimates (p= 0.2186).

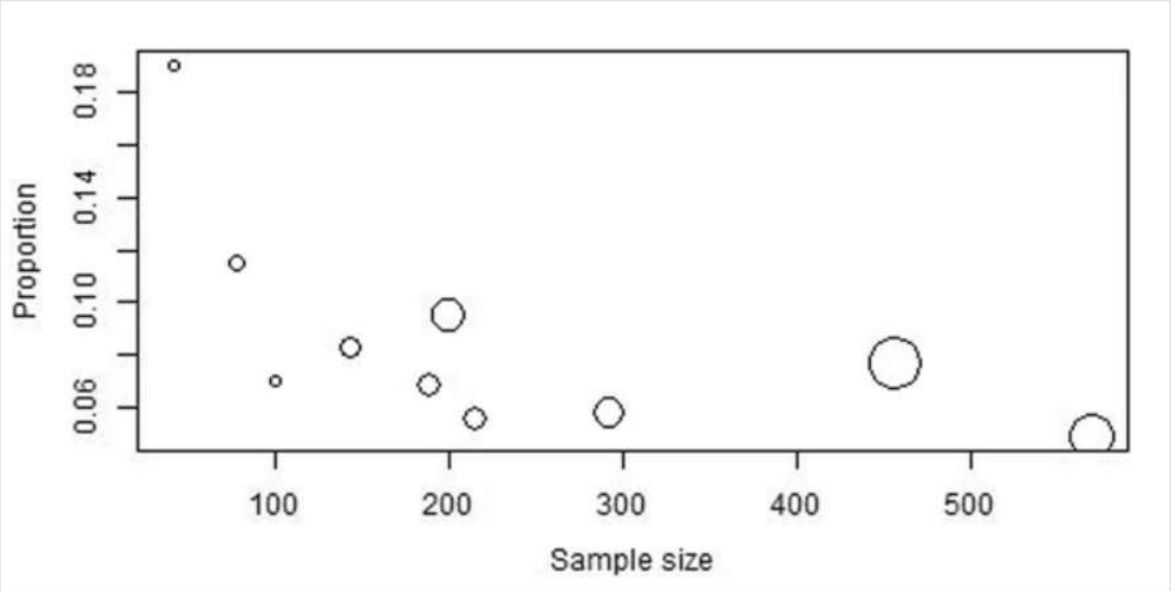


Figure 20: *Funnel plot to assess potential publication bias. The x axis shows the estimated prevalence (log odds) compared to the y axis which shows study precision in the seven selected studies.*

Finally, as before, a funnel plot (**Figure 21**) showed low evidence of asymmetry reflective of no significant publication bias (p=0.071).

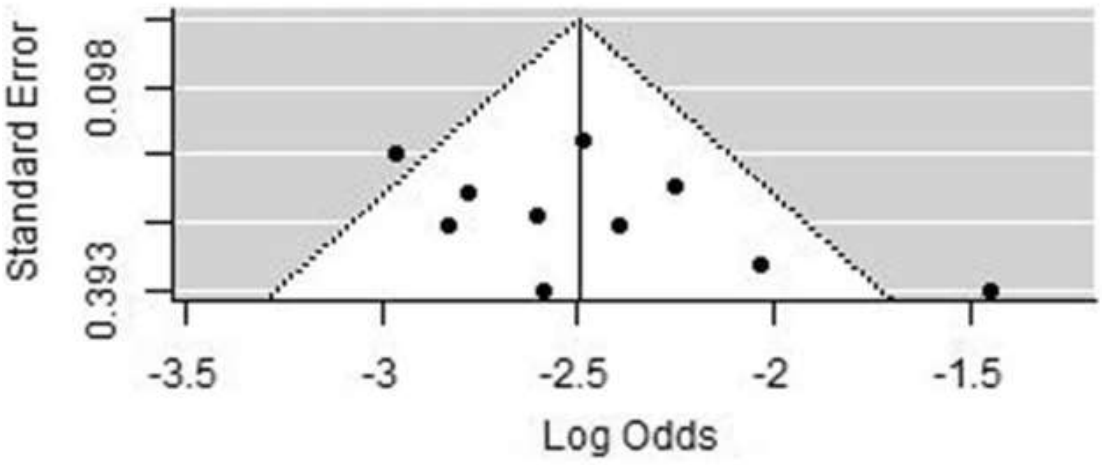


Figure 21: *Funnel plot which is symmetrical showing no significant publication bias.*

3.3.5 Discussion

Previously, a number of studies have attempted to report the prevalence of family history in CH patients. Despite this, the exact prevalence of familial CH remains disputed, with some studies estimating a prevalence as low as 2.25% and others as high as 20% [297, 298]. Here, employing a robust systematic review and meta-analysis, we provide a more accurateprevalence of familial cluster headache of approximately 6.27%. To our knowledge, our analysis provides the most accurate estimation of familial CH to date.

Several epidemiology studies have reported higher prevalence of familial CH, possibly reflecting inflated estimations [95, 120, 298]. This disparity between studies is likely multifactorial. Notably, we excluded studies from our systematic review that lacked a confirmed clinical diagnosis in affected relatives. The high percentage of misdiagnosis or delay in diagnosis of CH by physicians is testament to the specialist clinical expertise required to provide an accurate diagnosis [118, 119, 488]. Our unpublished cohort was representative of this challenge, whereby a diagnosis of CH was inappropriately assigned to relatives by 18 (27.3%) probands. Therefore, clinical verification of a presumed diagnosis of CH in a relative should be a critical requirement in any study reporting family history.

The high degree of heterogeneity between studies included in our analysis is likely due to a number of factors including population stratification, differing reporting methods, an ambiguous definition of family history, variation in diagnostic criteria, and atypical phenotypes. Through ascertaining which studies adhered to strict eligibility criteria, we were able to homogenize data and derive a pooled estimate for the

frequency of family history in patients with CH. Of note, removal of the Leone et al (2001), study as an outlier noticeably reduced the heterogeneity in our analysis.

The considerably higher rate of family history in this cohort [20% (n=44/220)] was attributed to the mode of data collection. Probands who were directly interviewed reported a considerably higher rate of family history compared with those recruited by postal questionnaire. Furthermore, a significant proportion of relatives were previously undiagnosed, investigated as part of the study and subsequently received a diagnosis. As this study was conducted almost twenty years ago, the under-diagnosis of CH is not unsurprising. However, this may also be compounded by intra-familial clinical variability and presence of phenotypes atypical for CH. The documented preponderance of relatives with atypical cluster headache in these studies complicates this further [489, 490]. These cases are often omitted from epidemiological studies as they do not strictly fulfil diagnostic criterion, but perhaps represent part of a clinical spectrum associated with intergenerational genetic variation.

The high degree of variance in the reported estimates was illuminated further by a gender segregated analysis, which revealed that although the prevalence of family history was higher in females than in male probands, this difference was not significantly different. This conflicts with some reports which found a significant difference between gender (Kudrow and Kudrow 1994, Taga 2015). An explanation for this seemingly increased prevalence of family history in females is that CH is more common in males, therefore published studies tend to have larger numbers of male probands. Thus, the estimates of family history prevalence are more precise for males than females.

We conducted a moderator analysis which revealed that study size influences the estimated prevalence. This potentially explains the discordance in observed prevalence between genders as the median number of male probands was 54% higher than the number of female probands across the five studies in our segregated analysis. Therefore, while we can estimate the prevalence of family history in male CH patients with a high degree of confidence, we found no convincing evidence that there is an increased prevalence of familial CH in females. Ultimately, further studies with a larger number of female probands are needed to determine any difference between genders, though this will likely have logistical challenges.

Our results add to the body of evidence suggesting a familial aggregation of CH and a role of genetic variation in its aetiology. This is further supported by several reports of concordance of CH reported amongst monozygotic twins [287-290] and genetic studies demonstrating association with variation in candidate genes [312, 322, 325, 491]. It can be hypothesised that families share similar environmental risk factors which may also contribute to the development of the CH phenotype. However, it is difficult to ignore consistent evidence showing CH to be more common in related individuals than in the general population, implying a possible genetic predisposition. The exact contribution of familial risk to CH is not yet clearly understood and is complicated by complex pedigrees which often demonstrate reduced penetrance [302]. Large, sufficiently powered, population-based studies are needed to ascertain an accurate estimation of genetic risk. This would further inform genetic studies and provide optimal genetic counselling to sufferers and their families.

There are limitations to this study including a dependence on the interpretation on published data which limits our ability to explore clinical data in independent studies and provide a rigorous evaluation of factors influencing family history. Another

limitation is potential recall bias whereby patients with CH are more likely to recount symptoms of the condition in a relative than those without CH. Furthermore, restricting inclusion to studies where cluster headache was confirmed in a relative, while improving accuracy, removed larger, population-based studies from our analysis. The small number of studies were also confined to high income settings in North America and Europe which impedes generalisability.

In conclusion, from this systematic review and meta-analysis, the prevalence of family history in CH was estimated to be approximately 6.27%. Additionally, contrary to previous findings, I was unable to confirm higher rates of familial history in female suffers. These results provide a robust estimation of the prevalence of familial CH and support the hypothesis of a potential genetic risk factors predisposing to the condition.

3.4 The Clinical Characteristics of Familial Cluster Headache

Statement of contribution: My contribution involved collection of data, confirmation of family history and phenotyping of affected relatives and interpretation of the findings. Statistical analysis was carried out by Dr. Elham Nikram.

3.4.1 Introduction

The familial aggregation of cluster headache (CH) has been described across a number of populations [292, 294, 485]. A number of factors including population stratification and reporting methods may contribute to this discrepancy. The largest study, comprising of 1,720 CH patients, identified a positive family history in 75 patients, just over 4% of their cohort [489]. Recently, a systematic review of all published data reporting familial CH estimated a rate of 0-22%, with a median rate of 8.2% [492]. This is slightly higher than my meta-analysis which suggested a rate of approximately 6.27% [493]. Overall, the cumulative evidence indicates that the number of affected individuals within families exceeds the estimated prevalence of CH occurring sporadically within the general population (1 in 1000 people)[122].

Complex inheritance patterns, poor penetrance, intra-familial clinical heterogeneity, and the existence of atypical phenotypes within families impede familial genetic studies [489, 490]. The majority of genetic studies to date focus on candidate genes. For example, *HCRTR2* involved in the orexinergic system, has previously shown an association [318-320]. However, additional population based studies have failed to reproduce these findings [309, 310, 321]. Other plausible candidates have also demonstrated an association, including *CLOCK* , *ADH4* and *ANO3* [311, 322, 494]. These findings have yet to be replicated [76, 307-310, 323].

The majority of familial CH cases exhibit an autosomal dominant mode of inheritance, however, cases of autosomal recessive inheritance are also described[303]. Penetrance appears to be lower in women than in men, with prominent father-to-son transmission [301, 303, 489, 495]. This lack of consistency across pedigrees may indicate loci heterogeneity - the cumulative effect of more than one variant or the influence of genetic and environmental modifiers.

The phenotypes of familial and sporadic CH appear to have similar clinical features [300, 485, 486]. An earlier age of onset has been observed in the relatives of sufferers [295, 300]. Sporadic CH shows a clear male preponderance compared to familial CH where the gender ratio appears lower [298, 301, 485, 490, 495]. No study had performed an in-depth interrogation of the precise clinical characteristics specific to familial CH.

3.4.2 Aims

1. Estimate the occurrence of a family history in our cohort of CH patients
2. Identify the likely mode of inheritance in these families.
3. To delineate the differing clinical parameters between patients with familial CH compared to those with the sporadic form.

3.4.3 Methods

3.4.3 (a) Cohort collection

This is described in detail in the methodology section (Section 2.2.2).

3.4.3 (b) Statistical analysis

To identify differences in clinical characteristics, cases of familial CH were compared to those with sporadic CH. In this analysis, descriptive statistics were expressed as a mean with standard deviation (SD). Before starting the analysis, missing values were accounted for using imputation techniques based on random forests. As the dataset

was highly imbalanced, the ROSE algorithm was used to balance the data [457]. The ROSE function creates an artificially balanced sample according to a smoothed bootstrap approach. Univariate and multivariate analysis was then performed using the ROSE-adjusted sample (Rose sample).

In the univariate analysis, a Mann-Whitney U nonparametric test was utilised for continuous data and a Fisher exact or Chi2 test for categorical data. For multivariate analysis, the LASSO algorithm was used to select relevant explanatory variables [458]. The LASSO performs automatic variable selection and has the capacity to select groups of correlated variables. A logistic regression model was then fitted with the variables selected by the LASSO. The analysis was performed using R; the random Forest, ROSE and glmnet packages were used [431, 459]. The threshold for statistical significance was set to $p \leq 0.05$.

3.4.4 Results

A total of 645 patients were included in the study. Of these, 456 (70.7%) were male. A family history of CH was reported in 66 patients (10.2%), the remainder were categorised as sporadic CH and used as controls. Probands were excluded because their affected relative(s) were deceased and lacked an official diagnosis (n=7), they did not fulfil the ICHD3β diagnostic criteria (n=6), and they declined participation or were uncontactable (n=5). Overall, forty-eight (7.4%) individuals had a confirmed family history of CH.

In the familial CH cohort, twenty-seven patients had episodic CH and twenty-one had chronic CH. The mean age of cases was 48.9 years (SD 12.05) and the mean age of onset was 28.48 years (SD 13.09). The mean follow-up time at clinic was 7.08 years (SD3.8) years. The mean duration of attacks was 67.2 minutes (SD 43.6) and the

mean frequency of attacks per day was three (SD 2). Only one patient lacked autonomic symptoms but experienced restlessness. In terms of concomitant headache, fifteen cases had concurrent migraine and three were diagnosed with a concomitant SUNCT (Short-lasting unilateral neuralgiform headache with conjunctival injection and tearing).

Four affected family members were observed in three family pedigrees, 11 families had three affected individuals and the remaining families consisted of only two affected individuals. One set of concordant monozygotic twins with no other affected family members also featured in the cohort. The most common mode of inheritance observed was consistent with autosomal dominant transmission, observed in forty families. However, of these, nine families exhibited evidence of reduced penetrance. The remaining eight had a pattern more consistent with an autosomal recessive pattern of inheritance. Transmission from parent to child was the most frequent mode of inheritance. In one family, both parents of the proband suffered from CH. All pedigrees are available in appendix (section 3.4.3).

3.4.4 (a) Univariate analysis

Univariate analysis of ROSE-adjusted dataset identified several significant variables relevant to the familial CH group. These included a younger age of onset (27.53 years, SD 14.25) compared to the sporadic group (31.80 years, SD 14.27, $p<0.001$). Attack duration was shorter in familial CH group (70.35 +/- 49.42 minutes versus 91.49 +/- 90.86 minutes, $p <0.001$). Autonomic symptoms were more prominent in patients with a family history of CH including eyelid oedema (176 [56.23%] vs 128 [38.55%] ,$p <0.001$), conjunctival injection (270 [86.26%] vs 247 [74.39%],$p < 0.001$), lacrimation (283 [90.41%] vs 281 [84.63%], $p =0.03$), nasal blockage (278 [88.81%] vs 218 [65.66%] $p<0.001$), facial sweating (201 [64.21%] vs 175 [52.71%], $p =0.003$), or

flushing (142 [45.36%] vs 123 [37.04%], p =0.03). An occipital (42[13.41%] vs 82 [24.69%], p <0.001) and frontal (86 [27.47%] vs 119 [35.84%], p =0.02) location of pain was more frequently identified in the sporadic group. Concomitant SUNCT occurred more frequently in the familial group (22 [7.02%] vs 8 [2.40%], p =0.009). In addition, a poor response to high-flow oxygen as a treatment was significant in those with a positive family history (response rate of 65 [20.76%] vs 248 [74.69%], p= 0.002) (**table 16**).

Table 16: Baseline demographics and clinical characteristics of cohorts, with corresponding univariate analysis following imputation and re-balancing of cohorts.

Cohorts	Demographics and Clinical Characteristics		Imputed and re-balanced cohorts & Univariate Analysis		
	Familial CH (n=48)(%)	Sporadic CH (n=597)(%)	Familial CH ROSE sample (n=313)(%)	Sporadic CH ROSE sample (n=332)(%)	*P value*
Age	48.91+/-12.05	49.49+/-12.46	47.93+/-12.91	49.94+/-13.56	0.09
Gender M:F	35 : 13	421:176	225:88	237:95	0.95
Age of onset	28.48+/-13.09	31.29+/-13.13	27.53+/-14.25	31.80+/-14.27	**<0.001**
Chronic	21 (43.75)	285 (47.73)	137 (43.76)	166 (50.00)	0.13
Site					
Orbital	33 (68.8)	419 (70.18)	228 (72.84)	227 (68.37)	0.24
Frontal	14 (29.1)	203 (34)	86 (27.47)	119 (35.84)	**0.02**
Temporal	22 (45.8)	304 (50.9)	147 (46.96)	171 (51.50)	0.28
Parietal	7 (14.5)	106 (17.7)	48 (15.33)	70 (21.08)	0.07
Occipital	9 (18.75)	122(20.43)	42 (13.41)	82 (24.69)	**<0.001**
Cheek	14 (29.2)	136 (22.8)	90 (28.75)	68 (20.48)	**0.018**
Teeth	4 (8.3)	59 (9.88)	33 (10.54)	42 (12.65)	0.47
Ear	4 (8.3)	59 (9.88)	24 (7.66)	35 (10.54)	0.25
Autonomics					
Absence of Autonomics	1 (2.1)	13 (2.2)	3 (0.95)	13 (3.91)	0.62
Ptosis	28 (58.3)	345 (57.8)	207 (66.13)	211 (63.55)	0.54
Eyelid oedema	21 (43.8)	213 (35.7)	176 (56.23)	128 (38.55)	**<0.001**
Conjunctival Injection	34 (70.8)	401 (67.2)	270 (86.26)	247 (74.39)	**<0.001**
Miosis	1 (2.1)	22 (3.7)	49 (15.65)	35 (10.54)	0.07
Lacrimation	39 (81.25)	472 (79.1)	283 (90.41)	281 (84.63)	**0.03**
Nasal blockage	36 (75)	350 (58.6)	278 (88.81)	218 (65.66)	**<0.001**
Rhinorrhoea	30 (62.5)	364 (60.9)	245 (78.27)	244 (73.49)	0.18
Facial Sweating	23 (47.9)	295 (49.4)	201 (64.21)	175 (52.71)	**0.003**
Flushing	19 (39.6)	231 (38.7)	142 (45.36)	123 (37.04)	**0.03**
Aural Fullness	8 (16.7)	105 (17.6)	88 (28.11)	71 (21.38)	0.06
Agitation	40 (68.9)	498 (83.4)	288 (92.01)	287 (86.44)	**0.03**
Frequency / Duration of attacks					
Average Attacks per day	3.08+/-2.18	2.99+/-2.18	3.11+/-2.56	2.96+/-2.29	0.50
Average Duration (mins)	67.22+/-43.56	94.97+/-132.36	70.35+/-49.42	91.49+/-90.86	**<0.001**
Associated Headaches					
Migraine	15 (31.3)	180 (30.2)	85 (27.15)	97 (29.21)	0.62
TACS	3 (6.25)	20 (3.3)	22 (7.02)	8 (2.40)	**<0.009**
Treatment Response					
Response to Oxygen	9 (18.7)	84 (14.1)	65 (20.76)	248 (74.69)	**0.002**
Response to sc Sumatriptan	6 (12.5)	29 (4.8)	85 (27.15)	80 (24.09)	0.42
Intractable to preventative treatment	24 (50)	244 (40.8)	227 (72.52)	217 (65.36)	0.06

Abbreviations: CH: Cluster Headache, M: male, F: female, sc: subcutaneous, TACS: Trigeminal Autonomic Cephalalgia

3.4.4 (b) Multivariate analysis

Results of multivariate analysis are summarised in **table 17**. Consistent with univariate analysis, we identified a significant association between prominence of nasal blockage and the familial subgroup (OR 4.06, 95% CI 2.600-6.494; p<0.001). Concomitant SUNCT was associated with familial CH (OR 3.76, 95% CI; 1.572-9.953; p=0.004). Correction for multivariate modelling an odds ratio close to one for age of onset (OR 0.98, CI 0.971-0.996%, p <0.009) and attack duration (OR 0.997, 0.994-0.999 p 0.012). This is owing to the large SD for both familial CH and sporadic CH for these variables (**Figures 22** and **23**, **Tables 17** and **18**).

Table 17: Summary of significant variables identified on multivariate analysis.

Predictive factor	OR	95% CI	p value
Age of onset	0.98	0.971-0.996	0.009
Presence of nasal blockage	4.06	2.600-6.494	<0.001
Attack duration	0.997	0.994-0.999	0.012
Associated SUNCT	3.76	1.572-9.953	0.004

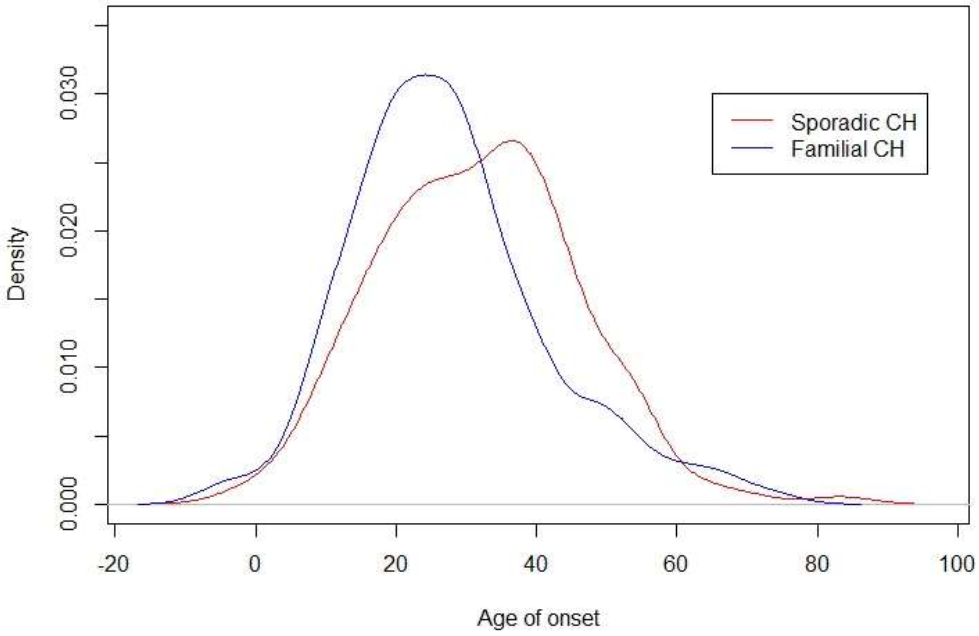


Figure 22: *Density plot of 'age of onset' using ROSE sample.*

Table 18: Statistical details for Age of onset on ROSE sample.

	Median	Mean (SD)
Familial CH (n=313)	26.3	27.5(14.3)
Sporadic CH (n=332)	31.7	31.8 (14.3)

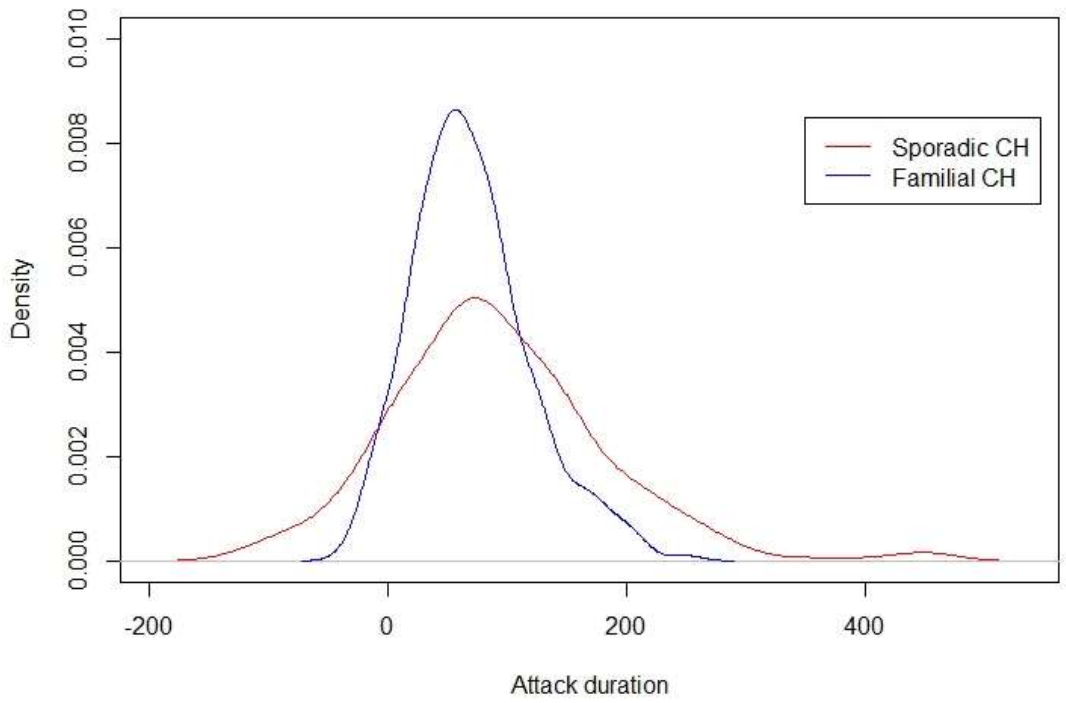


Figure 23: *Density plot of 'attack duration' using ROSE sample*

Table 19: Statistical details for 'attack duration' on ROSE sample.

	Median	Mean (SD)
Familial CH (n=313)	26.3	27.5(14.3)
Sporadic CH (n=332)	31.7	31.8 (14.3)

3.4.5 Discussion

This study identified a possible family history of CH in 10.2% of cases. Further evaluation of suspected affected relatives demonstrated a familial rate of 7.44%. A diagnosis of CH was incorrectly attributed by probands to five individuals who fulfilled the ICHD3b criteria for migraine. Similarly, one relative was excluded due to atypical

features of CH precluding clinical confirmation. As CH is a clinical diagnosis, this disparity reflects the documented diagnostic challenges associated with headache disorders, often requiring specialist input[118]. Furthermore, it highlights the weaknesses of a family history taken by proxy and the essential requirement for clinical validation to provide accurate rates of family history in headache epidemiological studies.

My findings are similar to rates reported in several studies where a diagnosis of CH was confirmed in an affected relative [297, 302, 486, 487] and the cumulative estimates of 6.27-8.2% [492, 493]. This provides further evidence for the role of heredity in the aetiology of CH. The extent of this, and the influence of environmental factors on the development of disease, remains unclear. Previously, a relative risk as high as 45.6-fold was predicted for individuals with a first degree relative with CH [495]. Other studies describe a much lower familial prevalence [301]. These findings should be examined in the context of an overall increase in the prevalence of CH due to recent improvements in awareness of the condition and adherence to diagnostic criterion [115, 120].

The presence of relatives with atypical CH within families, who perhaps reflect part of a clinical spectrum, also impede a precise evaluation of the rate of familial CH [490]. Similar to our cohort, these cases are often omitted from epidemiological studies as they do not strictly fulfil diagnostic criterion, but perhaps carry the same genetic risk with alternate modifiers. A later age of onset may also hinder the recognition of familial cases. In contrast, shared environmental risk factors may influence the development of the phenotype and therefore contribute to familial clustering. Nevertheless, consistent evidence indicating a higher incidence of CH in families with other affected members compared with the general population suggests a predisposing genetic risk which remains unidentified. To date, there has been only one hypothesis-free genetic familial study that examined linkage in five Danish pedigrees. It did not produce a

significant LOD score but showed a suggestion of linkage at loci on chromosomes 2, 8 and 9. Unfortunately, this was not replicated when extended to the entire cohort [321].

A review of pedigrees revealed a pattern most consistent with an autosomal dominant mode of inheritance in the majority of cases (40/48 families). Some families exhibited evidence of reduced penetrance, however, this could also be reflective of a cumulative effect of a number disease-associated variants. The remaining pedigrees, more consistent with an autosomal recessive inheritance pattern, were small in size and may also represent a dominant mode of inheritance with reduced penetrance. The apparent incongruity in inheritance patterns across families correlates with previous familial studies in CH [300, 302, 303]. Explanations for this includes reduced penetrance, loci heterogeneity, genetic pleiotropy, and the presence of modifying variants, which augment or attenuate the effect of inherited pathogenic mutations.

In contrast to previous studies, we did not identify a higher proportion of females amongst familial cases [487, 496]. This is possibly reflective of the evolving demographics of CH in more recent years, with a considerable increase in the diagnosis of female patients [123]. In earlier studies, it is possible that an underrepresentation of females provided insufficient power for gender segregation analysis [493]. A younger age of onset has been observed in familial CH, particularly in female patients [486, 487]. The possibility of anticipation has previously postulated, however, this would require large pedigrees exhibiting a decreasing age of onset across successive generations [300]. This difference may indicate an earlier recognition of familial CH in patients acquainted with the condition in their relatives. It is also possible that variability in age of onset is determined by distinct genetic variants inherited within families. We did not identify a statistically significant earlier age of onset on multivariate analysis. Larger, well-designed studies are required to established this definitively.

The autonomic symptom of nasal blockage was significantly more prevalent in cases of familial CH. Larger studies are required to examine this association however it raises interest in genetic candidates associated with pain conditions with prominent dysautonomia such as *SCN9A* [OMIM 603415] in paroxysmal extreme pain disorder (PEPD) and *SCN11A* [OMIM 604385] in familial episodic pain syndrome (FEPS) [497, 498].

Patients with familial CH were also more likely to have a concomitant SUNCT, a TAC with an estimated prevalence of 1.2/100,000 people [499]. We see a higher volume of such patients with this condition at our tertiary referral headache clinic, but considering the rarity of this disorder and CH, their co-existence in familial cases is unlikely to be coincidental. Also, although SUNCT shares clinical features with CH, these conditions are clearly phenotypically distinct. SUNCT presents with considerably shorter attacks (1-600 seconds) and responds to different treatments including intravenous lidocaine and prophylactic lamotrigine [500].

Patients with one headache disorder are at higher risk of developing another form of headache, possibly reflecting a predisposition to primary headache due to central sensitization of the pain matrix. The co-occurrence of more than one TAC in a patient is unusual but has been observed [501-508]. It is plausible that this overlap implies a shared pathophysiological mechanism between both syndromes leading to the activation of trigeminovascular system. Furthermore, activation of the posterior hypothalamus in functional imaging is evident in both CH and SUNCT, possibly representing a derangement in the regulation of hypothalamic neurotransmitters common to both conditions [509, 510]. The higher proportion of familial cases with SUNCT and CH suggests that genetic variation may be a common denominator driving this pathophysiological pathway, thus predisposing to both syndromes.

In conclusion, a confirmed family history in 7.44% of this cohort further supports the role of heredity in the pathophysiology of CH. Additionally, I found that nasal blockage and concurrent SUNCT is distinctly more common in familial cases, potentially suggesting that genetic variation may influence phenotype. Specifically, we did not replicate the findings of a younger age of onset or difference in the gender ratio in familial cases as previously demonstrated. A limitation to this study is the small cohort size due to the rarity of familial CH, and thus these findings require replication. Nonetheless, these results add to evidence indicating that genetic variation likely contributes to the development of CH. Further studies investigating the genetic architecture of CH are required to understand the genotype-phenotype correlation and its potential impact on mechanistic studies and therapeutic intervention.

Section IV Genetic Studies

4.1 Genome-Wide Association Study of Cluster Headache

4.1.2 Statement of Contribution

I obtained ethical approval for and oversaw the standard operational procedures at five CH recruitment sites across the UK. I recruited, phenotyped and collected DNA for CH cases with clinical colleagues at these sites. I extracted DNA from majority of cases with the assistance of Janice Yip (lab technician) and prepared cases for genotyping. I conducted the initial QC of the UK data. The remaining bioinformatics was led and completed by Dr. Prasanth Sivakumar. I established an international collaboration to facilitate the replication of results. A version of this chapter is currently under review in Annals of Neurology.

4.1.3 Introduction

Genome-wide association studies (GWAS) are an effective tool to detect common genetic variants, which predisposes to the development of complex disorders, with the ultimate goal of discovering underlying biological and mechanistic pathways. This approach is based on the “common disease, common variant” hypothesis which correlates allele frequency with population prevalence, proposing that common genetic variation impacts the risk of developing common disorders [15]. Causal variants for mendelian conditions are usually rare and highly penetrant with a large effect size whereas the effect size of common risk variants is small [511]. Therefore, no single mutation can predict risk but multiple common alleles in combination can account for a proportion of the heritability of complex disorders. Genome wide association analysis uses genetic markers (usually SNP’s) distributed across the whole genome to assess the differences between cases and controls in allelic frequency between cases and

controls. Under this framework, allelic differences at SNPs demonstrating a p-value of <5x10-8 indicates a genome wide significant association with the disorder under investigation [512].

The inheritance patterns and clinical features of CH are suitable for GWAS. Firstly, it has a frequency of 1/1000 therefore, although not common, it is not rare either. Linkage and familial studies are difficult in CH as large families involving several affected relatives are uncommon. Furthermore, available pedigrees often demonstrate evidence of reduced penetrance and as CH is purely a clinical diagnosis, accurate phenotyping to illustrate segregation is challenging, particularly in the presence of atypical symptoms and phenocopies. In GWAS, cases include only unrelated individuals which mitigates this issue. Secondly, candidate gene association studies have previously failed to provide reproducible results. In such studies, candidate genes were selected based on their putative pathophysiological role in CH. However as the aetiology of CH is not yet entirely understood, this methodology is unreliable. GWAS provides a hypothesis free approach, which can potentially lead to the conceptualization of alternative disease mechanisms.

GWAS have previously enabled an unprecedented understanding into the genetic risk factors for several other neurological conditions including migraine[78-80]. The first GWAS for migraine was published in 2010 identifying a genome-wide significant association on 8q22.1[78]. Subsequent studies identified additional SNPs associated with migraine risk, the vast majority of which replicated in independent cohorts indicating a robust association. This included a meta-analysis involving a large cohort of 59,674 cases and 318,326 controls, which identified 44 genome-wide significant SNPs at 38 distinct loci [513]. Expression quantitative trait loci (eQTL) and pathway analysis of these risk loci favoured an aetiological role for vascular mechanisms but

also implicated neuronal migration and ion homeostasis in migraine susceptibility[79, 513]. Despite this, the complexity of the underlying genetic architecture of migraine and associated functional consequences remains unsolved.

There are limitations to this approach. Firstly, compared with migraine that has a prevalence of 2.6% and 21.7%[514], CH occurs with a frequency of approximately 1/1000. Detection of variants with a small effect size requires a large cohort of cases. Therefore, collaboration across sites and the formation of large consortia is essential to a sufficiently powered study. Secondly, genome wide significant SNPs merely label a disease locus, indicating a causal variant is within a 'block' of linkage disequilibrium. The attribution of causality to a variant and its consequences requires further genetic and functional investigation. This challenge is evident in migraine studies where, a decade after the first GWAS and the identification of multiple replicable associations, there is a lack of translatable clinically relevant conclusions[515].

Compared to other neurological conditions with a comparable frequency, such as multiple sclerosis (MS), the CH GWAS field is trailing behind, further highlighting the need for this study. In 2007, the initial GWAS on MS was carried out and since then over 40 GWASs and several meta-GWASs have identified more than 200 susceptibility loci[516, 517]. In comparison, there has been only one published GWAS on CH to date, involving an analysis of 99 Italian cases. Ultimately, this study did not have sufficient power to demonstrate significance, but uncovered variants suggestive of significance in LD with variants in the *ADCYAP1R1* and *MME* genes, plausible candidates in CH susceptibility[343]. However, the results were not replicable in a larger Swedish candidate study[347]. Overall, an adequately powered GWAS to identify novel genetic risk variants for CH is overdue.

4.1.4 Aims and objectives

1. To perform the largest GWAS in CH to date in order to detect replicable novel genetic risk variants.
2. To explore the biological and functional consequences of these variants to gain further understanding into the aetiology of CH.
3. To replicate associations reported in previous CH GWAS and candidate association studies.

4.1.5 Methods

The analysis involved four stages. Firstly, I conducted a GWAS on UK CH cases and controls. Secondly, I performed a replication study of significant associations derived from previously published candidate association studies. Thirdly, through collaboration with the Karolinska Institutet neuroscience institute in Stockholm, a GWAS was conducted on an independent cohort of CH patients. Finally, the Swedish and UK cohorts were combined and GWAS repeated to detect additional loci and conduct a downstream analysis.

4.1.5 (a) Discovery Cohort (UK)

Participant Recruitment and Phenotyping

Information pertaining to the recruitment and phenotyping of the UK cohort and controls is provided in detail in the methods section (2.3.2, 2.3.4). The cohort demographics are summarised in **Table 21.**

DNA extraction and genotyping

This is information is detailed in methodology section 2.3.8.

Data Processing

Raw IDAT files were processed using GenomeStudio (Illumina). SAMtools and PLINK were used in subsequent data processing and analysis (34, 35). Only biallelic SNPs on chromosomes 1-22 common to the GSA and 1.2M Illumina arrays were retained for further analysis.

Quality Control

The steps involved in sample and variant QC are summarised in **table 22**. The full GWAS protocol and commands used is included in the appendix (Section IV, 4.1.5)

Sample QC

Call rate: Using PLINK[376] (--geno and –mind commands) samples with a high degree of missingness were removed from the analysis using a threshold of 0.02 to achieve a 98% genotype efficiency. This resulted in the removal of fifty-six cases and eighteen controls from the UK cohort.

Sex mismatch: This was achieved with PLINK[376] (-- check-sex command) to estimate X chromosome homozygosity rates and compared to expected sex. Two cases and three controls were removed due to sex mismatch.

Heterozygosity outlier: A scatterplot based on observed heterozygosity rate in each individual was created in R and outliers visualised. Two cases and two controls were removed at this step.

Relatedness: Pairwise kinship was revealed (-- genome command) in PLINK[376] and identity by descent (IBD) was visualised. For IBD, a pi-hat threshold of 0.2 was used to remove exclude cases. Overall, thirty-one cases and 10 controls were excluded at this stage.

Population stratification: This was corrected for using Peddy, whereby the ancestry of each sample is calculated utilising a support vector machine (SVM) trained on ancestry of individuals included in the 1000 Genomes Project[518]. Samples with genotypes predicted to be associated with samples denoted as Non-European in the 1000 Genomes project were visualized in an interactive plot and removed. This involved the elimination of sixty cases and thirty-four controls from the UK cohort (**Figure 23**).

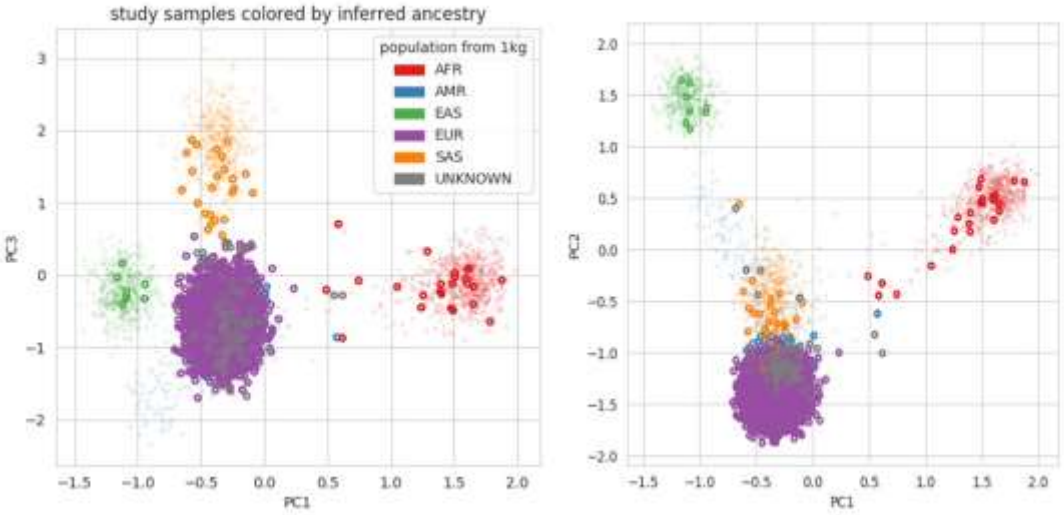


Figure 23: *Principal Component Analysis (PCA) plot generated using Peddy showing population structure of 852 CH cases and 5,614 controls. Study samples are coloured by inferred ancestry. Samples that deviated from the European control population (purple cluster) were excluded as likely to be not of European ancestry.*

SNP QC

Call rate: Using the PLINK --geno command and a filter of 0.02, SNPs that were missing in a high proportion of samples were removed.

HWE: SNPs which violated HWE (p value <1e−10 in cases and <1e−6 in controls) were removed.

Power calculation

This was calculated for 852 and 5614 controls using the Genetic Association Study (GAS) power calculator[519]. (**Figure 24**)

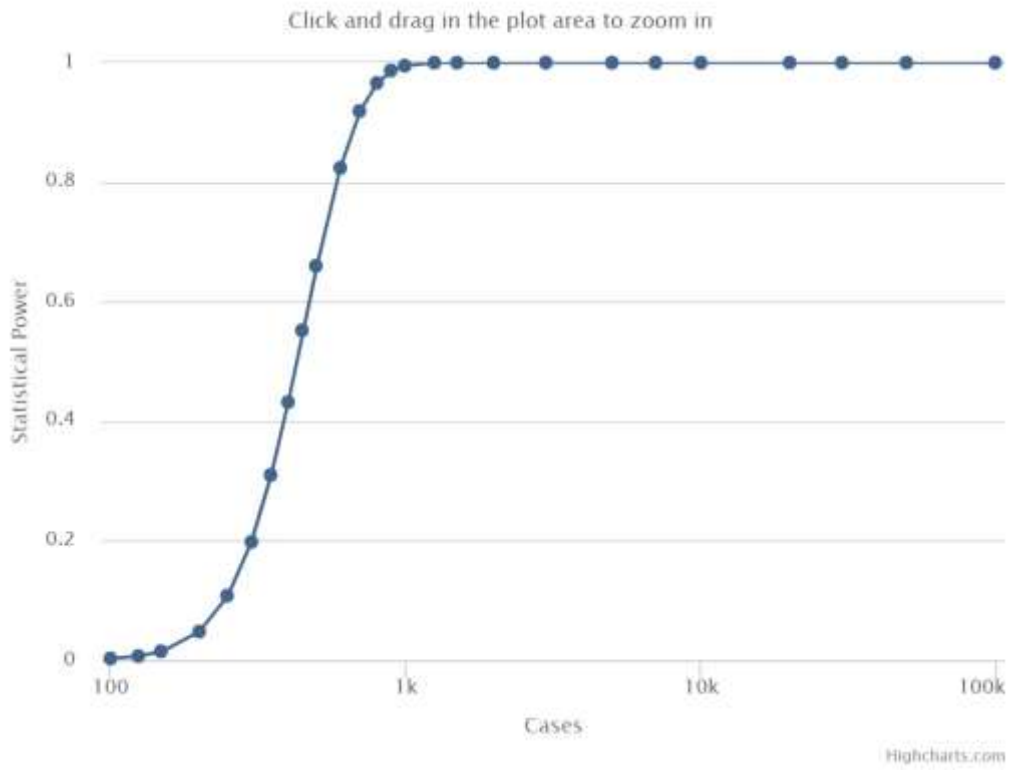


Figure 24 *Power calculation generated using Genetic Association Study (GAS) Power Calculator employing a log additive model, assuming a disease frequency of 0.001 with disease allele frequency of 0.4 and significance level of 5 x10^{-8} achieving, 80% power with a relative risk (RR) 1.42 of was achieved.*

Imputation

Before imputation, the HRC/1KG imputation preparation and checking system was employed to detect problems in the pre-imputed dataset related to strand, reference and alternate allele assignments and allele frequency differences (> 0.2) as compared to the Haplotype Reference Consortium panel v1.1[520]. Estimated haplotypes were

phased using Eagle v2.3, and imputation of cases and controls was conducted using the Michigan imputation server using the HRCv1.1 panel [378, 521]. Monomorphic SNPs and SNPs with an imputation quality score R2 of <0.3 or MAF < 0.01 were removed.

Single variant association testing

SAIGE was used to conduct single variant association testing employing a logistic mixed model [380]. The analysis was carried out on autosomal SNPs alone. Covariates used in the analysis included principal component vectors 1-20. SNPs with a p-value of $< 5x10^{-8}$, in cases versus controls, were described as exceeding genome wide significance. The SNP with the lowest p-value in each LD cluster at a significant locus was defined as the lead SNP. Results were illustrated with Manhattan and Q-Q plots which were generated using R v3.6.2[522]. Closer visualization of susceptibility loci was achieved using regional association plots generated by Locus Zoom[523]. Downstream analysis was conducted with R v3.6.2[522].

4.1.5 (b) Candidate Association analysis

To interrogate previous associations in candidate genes, I conducted a literature review and extracted previous associations achieving a significant p value in candidate association studies and GWAS associations suggestive of significance (**table 20**). Locus zoom plots were then generated to visualize these regions for evidence of an association[523].

Table 20: Candidate gene studies in CH showing a significant association.

Gene	SNP	Population	Cases	Controls	P value
CLOCK[311] (Circadian locomotor output cycles kaput)	rs12649507	Swedish	449	667	**0.02**
HCRTR2[527] (Hypocretin receptor 2)	rs2653349	Italian Grmane Meta-analysis	109 226 1167	211 266 1618	**0.0002** **0.007** **0.006**
ADH4[309, 528] (Alcohol dehydrogenase)	rs1126671 rs1126671 rs1800759	Italian Italian	110 54	203 200	**0.006** **0.03** **0.03**
ANO 3 [496] (Anoctamin 3)	rs1531394	Swedish	628	586	**0.0097**
GWAS[529]	rs10064717 rs12668955 rs147564881	Italian	99	360	1.9 x10-6 9.1 x10-6 2.5 x10-5

4.1.5 (c) Swedish Cohort

I established a collaboration with the headache research group at Karolinska University, who have previously published extensively on the genetics of CH [311, 323, 494, 524-530]. Recruitment, phenotype and genotype information was shared between our groups, under a data transfer agreement, and the Karolinska group conducted their own GWAS analysis independently using the same analysis protocol.

<u>Participant Recruitment and Phenotyping</u>

Patient recruitment of Swedish cases was primarily done at the neurology clinic at the Karolinska University Hospital (Stockholm, Sweden). Recruitment of patients occurred from 2014 to 2017. Ethical approval was obtained from the Regional Ethical Review Board in Stockholm, Sweden (registration number 2014/656-31), and informed consent from all participating individuals according to the Declaration of Helsinki. CH patients are part of a Swedish CH biobank, which has been previously described and characterized[531]. CH patients were diagnosed on the basis of the ICHD-3b[374]. The CH diagnosis were validated by specialist neurologists in addition to medical records and a diagnostic questionnaire[531]. Control genotype data was obtained from neurologically healthy controls from the multiple sclerosis consortium IMSE-II (Immunomodulation and Multiple Sclerosis Epidemiology Study) consisting of 1,226 individuals and genotyped on Infinium 24v1.0 GSA. Ethical approval was obtained from the Regional Ethical Review Board in Stockholm, Sweden (registration number 2011/641-31/4). The demographics for the UK and Swedish Cohort are summarised in **table 21**.

Table 21: Summary of clinical phenotype and demographics of cluster headache (CH) patients and controls.

Origin:	UK CH (n=852)	Swedish CH (n=591)
Gender:		
Male	546 (64%)	396 (67%)
Female	306 (46%)	195 (33%)
Smoking:		
Current smoker	204 (23.9%)	155 (26.2%)
Previous smoker	271 (31.8%)	237 (40.1%)
Never smoked	241 (28.2%)	160 (27.1%)
NA	136 (15.9%)	39 (6.6%)
Subtype:		
Episodic	448 (52.6%)	524 (88.6%)
Chronic	261 (30.6%)	64 (10.9%)
Uncharacterised	143 (16.8%)	3 (0.5%)
Concurrent Migraine:		
Migraine	195 (22.9%)	94 (15.9%)
No Migraine	460 (54.0%)	441 (74.7%)
Not available	197 (23.1%)	56 (9.5%)

Genotyping

Swedish cases were genotyped at the SNP&SEQ Technology Platform in Uppsala, Sweden, using the Infinium 24v1.0 GSA (Illumina), the same platform used for the UK cases. Swedish controls were genotyped at deCODE, Iceland using the Infinium 24v1.0 GSA (Illumina).

Quality Control (QC)

QC steps were the same for the UK and replication cohort are **Table 22.**

Table 22: Summary of independent UK and Swedish datasets and the steps taken for Sample and SNP Q.

COHORTS	UK CH n=1,003	UK controls n=463	1958bc n=2,699	NBS n=2,501	Swedish CH n=643	Swedish controls n=1,229
GENOTYPING						
Genotyping Centre	Human Genotyping Facility (HuGe-F)	Human Genotyping Facility (HuGe-F)	Wellcome Trust Sanger Institute	Wellcome Trust Sanger Institute	SNP&SEQ Technology Platform, Uppsala Universitet	deCODE, Iceland
Genotyping Array	Illumina Infinium® GSA-24v1.0	Illumina Infinium® GSA-24v1.0	Illumina 1.2M (custom) chip	Illumina 1.2M (custom) chip	Illumina Infinium® GSA-24v1.0	
SAMPLE QC						
Samples with call rate > 0.98	947 (-56)	5,663 (-18)			643 (-0)	1,218 (-11)
No sex mismatch	945 (-2)	5,660 (-3)			640 (-3)	1,203 (-15)
Not heterozygosity outlier	943 (-2)	5,658 (-2)			631 (-9)	1,185 (-18)
Relatedness IBD < 0.2	912 (-31)	5,648 (-10)			628 (-8)	1,179 (-6)
Population stratification European	852 (-60)	5,614 (-34)			591 (-32)	1,134 (-45)
For analysis	**852**	**5,614**			**591**	**1,134**
SNP QC						
Shared biallelic SNPs	119,527				549,330	
SNPs with call rate > 0.95	118,159 (-1,368)	119,526 (-1)			549,119 (-211)	549,167 (-163)
SNPs not deviating from HWE ($<10^{-6}$)	116,891 (-1,268)	119,463 (-63)			548,961 (-158)	549,167 (-0)
SNPs with shared call rate > 0.98	115,200				549,070 (-260)	
SNPs with non-sig different call rate cases vs controls (p < 0.00001)	113,445 (-1,755)				548,603 (-467)	
Imputation checker tool pass	109,898 (-3,547)				532,979 (-15,624)	

Abbreviations: CH=Cluster headache, GSA = Global screening array, HWE = Hardy-Weinberg equilibrium, IBD = Identity-by-descent, NBS= National blood service, QC = Quality control, SNP = Single nucleotide polymorphism, UK = United Kingdom, 1958bc = 1958 birth cohort

4.1.5 (d) Combined analysis

Merging of UK and Swedish cohort

The UK and Swedish datasets, of 1,443 cases and 6,748 controls were combined using shared genotyped biallelic SNPs, with further data processing and QC following the same steps as in the UK cohort methodology described above and summarised in **Table 23.**

Table 23: Summary of QC steps taken when forming the merged cohort.

Initial Dataset	Cases (n=1,443)	Controls (n=6,748)
Shared biallelic SNPs	117,308	
SNPs with call rate > 0.98	116,517	116,517
SNPs not deviating from HWE ($\geq 10^{-6}$)	114,944	114,944
SNPs with shared call rate > 0.98	114,780	
SNPs with non-sig different call rate cases vs controls ($p < 0.00001$)	113,266	
Imputation checker tool pass	113,168	

Abbreviations: HWE = Hardy-Weinberg equilibrium, QC=Quality control

After QC, the final number of SNPs included in the combined analysis was 7,249,346

Gene-based association testing

To aid the identification of causal variants, gene-based association analysis was carried out using MAGMA through FUMA v1.3.5e[532, 533]. This process involved the computation of gene-based p-values through correlation with SNPs located within these genes. This allowed for the prioritization of candidate genes based on the mean association of all SNPs accounting for linkage disequilibrium. To ensure inclusion of regulatory regions, gene windows included 35kb upstream and 10kb downstream of the annotated gene's start and end sites.

Functional variant annotation and prediction

Ensembl Post-GWAS analysis pipeline (POSTGAP) was used for variant annotation and functional consequence prediction of SNPs exceeding genome wide significance [534]. The lead SNP from each associated loci was annotated. Provided metrics included alternate allele frequency by population, Variant Effect Predictor (VEP) annotation including the Combined Annotation Dependent Depletion (CADD) score, and Genomic Evolutionary Rate Profiling (GERP) score annotation[535, 536]. POSTGAP, using 1000 Genomes genotypes, also extracts all variants in LD with identified associated variants using these clusters to provide analysis on variant-gene interactions, regulatory activity and expression through the accrual of data from FANTOM, DHS, RegulomeDB and GTEX[537-539].

Pathway analysis

Pathway analysis involved an enrichment analysis of protein-coding regions within 1 Mb window surrounding lead SNPs in individual pathways. This was achieved with gprofiler2 R package[540], with a custom background of protein-coding genes (set size 17,937) incorporated from Ensembl BioMart package[541]. Established pathways and complexes were sourced from Gene Ontology, KEGG and Reactome[542-544]. Only pathways with a multiple testing-adjusted p-value < 0.05 (FDR) were kept for further analysis.

Gene expression and expression quantitative trait loci (eQTL) analysis

The expression of significant genes identified in gene-based association testing were analysed. GTEx v8 was used to investigate tissue expression profiles and the temporality of expression in brain was examined using the Human Brain Transcriptome dataset [64, 539]. Relevant expression quantitative trait loci were

deciphered implementing FUMA[533]. EQTLs with multiple testing correction of FDR < 0.05 were retained for further analysis.

Genetic correlation analysis

Cross-trait LD score regression allows an estimation of the extent of genetic correlation between complex traits and disorders of interest. This can provide potential causal inferences and aetiological insights. To achieve cross this, summary statistics were obtained from the UK Biobank GWAS database for neurological, psychological and sleep disorders respectively. The analysis was conducted using LD score regression (LDSC). Pre-computed LD scores were provided and SNPs with imputation quality score of < 0.7 were removed. SNPs common to both the reference HRC site list v1.1 and the recommended SNPs in LDSC package resources list, were retained for analysis[545].

Genetic colocalisation analysis

For regions of significance suggestive of an overlap with migraine susceptibility loci, Bayesian colocalisation analysis was employed to confirm common shared risk loci between CH and migraine. R package coloc was used with the migraine GWAS from the UK Biobank GWAS database [546]. Causal regions were described as the range of positions of SNPs in LD $r^2 > 0.7$ with the lead SNP. Five hypotheses were tested: H0 – neither CH nor migraine had a genetic association within the tested region, H1 – only CH had a genetic association within the tested region, H2 – only migraine had a genetic association within the tested region, H3 – both CH and migraine had a genetic association within the tested region, but did not share causal variants, H4 – both CH and migraine shared a single causal variant with the tested region

4.1.6 Results

4.1.6 (a) UK Cohort

The quantile-quantile (QQ) plot (**figure 25**) showed no evidence of significant population stratification.

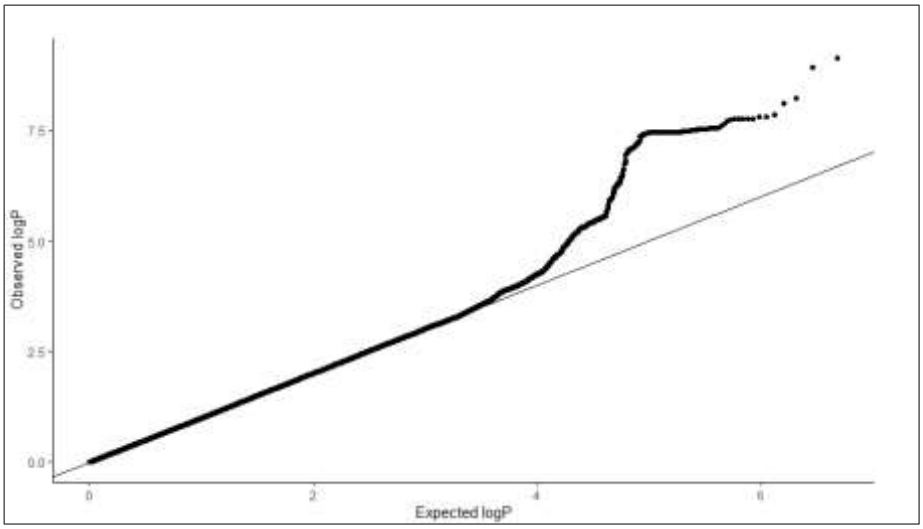


Figure 25: *Quantile-quantile (QQ) plot showing SNP p-values in GWAS analysis versus expected p-values. The straight line in the Q-Q plot indicates the distribution of SNPs under the null hypothesis.*

This was consistent with the calculated genomic inflation factor (λ score) of 1.04. In total, 86 SNPs passed genome wide significance threshold p-value < $5x10^{-8}$ and clustered in 3 independent loci (**Figure 26**). Two were located on chromosome 2 (chr2q13, chr2q33) and one was on chromosome 6 at a locus previously associated with migraine and headache (**Figure 26**, **Table 24**).

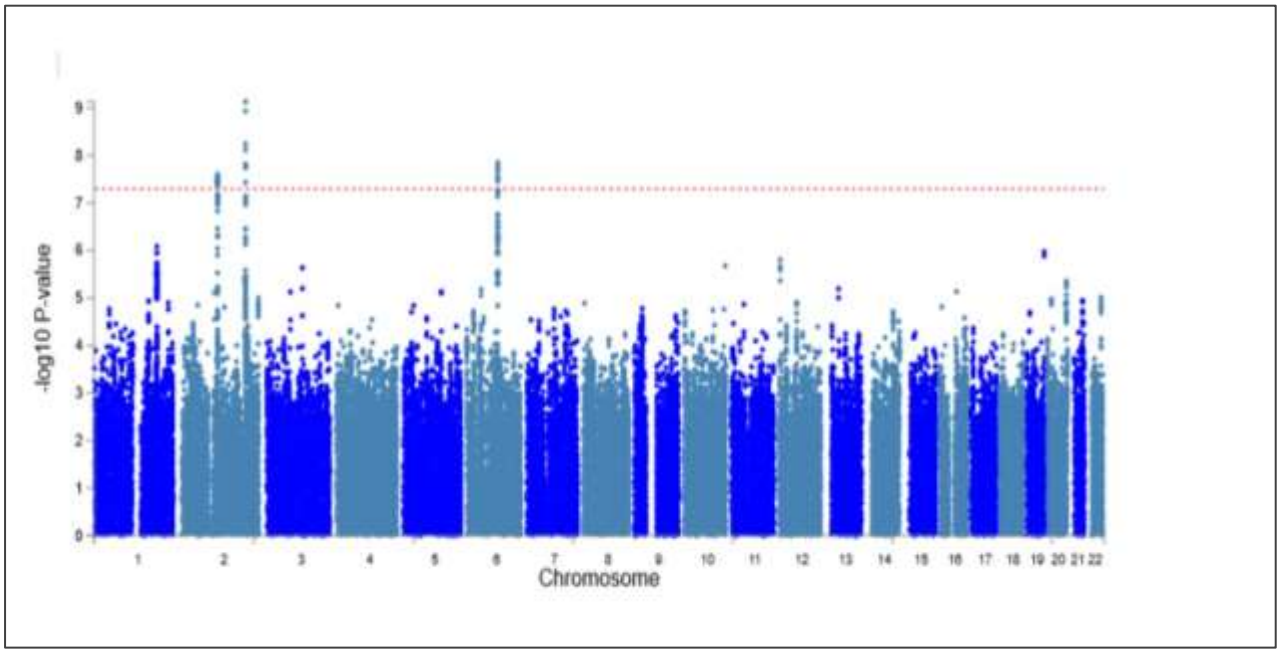


Figure 26: *Manhattan plot of the UK cohort CH association analysis, generated by FUMA, which highlights genome-wide significant associations at chromosome 2 and chromosome 6. The broken red line indicates the threshold for genome wide significance.*

4.1.6 (b) Candidate gene screening

A total of 852 cases and 5,614 controls of European ancestry were included after quality control. Regional plots did not reproduce associations as previously demonstrated in smaller candidate gene studies involving *CLOCK, HCRTR2, ADH4* or *ANO3*. Furthermore, associations demonstrated in Italian GWAS were not replicated in our study. (**Figure 27**)

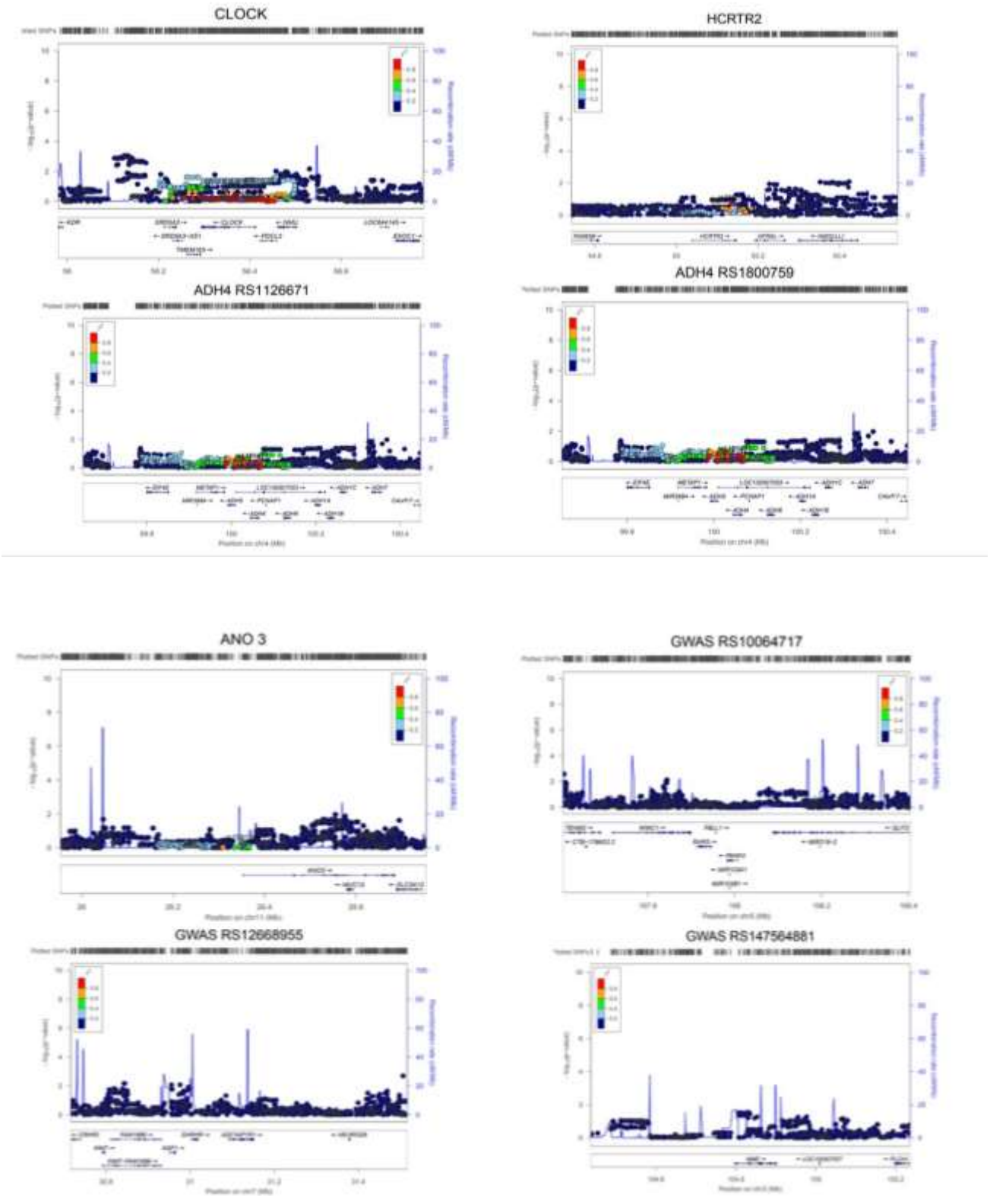


Figure 27: *Regional association plots of previous associations in candidate gene studies on CH, showing no evidence of association in the in 852 UK cases and 5614 controls.*

4.1.6 (c) Swedish Cohort

A total of 591 cases and 1,134 Swedish controls were included after quality control. 54 SNPs reached genome-wide significance (p-value< $5x10^{-8}$). There were two independent loci located on chromosome 2 (chr2q13, chr2q33). The lead SNPs at each locus included rs6671564 (p=4.9 $x10^{-5}$) and rs72825689 (p=1.07$x10^{-8}$). (**Figure 28 and 29**)

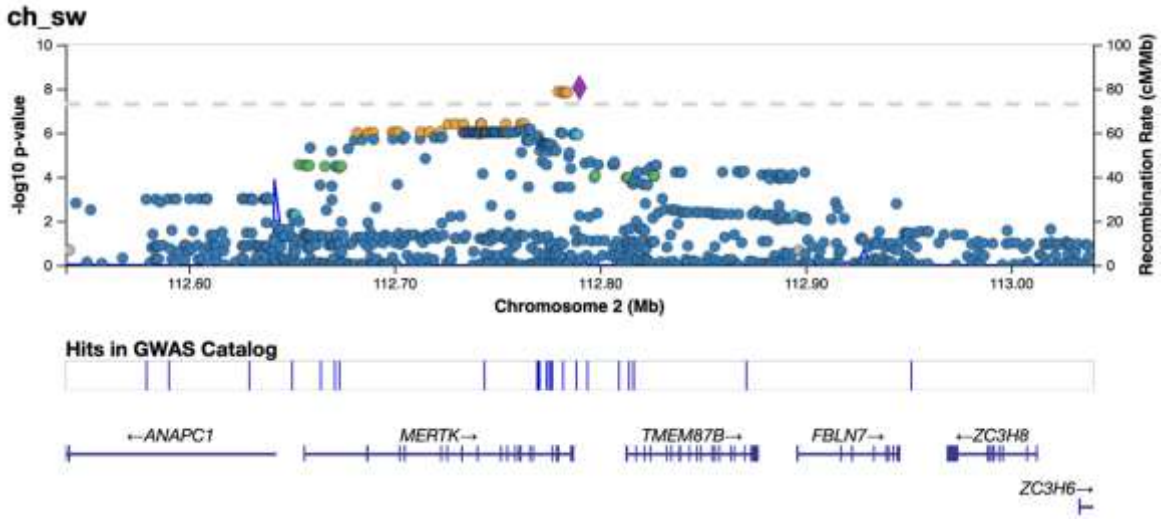


Figure 28: *Regional association plot showing replication locus on Chr 2 (rs72825689): The lead SNP (rs72825689) (p = 8.84x10^{-9}) overlaps the MERTK gene. SNP positions, recombination rates, and gene boundaries are based on GRCh37/hg19*

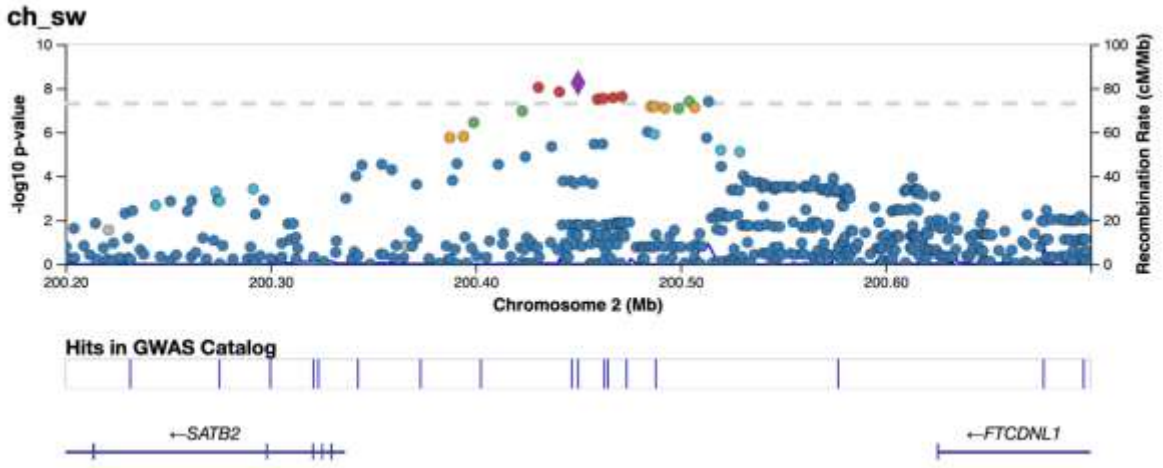


Figure 29: *Regional association plot showing replication locus on Chr 2 (rs4675692): The lead SNP (rs4675692) p-value below genome-wide significance (p = 5.6 x10^{-9}) at chromosome 2 overlies the long*

intergenic non-coding RNA LINC01877. SNP positions, recombination rates, and gene boundaries are based on GRCh37/hg19.

4.1.6 (d) Combined Analysis

The combined association analysis identified 248 SNPs linked to cases with genome wide significance (p<5x10-8) (**Figure 30**, **Table 24**) with a λ score of 1 (**Figure 31**) Two loci on chromosome 2 had significant independent association with CH in both cohorts and on combined analysis. The lead SNP with the strongest association (rs6435024, p=8.5x10-17) is located in a region without any protein coding genes but includes a long intergenic non-coding RNA *LINCO1877* (**Figure 32**). The second lead SNP, rs10186291, is an intronic variant in the MER Proto-Oncogene, Tyrosine Kinase (*MERTK,* [OMIM 604705]) gene (p=2.19x10-14) (**Figure 33**). A third locus was located on chromosome 1q41 with a lead SNP, rs6687758 (p=4.23x10-8). The region does not contain any known genes (**Figure 34**). The closest gene is LINC01655, a long non-coding RNA, located 151 kb from rs6687758. The lead SNP at chromosome 6, rs2273621, did not exceed genome wide significance but was suggestive of significance ($5.15x10^{-8}$). Genes in this region include UFM1 specific Ligase 1 (*UFL1* [OMIM 613372]) and Four and a Half LIM Domains 5 (*FHL5* [OMIM 605126]) (**Figure 35**).

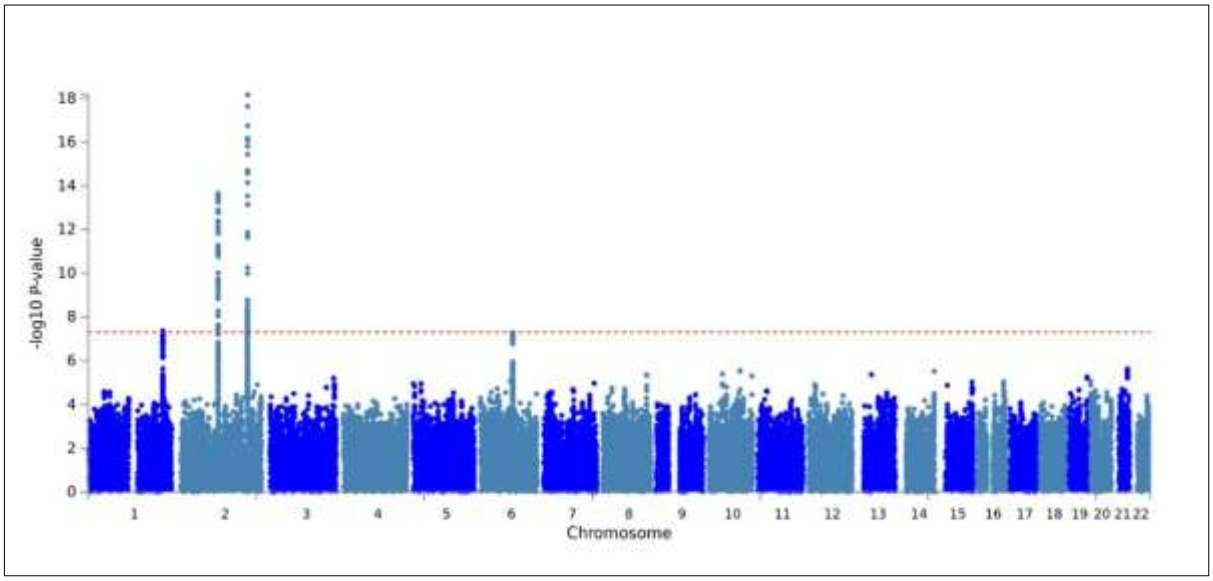


Figure 30: *Manhattan plot of the combined cohort CH association analysis, showing loci exceeding genome-wide significant associations at chromosome 2 and chromosome 1 and a signal suggestive of significance at chromosome 6. The broken red line indicates the threshold for genome wide significance.*

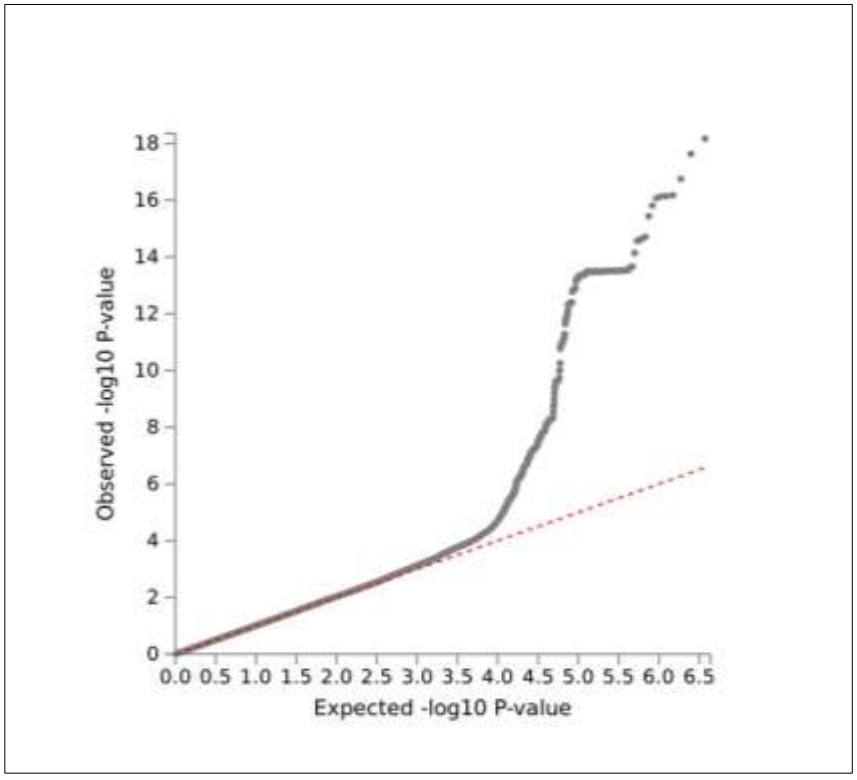


Figure 31: *Quantile-quantile (QQ) plot for combined analysis showing SNP p-values in GWAS analysis versus expected p- values. The straight line in the Q-Q plot indicates the distribution of SNPs under the null hypothesis.*

Table 24: Summary of Lead SNPs at each locus associated with cluster headache (CH).

TABLE 4.1.5: Summary of Lead SNPs at each locus associated with cluster headache (CH)				
Region	***LINC01655/DUSP10***	***MERTK***	***LINC01877 / SATB2***	***FHL5***
DISCOVERY PHASE (UK)				
rsID	rs6687758	rs4519530	rs6435024	rs9386670
Variant details	1:222164948:A/G	2:112759182:T/C	2:200512641:T/G	6:97060688:C/A
Odds Ratio (CI)	1.27 (1.12-1.45)	1.39 (1.24-1.55)	1.52 (1.32-1.76)	1.4 (1.25-1.57)
p-value	$3.29x10^{-4}$	$2.49x10^{-8}$	$7.55x10^{-9}$	$1.27x10^{-8}$
AF Cases	0.23	0.67	0.6	0.38
AF Controls	0.19	0.6	0.54	0.3
COMBINED ANALYSIS (UK and Sweden)				
rsID	rs6687758	rs10186291 ($r^2 = 1$ with rs4519530)	rs6435024	rs2273621 ($r^2 = 0.97$ with rs9386670)
Variant details	1:222164948:A/G	2:112748514:A/G	2:200512641:T/G	6:97058553:A/G
Odds Ratio (CI)	1.34 (1.21-1.49)	1.42 1.30-1.55)	1.62 (1.44-1.81)	1.28 (1.17-1.40)
p-value	$4.23x10^{-8}$	$2.19x10^{-14}$	$8.5x10^{-17}$	$5.15x10^{-8}$
AF Cases	0.25	0.7	0.62	0.39
AF Controls	0.19	0.62	0.56	0.33

Table footnote: SNP = Single nucleotide polymorphism, Chr = Chromosome, AF = Allele frequency, OR = Odds ratio.

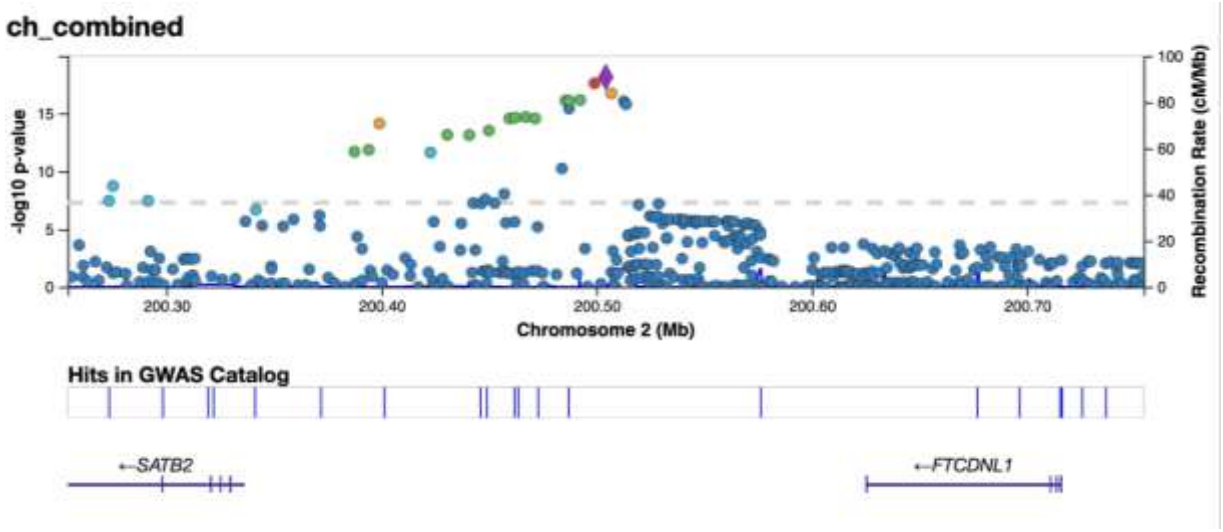


Figure 32: *Regional association plot using imputed SNP data, showing the lead SNP rs6435024 at chromosome 2 overlies the RNA LINC01877. SNP position, recombination rates, and gene boundaries are based on GRCh37/hg19*

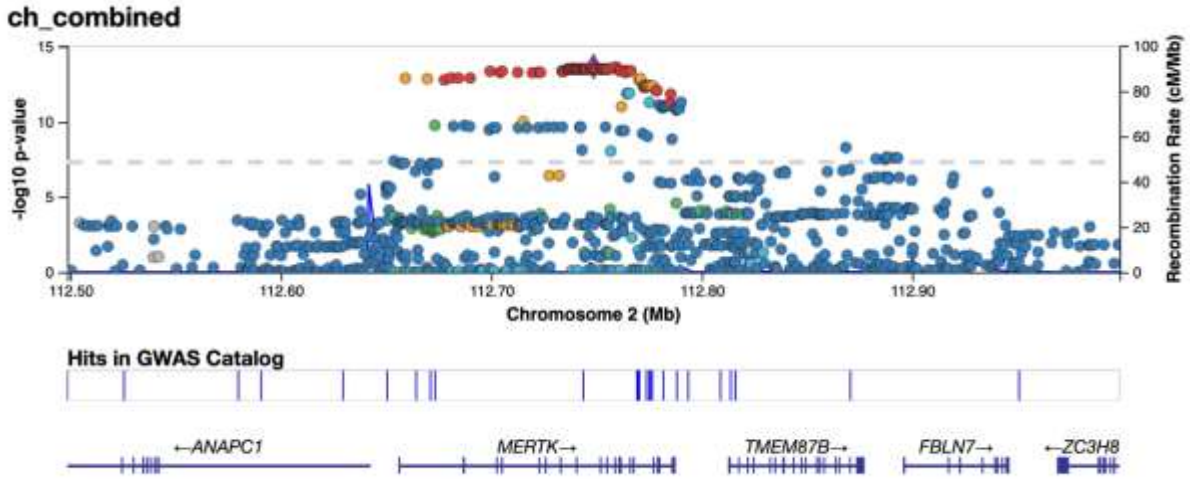


Figure 33: *Regional association plot using imputed SNP data, showing the lead SNP rs10186291 at chromosome 2 overlies the MERTK. SNP position, recombination rates, and gene boundaries are based on GRCh37/hg19*

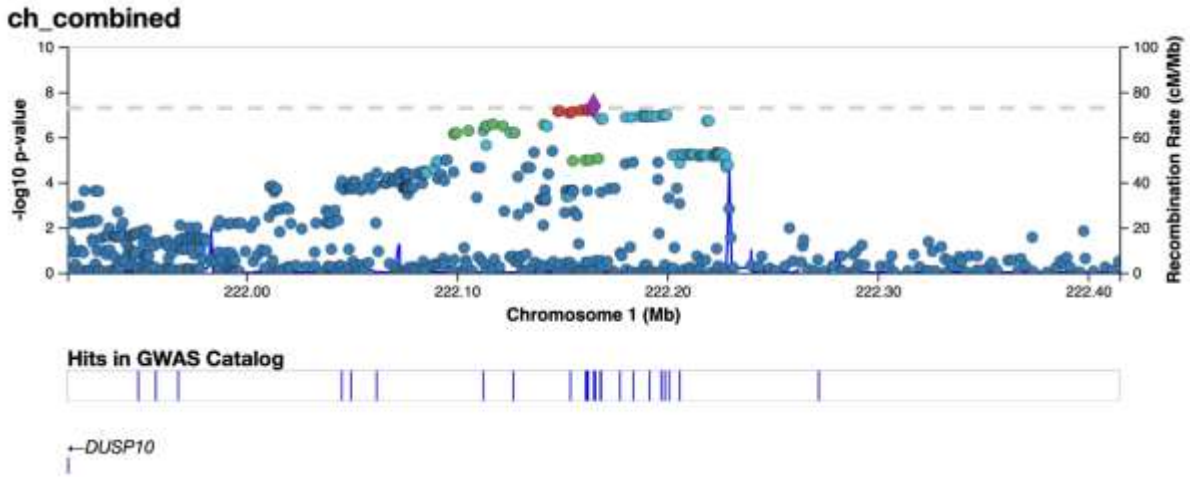


Figure 34: *Regional association plot using imputed SNP data, showing the chromosome 1q41 locus with lead SNP rs6687758. SNP position, recombination rates, and gene boundaries are based on GRCh37/hg19*

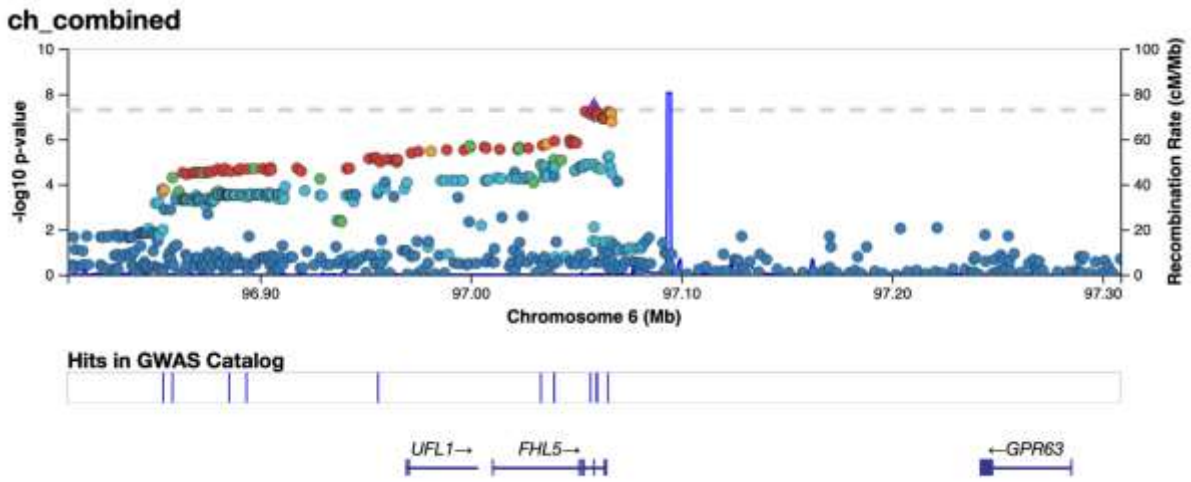


Figure 35*: Regional association plot using imputed SNP data, showing the chromosome 6 locus with lead SNP rs9386670 which is just below genome wide significance. SNP position, recombination rates, and gene boundaries are based on GRCh37/hg19*

4.1.6 (d) (i) Functional variant annotation and prediction

All lead SNPs were located in non-coding regions of the genome, but four variants in high LD with the lead SNP ($r^2 > 0.9$) were exonic variants with moderate impact. Two of these variants were missense variants in *MERTK* (rs7604639, rs2230515), and two missense variants (rs2273621, rs9373985) in *FHL5* gene. Both *FHL5* variants had high CADD scores of 23.6 and 15.5 respectively and rs9373985 showed a high level of mammalian conservation, with a GERP score of 3.25. Variants in *MERTK* had low in-silico pathogenicity predictions.

4.1.6 (d) (ii) Gene-based association testing

Gene-based testing used the mean association signal from all SNPs within each gene, accounting for LD. 19,007 genes were analysed. A multiple testing-corrected p-value threshold of 2.63×10^{-6} was applied to identify genes significantly associated with CH. Five candidate genes passed this threshold: *MERTK* ($p=2.22 \times 10^{-14}$), Transmembrane

Protein 87b (*TMEM87B*, [OMIM 617203], [p=5.12x10^{-13}]), Anaphase-Promoting Complex, Subunit 1 (*ANAPC1* [OMIM 608473], [p=4.39x10^{-10}]), Fibulin 7 (*FBLN,* [OMIM 611551] [p=3.48x10^{-8}], and *FHL5* (p=6.21x10^{-7}). *UFL1* (p=8.46x10^{-6}) was below genome wide significance.

4.1.6 (d) (iii) Gene expression and expression quantitative trait loci (eQTL) analysis

All of the candidate genes (*MERTK, UFL1, ANAPC1, TMEM87B, FHL5*, and *FBLN7*) are expressed in the human brain. (**Figure 36**)

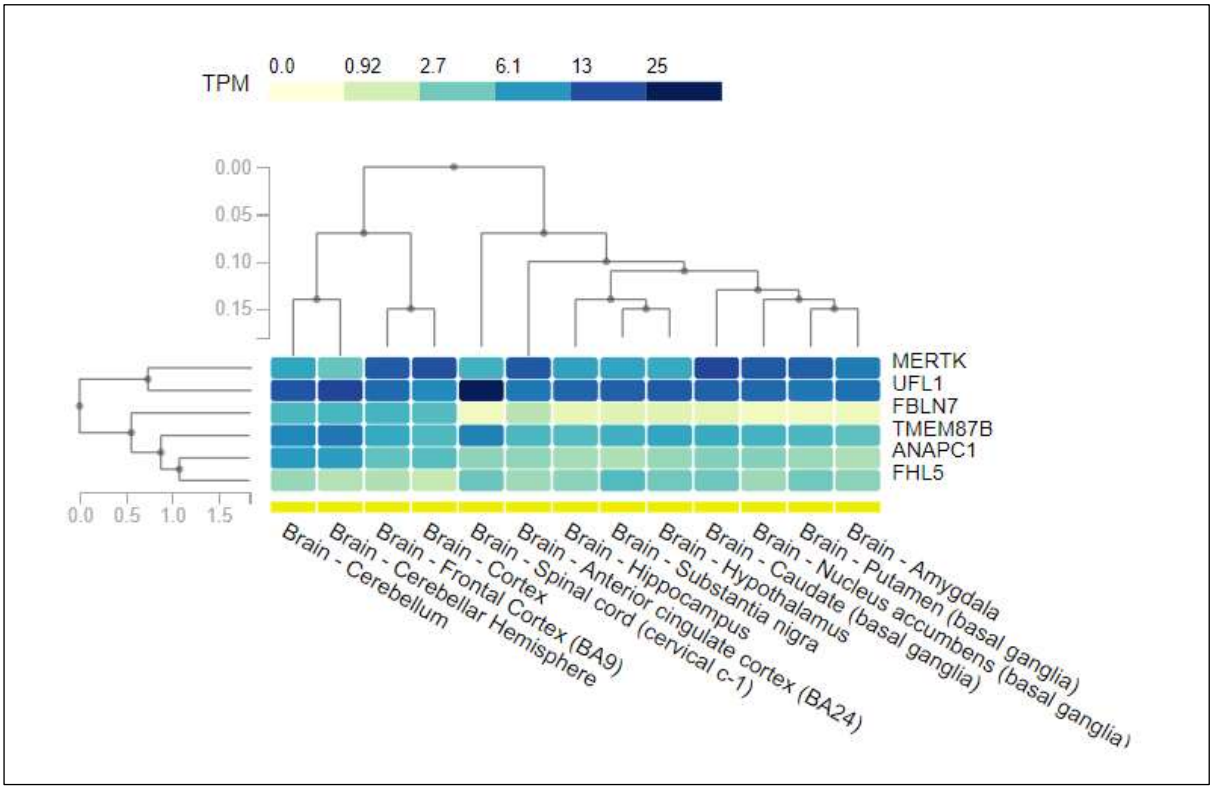


Figure 36: *Heatmap generated from GTEx (The Genotype-Tissue Expression Project) showing all genes identified in gene-based association analysis (MERTK, UFL1, ANAPC1, TMEM87B, FHL5 and FBN7) are expressed in the brain (median TMP >3)*

Cell type analysis showed *MERTK, TMEM87B, UFL1, ANAPC1* have the highest RNA expression in brain support cells, namely microglia and astrocytes. (**Figure 37**)

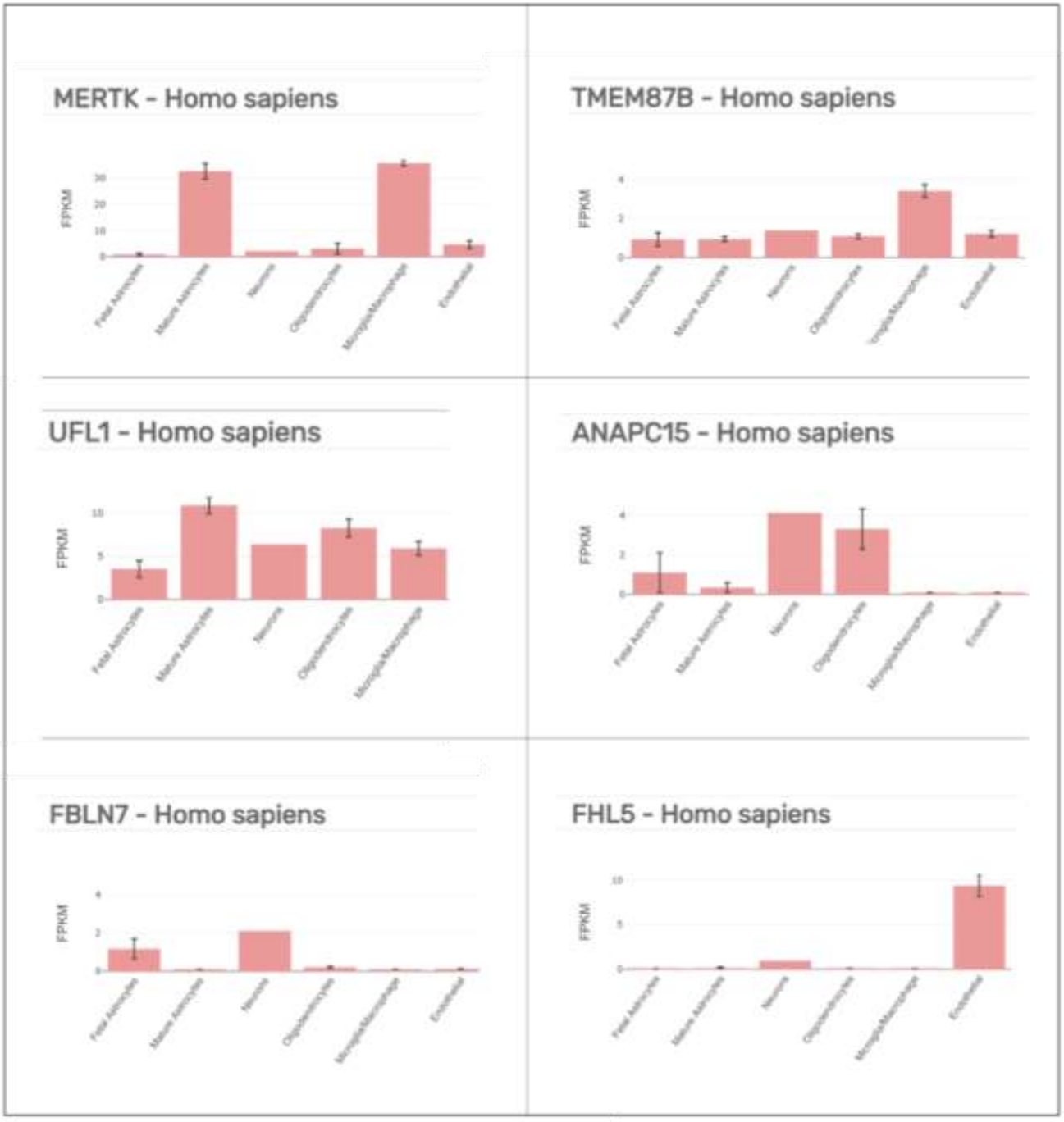


Figure 37: *Cell type expression analysis generated with Brain RNA-Seq showing MERTK, FHL5, ANAPC15, FBLN7, TMEM87B, UFL1.*

FHL5 had minimal expression in brain but highly expressed in tibial artery tissue. *MERTK, UFL1, TMEM87B, FBLN7, ANAPC1* and were shown to be highly expressed in the adult brain, particularly the hippocampus and neocortex. (**Figure 38**)

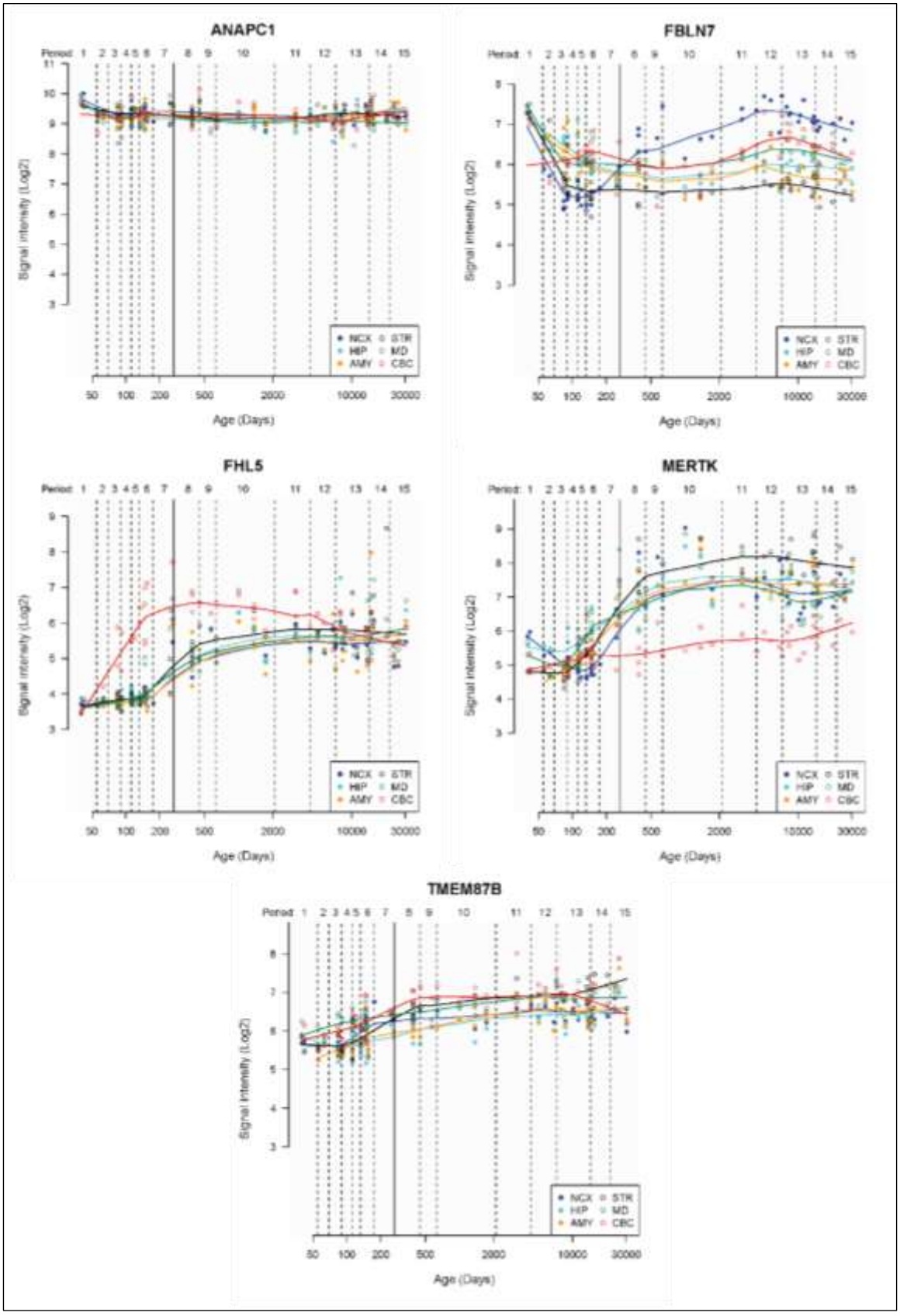


Figure 38: *Temporal analysis generated using Human Brain Transcriptome showing MERTK, FHL5, TMEM87B, FBLN7, ANAPC1 are highly expressed in the adult brain, particularly in the hippocampus and neocortex.*

Using lead SNPs, (rs6687758, rs10186291, rs6435024 and rs2273621) SNP-gene pairs were identified across all tissues in databases available through FUMA. Overlapping eQTLs significant for neurological, vascular and immune system tissues were observed **(Table 25).**

Table 25: Expression Quantitative Trait Loci (eQTL) for lead SNPs at cluster headache susceptibility loci as derived from FUMA.

Chr	Symbol	Position	Database	Tissue	p value	FDR
Neurological eQTLs						
2	*MERTK*	112681991	GTEx/v8	Brain Caudate basal ganglia	8.41×10^{-13}	2.35×10^{-8}
				Brain Cortex	3.86×10^{-9}	3.21×10^{-5}
				Brain Nucleus accumbens	3.88×10^{-9}	3.08×10^{-5}
				Brain Hippocampus	4.30×10^{-8}	0.0004247
				Brain Putamen basal ganglia	1.04×10^{-7}	0.0004146
		112653715		Brain Nucleus accumbens	1.01×10^{-6}	3.08×10^{-5}
		112681991		Anterior cingulate cortex BA24	2.06×10^{-6}	0.0062551
				Cerebellar Hemisphere	4.64×10^{-6}	0.0101166
				Frontal Cortex BA9	5.69×10^{-6}	0.0191458
				Nerve (Tibial)	7.11×10^{-5}	8.83×10^{-36}
2	*RGPD8*	112715142			0.0001357	1.39×10^{-86}
1	*DUSP10*	222219753			4.89×10^{-6}	0.0049572
2	*FTCDNL1*	199908378			0.0002804	1.12×10^{-31}
2	*TMEM87B*	112816348			3.71×10^{-19}	5.35×10^{-15}
2	*ZC3H8*	112715142			3.15×10^{-5}	1.28×10^{-27}
2	*FBLN7*	112899915			5.02×10^{-14}	3.40×10^{-11}
		112887685		Brain Cortex	9.82×10^{-7}	6.11×10^{-7}
		112790446		Cerebellar Hemisphere	1.10×10^{-6}	1.65×10^{-5}
2	*ZC3H6*	112748704		Frontal Cortex BA9	7.89×10^{-6}	0.02422
Vascular eQTLs						
2	*FBLN7*	112875614	GTEx/v8	Artery Tibial	4.05×10^{-21}	2.92×10^{-60}
		112875614		Artery Aorta	2.27×10^{-16}	7.83×10^{-20}
2	*MERTK*	112681991		Artery Aorta	8.21×10^{-25}	1.50×10^{-19}
		112681991		Artery Tibial	2.93×10^{-20}	1.93×10^{-15}
		112681991		Artery Coronary	1.06×10^{-9}	1.59×10^{-05}
2	*POLR1B*	112899915		Artery Tibial	4.14×10^{-5}	1.37×10^{-73}
2	*RGPD8*	112715142		Artery Aorta	1.02×10^{-9}	4.39×10^{-70}
		112715142		Artery Tibial	1.92×10^{-8}	1.31×10^{-87}
2	*SATB2*	199686506		Artery Tibial	0.00011^{-8}	0.0031042
		199756278		Artery Aorta	0.000122	0.0082404
2	*TMEM87B*	112776726		Artery Aorta	4.67×10^{-11}	3.59×10^{-07}
		112779732		Artery Tibial	1.77×10^{-8}	1.40×10^{-13}
2	*ZC3H8*	112750714		Artery Coronary	2.16×10^{-5}	0.0009504
Immune eQTLs						
2	*MERTK*	112788380	eQTLcatalogue	Monocyte CD14	1.29×10^{-14}	6.43×10^{-11}
		112781917		Monocyte	1.58×10^{-11}	7.90×10^{-8}
		112681991	GTEx/v8	Spleen	8.27×10^{-23}	1.34×10^{-17}
2	*NT5DC4*	112795908	eQTLcatalogue	T-cell CD8	6.90×10^{-6}	0.0344919
2	*RGPD8*	112659110	GTEx/v8	Spleen	3.34×10^{-9}	1.90×10^{-35}
		112673147	eQTLcatalogue	Neutrophil	6.69×10^{-8}	0.0003343
2	*TMEM87B*	112798941		T-cell	3.16×10^{-37}	1.58×10^{-33}

4.1.6 (d) (iv) Pathway analysis

15 pathways were significantly enriched for genes in candidate regions ($p< 0.05$). These included cytokine activity (GO:0005125, p=0.00086), cytokine-cytokine receptor interaction (KEGG:04060, p=0.0051), interleukin-36 (IL-36) pathway (REAC:R-HSA-9014826, p=0.0013), positive regulation of I-kappaB kinase/NF-kappaB signaling (GO:0043123, p=0.036), and interleukin-1 (IL-1) receptor binding (GO:0005149, $p=7.2x10^{-7}$).

4.1.6 (d) (v) Genetic correlation analysis

I calculated a SNP-based heritability for CH of 27.6% (SE=6.1%). A significant correlation between CH and migraine (RG=0.42, SE=0.12, p=0.0005), diagnosed stroke (RG=0.84, SE=0.31, p=0.007), depression (RG=0.25, SE=0.10, p=0.0012), bipolar disorder (RG=0.35, SE=0.1, p=0.0006) and insomnia (RG=0.39, SE=0.11, p=0.032) was also identified.

4.1.6 (d) (vi) Migraine GWAS overlap

The chromosome 6 locus identified in the UK analysis remained suggestive of association, with the lead SNP (rs2273621) p-value below genome-wide significance ($p = 5.15x10^{-8}$). This loci overlaps with a previously established loci in GWAS of migraine and headache[17]. Using, colocalization analysis, I confirmed that this association signal resulted from the same causal variant as the UK Biobank migraine study (posterior probability for shared causal variant at chr6q16: 94.9%). Therefore, I sought to investigate whether this association could be representative of concurrent migraine in our cohort. This information was available for 655 patients, of which 195 had co-existing migraine. Fisher's exact tests for the alternate allele frequency across

the two groups in the lead SNPs showed no significant differences (rs9386670; p-value = 0.93, OR = 1.02 [0.71 - 1.47]). This suggests that the association signals for the lead SNPs were not driven by the presence of concomitant migraine in our cohort.

4.1.7 Discussion

In this study, for the first time, susceptibility loci for CH with moderate to high effect sizes were identified and replicated in two independent cohorts. The strongest association was observed for a region on chromosome 2 which contains a long intergenic non-coding RNA *LINC01877. LINC01877* is highly expressed in brain, most abundantly in the hippocampus and hypothalamus. 177kb proximal to the lead SNP, the Special AT-rich sequence-binding protein 2 (*SATB2*, [OMIM 608148]) gene and its antisense equivalent (*SATB2- AS1*) provide possible candidates. Mutations causing haploinsufficiency in this gene are associated with SATB2 associated syndrome (SAS), a disorder characterised by neurodevelopmental delay, craniofacial abnormalities, intellectual disability and behavioural problems[547]. In the developing brain, SATB2 is primarily required for cell-type specification of the upper layer pyramidal neurons in the neocortex and formation of the corpus callosum[548, 549]. In adult mice, it has been shown to be expressed in the hypothalamus (with a strong expression in the lateral hypothalamus and the paraventricular hypothalamic nucleus) as well as in the A12 cell group of dopaminergic neurons.[550] It is also highly expressed in the hippocampus in the adult brain and regulates synaptic plasticity impacting memory[551-553]. SATB2 regulates the neurotransmitter phenotype of sympathetic neurons, a process which is modulated through its interaction with neuropoietic cytokines[554]. Conditional knockout mice exhibit abnormalities in structures with a proposed role in nociceptive processing, showing loss of barrels in layer IV of the somatosensory cortex and a paucity a of thalamocortical projection axons[555-557].

Common variation in this region, as well as SATB2 regulated genes, have been identified as a risk factor for schizophrenia in several GWAS[558-562]. Furthermore, interrogation of our association analysis identified a significant genetic correlation with depression and bipolar. The co-occurrence of depression with CH is well documented[88, 127]. These findings are consistent with previous evidence showing sharing of common risk variants between psychiatric and headache disorders, possibly implying an overlap of pathogenic processes, at least at a genetic level, in these conditions[563].

The lead SNP of a second independently significant region on chromosome 2 lies in an intronic region of the mer tyrosine kinase proto-oncogene *MERTK* and is in high LD with two missense mutations also reaching genome-wide significance. MERTK constitutes one of 'TAM receptors', consisting of Tyro3, Axl and MERTK, regulators of microglial function and the phagocytosis of apoptotic cells across several tissues. Homozygous mutation in this gene are associated with retinitis pigmentosa a condition often associated with headache[564-566]. As illustrated in our cell type expression analysis, MERTK is highly expressed in microglia and Mer deficient mice exhibit an aggregation of apoptotic cells in neurogenic regions of the CNS[567, 568]. MERTK is involved in two different physiological processes suspected to be involved in cluster headache pathogenesis. Firstly, it has a clear role in neuro-inflammation and a loss of MERTK function has previously been associated with increased risk for Multiple Sclerosis[569-571]. Furthermore, MERTK phagocytic pathways also mediate astrocyte elimination of excess synapses in the adult brain, critically regulating synapse remodelling through neural circuit refinement [572]. Using Transcranial Magnetic stimulation, increased cortical excitability has been found in cluster headache patients[573]. This increase could directly result from the disrupted MERTK regulation

of plasticity. Several prophylactic treatments used in CH, in particular anti-epileptics, are known to reduce neural excitability indicating a possible role in pathophysiology. Another potent way to regulate neural excitability is deep brain stimulation, which has proved to be an effective treatment for cluster headache. Another potent way to regulate neural excitability is deep brain stimulation, which has proved to be an effective treatment for cluster headache[574].

An additional significant association identified on chromosome 6 correlates with a locus previously implicated in migraine and headache[17, 77, 575, 576]. Detected vascular eQTLs are also reflective of expression patterns at migraine associated loci [17]. Concomitant migraine is often associated with CH, with a frequency that varies considerably from 0% to 65% [577]. Although, phenotypically distinct, CH may be misdiagnosed as migraine, and vice a versa. A considerable misclassification of cases is needed to create false levels of genetic correlations (15 to 30% uni- or bidirectional correlations respectively)[578]. However, this association was notably stronger in the UK cohort. Therefore, as an additional step in our analysis, 655 UK patients were phenotyped to re-assess the significance of this association in CH patients with and without migraine. Overall, 194 (29%) cases had concurrent migraine. This association was not dependent of the presence of co-existent migraine (p=0.93), indicating possible pleiotropy at this locus. The lead SNP driving the association at chromosome 6 is an intronic variant in the FHL5 gene, and also overlaps the UFL1 gene, which encodes for a protein constituting part of the ubiquitin- fold modifier 1 (UFM1) conjugation system involved in the apoptosis and trafficking of vesicles in the endoplasmatic reticulum [579].

Cell type analysis shows enhanced microglial expression. Neuro-inflammation has previously been implicated in several pain disorders[580] and microglia have been shown to mediate the generation of neuropathic pain through the amplification of excitatory neuronal currents and attenuation of inhibitory currents [82, 581]. Microglia also influence central sensitisation events in chronic pain[582, 583] and are responsible for synaptic pruning in normal brain development modulating functional connectivity[584-586]. Defective pruning by microglia of complement tagged synapses may impair CNS connectivity predisposing to conditions such as epilepsy and autism[572, 585-588]. Connectivity defects have been demonstrated in CH imaging studies[316, 317, 589, 590]. Although unclear, it is possible microglial dysfunction may potentially dysregulate synaptic elimination and plasticity leading to connectivity impairment in CH patients. It is also worth noting that microglia are potential therapeutic targets for agents currently used in the management CH. For example, Verapamil, a calcium channel blocker, often the prophylactic agent of choice in CH [238-240] exhibits neuroprotective action through the inhibition of microglial PHOX activity, mediating generation of reactive oxygen species, via binding to its catalytic subunit gp91 [591, 592]. Similarly, Valproic acid, a histone deacetylase inhibitor commonly used in CH prophylaxis, triggers microglial apoptosis inhibiting over-activation [593].

Overall, our results implicate several immunological processes in the pathogenesis of CH, with several immune eQTLs reaching. Furthermore, gene set enrichment analysis were predominantly significant for pathways involved in cytokine activity, in particular the regulation of IL-1 and IL-36. The role of cytokines in the generation of headache is evidenced by elevated IL-6 in migraine and tension type headache[594, 595]. Enhanced nociceptive neuronal responses of the trigeminal nucleus caudalis and subsequent hyperalgesia is also observed following the injection of recombinant human IL-1 beta

into the cerebrum of rats[596]. The impact of IL-1 in mediating trigeminal inflammatory hyperalgesia is also demonstrated by nociceptive A-delta-TRG neurons innervating the facial skin via IL-1beta paracrine action within trigeminal ganglia[597].

Several candidate genes identified in our analysis appear to influence the CREB (c-AMP-responsive element binding protein) pathway. *MERTK* activates transcription factors *STAT6* (which was suggestive of significance in the gene based association analysis of the discovery UK cohort) and CREB [598]*. FHL5* is also an activator of CREB and CREM[599]. CREB is also a transcription factor for *UFL1*[600]. The CREB transcription pathway has been implicated in the sensitization of nociceptive cells and meningeal pain hypersensitivity[601]. Its role in pain transmission is also apparent in the activation of CREB in the trigeminal ganglion in vitro only after the direct stimulation of nociceptive neurons[602]. Triptans, often used to treat CH, have been shown to reduce the activity of CREB within the trigeminal system, inhibiting central sensitization and brainstem nociceptive neurons[603]. Notably, CREB is critical for both the maintenance of timing in the suprachiasmic nucleus and light entrainment of the circadian clock and *MERTK* expression decreases during sleep[604, 605]. This is relevant in the context of the striking circadian periodicity and frequent nocturnal attack associated with CH[604, 606].

There are limitations to this study. Firstly, ensuring a phenotypically homogenous cohort is challenging as CH is reliant upon clinical diagnosis. In this study, cohorts were carefully phenotyped to minimise possible confounding with migraine which is phenotypically distinct. Secondly, genotyping of the UK controls on a different array platform introduces a potential confounder. The independent replication of loci in the Swedish cohort, which were performed on cases and controls genotyped on the same

array (GSA), reduces the likelihood of spurious associations. Finally, CH is a relatively rare disorder, therefore collecting samples to achieve sufficient power is difficult. A larger study or meta-analysis is required to derive additional associations with variants with a lower effect size. This study is ongoing.

For the first time, this study identified replicable genome-wide-significant associations which contribute to a genetic predisposition in CH. These loci exhibit a significant genetic correlation with migraine, and depression, bipolar disorder, stroke and insomnia. Microglial expression appears predominant amongst candidate genes and pathway analysis implicated cytokine and immune activity as a pathogenic driver. Future targeted sequencing of significant loci will further annotate and fine map these regions to help identify pathogenic variants and facilitate functional and mechanistic studies. This in combination with deep phenotyping has the potential to provide genotype-phenotype correlations, unprecedented insights into the pathophysiological pathways underlying CH and possible therapeutic targets.

4.2 Genome-Wide Linkage Analysis of Familial Cluster Headache

4.2.1 Statement of Contribution

I established a collaboration with Dr. Christina Sjostrand (Karolinska University Hospital) and Dr. Laura Southgate (KCL) to obtain access to this cohort. I prepared samples for genotyping and whole-exome sequencing and conducted the linkage and familial segregation analysis.

4.2.2 Introduction

As outlined in section 3.3.4, meta-analysis indicates a familial rate of CH of approximately 6.27%[493]. Despite this, there has only been a few family-based studies to investigate the genetics of CH, the majority of which use a candidate gene approach, targeting specific regions of the genome[309, 328, 607]. These attempts have been impeded by certain features of CH including a lack of large multiplex pedigrees, reduced penetrance, locus heterogeneity, atypical presentations and the absence of a diagnostic test.

Genome-wide linkage analysis allows the identification of chromosomal regions shared by affected individuals manifesting a phenotype of interest within a family. This method has been successful in the detection of causal genes in conditions exhibiting mendelian inheritance patterns and has been applied to other genetically determined headache conditions. For example, it played a fundamental role in the isolation of the genes responsible for FHM including *CACNA1A, ATP1A2* and *SCN1A*[12, 608, 609]. It has also been used to identify susceptibility loci in both migraine with aura and without aura[610-612]. Similarly, the co-occurrence of migraine with aura and other conditions including occipito-temporal lobe epilepsy and CADASIL have been linked to loci on chromosome 9q21–q22 and 19 respectively[613, 614]. Linkage analysis has also been used to identify regions associated with specific migraine features such as photophobia-phonophobia (5q21) and severity of phenotype[615, 616].

To date, there has been only one study of familial CH employing genome-wide linkage analysis. This involved parametric and non-parametric linkage analysis of five multi-generational Danish kindreds. It did not identify a significant LOD score but four linkage peaks suggestive of significance were identified, two on chromosome 2 and one on chromosome 8 and 9[617]. These loci were not reproducible when extended to a

larger cohort of thirty-three Danish and Italian families. Authors suggested that, assuming genetic homogeneity, the study had sufficient power to detect linkage. Therefore, the lack of a single locus achieving significance may imply that CH is not determined by a single gene[617]. In contrast to mendelian conditions, the success of linkage analysis in complex human disease is limited[618]. Nevertheless, it has previously provided genetic insights into mendelian subsets of complex conditions such as Alzheimer's disease and type I diabetes and therefore is a worthwhile approach to unravelling the genetics of familial CH[619, 620].

4.2.3 Aims and objectives

1. To run genome-wide linkage on a cohort of Swedish families with inherited CH to map susceptibility loci.
2. To attempt to replicate linkage peaks identified on chromosome 2, 8 and 9 in a Danish CH cohort.
3. To use WES to fine map regions within linkage peaks to identify a causal gene.

4.2.4 Methods

4.2.4 (a) Patient Recruitment

Patient samples were obtained through collaboration with the Department of Neurology at Karolinska University Hospital. Recruitment was conducted in 1997 by my collaborator Professor Christina Sjostrand, consultant neurologist. For completeness, I will briefly summarize the recruitment process.

All cases diagnosed with CH in accordance with the 2nd edition of The International Classification of Headache Disorders [352]. Patients who reported a family history in at least one first or second-degree relative were recruited into the study. Subsequent patients could opt into the study through the patient support network website

www.migran.org, where the study was promoted. These patients and their relatives were phenotyped by a neurologist to validate the diagnosis of CH and where possible, additional relatives were contacted to confirm their unaffected status. Deceased relatives with a postulated diagnosis based on clinical details provided by proxy were also included in pedigrees. All families were of Swedish ancestry except for one (S8) which was of afro-american origin. Patients were assigned a diagnosis of 'probable' CH if they had symptoms consistent with CH but did not fulfil one aspect of the diagnostic criteria. These cases were followed longitudinally and further interrogated by the treating neurologist prior to inclusion in the genetic study. Relatives with atypical features of CH or those with new symptoms were re-phenotyped. The presence of migraine in unaffected relatives was also documented. All patients gave informed consent for the use of their sample in this study which was conducted under the approval of the local ethics committee. A standard UCL data transfer agreement was obtained for the transfer of samples.

4.2.4 (b) DNA extraction and genotyping

DNA for the majority of samples was extracted from blood samples at the Karolinska Institutet using a modified salting out method[621]. Additional samples were extracted as outlined in methods section. All samples were genotyped as described in methodology.

4.2.4 (c) Linkage analysis

Genome-wide linkage analysis was conducted using MERLIN[382] software in a standard manner as outlined in the methods section. Loci suggestive of significant underwent further fine mapping to confirm segregation of haplotype using Haplopainter 1.043[622]

4.2.4 (d) Parametric linkage analysis

I conducted parametric linkage analysis, under an autosomal dominant model of inheritance with a 90% penetrance and a disease allele frequency of 0.001%. A cumulative maximal heterogeneity logarithm of the odds score (HLOD) was calculated across families.

4.2.4 (e) Nonparametric linkage analysis NPL

I also carried out nonparametric linkage analysis using statistics built in Merlin. I used the NPL analysis which calculates sharing between affected individuals using Whittemore and Halpern NPL statistics and Kong and Cox linear model and also the EXP option which calculates nonparametric LOD scores using the Kong and Cox exponential model. Loci suggestive of significant underwent further fine mapping to confirm segregation of haplotype using Haplopainter 1.043[622]

4.2.4 (f) Whole exome sequencing (WES)

In pedigrees with a segregating haplotype, two related affected individuals were selected for WES (Agilent SureSelect V6+UTR; >30x coverage, min 10x > 92%) on the Illumina HiSeq3000 as described in methodology.

4.2.4 (g) Prioritisation of variants

A detailed prioritisation strategy is outlined in the methods section. As all pedigrees had an autosomal dominant mode of inheritance, only shared heterozygous variants were included for further analysis. Variants were then prioritised based on (i) A population frequency of <0.05% in gnomAD [623] (ii) functional impact (causing amino acid or splice site change) (iii) conservation across species (iii) predicted pathogenicity using in-silico predictors SIFT/Polyphen2.

4.2.5 Results

Six families were excluded from the analysis as relatives did not fulfil the diagnostic criteria for CH due to atypical features. These included bilateral pain, attacks prolonged beyond 3 hours, abnormal temporality of attacks and a lack of autonomic symptoms and restlessness or agitation. Overall, fifteen families were included in the study consisting of sixty-six individuals, thirty-three males and thirty females. Forty–one individuals were affected with CH and twenty-five were unaffected relatives. (**Figure 39**). In terms of demographics of the forty-one affected individuals; twenty-eight were male and thirteen were female. The average age of onset was 27 years (range 12-56, SD_/-11). Thirteen patients had a diagnosis of CCH, eighteen had ECH however this information was not available for the remainder of the cohort.

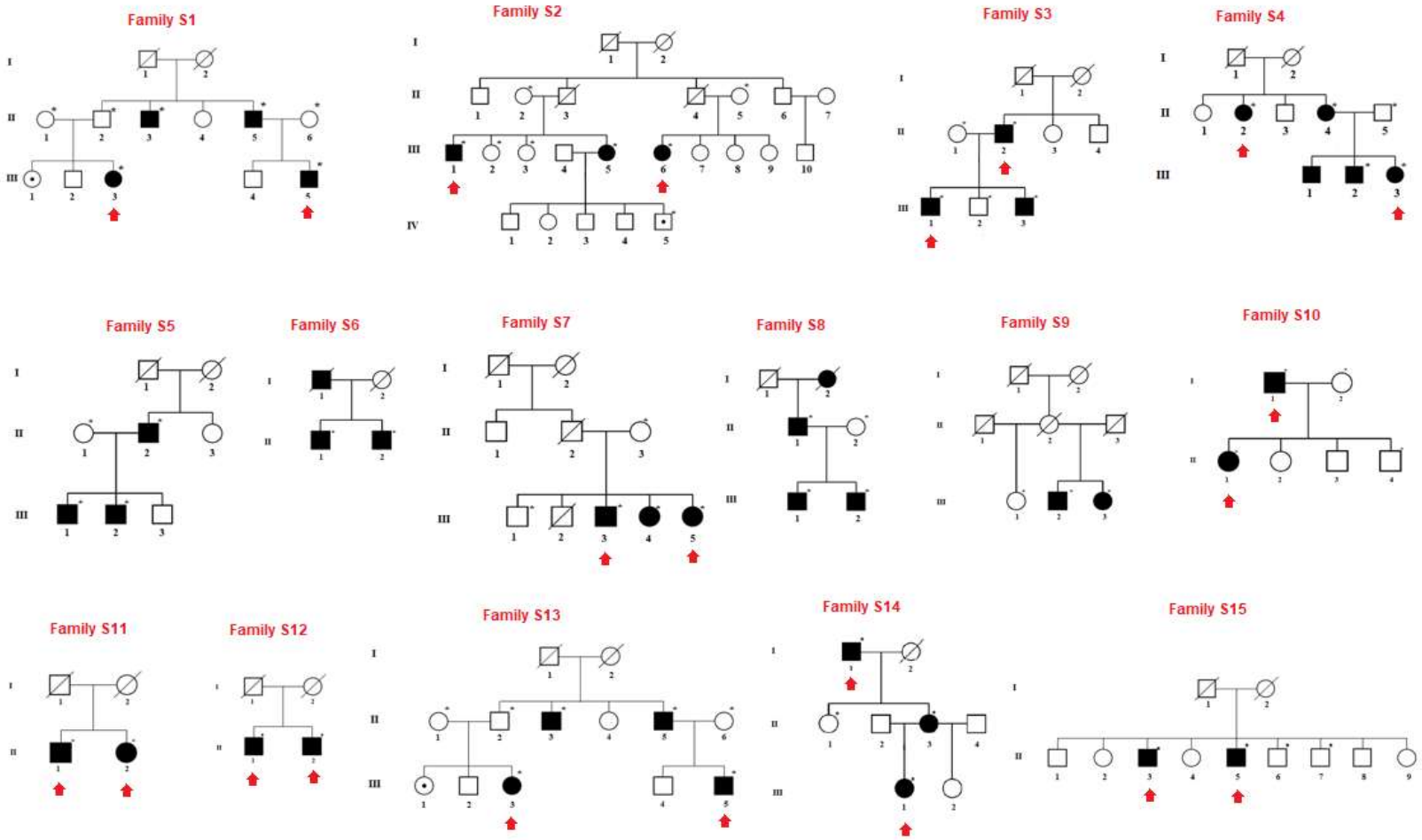


Figure 39: *Pedigrees of families S1-S15. Black indicates affected status. A black dote indicates migraine. Cases indicated with a star were genotyped for linkage. Cases that underwent WES are marked with a red arrow.*

4.2.5 (a) Linkage analysis

4.2.5 (a) (i) Parametric analysis

Genotype information from total of sixty-six individuals were included in the analysis. No locus achieved significance (HLOD >3) in parametric analysis, however two distinct loci had a HLOD of >2 and four additional loci had a HLOD of >1. (**Figure 40**, **Table 26**). The highest HLOD score of 2.41 (alpha 0.58) was located on chromosome 12q23 and fine mapping identified a haplotype that segregated across six pedigrees (S1, S2, S3, S10, S13, S14). In one family (S1), one unaffected individual (II:2) also carried the haplotype. However, as he was lost to follow-up I could not confirm if he developed CH after recruitment and he was therefore excluded from the analysis. This region, is delimitated by SNPS rs774047 and rs4761530 (chr12: 56,815,672-100554737; UCSC GRCh38/hg 38). Interrogation of this haplotype did not detect a founder effect. Of note, this locus overlapped with the region suggestive of significance on chromosome 12 (12:57497005- 59497710) in the GWAS (section 4.1).

A second linkage peak was observed on chromosome 2q24 achieving a HLOD score of 2.33 (alpha 0.63), with a haplotype that segregated in seven pedigrees (S1, S3, S4, S7, S11, S12, S15). This locus was between rs829963 and rs1344637 (Chr2:168745003-204124823; UCSC GRCh38/hg 38) and overlaps with GWAS (2:199802587- 200513620) susceptibility locus on chromosome 2 at approximately 197cM.

There were four regions with HLOD of > 1 including a locus on chromosome 17q22 (HLOD 1.43, alpha 0.47), an additional region on chromosome 12q21 (HLOD 1.41, alpha 0.43) (upstream from the GWAS locus), on chromosome 5q34 (HLOD1.26, alpha 0.34) and chromosome 6p25.1 (HLOD 1.22, alpha 0.4).

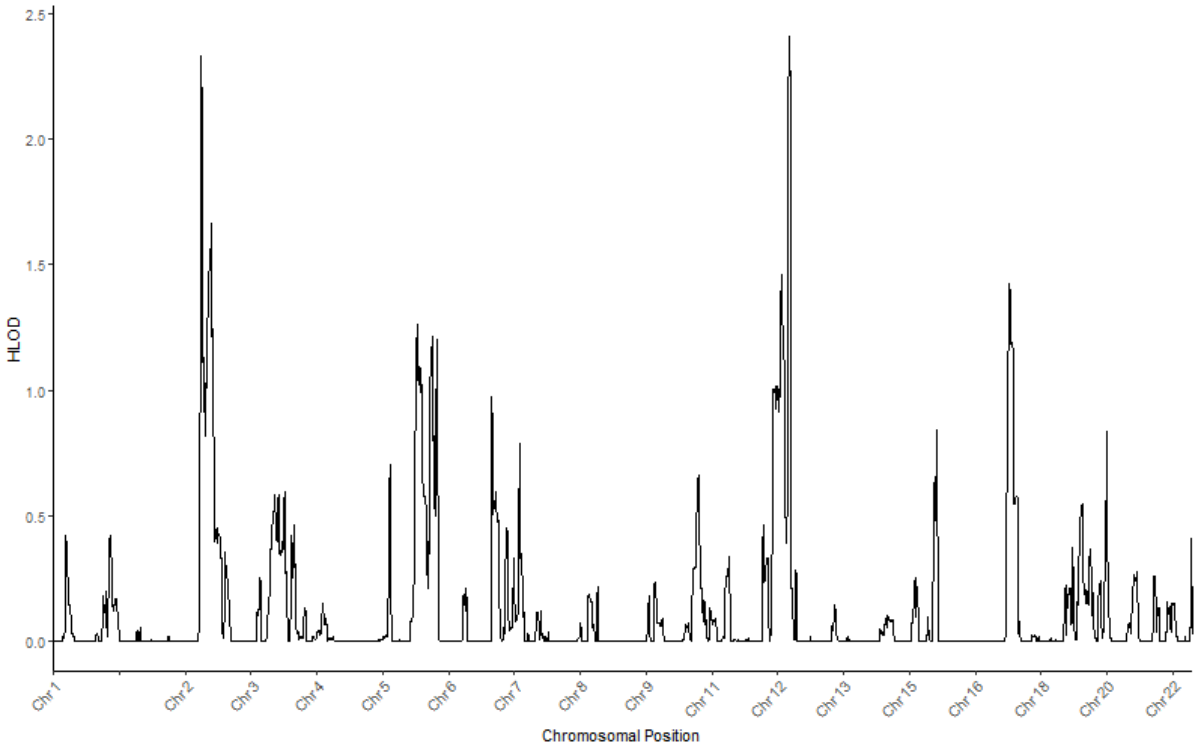


Figure 40: *HLOD score plot for genome-wide linkage analysis of fifteen CH families showing a linkage peak on 12q23 with HLOD score of 2.41 and second peak on 2q24 with HLOD score of 2.33. Chromosomal markers are indicated on the x-axis and HLOD score is shown on y-axis.*

Table 26: Summary of position and size of regions with HLOD score >1, in order of significance as detected on parametric linkage.

Chromosome / cM	Start point (GRCh38.p13)	Stop position (GRCh38.p13)	HLOD Score
12 (50cM-132cM)	56815672	100554737	2.41
2 (167cM-266cM)	168745003	204124823	2.33
17 (73-120cM)	56228296	67747327	1.43
12	66753823	78208198	1.41
5 (145cM-205cM)	159532911	169012471	1.26
6 (0cM-27cM)	1008724	2511841	1.22

4.2.5 (a) (ii) Non-parametric linkage

No loci reached significance, but three regions had a non-parametric LOD score of > 1 (max predicted for the families was 4.5). One region was on chromosome 2

overlapped with the results of parametric linkage. The remaining four were on chromosomes 1, 11, 19, and 22 (**Figure 41**).

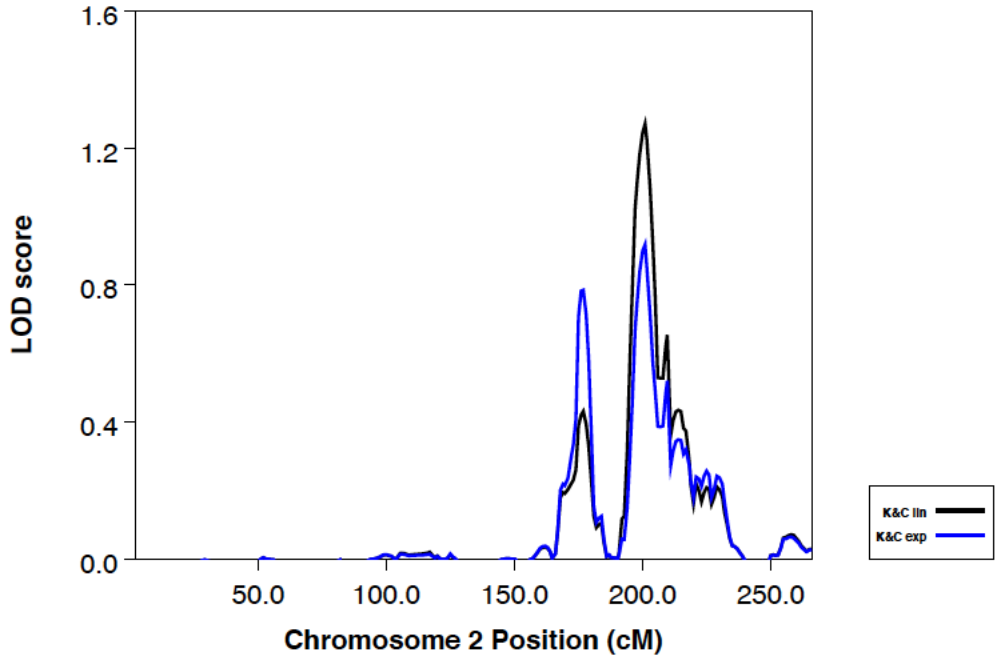


Figure 41: *Nonparametric results showing region on chromosome 2 which overlaps with region suggestive of linkage on parametric results.*

4.2.5 (b) Whole exome sequencing (WES)

WES was performed on two affected individuals in each of the families in whom a haplotype segregated at loci with a HLOD >2 (indicated with red arrows in **Figure 39**).

Chromosome 12 locus

For the most significant locus on chromosome 12 (56,815,672-100554737), twelve individuals across six families (S1, S2, S3, S10, S13, S14) underwent WES. Intrafamilial analysis was carried out by extracting only variants that were shared between the two affected relatives in this region. Identified variants were prioritized based on impact, frequency and consequence in accordance with the strategy described in methods. Following filtering, three variants remained (**Table 27**).

Two variants were identified in family S2. A missense variant in the Nuclear Receptor Subfamily 1, Group H, Member 4 gene (*NR1H4* [OMIM 603826]) which is poorly expressed in brain. A second missense mutation was observed in Fyve, Rhogef, And Ph Domain-Containing Protein 6 *(FGD6* [OMIM 613520]). In-silico predictions, indicated potential pathogenicity. In family S3, a missense variant in Apoptotic Protease Activating Factor 1 (*APAF1* [OMIM 602233]) was present in both affected individuals. It was expressed in brain but in-silico predictions implied tolerance. No candidate replicated in more than one family.

Table 27: Segregating variant identified through WES at chromosome 12 locus (56,815,672-100554737).

Fam	Gene	Position	AA	rsID	Variant	SIFT	Polyphen	Gnomad	Brain (GTEX)
S2	*NR1H4*	100534946 C/T	H/Y	rs189042762	Missense	T (0.88)	B (0.25)	0.000264	N
S2	*FGD6*	95094662 G/C	A/V	rs377183623	Missense	D (0)	PD (1)	0.00004879	Y
S3	*APAF1*	98648639 A/G	Q/R	rs770595178	Missense	T (0.24)	B (0.01)	0.00002847	y

Abbreviations: AA (Amino Acid) , B (Benign), Fam (Families), N (No), PD (Probably Damaging), T (Tolerated), Y (Yes)

Chromosome 2 Locus

Fourteen individuals across seven families (S1, S3, S4, S7, S11, S12, S15) underwent WES. Three variants were prioritised (**Table 28**). A missense mutation in SLC25A12 was found in two affected relatives in family S1. This variant is rare with a frequency of 0.000008124 in gnomad. It is highly expressed in brain and was categorised as deleterious by SIFT and probably damaging by Polyphen. Two variants were identified in S4. A missense variant in Low Density Lipoprotein Receptor-Related Protein 2 (LRP2 MIM 600073) which is expressed in brain and a missense variant in Myosin 3B (MYO3B MIM 610040). Interfamilial segregation was not observed.

Table 28: Segregating variant identified through WES at chromosome 2 locus (168745003-204124823)

Fam	Gene	Position	AA	rsID	Variant	SIFT	Polyphen	gnomAD	Brain (GTEX)
S1	*SLC25A12*	171787637 T/C	Q/R	rs121434396	Missense	D (0)	PD (0.98)	0.000008124	y
S4	*LRP2*	169244759 T/C	I/V	rs149853330	Missense	T (0.22)	B (0.083)	0.000386	Y
S4	*MYO3B*	170653083 T/A	S/T	rs201654883	Missense	T (0.66)	B (0)	0.002137	N

Abbreviations: AA (Amino Acid) , B (Benign), Fam (Families), N (No), PD (Probably Damaging), T (Tolerated), Y (Yes),

4.2.6 Discussion

I established an international collaboration to gain access to a cohort of CH families, phenotyped across several generations, including multiplex pedigrees suitable for linkage analysis. In this study, I sought to detect genetic locations implicated in CH predisposition by conducting genome- wide linkage analysis on fifteen families including forty-one affected individuals. No region with a significant HLOD score was identified across the genome however two areas of potential interest were detected, one on chromosome 12 (HLOD 2.4) and one on chromosome 2 (HLOD 2.33). Interestingly, the region on chromosome 2 replicated a locus suggestive of significance previously demonstrated using non-parametric linkage analysis in a Danish kindred with CH[617]. Both loci also overlap with regions identified in the GWAS study. Whist promising these regions are large and further fine-mapping or additional families are required.

For this study, I decided to focus on regions directly below the linkage peaks with highest proportion of linked families. WES of two affected relatives in each family was utilised to further interrogate these regions and identify variants in a common gene. Overall, six rare variants shared between two affected relatives were prioritized. The most interesting of these was a rare missense mutation in *SLC25A12* on chromosome 2, which encodes a protein involved in the transfer of aspartate in exchange for

glutamate from mitochondria to cytosol. *SLC25A12* is highly expressed in the frontal cortex of the brain and homozygous variants in this gene cause developmental and epileptic encephalopathy. However, rare variants in this gene were not present in other families with the segregating haplotype.

The lack of a solitary disease locus in this study may reflect locus heterogeneity and is consistent with suggestions that familial CH is unlikely to be caused by mutations in a single gene. This observation is reflective of other forms of inherited headache disorders such as FHM that can occur due to variation in at least three causative genes including *CACNA1A, ATP1A2* and *SCN1A*[11, 624, 625].

There are limitations to this study. Firstly, large multiplex families with several affected individuals are optimal for linkage analysis. These families are rare in CH due to reduced penetrance and barriers to precise phenotyping such as atypical phenotypes and frequent misdiagnosis [626, 627]. Ideally, families for future linkage studies should be collected prospectively to facilitate deeper phenotyping and extended pedigrees. Also linkage analysis is less effective in complex polygenic disorders, due to limited power to identify the effect of common alleles with modest effects on determining phenotype[618, 628]. Similarly, the prioritization strategy used to stratify variants was devised to detect very rare variants with a large effect and therefore may have excluded lower impact variants contributing to CH predisposition. Finally, WES will detect predominantly exonic variants and miss more complex variants such as repeat expansions which are demonstrated in linkage analysis as demonstrated elsewhere[629]. In conclusion, this study suggests that CH is likely to be a disorder compounded by locus heterogeneity.

4.3 An Exploratory Study of Familial Cluster Headache using Whole Exome Sequencing.

4.3.1 Statement of contribution

My contribution involved establishing an international consortium of CH centres for recruitment to the study. I recruited, phenotyped and collected samples from participants in the UK. I prepared samples for WES and carried out the analysis.

4.3.2 Introduction

The introduction of WES studies has resulted in the rapid identification of rare mutations pinpointing the cause and underlying mechanistic pathways of several Mendelian disorders[630]. Conventionally, this is achieved through the prioritisation of variants that are rare in population databases, are predicted to affect protein structure, have a functional impact as indicated by in-silico tools and have a postulated role in disease aetiology[631]. As previously discussed, there is considerable evidence to suggest a high rate of heritability in CH[295, 626 294]. This was confirmed by my systematic review and meta-analysis that found a familial rate of approximately 6.27%[493]. Autosomal dominant modes of transmission, often with reduced penetrance, are commonly reported in these families[286]. Despite this, there has been only one hypothesis free, genome-wide familial study which identified regions of interest utilising linkage analysis[617].

4.3.3 Aims and objectives

The purpose of this study is to perform the first WES study on cases of familial CH to attempt to capture potential candidate genes for further functional validation and mechanistic studies. To achieve this, I employed WES to perform segregation analysis

on large multiplex families affected with CH, to permit the identification of potentially causal variants. I then screened a larger cohort of cases of familial CH to detect additional segregating and potentially pathogenic variants in these genes.

4.3.4 Methods

Sample collection

As outlined in methods, cases of familial CH were recruited through international collaboration. A proportion of cases were derived from previously published cohorts with stored DNA that were collected by Dr. Laura Southgate at Kings College, London. Details pertaining to the recruitment process and demographics of these cases can be found elsewhere[299, 626].

WES and data processing

As described in methods section, all patients underwent WES and bioinformatics processing of raw data was conducted by the bioinformatics team at UCL.

Pedigree selection and Prioritisation of Variants

To identify candidate genes, eight families were selected for the initial segregation analysis. All affected individuals had a confirmed diagnosis of CH, as validated by a consultant neurologist. Each family required a minimum of three affected individuals including at least one pair of second- degree relatives. All families consisted of between three and six affected individuals including relatives which ranged from parent-child relations to cousins. As reduced penetrance is frequently encountered in familial CH, an affected only analysis was conducted to avoid the exclusion of poorly penetrant variants in relatives who were currently unaffected.

The details of the bioinformatics pipeline used for the prioritisation of variants is described in the methods section. In brief, quality control parameters were first assessed to filter out false positive / artefactual variants. Variants were retained in accordance with the relevant inheritance pattern. Families 1-8 demonstrated an autosomal dominant mode of inheritance therefore only heterozygote variants were included in the analysis. Variants were then tiered based on frequency, impact, expression and scores derived from in-silico predictors.

Cohort screening

To detect additional cases with variants in candidate genes, as derived from the initial analysis of families 1-8, all 237 affected individuals from 137 families with a confirmed diagnosis of CH were screened. Variants in candidate genes were prioritised in accordance with the above strategy and checked for segregation in families.

4.3.5 Results

In total, 237 affected individuals from 137 families were included in the study. This included 1 family with 6 affected individuals, 5 families with 4 affected individuals, 8 families with 3 affected individuals, 63 with 2 affected individuals and 59 singletons with a self-reported family history of CH. The contribution of each site is summarized in **Table 29**.

Table 29: Origin of cases and contributions of study groups involved in international CH collaboration.

Origin	Clinical Centre	Collaborator	Study Group	Number of Families
UK	NHNN	Houlden	Synaptopathies	47
Leiden, The Netherlands	Leiden University Medical Centre	Prof Arn MJM van den Maagdeberg	LUCA	24
Stockholm, Sweden	Karolinska University Hospital	Prof Christina Sjöstrand	N/A	19
Copenhagen, Denmark.	Rigshospitalet-Glostrup, University of Copenhagen	Prof Rigmor Jensen	N/A	7
UK, London	Kings College London	Dr. Laura Southgate and Prof Richard Trembath	N/A	36
Belgium, Ghent	Ghent University Hospital	Prof Koen Paemeleire	N/A	2
Greece	Glyfada Headache Centre	Dr Michael Vikelis	N/A	2

4.3.5 (a) Whole exome sequencing of CH families

Variant Prioritization: Family 1

This family (**Figure 42A**) includes three affected males of Danish origin, across two generation. (II:3, II:5, III:1) and was previously part of a linkage study which attained a LOD score suggestive of significance on chromosome 2[617]. Recruitment occurred in the 1990's and unfortunately longitudinal follow-up information on individuals II:2 and III:2-9 is not available. Therefore, I was unable to clarify the affected status of additional family members. Overall, thirty-four variants of high or moderate impact with an MAF <0.05 were shared between all affected individuals. As a further step, variants with AC of >50 in ION database, or variants predicted as tolerated and benign

by in-silico predictors were excluded. Following filtering, variants in four genes remained and were further prioritised based on their expression in brain leaving three potential candidates (**Figure 42b**, **Table 30**). The first, an inframe deletion in *CACNA1A* occurs in the 46/47 exon, is rare in general population (gnomAD AF of 0.0003276). The second, Solute Carrier Family 2 (*SLC2A8*, [OMIM 605245]) which encodes for GLUT8 is also highly expressed in brain but is broadly expressed in other tissues including the testis and adrenal glands. In-silico predictions of this variant infer pathogenicity (deleterious in SIFT and possibly damaging in Polyphen) however gnomAD constraint scores imply relative tolerance for missense mutations (Z =0.35, o/e =0.94.) The third variant is in Family with Sequence Similarity 178 Member B gene, (*FAM178B*, [HGNC: 28036]) and is in the region of linkage on chromosome 2 identified previously. *FAM178B* is highly expressed in the spinal cord but is also expressed in brain, most highly in the substantia nigra.

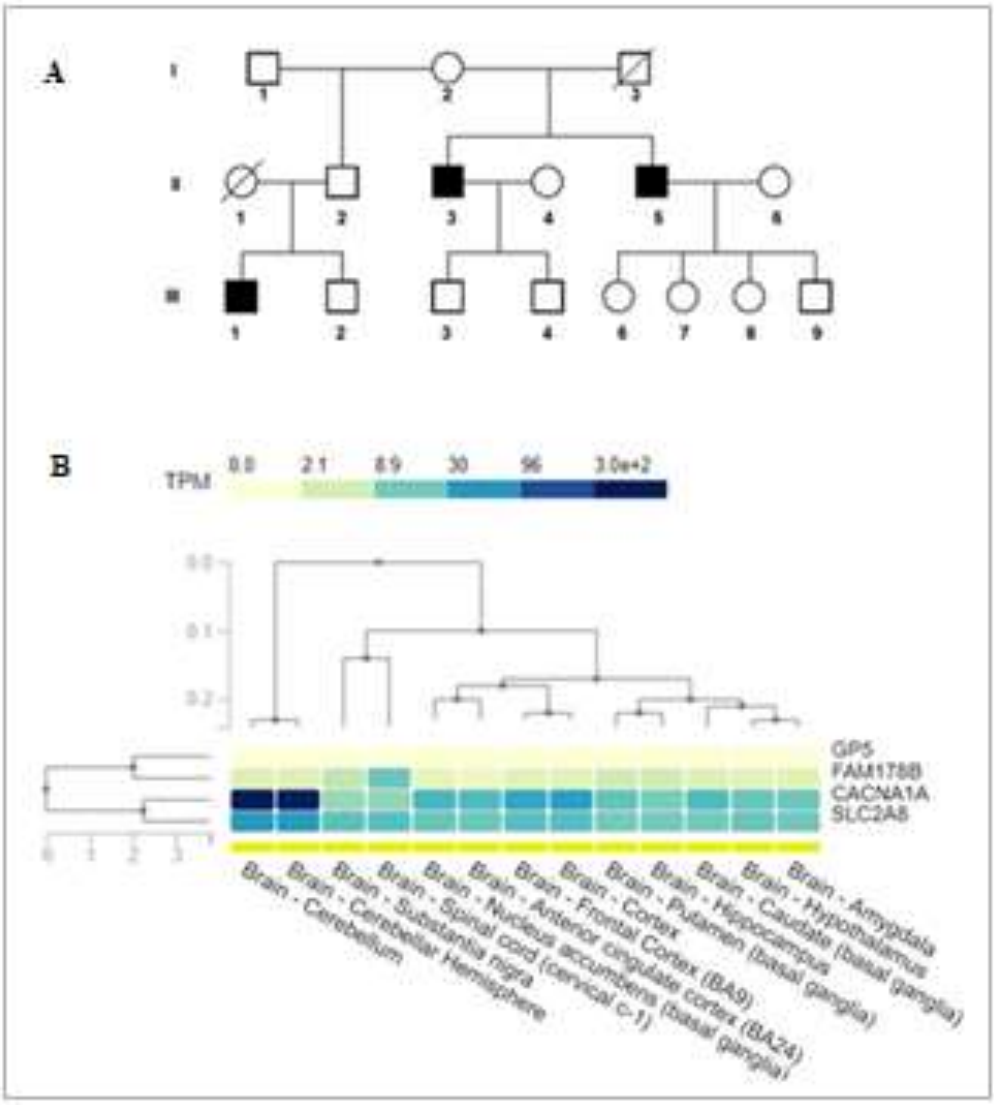


Figure 42A: *Pedigree of family 1 showing three affected male family members. B: Heatmap illustrating expression in brain of candidate genes generated with GTEX showing CACNA1A and SLC2A8 are highly expressed throughout the brain with FAM178B expressed primarily in the substantia nigra and spinal cord.*

Variant Prioritization: Family 2

This Danish family (**Figure 43A**), included four affected individuals (II:3, II:4, III:2, III:3). Forty-two variants with moderate or high impact were shared between all affected members. Thirty-one variants were removed as they were predicted to be tolerated or benign by in-silico predictors. Of the remaining variants, five had a high frequency in ION exomes. Following filtering, variants in six potential candidate genes remained.

Of these, five were expressed in brain (**Figure 43B**). A missense variant in Dishevelled Associated Activator Of Morphogenesis 2 gene (*DAAM2*, [OMIM: 606627]) segregated in the affected individuals. This variant is rare with an AC of 8 in Non-Finnish Europeans (NFE) in gnomAD and encodes a protein that regulates the WNT signaling pathway, which is required for myelination in the central nervous system. A novel missense variant in the highly conserved Clathrin Heavy Chain gene (*CTLC* [OMIM 118955]) was also included. This gene encodes for clathrin which is critical for the intracellular trafficking of receptors and endocytosis of several macromolecules. A missense variant in the Imp U3 Small Nucleolar Ribonuclear Protein 3 gene (*IMP3*, [OMIM 612980]) also segregated with the phenotype and is highly expressed in brain but is also broadly expressed in other tissue and has low constraint scores (z= -0.77). For these reasons, it was excluded from further cohort analysis. A rare variant in Apoptosis-Enhancing Nuclease (*AEN,* [OMIM 610177]) was also prioritised. A variant in the Eya Transcriptional Coactivator And Phosphatase 2 gene (*EYA2*, [OMIM 601654]) was excluded due its high frequency (AC of 97 on gnomAD), as was DENN Domain Containing 2C, (*DENND2C*, [HGNC 24748]), as it is minimally expressed in brain (**Figure 43B**).

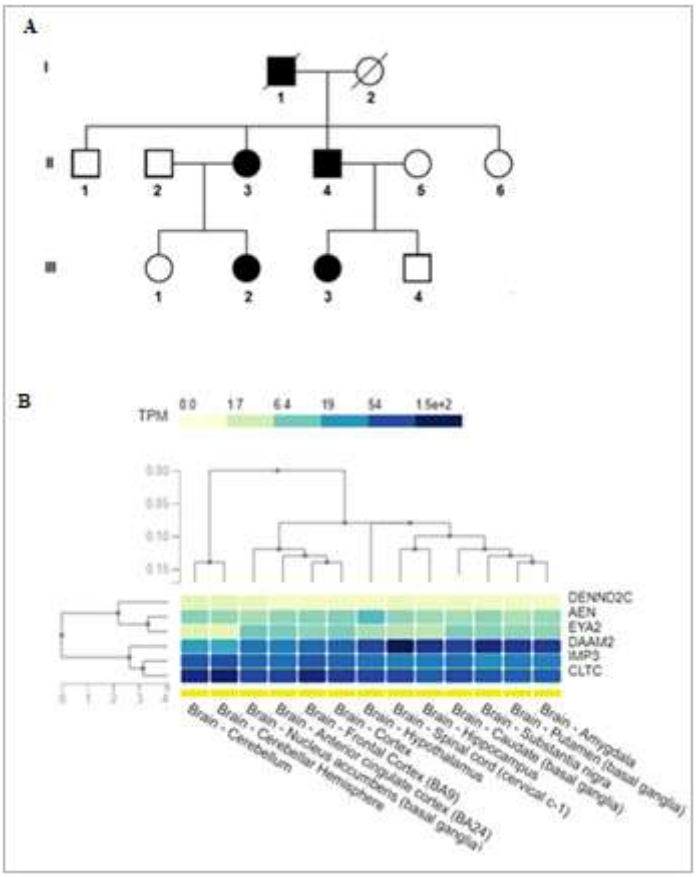


Figure 43A: *Pedigree of family 2 showing four affected individuals.* **B***: Heatmap illustrating generated with GTEX illustrating expression levels of identified genes in brain. DENND2C is poorly expressed in brain and was therefore excluded from downstream analysis.*

Variant Prioritization: Family 3

Family 3 (**Figure 44A**) was also of Danish origin and consisted of five affected individuals however only II:2, II:5, III:2 were available for WES. The filtering strategy identified variants in eleven genes of interest, five of which were highly expressed in brain (**Figure 44B**). These included a variant in Fat Atypical Cadherin 1,(*FAT1,* [OMIM 600976]) which was rare with a gnomAD AC of 3 in NFE, however constraint scores indicate missense variation is well tolerated in this gene (z= -0.43). A variant in tubulin-gamma complex-associated protein 2 (*TUBGCP2,* [OMIM 61781]) was also prioritized. This gene encodes for a protein necessary for microtubule nucleation at

the centrosome. A variant in a paralog of ataxin 2, Ataxin 2-Like gene (*ATXN2L,* [OMIM 607931]) and rare variant (gnomAD AC of 7 in NFE) in Brisc And Brca1 A Complex, Member 1, (*BABAM1,* OMIM 612766]) which is involved in double-strand DNA repair were prioritised.

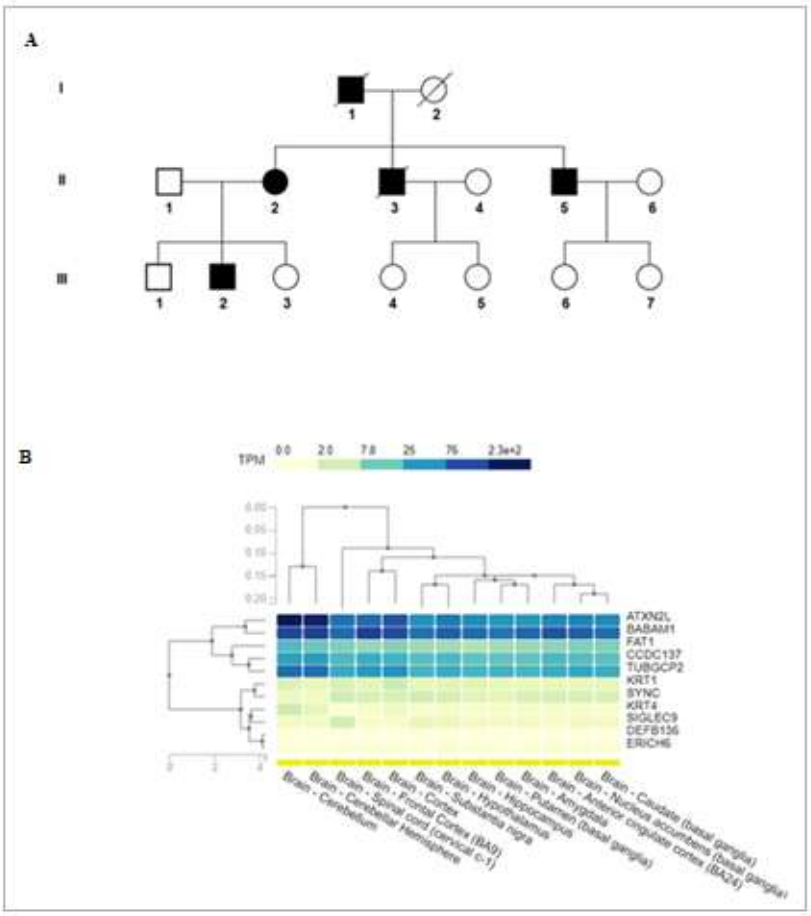


Figure 44A: Pedigree of family 3 showing five affected individuals. **B**: Heatmap illustrating generated with GTEX illustrating expression levels of identified genes in brain. Five of the eleven candidates were expressed in brain and was therefore included in cohort analysis.

Variant Prioritization: Family 4

Four affected individuals (I:2, II:4, II:5, III:5) across three generations were available for WES in this Danish family. As shown in pedigree (**Figure 45A**) there was a clear autosomal dominant mode of transmission therefore heterozygote variants with a MAF <0.05 were retained. A total of thirty-nine moderate or high impact variants segregated

with disease. Twenty-nine were excluded due to frequency and tolerated or benign in-silico predictions. Of the remaining variants, four were expressed in brain and therefore included in cohort analysis. Of these, variants in two genes highly expressed in brain segregated with the phenotype (**Figure 45B**). These included rare variants in heat-shock transcription factor 2 (*HSF2*, [OMIM 140581]) which encodes a protein which acts as a molecular chaperone at several stages of protein biogenesis and a novel mutation in the highly conserved (gnomAD z score = 4.64) Calcium Channel, Voltage-Dependent, T Type, Alpha-1g Subunit gene (*CACNA1G,* [OMIM: 604065]). Other qualifying genes included A4GALT (Alpha-1,4-Galactosyltransferase, OMIM: 607922), which encodes a protein which acts as a catalyst for the transfer of galactose to lactosylceramide to form globotriaosylceramide, and the tRNA-histidine guanylyltransferase 1-like gene (*THG1L,* [OMIM 618802]). Homozygous mutataions in THG1L have previously been implicated in autosomal recessive spinocerebellar ataxia 28.

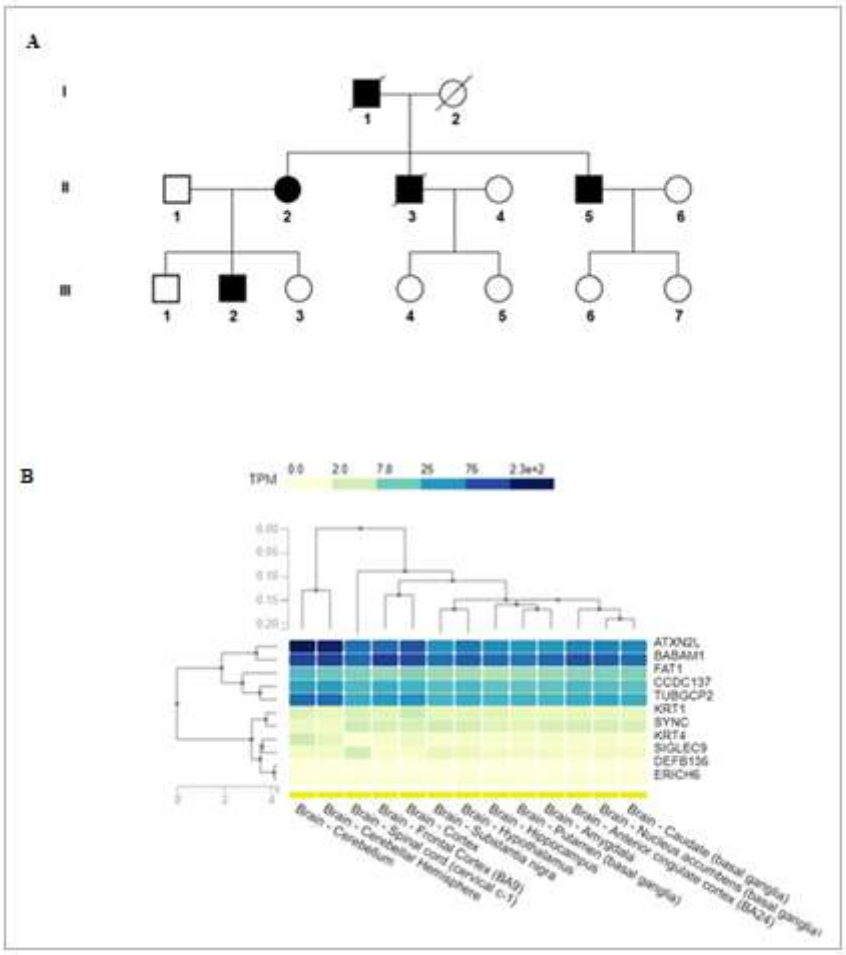


Figure 45A: *Pedigree of family 4 showing four affected individuals.* ***B:*** *Heatmap illustrating generated with GTEX illustrating expression levels of identified genes in brain. Four candidates were expressed in brain and therefore included in cohort analysis.*

Variant Prioritization: Family 5

Family 5, also of Danish descent, consisted of seven affected individuals (II:2, III:2, III:5, IV:1, IV:2, IV:3, IV:4), six of whom underwent WES. The pedigree (**Figure 46A**) reflected an autosomal dominant mode of transmission and therefore only heterozygote variants were retained for further analysis. A total of eight moderate or high impact variants segregated with disease. Five of these were excluded based frequency and in-silico pathogenecity prediction tools. Of the remaining variants, two were abundantly expressed in brain (**Figure 46B**). These included the Lysophosphatidic Acid Receptor 3 gene (*LPAR3*, [OMIM: 605106]) and a novel

mutation in the Purinergic Receptor P2X 6 gene (*P2RX6* [OMIM 608077]) was also prioritized. The Spermatogenesis Associated 17 gene (*SPATA17* OMIM: 611032) was not highly expressed in brain and was therefore removed from downstream analysis.

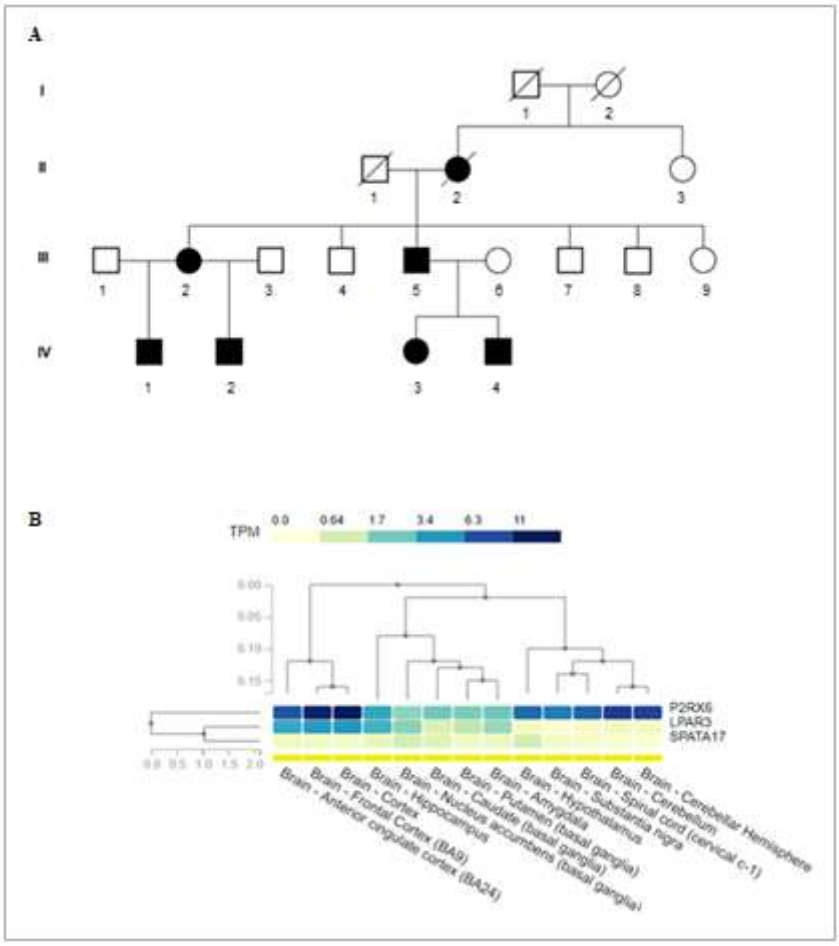


Figure 46A: *Pedigree of family 5 showing seven affected individuals.* **B:** *Heatmap illustrating generated with GTEX illustrating expression levels of identified genes in brain. Two candidates (LPAR3 and P2RX6) were expressed in brain and therefore included in cohort analysis.*

Variant Prioritization: Family 6

Three affected (II:1, III:5, IV:5) males from this Dutch family (**Figure 47A**) underwent WES. Again, the inheritance pattern was autosomal dominant. Fourty-one variants of high or moderate impact with an MAF <0.05 were shared between all affected individuals. The same filtering strategy was used in variant prioritisation with the removal variants based on frequency and predicted pathogenecity. Following filtering, variants in eight genes were included, and expression analysis confirmed that 7 were expressed in the CNS (**Figure 47B**). These included the Mitochondrial Fission Regulator 1 Like gene (*MTFR1L*, [HGNC 28836]), the Paternally Expressed 3 gene (*PEG3* [OMIM 601483]), the AT-Hook DNA Binding Motif Containing 1 gene, (*AHDC1*, [OMIM 615790]), the Phospholipase A2 Group IVB gene (*PLA2G4B*, [OMIM 606088]), FGR Proto-Oncogene (*FGR*, [OMIM 164940]), Von Willebrand Factor A Domain Containing 5A gene, (*VWA5A*, [OMIM 602929]) and Tuftelin 1 (*TUFT1*, [OMIM 600087]).

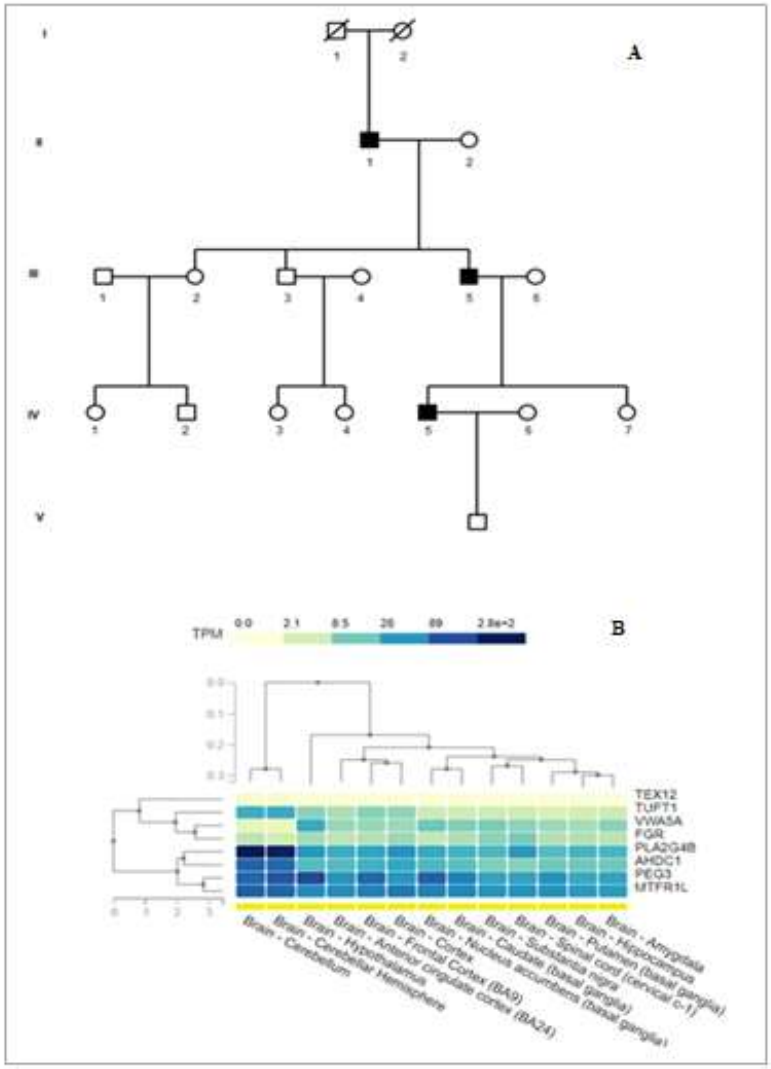


Figure 47A: *Pedigree of family 6 showing three affected individuals*. **B:** *Heatmap illustrating generated with GTEX illustrating expression levels of identified genes in brain. Seven candidates were expressed in brain and therefore included in cohort analysis.*

Variant Prioritization: Family 7

This was a Swedish family consisting of four affected individuals (II:3, II:5, III:3, III:5) presenting with CH who underwent WES. As shown in pedigree (**Figure 48A**) there was an autosomal dominant mode of transmission. A total of fifty-four moderate or high impact heterozygote variants segregated with disease. The majority of these were excluded due to an allele frequency above the cut-off point of the filtering strategy or higher than expected frequency in the ION in-house exomes. Additional variants were

removed as they were predicted to be tolerated and benign in-silico on SIFT and Polyphen respectively. Of the remaining variants, 2 were expressed in brain including the Set Domain-Containing Protein 1b gene (*SETD1B,* OMIM 611055) and Glutamate Receptor, Ionotropic, N-Methyl-D-Aspartate 3a gene (*GRIN3A,* OMIM 606650) **(Figure 48B).**

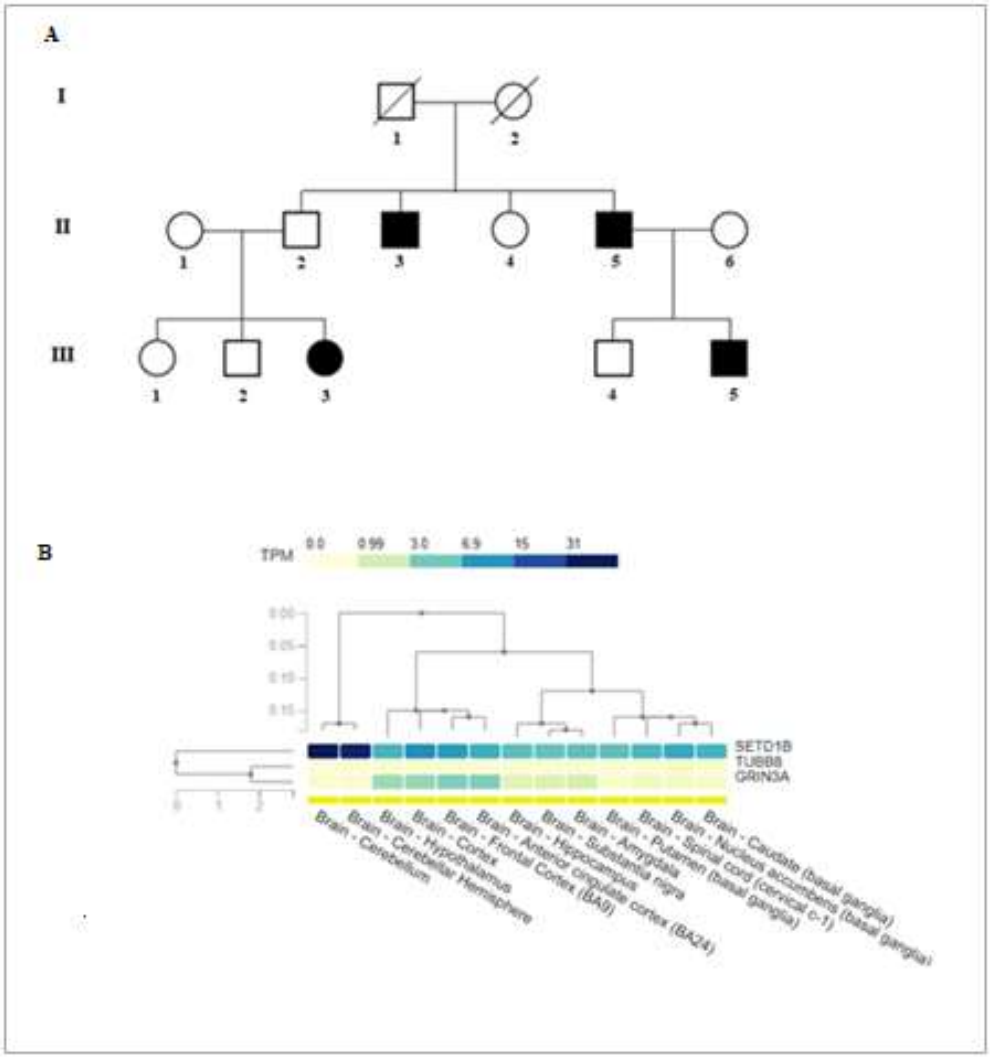


Figure 48A: *Pedigree of family 7 showing three affected individuals*. **B:** *Heatmap illustrating generated with GTEX illustrating expression levels of identified genes in brain. Two candidates (SETD1B and GRIN3A) were expressed in brain and therefore included in cohort analysis.*

Variant Prioritization: Family 8

This was a Danish family consisting of four affected individuals (I:2, II:3, III:4, III:7) with an autosomal dominant mode of inheritance (**Figure 49A**). Overall, sixty-two heterozygote moderate or high impact variants with a MAF <0.05 segregated with disease. Following employment of filtering strategy, six genes were analysed further for expression analysis and five were highly expressed in brain and included in cohort screening (**Figure 49B**).

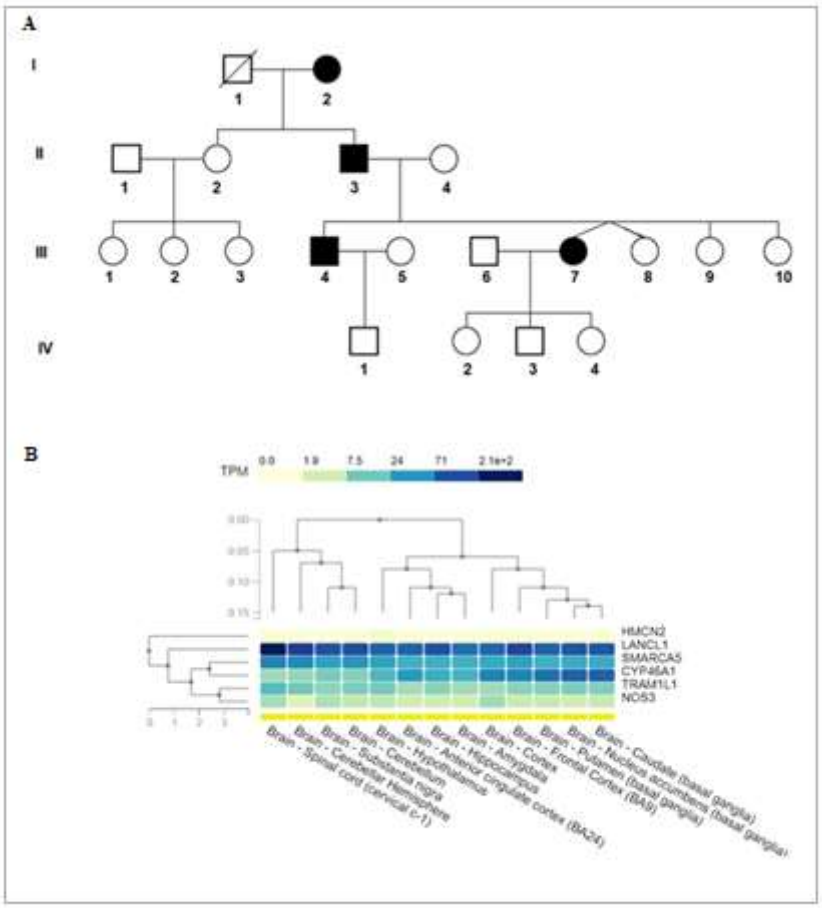


Figure 49A: *Pedigree of family 8 showing four affected individuals.* **B**: *Heatmap illustrating generated with GTEX illustrating expression levels of identified genes in brain. Five candidates were expressed in brain and therefore included in cohort analysis.*

In total, segregation analysis in families 1-8 identified variants in thirty genes expressed in brain (**Table 30**). The majority of these were of moderate impact representing rare non-synonymous SNV and ten were novel on gnomAD. Seven of

the genes identified were previously associated with a neurological phenotype. These included *CACNA1A* (FHM1, EA2, SCA6), *CLTC* (Autosomal Dominant mental retardation), *TUBGCP2* (Autosomal Recessive Pachygyria, microcephaly, developmental delay and seizures), *THG1L* (SCAR 28), *CACNA1G* (SCA42) and *A4GALT* (Xia Gibbs syndrome).

Table 30: List of prioritised variants from WES analysis of families 1-8

Fam	CHR	Gene	Transcript	Mutation	Nucleotide	Amino Acid	rsID	SIFT	PolyPhen	gnomAD AF	ION Hets	CH Hets
1	2	*FAM178B*	ENST00000393526.6	Missense	c.128T>G	p.Leu43Arg	rs200958044	D(0)	PD(0.999)	0.0001954	5	5
1	9	*SLC2A8*	ENST00000373360.7	Missense	c.251G>A	p.Gly84Glu	rs745733061	D(0)	PD(1)	-	3	3
1	19	*CACNA1A*	ENST00000360228.10	Missense	c.6647_6658del	p.His2216_His2219del	rs770368215	-	-	0.0003276	5	3
2	6	*DAAM2*	ENST00000274867.8	Missense	c.313C>T	p.Arg105Cys	rs376069544	D(0)	PD(0.938)	0.00002456	6	6
2	15	*AEN*	ENST00000332810.3	Missense	c.115C>T	p.Arg39Trp	rs756247681	D(0)	PD(0.993)	0.00003679	9	6
2	17	*CLTC*	ENST00000269122.7	Missense	c.2699A>G	p.Tyr900Cys	-	D(0)	PD(0.999)	-	7	7
3	4	*FAT1*	ENST00000441802.6	Missense	c.6743C>T	p.Pro2248Leu	rs367776554	D(0)	PD(1)	2.85E-05	4	4
3	10	*TUBGCP2*	ENST00000252936.7	Missense	c.1472C>T	p.Ala491Val	rs149207468	D(0)	PD(1)	6.09E-05	4	4
3	16	*ATXN2L*	ENST00000325215.10	Missense	c.379C>T	p.Leu127Phe	.	D(0)	PD(0.997)	-	4	4
3	19	*BABAM1*	ENST00000359435.8	Missense	c.833A>G	p.Tyr278Cys	rs767310034	D(0)	PD(0.943)	2.04E-05	8	4
4	5	*THG1L*	ENST00000231198.11	Missense	c.260C>T	p.Ala87Val	-	D(0)	PD(0.998)	-	6	7
4	6	*HSF2*	ENST00000368455.8	Missense	c.338C>T	p.Ser113Phe	rs745668853	D(0)	PD(0.969)	2.08E-05	7	6
4	17	*CACNA1G*	ENST00000352832.9	Missense	c.3820C>A	p.Arg1274Ser	-	D(0)	PD(0.999)	-	6	4
4	22	*A4GALT*	ENST00000249005.3	Missense	c.290C>T	p.Ser97Leu	rs146544312	D(0)	Pos D (0.506)	2.03E-05	6	6
5	1	*LPAR3*	ENST00000370611.3	Missense	c.784G>A	p.Asp262Asn	-	D(0)	PD(0.997)	8.15E-06	6	6
5	22	*P2RX6*	ENST00000401443.5	Missense	c.645C>A	p.Phe215Leu	-	D(0)	PD(0.981)	-	6	6
6	1	*MTFR1L*	ENST00000464008.6	Missense	c.83A>G	p.Tyr28Cys	rs1031564497	-	-	1.34E-05	3	3
6	1	*AHDC1*	ENST00000247087.10	Missense	c.4237C>T	p.Arg1413Trp	rs777716141	D(0)	PD(0.952)	3.49E-05	3	3

6	1	*FGR*	ENST00000374003.7	Missense	c.457G>A	p.Asp153Asn	rs1034293860	D(0.)	PD(0.938)	-	3	3
6	1	*TUFT1*	ENST00000353024.4	Missense	c.274C>T	p.Arg92Trp	rs150612239	D (0)	Pos D(0.855)	5.28-05	10	5
6	15	*PLA2G4B*	ENST00000452633.5	Splice Acceptor	c.1948-2del	-	-	-	-	0.0000583	9	5
6	19	*PEG3*	ENST00000326441.14	Missense	c.3713C>G	p.Ser1238Cys	rs370699815	D(0)	PD(0.99)	3.26E-05	5	5
6	11	*VWA5A*	ENST00000361352.9	Missense	c.789G>A	p.Met263Ile	rs756944339	D(0)	Pos D (0.53)	1.63E-05	4	4
7	12	*SETD1B*	ENST00000267197.9	Missense	c.517A>C	p.Ile173Leu	-	D(0.04)	B(0.391)	-	4	4
7	9	*GRIN3A*	ENST00000361820.3	Missense	c.2309G>A	p.Gly770Asp	rs181715524	D(0.02)	PD(0.987)	3.9E-06	10	7
8	2	*LANCL1*	ENST00000233714.8	Missense	c.26C>T	p.Pro9Leu	rs1380145499	D(0.05)	PD(0.998)	4.06e-06	4	4
8	4	*TRAM1L1*	ENST00000310754.5	Missense	c.803C>T	p.Ser268Phe	-	D(0)	PD(0.936)	-	4	4
8	4	*SMARCA5*	ENST00000283131.3	Missense	c.163G>T	p.Asp55Tyr	rs1243527828	D(0.4)	Pos D (0.713)	-	4	4
8	14	*CYP46A1*	ENST00000261835.7	Missense	c.1221C>G	p.Asp407Glu	rs375146687	D(0.4)	Pos D (0.609)	8.12E-06	4	4
8	7	*NOS3*	ENST00000297494.7	Missense	c.466G>A	p.Glu156Lys	rs141456642	D(0.1)	Pos D (0.547)	0.000234	10	7

Abbreviations: Fam: Family. Chr: Chromosome. D: Deleterious, PD: Probably damaging, Pos D: Possibly Damaging, ION: Institute of Neurology Exomes, Hets: Heterozygotes

4.3.5 (b) Cohort Screening

In total, 100,549 variants with a minor allele frequency of <0.05 and a high or moderate impact as defined by sequence ontology were extracted. Variants in candidate genes (derived from segregation analysis of families 1-8) underwent prioritization. Following filtering, 136 variants remained.

Of the thirty genes included for screening (**Table 31**), rare variants in four genes segregated in more than one family and fulfilled the prioritization criteria (**Table 31**). This included three missense variants in *FAT1* which demonstrated segregation with affected status in three families respectively, including twelve affected individuals, of Swedish, Danish and Dutch descent. All three variants were classified as deleterious or probably damaging by SIFT and polyphen. Rare variants in *FAT1* were also found in two singletons of British and Danish descent. Three of the five variants (p.Ile1712Thr, p.Glu2401Gln, p.Asp1836Asn) were novel and two were rare (p.Val1109Leu, p.Pro2248Leu). Variants in *CACNA1G* (p.Arg1274Ser, p.Arg1747His) also segregated in two families, one Danish and one Dutch, involving six affected individuals. A third variant (p.Pro519Thr) was identified in a singleton however, I was unable to contact additional family members to establish segregation. All three *CACNA1G* variants were novel and predicted as damaging. Variants in *SETD1B* also segregated in a Swedish (p.Ile173Leu) and British (p.Ser1306Cys) family. These variants were rare with an allele frequency of 0.0005417 and 0.0001278 respectively. Both were categorised as deleterious by SIFT but benign on polyphen. Finally, two variants in *FAM178B* (p.Leu43Arg, p.Met535Ile) segregated in two Danish families involving five affected individuals.

Table 31: Candidate Genes derived from cohort screening

Table 4.3.3 : Candidate Genes derived from cohort screening													
Chr	Gene	Fam	Pro	Ind	Mutation	Transcript	Variant	AA	Patient IDs	rsID	gnomAD	SIFT	Polyphen
4	*FAT1*	3	2	13	Missense	ENST00000441802.6	c.5135T>C	p.Ile1712Thr	6924,6927,6928,6929,8337,S0027	-	-	D (0)	PD (0.94)
					Missense	ENST00000441802.6	c.6743C>T	p.Pro2248Leu	7043, 7046, 8347	rs367776554	2.85E-05	D (0)	PD (1)
					Missense	ENST00000441802.6	c.7201G>C	p.Glu2401Gln	N64512, N64533	-	-	D (0.01)	PD (0.988)
					Missense	ENST00000441802.6	c.5506G>A	p.Asp1836Asn	106160	-	-	D (0.01)	PD (1)
					Missense	ENST00000441802.6	c.3325G>C	p.Val1109Leu	6954	rs200891145	9.83E-05	D (0.1)	B (0.3)
17	*CACNA1G*	2	1	7	Missense	ENST00000352832.9	c.1555C>A	p.Pro519Thr	105742	-	-	D (0.03)	Pos D (0.663)
					Missense	ENST00000352832.9	c.3820C>A	p.Arg1274Ser	6958, 6959,6960, 6961	-	-	D (0)	PD (0.99)
					Missense	ENST00000352832.9	c.5240G>A	p.Arg1747His	N65788 N67112	-	-	D (0)	PD (0.93)
9	*SETD1B*	2	0	6	Missense	ENST00000267197.9	c.517A>C	p.Ile173Leu	20529,20532, S0183,20528		0.0005417	D (0)	B (0.012)
					Missense	ENST00000267197.9	c.3917C>G	p.Ser1306Cys	65684, 67530	rs201434489	0.0001278	D (0.04)	B (0.391)
2	*FAM178B*	2	0	6	Missense	ENST00000393526.6	c.128T>G	p.Leu43Arg	7078,7079,S0016		0.0001954	D (0)	PD (0.99)
					Missense	ENST00000490605.2	c.1605G>A	p.Met535Ile	7971, 7972		6.61E-06	D (0.1)	B (0.26)
Abbreviations: AA: Amino acid,B: Benign, D: Deleterious, Fam: Number of Families, Ind: Affected individuals, PD: Probably damaging, Pos D: Possibly Damaging, Pro: Number of probands.													

Abbreviations: AA: Amino acid, B: Benign, D: Deleterious, F: Number of Families, Ind: Affected individuals, PD: Probably damaging, Pos D: Possibly Damaging, Pro: Number of probands.

4.3.6 Discussion

Evidence of familial cases of CH ,and recurrent reports of autosomal dominant inheritance patterns in these families, supports the possibility of the existense of a monogenic form of the disorder caused by rare genetic variants with a high impact on phenotype[493]. Based on this assumption, this study sought to identify candidate genes for familial cluster headache through the examination of rare, putative pathogenic variants segregating in affected families. To achieve this, I screened for genes with two or more rare non-synonymous variants segregating in more than one family. Clearly two such hits is inadequate to attribute causality, and this is further complicated by evidence of reduced penetrance in some families and the possibility of loci hterogeneity. However, this approach provides a framework for the identification of candidates for future cohort, functional and mechanistic exploration.

Initial evaluation of families 1-8 prioritized interesting candidates. These included variants in genes implicated in mendelian neurological disorders such as FHM (*CACNA1A*), Cerebellar ataxia (*CACNA1G, THG1L, CACNA1A*) and developmental delay with seizures (*SETD1B, TUBGCP2*)[11, 632-635]. Other genes of interest identified have a plausible etiological role. These included, *LPAR3* which encodes a protein that mediates lysophosphatidic acid-evoked calcium mobilisation, is highly expressed in the trigeminal ganglion and is involved in the initiation of neuropathic pain[636 637, 638]. Similarly, A novel mutation in *P2RX6* was also prioritized and encodes a receptor in an ATP-depenedent ligand-gated ion channel. Purinergic signalling has a hypothesised role in the etiology of migraine and peripheral dura vascular sensitisation and therefore presents an interesting candidate [639, 640]. Furthermore, variants in some

segregating genes (*FAM178B, FAT1*) are associated with lithim responsiveness, a treatment used in CH[136, 641].

Following cohort screening, tiered variants in four genes (*FAT1, CACNA1G, SETD1B, FAM178B*) segregated in more than one family. Of these *FAT1* and *CACNA1G* provided the most compelling evidence with the highest number of affected individuals, very low frequency on population databases and favourable pathogenecity predictions.

The *FAT1* variants segregated in three families and were also found in two affected singletons. All variants represented missense mutations that were rare or novel on gnomAD and predicted to be pathogenic however contraint scores show relative tolerance for such variation. FAT1 is a member of the atypical cadherin family, with a large extracellular domain that is comprised of thirty-four cadherin domains and extracellular cadherin motifs [642, 643]. Cellular localisation indicates that it plays a critical role in cell-cell adhesion, cell polarity and migration[642]. Loss of FAT1 function in humans and mice results in glomerular nephropathy reduced epithelial cell adhesion and effacement of podocyte foot processes[644]. It is highly expressed throughout embryonic neurodevelopment and FAT1 deficient mouse models exhibit severe defects of the nervous system and defective eye development.[645] It regulates actin dynamics and assebly through Ena/VASP signalling pathways and also binds to beta-catenin and synaptic scaffolding proteins Homer 1 and Homer 3[642, 646, 647]. Although the exact function of FAT cadherins in the nervous system remains unclear, they appear to play a critical role in neural tube closure and the creation of complex neural circuits through influencing proliferation, migration and differentiation. Fat4 works in

combination with FAT1 to regulate the migration of neurons to the cortical plate. Downregulation of FAT1 has been shown to impair radial migration in the developing cortex resulting in the abnormal accumulation of differentiated neurons below the cortical plate[648]. FAT1 mutations have been linked to severe neuropsychiatric disorders including schizophrenia, bipolar disorder, autism spectrum disorders, facioscapulohumeral muscular dystrophy (FSHD) and SCA[649-652].Interestingly, variation in FAT1 is associated with susceptibility to bipolar disease[649]. This association was derived follwing several linkage studies which indicated a susceptibility locus for bipolar disease on chromosome 4q35 and has been replicated in an independent cohort[653-656]. Similiarites exist between bipolar disease and CH, and the conditions co-occur in approximately 6.6% of patients[657]. Both exhibit perioidicity with postulated hypothalamic dysfunction, have distinct relationships with sleep and neuroendocrine abnormalities[658]. Furthermore both disorders respond to treatment with Lithium[136, 659]. Interestingly, in mice lithium induced downregulation of FAT1 and upregulation of ENAH and CATNB that code for the FAT1- associated proteins β-catenin and Ena/VASP indicating a direct link between Lithium and the FAT1 signalling pathway[649]. *FAT1* mutations have also been implicated in Autism Spectrum Disorders (ASD). Rare de-novo mutations have been identified in probands with ASD and damaging missense mutations segregated in affected individuals in three muliplex families[660 661 650]. Considering the establised role of FAT cadherins in dendrite orientation, it is possible that FAT1 dysfunction leads to alteration in neural circuit organization in CH.

Rare potentially pathogenic variants in a second candidate *CACNA1G*, segregated with disease in six affected individuals across two families, with a seventh variant identified in one singleton. *CACNA1G* encodes the voltage-dependent T-Type calcium

channel subunit alpha-1G (Cav3.1) which forms four sensor domains and a pore domain[662]. Six transmembrane (S1- S6) segments are located within the protein's four (I-IV) homologous repeats (**Figure 50**).

It is expressed highly in the brain especially in the amygdala, subthalamic nuclei, cerebellum and thalamus [663]. T-Type calcium channels are part of a 'low-voltage activated' category of channels which open at relatively negative potentials and undergo voltage-dependent inactivation[662]. These channels have pace-making roles in cardiac nodal cells and central neurons and regulate calcium signalling in vascular smooth muscle.[662]

CACNA1G mutations have previously been associated with a number of neurological phenotypes. Heterozygous mutations, within segment S4 of the IV repeat, cause SCA42, a slowly progressive pure cerebellar ataxia[664, 665]. Similarly de-novo variants, in S6 of repeat IV are responsible for early-onset syndromic cerebral ataxia with associated cognitive impairment, dysmorphism, microcephaly and epilepsy[632]. Variants in this gene also appear to function as genetic modifiers in epilepsy caused by the voltage-gated sodium channel *SCN2A*, impacting the severity of seizure phenotypes [666]. In our families, mutations occurred within the transmembrane segment S1 of repeat III (p.Arg1274Ser), which contributes to the voltage sensor domain, and helical S5 of repeat IV, which forms part of the pore domain. An additional, potentially pathogenic variant was also observed in the cytoplasmic domain between repeat I and II (p.Pro519Thr) in a singleton (**Figure 50**).

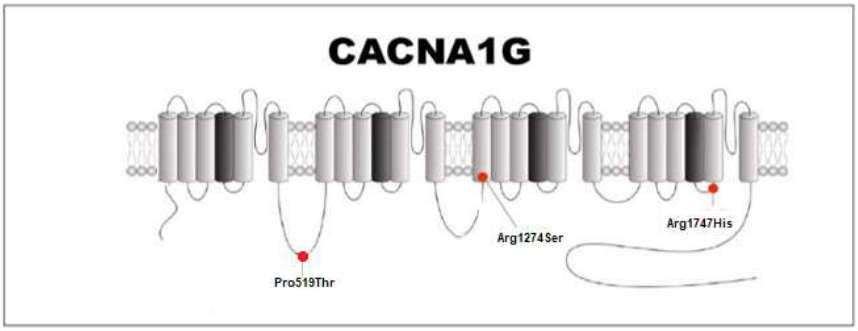


Figure 50: *Structure of CACNA1G protein showing four repeat domains consisting of six transmembrane segment. Red dots indicate locations mutations identified in CH families.*

In the context of CH, *CACNA1G* provides an interesting candidate for a number of reasons. Firstly, calcium channels have previously been implicated in headache conditions, namely *CACNA1A* mutations in FHM[11]. Secondly, Verapamil which is frequently used as the prophylactic agent of choice in CH, blocks $Ca_V3.1$ T-type Ca^{2+} channels[239, 667]. Thirdly, thalamic burst firing is also regulated by these channels[668]. Cortical low-frequency oscillations have been demonstrated in individuals with neuropathic pain, a process caused by thalamic burst firing[668-671]. Finally, the role of $Ca_V3.1$ T-type Ca^{2+} channels in the generation trigeminal neuropathic pain has been illustrated in knock-out CaV3.1 mouse models[672, 673]. Furthermore, ABT-639, a selective T-type Ca^{2+} channel blocker, reduces neuropathic pain in rat models[674].

In conclusion, this is the first study utilising WES to investigate familial CH. Using segregation analysis, I identified an enrichment of rare variants in *FAT1* and *CACNA1G* in these families. These finding require further replication in a larger cohort, variant validation and functional characterisation. However, this study provides the first steps towards elucidating the genetic architecture underlying familial CH.

4.4 Rare Variant Association analysis

4.4.1 Statement of Contribution

I collected and phenotyped the UK CH cases and established international collaborations to recruit additional cases and boost power. I prepared samples for WES and conducted the analysis under the supervision of Dr. Jana Vandrovcova.

4.4.2 Introduction

Evolutionary theory dictates that deleterious variants are inevitably rare, as they undergo natural selection, and loss of function variants are even rarer[395, 675, 676]. It is possible that, at least in a proportion of cases, low frequency variants (0.5% <MAF<5%) and rare variants (MAF <0.5%), that are not detected in GWAS predispose to CH . It remains unclear whether such 'rarer' variants may have a frequency just below the threshold for detection in GWAS or are very rare with a large effect. This could explain familial CH, which appears to have an autosomal dominant inheritance pattern, possibly reflecting a monogenic disorder or a combined burden of rare variants[297]. The paroxysmal nature of TACs may reflect the involvement of genes encoding ion channels and/or their machinery. I therefore hypothesised that these 'channelopathy genes' were plausible candidates. Rare variant association studies (RVAS), on the other hand, provide a hypothesis free approach to directly identify causal genes. This approach works optimally where each causal variant is anticipated to explain only a proportion of affected individuals in the study, but several causal alleles in the same gene has a larger cumulative affect[677]. Previously this method has been used in combination with GWAS to successfully identify rare variants in Triggering Receptor Expressed On Myeloid Cells 2 (*TREM2*, [OMIM 605086] *)* and Phospholipase D Family, Member 3 (*PLD3,* [OMIM 615698]) as a risk for Alzheimer's

disease [678, 679]. The decreasing cost of whole exome sequencing (WES) which enables the interrogation of all protein-coding variants in an individuals genome has made such analysis more accesible.

One impediment to this approach is the requirement of a sample size large enough to achieve statistical significance, particularly in a classical single-variant based association analysis. For this reason, based on the findings of an increased risk of concurrent SUNCT in familial CH (OR 3.76, 95%CI; 1.57-9.95, p0.04) (Section 3.4.4), and the possibility of a unifying pathogenic mechanism[472, 509]. I decided to conduct my analysis on a combined cohort of familial CH, Sporadic CH and SUNCT.

4.4.3 Aims and objectives

The purpose of this study is to identify rare variants which contribute to the development of TAC such as CH / SUNCT. This will involve three parts:

1. A hypothesis driven approach involving the screening of channelopathy genes highly expressed in brain.
2. A single variant association analysis
3. A rare variant burden analysis

4.4.4 Methods

4.4.4 (a) Sample collection

Details pertaining to sample collection are described in the methods (section 2.3.2). In brief, cases were recruited at UK CH sites across the UK and a proportion of the cases were obtained through international collaboration (**Table 32**). All cases were phenotyped by neurologist in accordance with diagnostic criteria (based on timing of recruitment this differed from IHS – ICHD3) and were of European ethnicity. Overall,

three cohorts of cases were included in the analysis (**Table 32)** For used a subset of the Queen Square Genetics (QSG) consortium controls that were generated using the same library capture and which consist of cases of rare-neurological disorders excluding all headache conditions.

Table 32: Cohorts used in RVAS

Cohort	Number of Cases
Familial CH	237
Sporadic CH	42
SUNCT	46
Queensquare Neurolgenetic Consortium Controls	3661

Abbreviations: CH: Cluster Headache, SUNCT: Short-lasting, Unilateral, Neuralgiform headache attacks with Conjunctival injection.

4.4.4 (b) Whole Exome sequencing

All exomes for cases and controls were generated using SureSelect-V4 or Sure select-V6 exome capture and sequenced using Illumina instruments generating 100bp paired-end reads. After removal of PCR duplicates (Picard) and reads without a unique mapping location, local realignment around Indels followed by variant calling with Haplotype Caller was conducted according to GATK (Genome Analysis Toolkit) best practices by the UCL Bioinformatics team[385]. Further information can be found in methods (section 2.3.8).

4.4.4 (c) Candidate gene analysis: Ion channelopathy genes

A list for genes involved in ion channel transmission was derived from genomic england panelApp[680] and “channel activity” genes as categorised by Gene Set

Enrichment Analysis (GSEA)[681]. Genes were then prioritised based on their expression profile in brain according to GTEX [539]. Overall, a list of 237 genes were selected for candidate analysis (appendix section IV, 4.4.4b). Non-synonymous, putative pathogenic variants in listed genes were examined for significance in the entire cohort. Significant variants were then checked for segregation in cases where family members were available.

4.4.4 (d) Quality Control (QC)

QC was conducted using bcftools v1.10 [379]. The protocol for quality control is detailed in methods (section 2.4.6a) and commands are available in appendix (Section IV, 4.4.4a).

Coverage

The majority of samples were generated using Sureselect-V4 which showed greater coverage compared with Sureselect-V6. Median exome coverage was 25.5x. Samples with less than 10x were removed leaving 323 cases and 3655 controls (**Figure 51**).

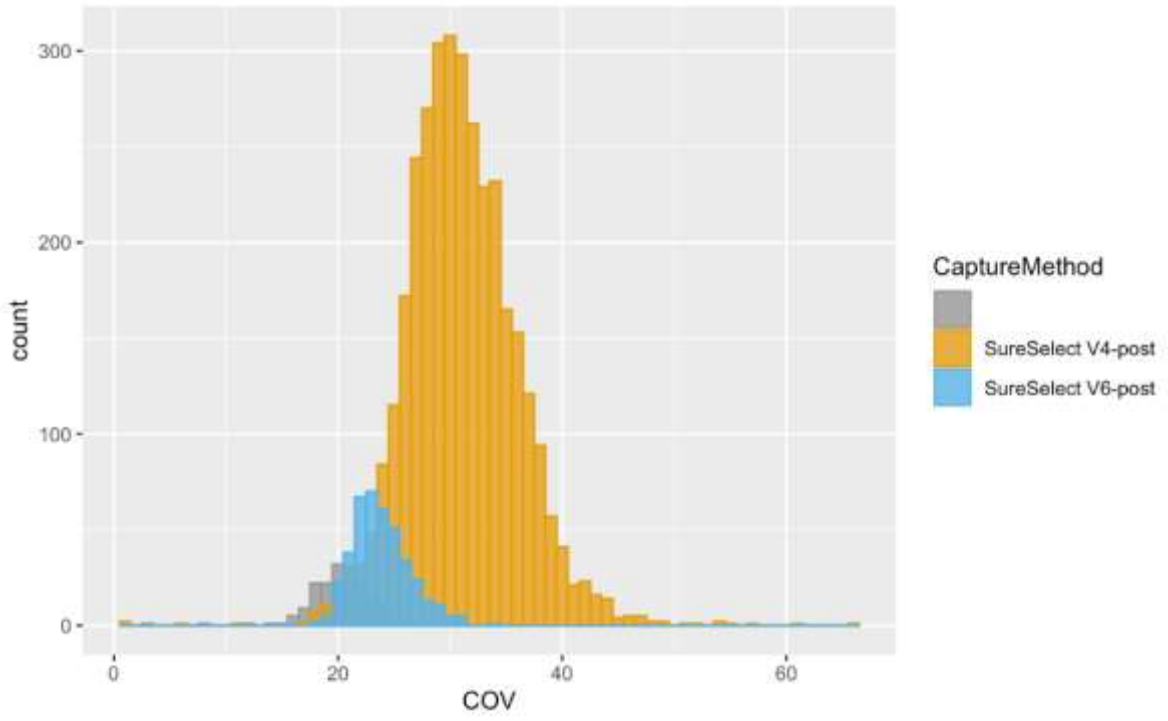


Figure 51: *Coverage per exome split by capture method for cases and controls. All samples with a coverage below 10x were removed. Samples with an unknown capture method are in grey.*

Contamination

Samples with a with contamination rate above the 3rd quartile (> 0.01) were excluded from further analysis. This step removed 1 case and 73 controls leaving 322 cases and 3582 controls (**Figure 52**).

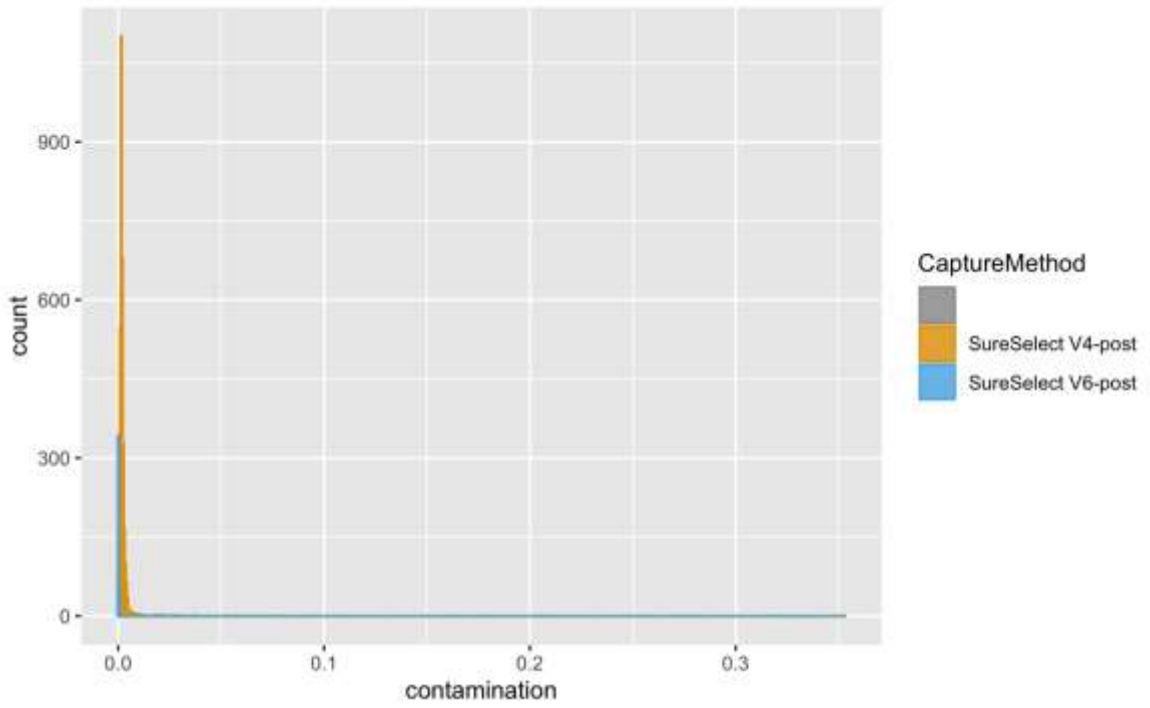


Figure 52: *Depicting contamination by capture method.*

Transition (Ti) to Transversion (Tv) Ratio

To avoid false positives in callset Ti/Tv ratio in all samples was checked. There were no outliers and all samples had a Ti/Tv ratio between 2.5 and 2.75.

Sex check

Sex check was then performed using X-chromosome heterozygosity rate using Peddy, whereby males are expected to have a heterozygosity rate of 0. Samples were then

filtered for ambiguous gender. A total of 320 cases and 3531 controls remained after this step.

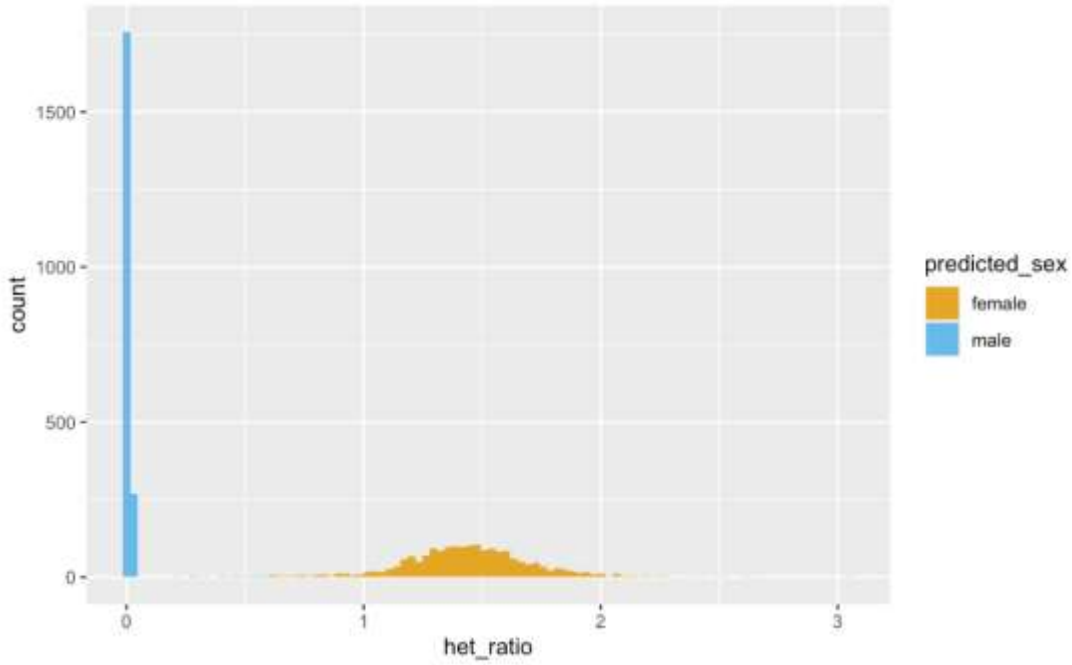


Figure 53: *Sex- check for cases based on X chromosome heterozygosity rate. Males are represented in blue and females are in orange.*

Ethnicity

Peddy was used to assess ancestry. Only cases of European ancestry were retained, excluding a large proportion controls leaving 300 cases and 1991 controls.

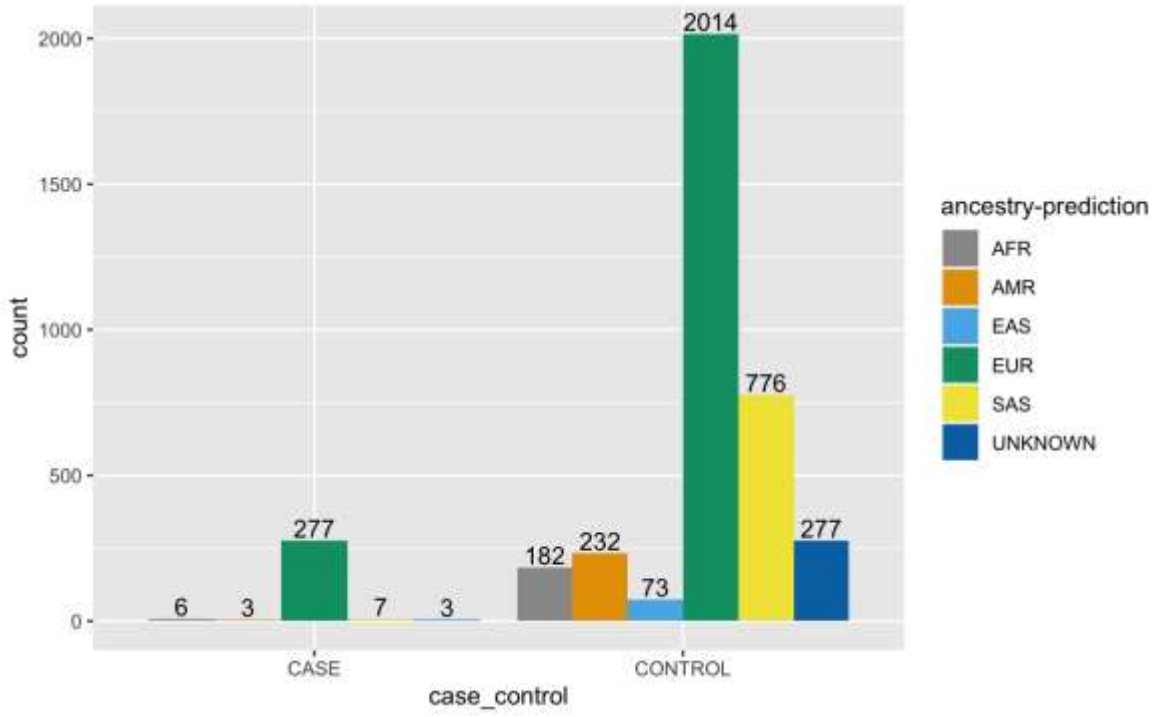


Figure 54: *Histogram generated with Peddy showing predicted ethnicity. Only samples with European ancestry (green) were retained.*

Relatedness

Relatedness was calculated as above and individuals with a relatedness score of <0.2 were removed. At this point, a large proportion of cases were removed owing to the familial cohort consisting of affected relatives. Following this step, 193 cases and 1262 controls remained.

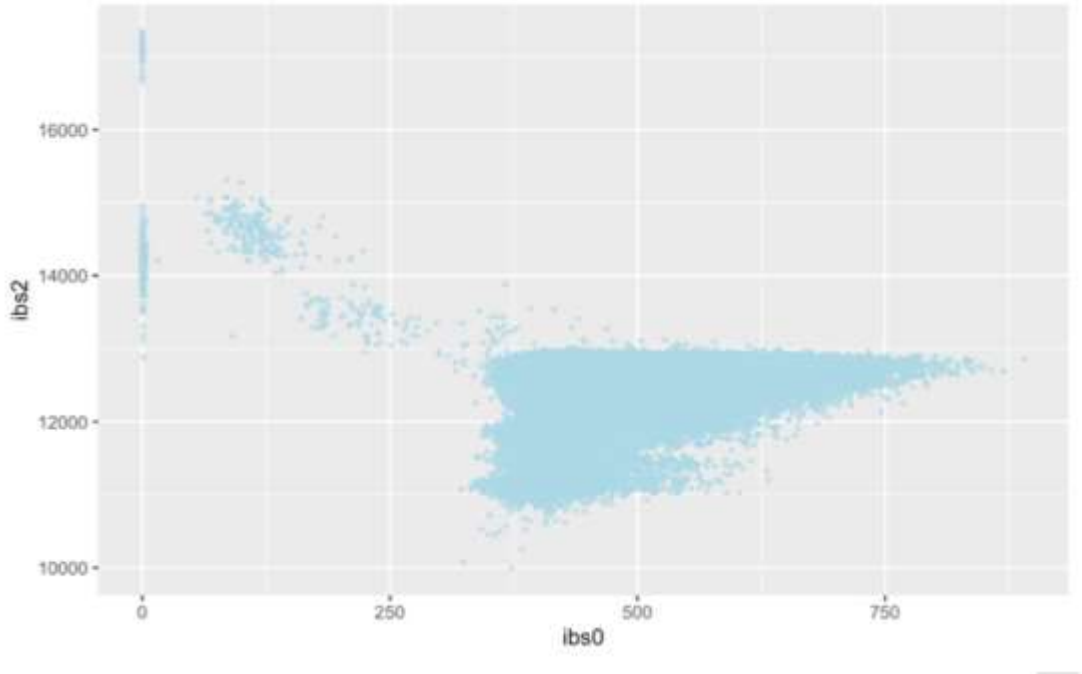


Figure 55: *Illustration of indentity-by-state of cases and controls, unrelated cases are represented by the larger cluster in blue.*

Further PCA correction

Despite all the QC steps listed above further systemic differences remained between cases and controls. These were removed using further PCA corrections which resultsed in final 174 cases and 457 controls. (these were due to finer population structure differences or batch effects) **Figure 56.**

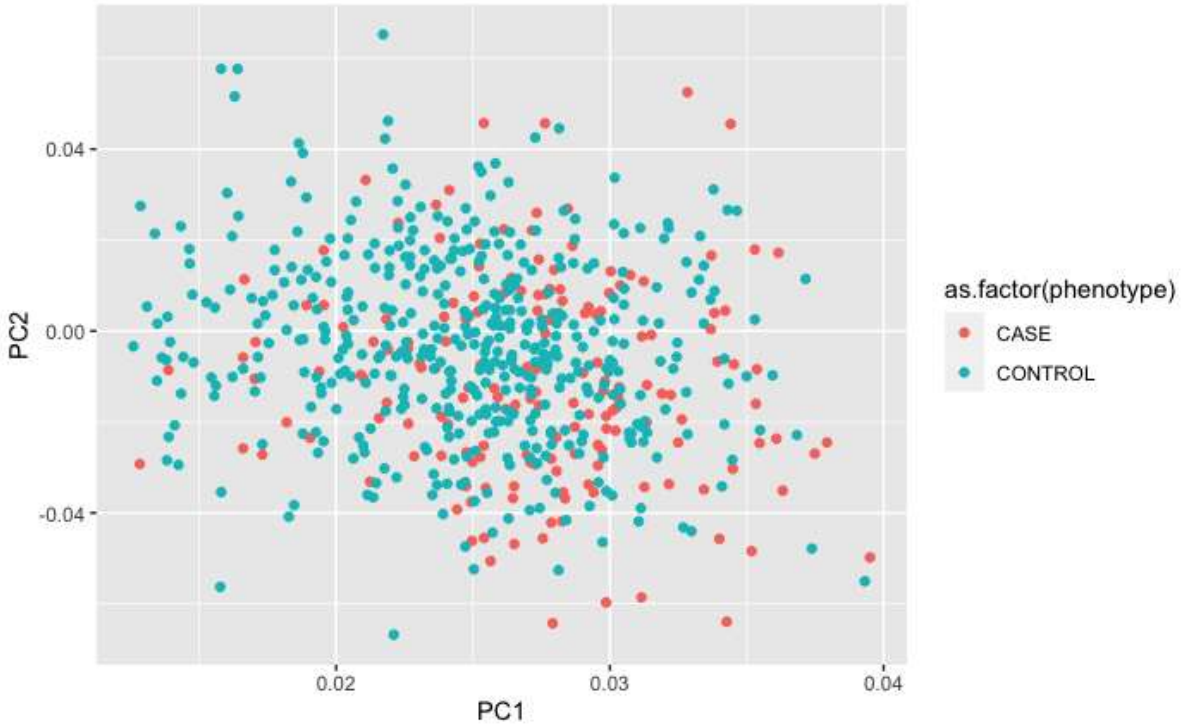


Figure 56: *Further PCA correction due to finer population differences. Cases are in red and controls are in green.*

Variant QC

Variant QC was conducted using bcftools. During VCF file generation all gentypes with DP < 10 or GQ < 20 were set to missing. After removal of problematic samples variants were annotated with call rate, variant quality score, HWE, excess heterozygosity and allele frequency. Variants with genotyping rate < 95% were removed as were variants with high missingness (> 5%) in either cases or controls separately.

Variants that did not pass quality filter were also removed. Overall, the initial number of 703,471 biallelic variants was reduced to 404,910 qualifying variants. Of these 64027 variants were common (MAF > 0.05).

Selection of likely deleterious variants

Using VEP[535], a merged VCF file for each cohort was filtered to include only variants with high and moderate impact as defined by sequence ontology.Variants were then filtered to retain only rare variants with a max AF of < 0.01. Max AF is defined as the highest allele frequency observed in any population from 1000 genomes, ESP or gnomAD.

4.4.5 Results

4.4.5 (a) Candidate gene results

I conducted a candidate gene study to look for pathogenic alterations in channelopathy genes by examining for an excess of rare variants in cases versus controls. No candidate reached statistical significance when corrected for multiple testing with a threshold of $p < 2x10^{-4}$ (0.05/237). The top 20 most significant candidates are summarised in **Table 33.**

Table 33: Candidate gene analysis

Chr	Location	Symbol	Cases Het	Cases Hom	Total AC	Control Het	Control Hom	Total AC	NumVar	Num PolyVar	Pvalue
9	2717510-2730037	*KCNV2*	3	0	3	1	0	1	10	4	0.0367053
20	63343223-63378401	*CHRNA4*	0	0	0	14	0	14	9	4	0.0463765
16	1153106-1221771	*CACNA1H*	6	0	6	8	0	8	23	7	0.0476122
12	13437942-13981957	*GRIN2B*	2	0	2	0	0	0	3	2	0.0498181
2	174747592-174787935	*CHRNA1*	1	0	1	1	0	1	5	3	0.127509
17	74842023-74861504	*GRIN2C*	2	0	2	5	0	5	7	4	0.218313
2	231056864-231172827	*PSMD1*	1	0	1	0	0	0	5	1	0.224181
11	3665587-3671384	*CHRNA10*	1	0	1	2	0	2	5	1	0.224181
5	161288429-161549044	*GABRB2*	1	0	1	0	0	0	1	1	0.224181
10	85599552-86366795	*GRID1*	1	0	1	0	0	0	7	1	0.224181
1	36795527-37034515	*GRIK3*	1	0	1	0	0	0	4	1	0.224181
18	79863668-79900184	*KCNG2*	1	0	2	0	0	0	4	2	0.224181
3	19148510-19535642	*KCNH8*	1	0	1	0	0	0	5	1	0.224181
2	154697855-154858354	*KCNJ3*	1	0	1	0	0	0	2	1	0.224181
10	76869601-77638369	*KCNMA1*	0	0	0	0	0	0	3	1	0.224181
12	51590266-51812864	*SCN8A*	1	0	1	0	0	0	6	1	0.224181
17	63938554-63972918	*SCN4A*	2	0	2	3	0	3	8	5	0.312582
16	24255553-24362801	*CACNG3*	1	0	1	1	0	1	2	2	0.398545
8	42752620-42796392	*CHRNA6*	1	0	1	1	0	1	6	1	0.398545

2	232539692-232548115	*CHRNG*	1	0	1	0	0	0	7	2	0.398545
9	98288109-98708935	*GABBR2*	1	0	1	4	0	9	10	2	0.398545
4	92303966-93810157	*GRID2*	1	0	1	1	0	1	5	2	0.398545
1	159998651-160070160	*KCNJ10*	1	0	1	1	0	1	5	2	0.398545
1	201039512-201112451	*CACNA1S*	3	0	3	7	0	7	29	9	0.425711
2	232525993-232536667	*CHRND*	2	0	2	2	0	2	8	3	0.534062
1	2019329-2030758	*GABRD*	0	0	0	0	0	0	4	2	0.534062
12	5043879-5046788	*KCNA5*	1	0	2	2	0	2	6	4	0.534062
8	72537225-72938349	*KCNB2*	1	0	1	2	0	2	7	3	0.534062
8	98426958-98432853	*KCNS2*	1	0	1	2	0	2	5	3	0.534062

Abbreviations: AC= allele count, Het= Heterozygous, Hom= Homozygous, NumVar= Number of variants, Num PolyVar= Number of polyvariants

4.4.5 (b) Single Locus Association Test

4.4.5 (b) (i) Power Calculation

I retrospectively performed a power calculation for single variant association study[519]. As CH has a prevalence of approximately 1/1000, to reach significance of $7x10^{-07}$(0.05 / 64027) 0.005 a sample size of 174 cases and 457 controls provides 58 % power with a relative risk of 1.5 for a MAF of 0.3.

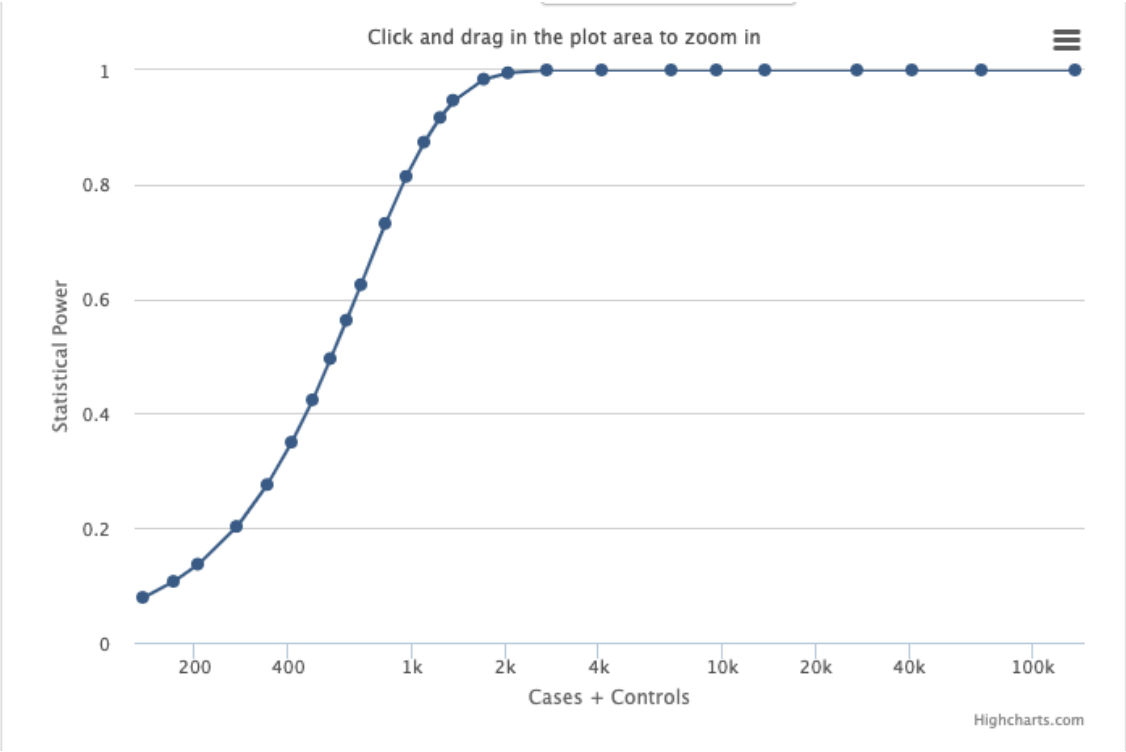


Figure 56: *Power calculation for rare variant association analysis involving 174 cases and 457 controls.*

4.4.5 (b) (ii) Single variant association results

A standard single locus association test was performed for variants with a MAF <0.05 using basic –assoc (Case-control [1df chi-square test] with adjusted p-values) and --fisher) functions as implemented in PLINK[376]. In addition, Single score test as

implemented in RVtests [415] was performed without covariates and using the first 4 PCA axis. Results from all analyses were similar and are presented for basic plink association test.

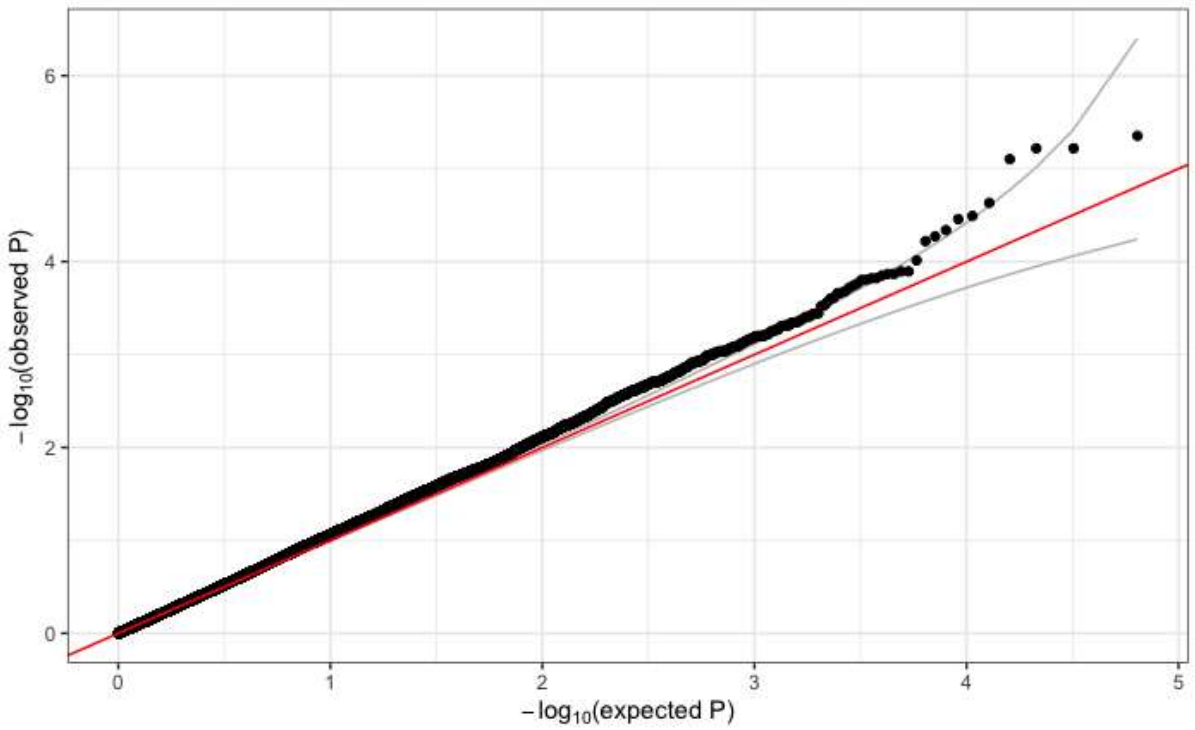


Figure 57: *Quantile – quantile (QQ) plot. Single locus association was calculated using plink and QQ plot was created using R[368]. A genomic inflation factor of 1.06 was calculated.*

As illustrated in the Manhattan plot below, no variant reached statistical significance. The top variants (unadjusted p-value < $1x10^{-04}$) derived from the analysis are shown in **Table 34**. Amongst variants with an unadjusted p-value < 0.001 were variants from loci identified in GWAS, namely two variants in *MERTK*.

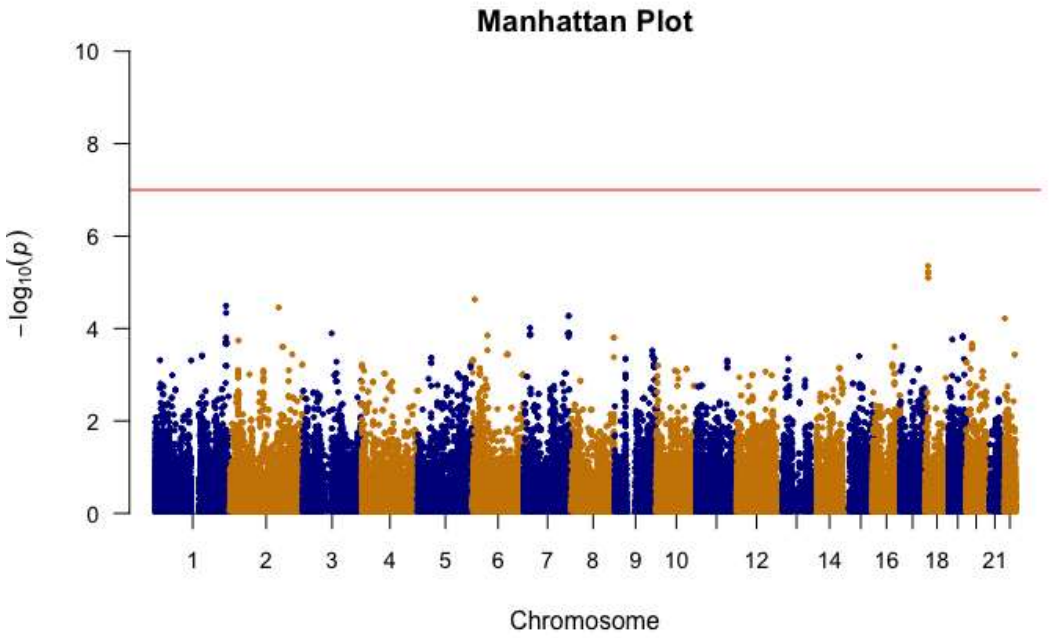


Figure 58: *Manhattan plot showing results of single variant association test using PLINK case-control association test*

Table 34: Top variants resulting from single variant association testing

CHR	SNP	P-value	FDR_BH	REF	ALT	AF_CASE	AF_CONTROL	Consequence	SYMBOL	HGVSc
18	rs3748414	4.44E-06	0.1264	C	T	0.192529	0.0973742	synonymous_variant	*APCDD1*	ENST00000355285.9:c.480C>T
18	rs3748415	6.05E-06	0.1264	G	A	0.192529	0.0984683	missense_variant	*APCDD1*	ENST00000355285.9:c.448G>A
18	rs3748413	6.05E-06	0.1264	C	G	0.192529	0.0984683	synonymous_variant	*APCDD1*	ENST00000355285.9:c.489C>G
18	rs3811368	7.90E-06	0.1264	C	T	0.196532	0.102198	3_prime_UTR_variant	*APCDD1*	ENST00000355285.9:c.*29C>T
6	rs2876098	2.34E-05	0.2992	T	G	0.229885	0.132385	missense_variant	*CAGE1*	ENST00000296742.11:c.437A>C
1	rs2056713	3.23E-05	0.3196	G	A	0.33046	0.216854	intron_variant	*ERO1B*	ENST00000354619.9:c.349-15C>T
2	rs113748016	3.50E-05	0.3196	G	A	0.106322	0.0438596	synonymous_variant	*KCNH7*	ENST00000328032.8:c.555C>T
1	rs1749589	4.60E-05	0.3679	T	C	0.328402	0.216484	splice_region_variant	*ERO1B*	ENST00000354619.9:c.348+3A>G
7	rs7791608	5.35E-05	0.3806	A	G	0.066092	0.150985	missense_variant	*KRBA1*	ENST00000319551.12:c.959A>G
22	rs5748648	6.03E-05	0.3863	G	A	0.091954	0.036105	missense_variant	*XKR3*	ENST00000331428.5:c.428C>T
7	rs7781669	9.71E-05	0.5058	A	G	0.571839	0.448889	intron_variant	*DNAH11*	ENST00000328843.10:c.495+53A>G

Abbreviations: AF=allele frequency, ALT= alternate, CHR = Chromosome, FDR = False Discovery Rate, Ref= Reference, SNP= Single Nucleotide Polymorphism.

4.4.5 (b) (iii) Rare variant tests

I used the software package RVTESTS to conduct a rare variant association test [415]. A joint VCF, containing the post-QC cases and controls was annotated. In total 103,378 rare variants were included in the analyses and variants were grouped across 16,031 protein coding genes. The cut-off for significance was $3x10^{-06}$(0.05/16,031). Various permutations of burden analysis were attempted including CMC and sequence kernel association tests (SKAT, SKATO). Analysis were performed with and without PCA covariates. For the purposes of this thesis, I am presenting SKATO results as it involves both burden and variance-component methods and reflects both trait-increasing and trait-decreasing variants. The most significant genes are summarized in **Table 35.**

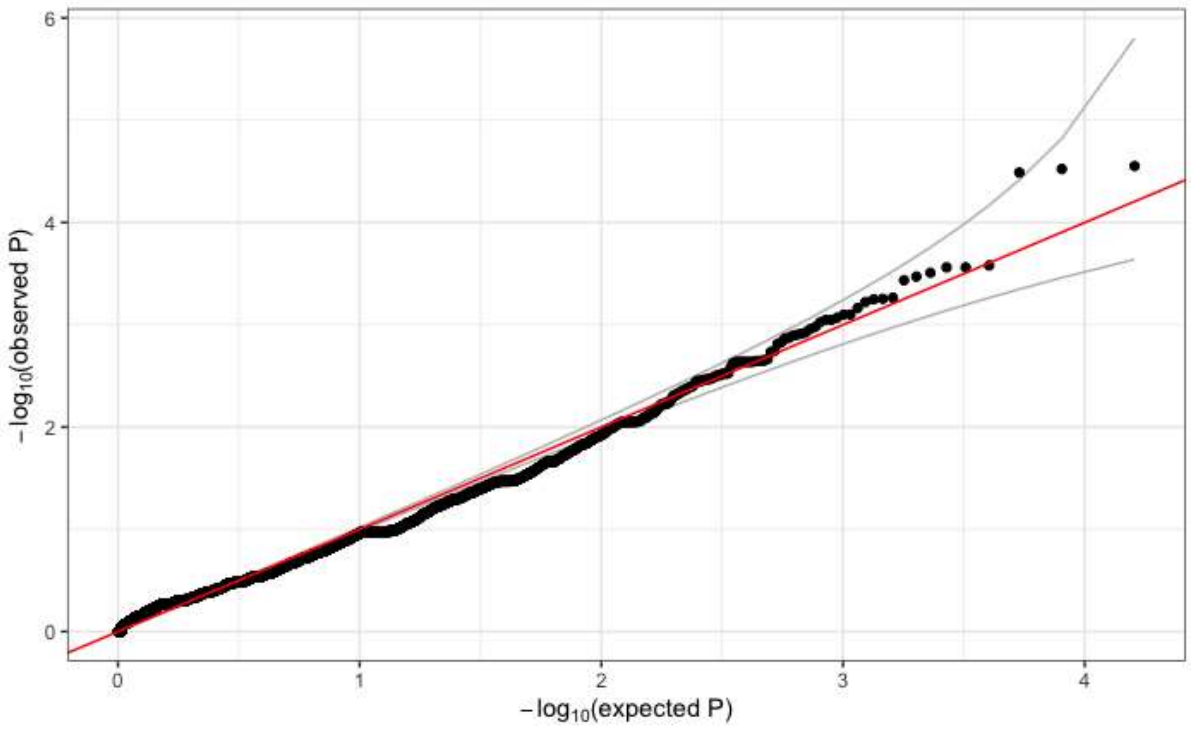


Figure 59: *Quantile-Quantile plot for SKATO analysis created with R*

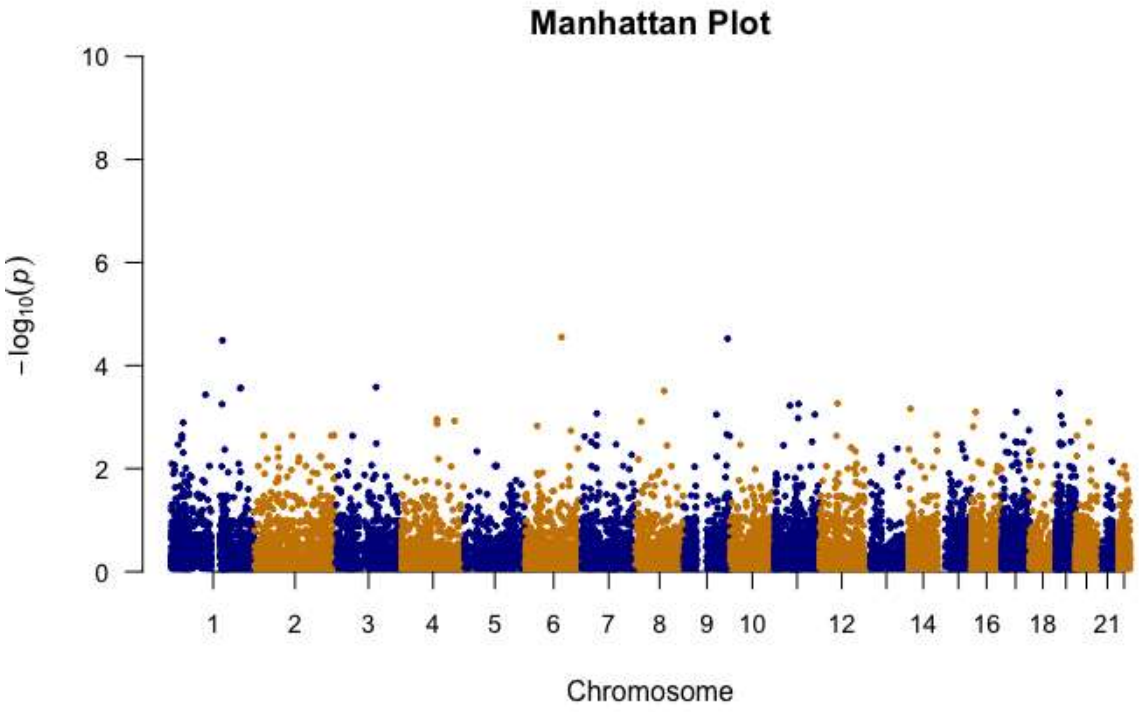


Figure 60: *Manhattan plot illustrating results of SKATO burden analysis.*

Table 35: Results from SKATO rare variant test.

Gene	NumVar	NumPoly Var	Q	Rho	Pvalue	Gene description
SMPD2	9	9	6050.24	0.1	2.80E-05	sphingomyelin phosphodiesterase 2
PRRC2B	33	33	13898.7	0.1	3.00E-05	Proline Rich Coiled-Coil 2B
SPRR3	3	3	15084	0	3.24E-05	small proline rich protein 3
IQCB1	3	3	6754.12	0.1	0.00026283	IQ motif containing B1
IL19	1	1	3485.71	0	0.00027447	interleukin 19
IL10	1	1	3485.81	0	0.00027447	interleukin 10
SLC7A13	9	9	4794.36	0.2	0.00030994	solute carrier family 7 member 13
ZNF562	2	2	3459.18	1	0.00033782	zinc finger protein 562
OLFM3	3	3	3669.45	1	0.00036687	olfactomedin 3
PDE1B	7	7	5634	0.2	0.00054338	phosphodiesterase 1B
KRTAP5-10	1	1	4333.91	0	0.00055708	keratin associated protein 5-10
SNX27	7	7	8961.13	0.4	0.00056289	sorting nexin 27
LARGE2	12	12	4779.89	0.2	0.00059942	LARGE xylosyl- and glucuronyltransferase 2
GMPR2	3	3	4005.9	0	0.00068647	guanosine monophosphate reductase 2
SNX29	8	8	4529.59	0	0.0007941	sorting nexin 29
DHX8	11	11	5179.25	0.1	0.00079692	DEAH-box helicase 8
URGCP	6	6	4297.76	0.1	0.00085125	upregulator of cell proliferation
ARHGEF12	13	13	12888.9	1	0.00089584	Rho guanine nucleotide exchange factor 12
TDRD7	6	6	3475.77	0.6	0.00089597	tudor domain containing 7
PALM3	10	10	5519.06	0	0.00094567	paralemmin 3

4.4.6 Discussion

As previously outlined (section 4.1), a GWAS approach successfully identified replicable susceptibility loci which contribute to the development of CH. However, these associations represent common variants (>MAF 5%) with a moderate effect size on genetic risk, but which account for only a fraction of the heritability of CH, which has a familial rate of approximately 6.27%[493] . Certainly, future larger meta-analysis of CH patients are likely to yield additional signals, however as these variants will have an even weaker effect size, the task of deciphering their functional consequences and disease pathways will be challenging. The purpose of this study was to identify rare variants with larger effect on phenotype, to potentially gain insights into the underlying biological pathways involved in TACs such as CH and SUNCT.

Following a stringent QC, I conducted a candidate gene study examining for the presence of rare variants in channelopathy genes. The hypothesis that TACs could result from variation in channel genes is supported by the fact that similar conditions such as FHM, and other migraine subtypes, have been categorised as channelopathies caused by variants in *CACNA1A, ATP1A2, SCN1A, KCNK18*[682]. My analysis did not identify relevant pathogenic variants in these genes.

I then performed a single variant association test in variants with MAF <0.05. Again, no variant reached statistical significance. A burden analysis using SKATO, also failed to achieve significant results. These predominantly negative findings are possibly reflective of the smaller size of this cohort and lack of suitable controls. One interesting finding derived from the study was the detection an overlap with GWAS. Rare variants in *MERTK* were identified amongst variants with an unadjusted p-value < 0.001. This is encouraging and will possibly reach significance in future analysis involving a larger cohort.

Section V: General Conclusions

This thesis has incorporated two distinct components to address pertinent issues pertaining to CH. A work package comprised of clinical observational studies aimed to improve the understanding of the phenotypic manifestations of CH, whilst a more prominent genetic section sought to identify genetic factors that predispose to its development. Each component is divided into four subsections that address a pertinent research question. The key points extrapolated from my findings are summarized below.

5.1 Clinical Conclusions

I examined the incidence of pituitary adenomas in CH and found that it is similar to that reported in the general population. This indicates that CH patients are unlikely to benefit from dedicated pituitary screening, contradicting some guidelines. Contrary to previous reports, atypical clinical features and intractability to treatment do not predict the likelihood of a pituitary lesion. These findings suggest that only patients with standard brain MRI findings or symptoms suggestive of a pituitary disorder require further investigation. A similar study in a secondary referral centre is required to further clarify the hypothesised link between pituitary disorders and CH. These results have the potential to influence future clinical imaging guidelines and improve the cost effectiveness and efficiency of the CH patient journey.

Secondly, I presented the largest series of PTH-CH described to date. This study illustrated the unique features and clinical course of PTH-CH. My analysis suggested that this form of CH is more severe than primary CH. Patients with this variant were

more likely to develop the chronic form, have more severe autonomic features, respond poorly to treatment, and suffer from concurrent chronic migraine. These results imply that this patient group require early specialist intervention and may benefit from neuromodulation. I also detected a positive family history more frequently in the PTH-CH group, suggesting trauma may act as a trigger in individuals that have a genetic susceptibility to CH. This is unsurprising considering the role of genetic variation, for example mutations in CACNA1A and APOE variants, on outcomes in traumatic brain injury (TBI)[683,684].

I examined the prevalence and clinical aspects of familial CH. Hereditary factors have long been postulated in the aetiology of CH, however the exact prevalence of familial CH has been the subject of much debate[120, 301]. I conducted a systematic review of 1,281 publications and carried out a rigorous appraisal of their methodology prior to their inclusion in a meta-analysis. This process observed a prevalence of family history in 6.27% of the included cohorts. I further delineated the phenotype of familial CH showing an overrepresentation of the autonomic symptom of nasal blockage. I also demonstrated that patients with familial CH appear to have a predisposition to developing a concurrent TAC, namely SUNCT. This possibly reflects a common genetic driver and mechanistic pathway underpinning both conditions.

5.2 Genetic Conclusions

In section two, I carried out the first sufficiently powered GWAS of CH, identifying genetic risk loci that were replicated in an independent cohort of Swedish CH patients. A combined analysis of both cohorts demonstrated three susceptibility risk loci, including two on chromosome 2 and one on chromosome 1. Recently, these loci were

also replicated by a third independent Dutch cohort at Leiden University (unpublished). The association effect sizes at these loci are higher when compared to those usually observed in GWAS (generally not exceeding 1.1-1.3). This presents the possibility that the genetic risk for CH is driven by a limited number of loci reflective of oligogenic inheritance, rather than the highly polygenic structure usually observed in complex traits.

A key finding of this study was the detection of an additional locus on chromosome 6 that was significant in the UK cohort, not replicated in the Swedish analysis, but then remained suggestive of significance on combined analysis. This overlaps a risk locus for migraine in the region of *FHL5* and *UFL.* These are both involved in the cAMP-responsive elements CREB6 and CREM, and the ubiquitin protease system respectively. They therefore present as interesting candidates[685, 686]*.* I demonstrated that this finding is independent of concurrent migraine in our cohort. This may indicate a shared genetic architecture underlying both migraine and CH explaining the overlapping pathophysiological features and efficacy of some treatments such as triptans in both conditions.

All genes identified through gene based analysis (*MERTK*, *TMEM87B*, *ANAPC1*, *FBLN7*, and *FHL5)* were highly expressed in brain with predominant microglial expression in *MERTK* and *TMEM87b*. Larger GWAS studies will have the power to detect more associated genes and therefore provide a more meaningful downstream analysis. However, these preliminary findings suggest a possible role for neuroinflammation in the aetiology of CH, a concept that to date has not been widely considered. Furthermore, pathway and gene set enrichment analysis implicate

immunological processes and cytokine activity as potential drivers for CH. This explanation also fits an existing paradigm surrounding headache pathogenesis: the cytokine theory of headache[687]. This theory has the benefit of explaining not only headache, but also the prostaglandins, leukotrienes, platelet activating, and vasoactive substances linked with headache and the associated incidence with depression[687].

Genetic correlation analysis identified a significant correlation with migraine, stroke, depression, bipolar disorder and insomnia. This is relevant for several reasons. Firstly, the correlation with migraine further supports the possibility of a common genetic driver that influences both conditions. The significance of stroke is also reminiscent of the vascular hypothesis derived from similar analysis in large migraine meta-analysis[77, 513].

Secondly, the correlation with depression and bipolar disorder may explain the higher prevalence of psychiatric conditions in individuals with CH when compared to the general population. This reiterates the previously demonstrated sharing of genetic risk variants between headache and psychiatric disorders[657, 688]. The comorbidity of these disorders has the potential to be leveraged to investigate neurobiological similarities and thus gain insights into the index condition.

Finally, the correlation with insomnia is unsurprising considering the distinct circadian rhythmicity associated with CH and the high proportion of these patients that experience disturbed sleep[689] . This adds to existing evidence to suggest that sleep disturbance is in fact part of the disorder with potential therapeutic implications[690 691].

Family-based analysis utilized in this thesis were less fruitful. Linkage analysis of fifteen families with CH failed to identify a region of significance, however, regions suggestive of significance overlapped a susceptibility locus identified in GWAS on chromosome 2 and replicated the findings of a previous linkage study in CH[321]. Additionally, a second region suggestive of linkage also overlapped a locus suggestive of significance on GWAS of the UK cohort. Finally, WES of families with CH lead to the identification of potential candidates. The most interesting of these is *CACNA1G* which has a demonstrated role in the modulation of pain in animal models [673]

5.3 Future Directions

The results derived from this thesis confirm the role of genetics in the aetiology of CH. These findings reiterate the heightened requirement for investment into genetic research in CH. Undoubtedly, as a continuation of the work presented in this thesis, large-scale genetic studies are likely to provide unprecedented insights into its origins. The identification of additional susceptibility loci in sporadic CH and the isolation of causal genetic variants in familial cases will help uncover the underpinning neurobiological mechanisms. This information could then be utilised to design mechanistic experiments with the ultimate goal of detecting potential targets for therapeutic intervention.

Observations from the clinical component of this thesis imply that the stratification of CH patients, based on clinical characteristics, into homogenous groups may optimise the efficacy of future genetic studies. This would involve separate analysis for the episodic and chronic subtypes, though a further pooling of cases based on clinical

characteristics may be beneficial. For example, as demonstrated in this thesis, the higher representation of a family history in PTCH-CH could infer that these forms of CH are caused by distinct genetic variations. Furthermore, the amalgamation of cases based on and the association between concurrent SUNCT and familial CH may also be advantageous.

In the future, different elaborations of genetic and functional methods need to be implemented to obtain informative results for CH. The GWAS data generated in this thesis has been incorporated within a larger international meta-analysis that is ongoing. This initiative may yield additional susceptibility loci for CH and provide a more meaningful downstream analysis. Further fine mapping at significant loci would aid the detection of causal-disease associated variants. This may involve the implementation of a bayesian approach in these regions to create of a series of credible sets of SNPs that, based on posterior probability, are likely to impact disease[692]. Priori knowledge of a gene's predicted function and its involvement in predefined biological pathways could also be used to then prioritise potential causative variants.

Downstream expression and pathway analysis obtained from GWAS (section 4.4) allude to the role of microglia and cytokine activity in CH. This is supported by their established involvement in the generation of neuropathic pain[82]. Although previously unexplored, this represents an avenue of inquiry that marries well with known pathophysiological concepts of CH. For example, the hypothalamus is thought to play a role in the generation of attacks. Hypothalamic regulated hormones such as

melatonin, prolactin, cortisol and growth hormone can be deranged in CH, and these hormones have been shown to regulate cytokine release[182, 693-698].

It is also plausible that genetic variation may lead to dysregulation of cytokine-related processes in the microglia of CH patients, and that aberrant activation of microglia may trigger an attack following normal stimulus such as from vasodilators and/or hypothalamus-secreted hormones. This temporary state of sensitisation by microglia may explain how normally benign stimuli such as alcohol or nitroglycerine trigger CH during a bout or episode and cannot persistently induce this effect[699]. While unproven, this hypothesis presents an opportunity for further investigation.

Regions suggestive of linkage on chromosome 2 and chromosome 12 in familial studies overlapped with GWAS susceptibility loci. While WES failed to identify a segregating variant driving this signal, these findings are unlikely to be coincidental. One explanation is that the prioritization strategy used to isolate variants was too restrictive considering the frequency of CH. In future, whole genome sequencing may be useful to further interrogate these regions for intronic mutations and copy number variants (CNV).

Mutations identified in candidate genes through WES require further characterization. The preliminary data generated in this thesis forms the bases for the PhD of an incoming student (Ms. Clarissa Rocca). She proposes to investigate the functional consequences of these variants through developing in-silico high-resolution structural models of the mutations and modelling them in xenopus oocytes and HEK293 cells. Functional evidence confirming the pathogenicity of these variants would support their

role in the aetiology of familial CH. Such findings would have the potential to influence treatment through the identification of t-type calcium channels as a potential target for pharmacological manipulation.

In summary, the comprehensive clinical and genetic data generated in this thesis should provide a robust road-map for future genetic and mechanistic studies. This will hopefully help to optimize current clinical practice, provide further insights into the aetiology of CH, and identify therapeutic targets that could improve outcomes for patients afflicted with a significantly debilitating neurological condition.

Appendix

Section III Appendix

3.3 A Systematic Review And Meta-Analysis of the Prevalence of Familial Cluster Headache

3.3.3 (a) Data Extraction

DATABASE	Search Term	Results
Pubmed	*("trigeminal autonomic cephalalgias"[MeSH] OR "Trigeminal Autonomic Cephalalgia" OR "Trigeminal Autonomic Cephalalgias" OR "TACS" OR "Cluster Headache"[MeSH] OR "Cluster Headache" OR "cluster headaches") AND ("family"[MeSH] OR "family" OR "familial" OR "hereditary" OR "heritability" OR "hereditability" OR "inherit" OR "inherited" OR "genetic" OR "genes"[MeSH] OR "genes" OR "gene")*	408
Medline	*(exp trigeminal autonomic cephalalgia/ or exp cluster headache/) OR (TACS or Cluster Headache*).mp AND (family or familial).mp. OR (hered* or heritability or inherit* or genetic or genes or gene).mp.*	391
EMBASE	*(Trigeminal autonomic cephalalgia/ or exp cluster headache/)OR (trigeminal autonomic cephalalgia* or TACS or cluster headache*).mp AND (family or familial or hered* or heritability or inherit or gene or genes or genetic*).mp.*	946
CINAHL	*(MH "Trigeminal Autonomic Cephalalgias+") OR (trigeminal autonomic cephalalgia* OR TACS OR cluster headache*) AND ((family or familial) OR (heredit* or heritability) OR inherit* OR (gene or genes or genetic*))*	283

3.3.3 (c) Script for Meta-analysis

```
######==== Meta analysis =====######
#Install and load packages
library(metafor)
library(meta)


#Read the data
dat=read.csv("Headache_data_precise.csv", header=T, sep=",")

#Non-transformed data
```

```
ies=escalc(xi=Num_affected, ni=Total_number, data=dat, measure="PR")
pes=rma(yi, vi, data=ies)
print(pes)
#Visually expect distribution of transformed data
d <- density(ies$yi)
plot(d)
#save the plot
png(filename="Non-transformed_density_plot.png",
    width = 371,
    height= 222)
plot(d)
dev.off()

dev.print(png, file = "Non-transformed_density_plot.png", width =
375, height = 222)
#Test to determine if the distribution is signficantly different
from normal
shapiro.test(ies$yi)
#Logit transformation
ies.logit=escalc(xi=Num_affected, ni=Total_number, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)
#Visually expect distribution of transformed data
d <- density(ies.logit$yi)
plot(d)
#Save the plot
png(filename="Logit-transformed_density_plot.png",
    width = 371,
    height= 222)
plot(d)
dev.off()
#Test to determine if the distribution is signficantly different
from normal
shapiro.test(ies.logit$yi)
#Double
ies.da=escalc(xi=Num_affected, ni=Total_number, data=dat,
measure="PFT", add=0)
pes.da=rma(yi, vi, data=ies.da)
pes=predict(pes.da, transf=transf.ipft.hm, targ=list(ni=dat$total))
print(pes)
#Visually expect distribution of transformed data
d <- density(ies.da$yi)
plot(d)
#Test to determine if the distribution is signficantly different
from normal
shapiro.test(ies.da$yi)

#The logit transformation provided the closest to a normal
distribution
print(pes.logit, digits=4)
confint(pes.logit, digits=2)

#Forrest plots
```

```
#Basic
pes.summary=metaprop(Num_affected, Total_number, Study, data=dat,
sm="PLO")
forest(pes.summary)
#Fancy
pes.summary=metaprop(Num_affected, Total_number, Study, data=dat,
sm="PLO")
forest(pes.summary,
       xlim=c(0,0.25),
       rightcols=FALSE,
       leftcols=c("studlab", "event", "n", "effect", "ci"),
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="maroon", col.diamond.lines="maroon",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)

#Save forrest plot
tiff(filename="All_eight_studies_forrest_plot.tiff",
    width = 3000,
    height= 2400,
    units = 'px',
    res = 300)

forest(pes.summary,
       xlim=c(0,0.25),
       rightcols=FALSE,
       leftcols=c("studlab", "event", "n", "effect", "ci"),
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="maroon", col.diamond.lines="maroon",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)
dev.off()

#NOICE - But significant study heterogeneity need outlier analysis
stud.res=rstudent(pes.logit)
```

```
abs.z=abs(stud.res$z)
stud.res[order(-abs.z)]
#Influential studies:
inf=influence(pes.logit)
print(inf); plot(inf)

#save the plots
png(filename="Diagnostic_plots_All_eight.png",
    width = 600,
    height= 800)
inf=influence(pes.logit)
plot(inf)
dev.off()


#Leone et al., may be outlier (>2)
L1O=leave1out(pes.logit, transf=transf.ilogit); print(L1O, digits=6)
#Write table for supplementary
L1O <- as.data.frame(L1O)
write.csv(L1O, file="Leave_one_out_analysis.csv")

#Funnel plot for study bias
#Make and save plot
png(filename="All_eight_studies_funnel_plot.png",
    width = 371,
    height= 222)
funnel(pes.logit, yaxis="sei")
dev.off()

#Moderator analysis:
#Number of participants
png(filename="Study_year_vs_effect_size_plot.png",
    width = 500,
    height= 299)

subganal.size=rma(yi, vi, data=ies.logit, mods=~Total_number,
method="DL")
pes.size=predict(subganal.size, newmods=c(0:2),
transf=transf.ilogit)
wi=1/sqrt(ies.logit$vi)
size=1+3 *(wi-min(wi))/(max(wi)-min(wi))
plot(ies.logit$Total_number, transf.ilogit(ies.logit$yi), cex=size,
xlab="Sample size",
     ylab="Proportion")
lines(0:2, pes.size$pred, col="navy")
lines(0:2, pes.size$ci.lb, lty="dashed", col="maroon")
lines(0:2, pes.size$ci.ub, lty="dashed", col="maroon")
dev.off()

print(subganal.size)

png(filename="Study_year_vs_effect_size_plot.png",
    width = 500,
    height= 299)
#Study year
```

```
metareg.year=rma(yi, vi, data=ies.logit, mods=~ Year, method="DL")
wi=1/sqrt(ies.logit$vi)
size=1+3*(wi-min(wi))/(max(wi)-min(wi))
pes.year=predict(metareg.year, newmods=c(1994:2020),
transf=transf.ilogit)
plot(ies.logit$Year, transf.ilogit(ies.logit$yi), cex=size, pch=1,
las=1, xlab="Publication year",
     ylab="Proportion")
lines(1994:2020, pes.year$pred, col="navy")
lines(1994:2020, pes.year$ci.lb, lty="dashed", col="maroon")
lines(1994:2020, pes.year$ci.ub, lty="dashed", col="maroon")
ids=c(1:17)
pos=c(1)
text(ies.logit$Year[ids], transf.ilogit(ies.logit$yi)[ids], ids,
cex=0.9, pos=pos)
dev.off()

print(metareg.year)
#Now again without Leone
#Read the data without Leone
dat=read.csv("Headache_data_precise_no_Leone.csv", header=T,
sep=",")
#Logit transformation
ies.logit=escalc(xi=Num_affected, ni=Total_number, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)
#Forrest
pes.summary=metaprop(Num_affected, Total_number, Study, data=dat,
sm="PLO")
forest(pes.summary,
       xlim=c(0,0.25),
       rightcols=FALSE,
       leftcols=c("studlab", "event", "n", "effect", "ci"),
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="maroon", col.diamond.lines="maroon",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)

#Save forrest plot
tiff(filename="All_seven_studies_forrest_plot.tiff",
    width = 3000,
    height= 2400,
    units = 'px',
```

```
    res = 300)

forest(pes.summary,
       xlim=c(0,0.2),
       rightcols=FALSE,
       leftcols=c("studlab", "event", "n", "effect", "ci"),
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="maroon", col.diamond.lines="maroon",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)
dev.off()

#Moderator analysis:
#Number of participants
png(filename="Study_year_vs_effect_size_plot.png",
    width = 500,
    height= 299)

subganal.size=rma(yi, vi, data=ies.logit, mods=~Total_number,
method="DL")
pes.size=predict(subganal.size, newmods=c(0:2),
transf=transf.ilogit)
wi=1/sqrt(ies.logit$vi)
size=1+3 *(wi-min(wi))/(max(wi)-min(wi))
plot(ies.logit$Total_number, transf.ilogit(ies.logit$yi), cex=size,
xlab="Sample size",
     ylab="Proportion")
lines(0:2, pes.size$pred, col="navy")
lines(0:2, pes.size$ci.lb, lty="dashed", col="maroon")
lines(0:2, pes.size$ci.ub, lty="dashed", col="maroon")
dev.off()

print(subganal.size)

png(filename="Study_year_vs_effect_size_plot.png",
    width = 500,
    height= 299)
#Study year
metareg.year=rma(yi, vi, data=ies.logit, mods=~ Year, method="DL")
wi=1/sqrt(ies.logit$vi)
size=1+3*(wi-min(wi))/(max(wi)-min(wi))
pes.year=predict(metareg.year, newmods=c(1994:2020),
transf=transf.ilogit)
plot(ies.logit$Year, transf.ilogit(ies.logit$yi), cex=size, pch=1,
las=1, xlab="Publication year",
```

```
     ylab="Proportion")
lines(1994:2020, pes.year$pred, col="navy")
lines(1994:2020, pes.year$ci.lb, lty="dashed", col="maroon")
lines(1994:2020, pes.year$ci.ub, lty="dashed", col="maroon")
ids=c(1:17)
pos=c(1)
text(ies.logit$Year[ids], transf.ilogit(ies.logit$yi)[ids], ids,
cex=0.9, pos=pos)
dev.off()

#Another funnel plot for study bias
#Make and save plot
png(filename="Seven_studies_funnel_plot.png",
    width = 371,
    height= 222)
funnel(pes.logit, yaxis="sei")
dev.off()
#Eggers regression test
regtest(pes.logit, model="rma", predictor="sei")

#######==== Gender separated analysis =====######
#Read the data
dat=read.csv("Headache_data_precise_MandF.csv", header=T, sep=",")
#Males
#Logit transformation
ies.logit=escalc(xi=Num_affected_M, ni=Total_number_M, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)

print(pes.logit, digits=4)
confint(pes.logit, digits=2)

pes.summary=metaprop(Num_affected_M, Total_number_M, Study,
data=dat, sm="PLO")

#Save forrest plot
tiff(filename="All_six_studies_forrest_plot_Males.tiff",
    width = 3000,
    height= 2400,
    units = 'px',
    res = 300)

forest(pes.summary,
       xlim=c(0,0.25),
       rightcols=FALSE,
       leftcols=c("studlab", "event", "n", "effect", "ci"),
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="maroon", col.diamond.lines="maroon",
       pooled.totals=FALSE,
```

```
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)
dev.off()

#Significant study heterogeneity need outlier analysis
stud.res=rstudent(pes.logit)
abs.z=abs(stud.res$z)
stud.res[order(-abs.z)]
#Influential studies:
inf=influence(pes.logit)
print(inf); plot(inf)

#save the plots
png(filename="Diagnostic_plots_All_six_Males.png",
    width = 600,
    height= 800)
inf=influence(pes.logit)
plot(inf)
dev.off()

#Taga et al., may be outlier (>2)
L1O=leave1out(pes.logit, transf=transf.ilogit); print(L1O, digits=6)
#Write table for supplementary
L1O <- as.data.frame(L1O)
write.csv(L1O, file="Leave_one_out_analysis_Males.csv")

#Females
#Logit transformation
ies.logit=escalc(xi=Num_affected_F, ni=Total_number_F, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)

print(pes.logit, digits=4)
confint(pes.logit, digits=2)

pes.summary=metaprop(Num_affected_F, Total_number_F, Study,
data=dat, sm="PLO")

#Save forrest plot
tiff(filename="All_six_studies_forrest_plot_Females.tiff",
    width = 3000,
    height= 2400,
    units = 'px',
    res = 300)

forest(pes.summary,
       xlim=c(0,0.25),
       rightcols=FALSE,
```

```
       leftcols=c("studlab", "event", "n", "effect", "ci"),
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="maroon", col.diamond.lines="maroon",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)
dev.off()

#Significant study heterogeneity need outlier analysis
stud.res=rstudent(pes.logit)
abs.z=abs(stud.res$z)
stud.res[order(-abs.z)]
#Influential studies:
inf=influence(pes.logit)
print(inf); plot(inf)

#save the plots
png(filename="Diagnostic_plots_All_six_Females.png",
    width = 600,
    height= 800)
inf=influence(pes.logit)
plot(inf)
dev.off()

#Taga et al., may be outlier (>2)
L1O=leave1out(pes.logit, transf=transf.ilogit); print(L1O, digits=6)
#Write table for supplementary
L1O <- as.data.frame(L1O)
write.csv(L1O, file="Leave_one_out_analysis_Females.csv")

#Again without Taga
#Read the data
dat=read.csv("Headache_data_precise_MandF_no_Taga.csv", header=T,
sep=",")
#Males
#Logit transformation
ies.logit=escalc(xi=Num_affected_M, ni=Total_number_M, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)

print(pes.logit, digits=4)
confint(pes.logit, digits=2)
```

```
pes.summary=metaprop(Num_affected_M, Total_number_M, Study,
data=dat, sm="PLO")

#Save forrest plot
tiff(filename="Five_studies_forrest_plot_Males.tiff",
    width = 3000,
    height= 2400,
    units = 'px',
    res = 300)

forest(pes.summary,
       xlim=c(0,0.3),
       rightcols=FALSE,
       leftcols=c("studlab", "event", "n", "effect", "ci"),
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="turquoise4", col.diamond.lines="turquoise4",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)
dev.off()


#Females
#Logit transformation
ies.logit=escalc(xi=Num_affected_F, ni=Total_number_F, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)

print(pes.logit, digits=4)
confint(pes.logit, digits=2)

pes.summary=metaprop(Num_affected_F, Total_number_F, Study,
data=dat, sm="PLO")

#Save forrest plot
tiff(filename="Five_studies_forrest_plot_Females.tiff",
    width = 3000,
    height= 2400,
    units = 'px',
    res = 300)


forest(pes.summary,
       xlim=c(0,0.4),
```

```
       rightcols=FALSE,
       leftcols=c("studlab", "event", "n", "effect", "ci"),
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="goldenrod1", col.diamond.lines="goldenrod1",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)
dev.off()

#Moderator analysis
#Males
#Logit transformation
ies.logit=escalc(xi=Num_affected_M, ni=Total_number_M, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)
#Number of participants
png(filename="Study_year_vs_effect_size_plot_Males.png",
    width = 500,
    height= 299)

subganal.size=rma(yi, vi, data=ies.logit, mods=~Total_number,
method="DL")
pes.size=predict(subganal.size, newmods=c(0:2),
transf=transf.ilogit)
wi=1/sqrt(ies.logit$vi)
size=1+3 *(wi-min(wi))/(max(wi)-min(wi))
plot(ies.logit$Total_number, transf.ilogit(ies.logit$yi), cex=size,
xlab="Sample size",
     ylab="Proportion")
lines(0:2, pes.size$pred, col="navy")
lines(0:2, pes.size$ci.lb, lty="dashed", col="maroon")
lines(0:2, pes.size$ci.ub, lty="dashed", col="maroon")
dev.off()

print(subganal.size)

png(filename="Study_year_vs_effect_size_plot_Males.png",
    width = 500,
    height= 299)
#Study year
metareg.year=rma(yi, vi, data=ies.logit, mods=~ Year, method="DL")
wi=1/sqrt(ies.logit$vi)
size=1+3*(wi-min(wi))/(max(wi)-min(wi))
```

```
pes.year=predict(metareg.year, newmods=c(1994:2020),
transf=transf.ilogit)
plot(ies.logit$Year, transf.ilogit(ies.logit$yi), cex=size, pch=1,
las=1, xlab="Publication year",
     ylab="Proportion")
lines(1994:2020, pes.year$pred, col="navy")
lines(1994:2020, pes.year$ci.lb, lty="dashed", col="maroon")
lines(1994:2020, pes.year$ci.ub, lty="dashed", col="maroon")
ids=c(1:17)
pos=c(1)
text(ies.logit$Year[ids], transf.ilogit(ies.logit$yi)[ids], ids,
cex=0.9, pos=pos)
dev.off()

print(metareg.year)
#Another funnel plot for study bias
#Make and save plot
png(filename="Seven_studies_funnel_plot_Males.png",
    width = 371,
    height= 222)
funnel(pes.logit, yaxis="sei")
dev.off()
#Eggers regression test
regtest(pes.logit, model="rma", predictor="sei")
#Females
#Logit transformation
ies.logit=escalc(xi=Num_affected_F, ni=Total_number_F, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)

#Number of participants
png(filename="Study_year_vs_effect_size_plot_Females.png",
    width = 500,
    height= 299)

subganal.size=rma(yi, vi, data=ies.logit, mods=~Total_number,
method="DL")
pes.size=predict(subganal.size, newmods=c(0:2),
transf=transf.ilogit)
wi=1/sqrt(ies.logit$vi)
size=1+3 *(wi-min(wi))/(max(wi)-min(wi))
plot(ies.logit$Total_number, transf.ilogit(ies.logit$yi), cex=size,
xlab="Sample size",
     ylab="Proportion")
lines(0:2, pes.size$pred, col="navy")
lines(0:2, pes.size$ci.lb, lty="dashed", col="maroon")
lines(0:2, pes.size$ci.ub, lty="dashed", col="maroon")
dev.off()

print(subganal.size)

png(filename="Study_year_vs_effect_size_plot_Females.png",
    width = 500,
```

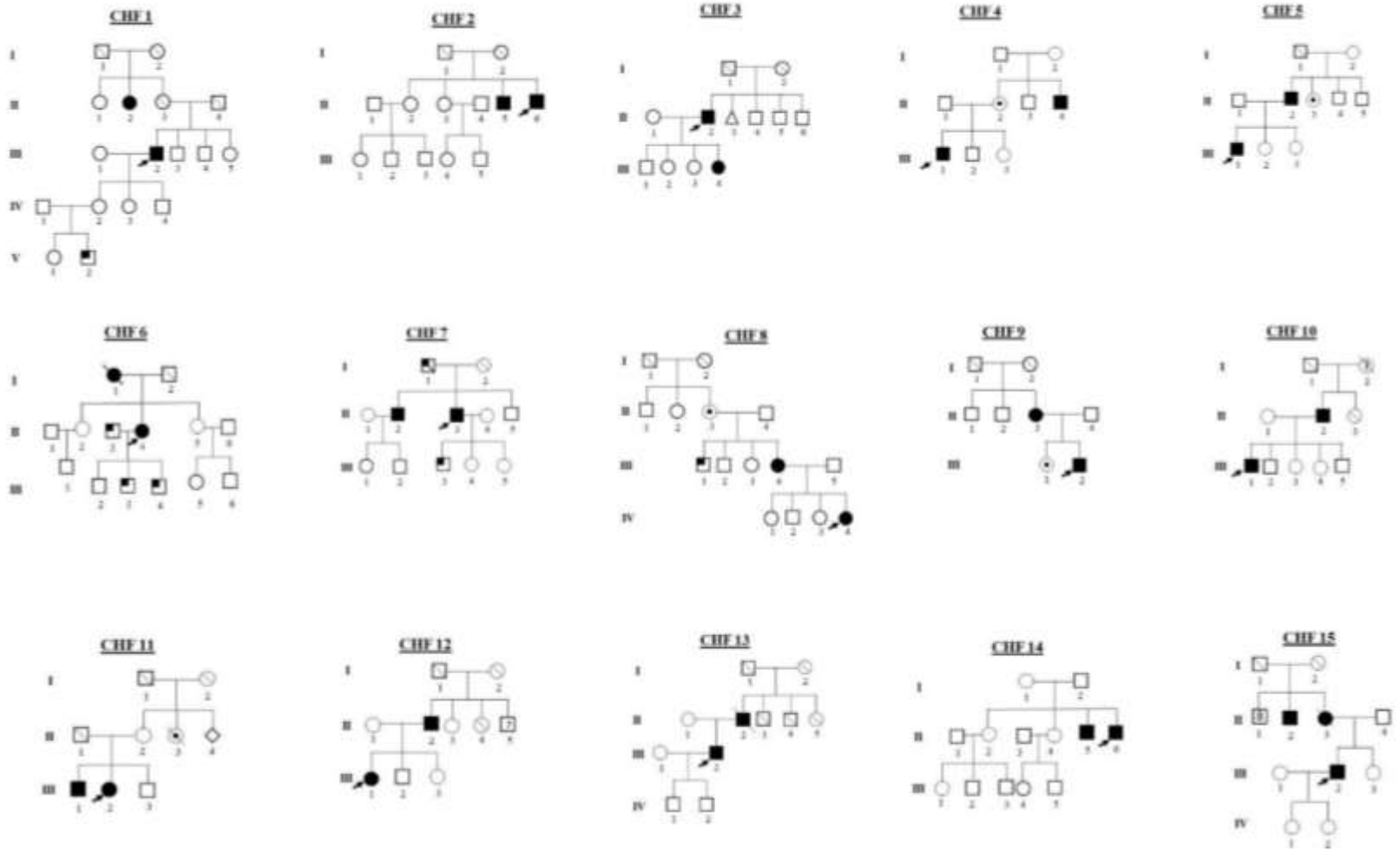
```
    height= 299)
#Study year
metareg.year=rma(yi, vi, data=ies.logit, mods=~ Year, method="DL")
wi=1/sqrt(ies.logit$vi)
size=1+3*(wi-min(wi))/(max(wi)-min(wi))
pes.year=predict(metareg.year, newmods=c(1994:2020),
transf=transf.ilogit)
plot(ies.logit$Year, transf.ilogit(ies.logit$yi), cex=size, pch=1,
las=1, xlab="Publication year",
     ylab="Proportion")
lines(1994:2020, pes.year$pred, col="navy")
lines(1994:2020, pes.year$ci.lb, lty="dashed", col="maroon")
lines(1994:2020, pes.year$ci.ub, lty="dashed", col="maroon")
ids=c(1:17)
pos=c(1)
text(ies.logit$Year[ids], transf.ilogit(ies.logit$yi)[ids], ids,
cex=0.9, pos=pos)
dev.off()

print(metareg.year)
#Another funnel plot for study bias
#Make and save plot
png(filename="Seven_studies_funnel_plot_Females.png",
    width = 371,
    height= 222)
funnel(pes.logit, yaxis="sei")
dev.off()
#Eggers regression test
regtest(pes.logit, model="rma", predictor="sei")

#Males and females as a subgroup analysis:
#Read the data
dat=read.csv("Headache_data_precise_MandF_subgroup_NL.csv",
header=T, sep=",")

#Logit transformation
ies.logit=escalc(xi=Num_affected, ni=Total_number, data=dat,
measure="PLO")
pes.logit=rma(yi, vi, data=ies.logit)
pes=predict(pes.logit, transf=transf.ilogit)
print(pes)


pes.logit.birthcohort=rma(yi, vi, data=ies.logit,
                          subset=Subgroup=="Male",
                          method="DL")
pes.logit.others=rma(yi, vi, data=ies.logit,
                     subset=Subgroup=="Female",
                     method="DL")
pes.birthcohort=predict(pes.logit.birthcohort, transf=transf.ilogit,
digits=5)
pes.others=predict(pes.logit.others, transf=transf.ilogit, digits=5)
dat.diffvar=data.frame(estimate=c(pes.logit.birthcohort$b,
pes.logit.others$b),
```

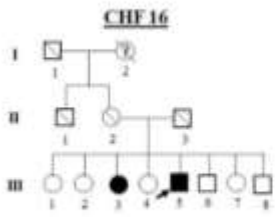
```
                        stderror=c(pes.logit.birthcohort$se,
pes.logit.others$se),
                        studydesign=c("Male", "Female"),
                        tau2=round(c(pes.logit.birthcohort$tau2,
                                     pes.logit.others$tau2), 3))
subganal.studydesign=rma(estimate, sei=stderror, data=dat.diffvar,
                         mods=~studydesign, method="FE")
pes.logit.studydesign=rma(estimate, sei=stderror, method="FE",
data=dat.diffvar)
pes.studydesign=predict(pes.logit.studydesign, transf=transf.ilogit)
print(pes.birthcohort, digits=6); print(pes.logit.birthcohort,
digits=3)
print(pes.others, digits=6); print(pes.logit.others, digits=3)
print(subganal.studydesign, digits=3)
print(pes.studydesign, digits=6)


pes.summary=metaprop(Num_affected, Total_number, Study, data=dat,
                     sm="PLO",
                     method.tau="DL",
                     method.ci="NAsm",
                     byvar=Subgroup,
                     tau.common=TRUE,
                     tau.preset=sqrt(subganal.studydesign$tau2))
#Save forrest plot
tiff(filename="Forrest_plot_all_studies_male_females.tiff",
     width = 3000,
     height= 2400,
     units = 'px',
     res = 300)

forest(pes.summary,
       xlim=c(0,0.4),
       rightcols=FALSE,
       leftcols=c("studlab", "effect", "ci"),
       leftlabs=c("Study", "Proportion", "95% C.I."),
       text.random="Combined prevalence",
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.diamond="maroon", col.diamond.lines="maroon",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)
dev.off()

forest(pes.summary,
       xlim=c(0,0.4),
       rightcols=FALSE,
       leftcols=c("studlab", "event", "n", "effect", "ci"),
```

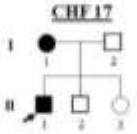
```
       leftlabs=c("Study", "Cases", "Total", "Prevalence", "95%
C.I."),
       xlab="Prevalence of FH in CH", smlab="",
       weight.study="random", squaresize=0.5, col.square="navy",
       col.square.lines="navy",
       col.diamond="goldenrod1", col.diamond.lines="goldenrod1",
       pooled.totals=FALSE,
       comb.fixed=FALSE,
       fs.hetstat=10,
       print.tau2=TRUE,
       print.Q=TRUE,
       print.pval.Q=TRUE,
       print.I2=TRUE,
       digits=2)

#NOICE - But significant study heterogeneity need outlier analysis
stud.res=rstudent(pes.logit)
abs.z=abs(stud.res$z)
stud.res[order(-abs.z)]
#Influential studies:
inf=influence(pes.logit)
print(inf); plot(inf)

#save the plots
png(filename="Diagnostic_plots_male_female_subgroup.png",
    width = 600,
    height= 800)
inf=influence(pes.logit)
plot(inf)
dev.off()

#Number of participants
png(filename="Studysize_vs_effect_size_plot_Males_and_females.png",
    width = 500,
    height= 299)

subganal.size=rma(yi, vi, data=ies.logit, mods=~Total_number,
method="DL")
pes.size=predict(subganal.size, newmods=c(0:2),
transf=transf.ilogit)
wi=1/sqrt(ies.logit$vi)
size=1+3 *(wi-min(wi))/(max(wi)-min(wi))
plot(ies.logit$Total_number, transf.ilogit(ies.logit$yi), cex=size,
xlab="Sample size",
     ylab="Proportion")
lines(0:2, pes.size$pred, col="navy")
lines(0:2, pes.size$ci.lb, lty="dashed", col="maroon")
lines(0:2, pes.size$ci.ub, lty="dashed", col="maroon")
dev.off()

print(subganal.size)

png(filename="Study_year_vs_effect_size_plot_Malesandfemales.png",
    width = 500,
    height= 299)
```

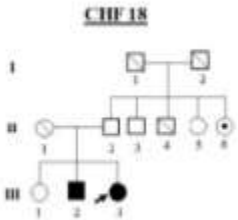
```
#Study year
metareg.year=rma(yi, vi, data=ies.logit, mods=~ Year, method="DL")
wi=1/sqrt(ies.logit$vi)
size=1+3*(wi-min(wi))/(max(wi)-min(wi))
pes.year=predict(metareg.year, newmods=c(1994:2020),
transf=transf.ilogit)
plot(ies.logit$Year, transf.ilogit(ies.logit$yi), cex=size, pch=1,
las=1, xlab="Publication year",
     ylab="Proportion")
lines(1994:2020, pes.year$pred, col="navy")
lines(1994:2020, pes.year$ci.lb, lty="dashed", col="maroon")
lines(1994:2020, pes.year$ci.ub, lty="dashed", col="maroon")
ids=c(1:17)
pos=c(1)
text(ies.logit$Year[ids], transf.ilogit(ies.logit$yi)[ids], ids,
cex=0.9, pos=pos)
dev.off()

print(metareg.year)
#Another funnel plot for study bias
#Make and save plot
png(filename="Studies_funnel_plot_Malesandfemales.png",
    width = 371,
    height= 222)
funnel(pes.logit, yaxis="sei")
dev.off()
#Eggers regression test
regtest(pes.logit, model="rma", predictor="sei")
#Leone et al., may be outlier (>2)
L1O=leave1out(pes.logit, transf=transf.ilogit); print(L1O, digits=6)
#Write table for supplementary
L1O <- as.data.frame(L1O)
write.csv(L1O, file="Leave_one_out_analysisMalesandfemales.csv")
```

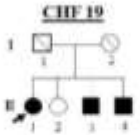
3.4.3 Figure 1: Pedigrees of families CHF1-CHF48

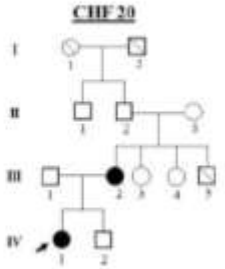


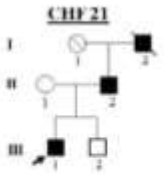

CHF 16

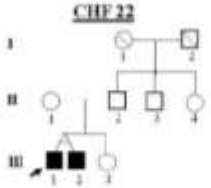

CHF 17

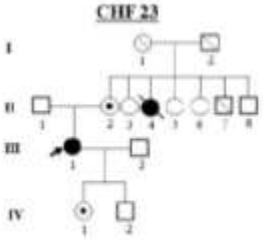

CHF 18

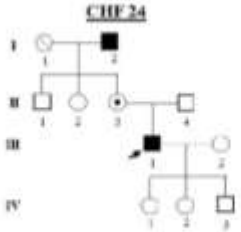

CHF 19

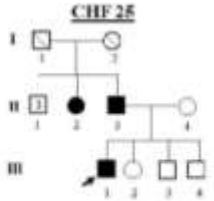

CHF 20

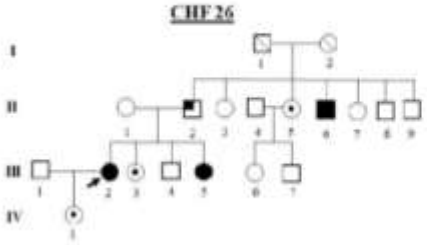

CHF 21

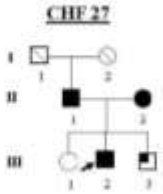

CHF 22

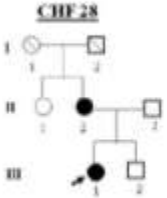

CHF 23

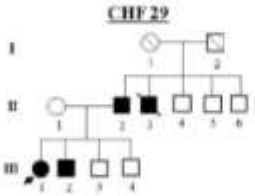

CHF 24

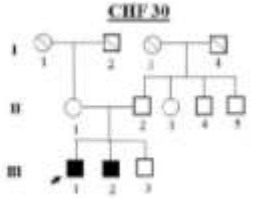

CHF 25

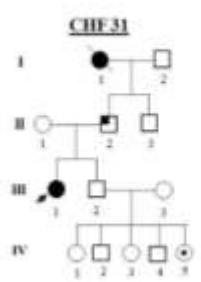

CHF 26

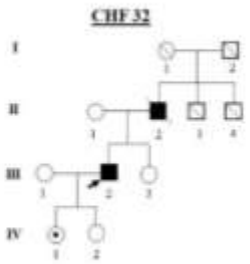

CHF 27

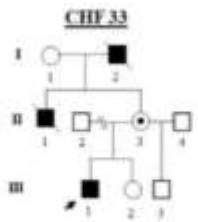

CHF 28

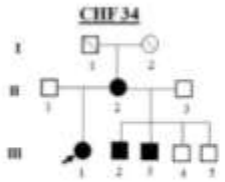

CHF 29

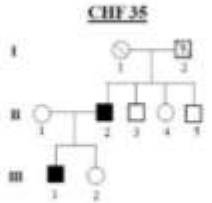

CHF 30


CHF 31


CHF 32


CHF 33


CHF 34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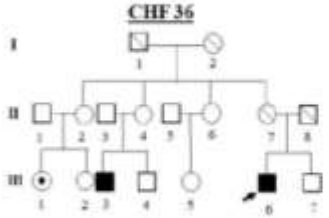
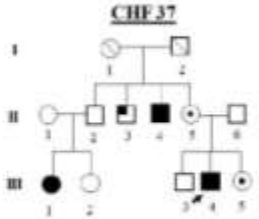

CHF 36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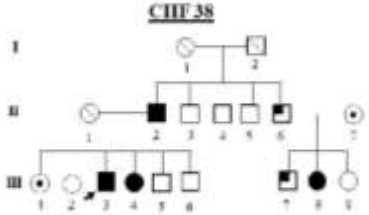
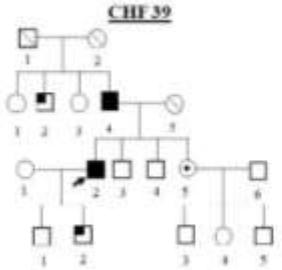
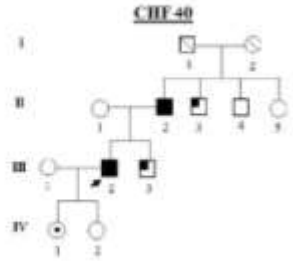
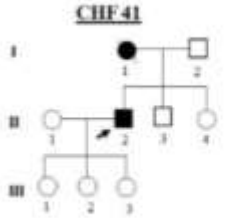
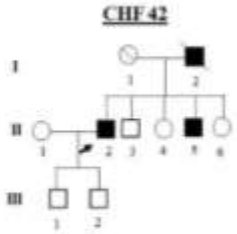
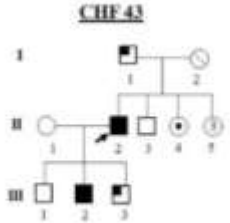

CHF 38

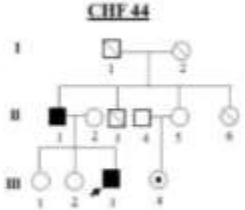

CHF 39

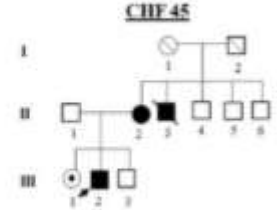

CHF 40

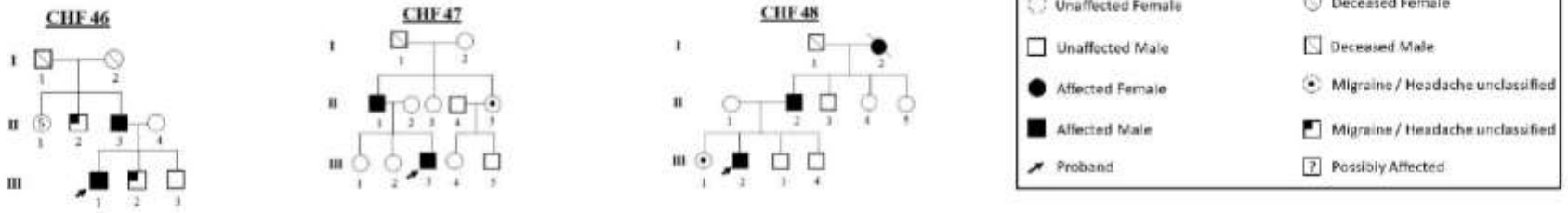

CHF 41


CHF 42


CHF 43


CHF 44


CHF 45


CHF 46
CHF 47
CHF 48
Unaffected Female
Unaffected Male
Affected Female
Affected Male
Proband
Deceased Female
Deceased Male
Migraine / Headache unclassified
Migraine / Headache unclassified
Possibly Affected

Section IV Appendix

4.1 Genome –Wide Association Study of Cluster Headache

Analysis protocol Cluster headache (4.1.5)

Quality control before merging cases and controls

#Remove variants with call rate < 95%

```
plink --bfile infile --geno 0.05 --make-bed --out outfile
```

#Remove individuals with call rate < 98%

```
plink --bfile infile --mind 0.02 --make-bed --out outfile
```

#Remove individuals with inconsistent sex information

#Calculate sex based on X-chromosome

```
plink --bfile inputfile --check-sex --out outputfile
```

Extract all individuals where assigned sex does not match sex based on X chromosome

```
grep PROBLEM outputfile.sexcheck | awk '{print $1,$2}' > fail-sexcheck.txt
```

#Remove the individuals from the dataset

```
sh plink --bfile inputfile --remove fail-sexcheck.txt --make-bed --out outputfile
```

#Remove variants with call rate < 98%

```
plink --bfile infile --geno 0.02 --make-bed --out outfile
```

#Remove variants deviating from the Hardy-Weinberg equilibrium (HWE) :Cases: HWE < 1e-10 Controls: HWE < 1e-6

-

```
plink --bfile inputfile --hwe 1e-6/1e-10 --make-bed --out outputfile
```

#Merge cases and controls and QC

#Remove variants with call rate < 98%

```
plink --bfile infile --geno 0.02 --make-bed --out outfile
```

#Removing individuals with call rate < 98%

```
plink --bfile infile --mind 0.02 --make-bed --out outfile
```

#Remove monomorphic variants/check that there are no monomorphic variants left

```
plink --bfile inputfile --maf 0.0000001 --make-bed --out outputfile
```

#Remove variants deviating from the Hardy-Weinberg equilibrium (HWE)

```
plink --bfile inputfile --hwe 1e-6 --make-bed --out outputfile
```

#Remove individuals with outlying heterozygosity rate

#Calculate heterozygosity rate and missingness with plink

```
plink --bfile inputfile --het --out outputfile
```

```
plink --bfile inputfile --missing --out outputfile
```

#Make scatterplot in R, based on observed heterozygosity rate per individual and exclude individuals with outlying heterozygosity rate. Especially high heterozygosity rates are problematic as they indicatecontamination.

#Remove duplicates/monozygotic twins

#Create a pruned dataset

```
plink --file inputfile --exclude high-LD-regions.txt --range --indep-pairwise 50 5 0.2 --out outputfile
```

#Calculate relatedness on the pruned dataset

```
plink --bfile inputfile inputfile --extract output_from_last_run.prune.in --genome --out outputfile
```

Remove one individual from each pair with PI_HAT > 0.98. Preferably remove controls before cases.

#Population stratification using the pca command in plink to run principal components analysis on the pruned dataset from the previous step. Use the first 20principal components to exclude outlying individuals.Then ran *peddy.*

#Remove variants with different genotyperate between cases and controls

```
plink --bfile inputfile --test-missing --out outputfile
```

#Remove all variants with p-value < 0.00001.

#Pre-imputation quality control/preparation

#Set heterozygote, haploid variants to missing

```
plink --bfile inputfile --set-hh-missing --make-bed --out outputfile
```

#Run HRC/1KG Imputation Preparation and Checking tool"

#Unzip with gunzip. Frequency files are created with :

```
plink ---bfile inputfile --freq --out outputfile
```

#Run the tools as follows: HRC

`perl HRC-1000G-check-bim.pl -b <bim file> -f <frequency file> -r HRC.r1-1.GRCh37.wgs.mac5.sites.tab -h`

: `<population>`

#The cleaned/updated binary files (one per chromosome) are now converted to vcf format using plink2, gbzip and tabix.

```
for i in `seq 1 23`;
do

    plink --bfile updated-file-chr${i} --keep-allele-order --recode vcf-iid --out updated-file-chr${i}
    bgzip updated-file-chr${i}.vcf

    tabix -p vcf updated-file-chr${i}.vcf.gz
```

Imputation with Michigan Imputation Server/ HRC v1.1 and re-phasingusing Eagle (implemented by the imputation server).

#Post-imputation quality control

#Create a list of monomorphic sites and subset polymorphic variants to minimize the size of output files.

```
#!/bin/bash

for i in `seq 1 22`;
  do

  bcftools view -q1.0:major -S ID_order_chrX.txt chr$(i).dose.vcf.gz -O u | bcftools query -f '%CHROM\t%P
OS\t%REF,%ALT\n' >> STUDY_PANEL_imputed.monomorphic.txt
```

#Generate VCFs with only polymorphic variants

```
#!/bin/bash

for i in `seq 1 22`;
  do

  bcftools view -c 1:minor -S ID_order_chrX.txt chr${i}.dose.vcf.gz -O z > chr${i}.imputed.poly.vcf.gz
  tabix -p vcf chr${i}.imputed.poly.vcf.gz
```

#Analysis

#Run SAIGE

#Step 1

```
Rscript /path/to/_fitNULLGLMM.R \

--plinkFile=/path/to/pruned_plinkfile \

--phenoFile=/path/to/phenotypefile \

--phenoCol=Phenotype \

--covarColList=Sex,PC1,PC2,PC3,PC4 \

--sampleIdColinphenoFile=ID_col \

--traitType=binary
```

```
--skipModelFitting=FALSE \

--outputPrefix=prefix

--nThreads=2
```

#Obtain logfiles

```
logfile_step1=.Phenotype_step1.log

{

Rscript...

--plinkFile..

...
```

```
--nThreads=2

} 2>&1 | tee «$logfile_step1»
```

Step 2

#SPAGMMATtest function

```
Rscript path/to/_SPAGMMATtest \
--vcfFile=dosagefile.vcf.gz \
--vcfFileIndex=dosagefile.vcf.gz.tbi \
--vcfField=DS \
--chrom=1 \
--minMAC=1 \
--sampleFile=samplefile.txt \
--GMMATmodelFile=Phenotype_prefix.rda \ # The outputfile from step 1
--varianceRatioFile=Phenotype_prefix.varianceRatio.txt \ # The outputfile from step 1
--SAIGEOutputFile=Outputfile.txt \ # Desired name of the output result file
--numLinesOutput=2 \
--IsOutputAFinCaseCtrl=TRUE #
```

4.4 Rare Variant Association Analysis

4.4.4a Rare Variant Association Study / QC

#Cases

```
gvcf <- fread("COHORT3/cohort3.gvcf.list", header = F)

s <- fread("COHORT3/cohort3.samples", header = F)

asl %<>% filter(LogID %in% s$V1)

ch <- asl %>% filter(LogID %in% p$LogID)

ch$case_control <- "CASE"

con <- asl %>% filter(!(LogID %in% p$LogID))

con$case_control <- "CONTROL"

dat <- rbind(ch, con)

dat %<>% select(LogID, Pi, `Original Sample ID`, Researcher, DataDir, CaptureMethod, case_contr
ol)

table(dat$case_control)
```

```
   CASE CONTROL
    325    3661
```

#Controls

```
#add coverage and contamination

ts <- fread("~/OneDrive - University College London/KOIOS and stuff/Tracked_211020.csv")

dat <- left_join(dat, ts %>% select(LOGID, contamination, mean_coverage, total_reads, gvcf), by
=c("LogID"="LOGID"))

m <- fread("INFO/exome_wgs_metrics/exome.metrics.txt")

m1 <- fread("INFO/exome_wgs_metrics/wgs.2.metrics.txt")

m2 <- fread("INFO/exome_wgs_metrics/wgs.metrics.txt")

m <- rbind(m, m1, m2)

colnames(m) <- c("GENOME_TERRITORY", "corrected_MEAN_COVERAGE", "SD_COVERAGE", "MEDIAN_COVERAGE
", "MAD_COVERAGE", "PCT_EXC_MAPQ", "PCT_EXC_DUPE", "PCT_EXC_UNPAIRED", "PCT_EXC_BASEQ", "PCT_EX
C_OVERLAP", "PCT_EXC_CAPPED", "PCT_EXC_TOTAL", "PCT_1X", "PCT_5X", "PCT_10X", "PCT_15X", "PCT_2
0X", "PCT_25X", "PCT_30X", "PCT_40X", "PCT_50X", "PCT_60X", "PCT_70X", "PCT_80X", "PCT_90X", "P
CT_100X", "HET_SNP_SENSITIVITY", "HET_SNP_Q")

m %<>% mutate(LogID = gsub("/mnt/qsg-results/pipeline/","", GENOME_TERRITORY)) %>%

  mutate(LogID = gsub("/.*","", LogID))

dat <- left_join(dat, m %>% select(LogID, corrected_MEAN_COVERAGE), by="LogID")

dat %<>% mutate(COV = ifelse(is.na(corrected_MEAN_COVERAGE), mean_coverage, corrected_MEAN_COVE
RAGE))

summary(dat$COV)
```

```
   Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
   1.14   26.28   29.77   29.80   33.38   65.63
```

```
table(dat$CaptureMethod, dat$DataDir)
```

```
                            cbettencourt-ATX-20160122-Macrogen_2016 cbettencourt-ATX-20170831-M
acrogen_2017
                                                                  0
0
  SureSelect V4-post                                           1402
670
  SureSelect V6-post                                              0
0
  TruSeq DNA PCR Free,350bp                                       3
0


                            cbettencourt-ATX-20170831-Macrogen_2017_2 cbettencourt-ATX-20180321
-Macrogen_2018
                                                                    0
0
  SureSelect V4-post                                              477
845
  SureSelect V6-post                                                0
0
  TruSeq DNA PCR Free,350bp                                         0
0


                            hhoulden-sefthymiou--20190507-HN00106571 sefthymiou--20190214-Macro
gen_1812KHX-0011
                                                                179
0
  SureSelect V4-post                                              0
0
  SureSelect V6-post                                              0
410
  TruSeq DNA PCR Free,350bp                                       0
0
```

```
dat %<>% filter(CaptureMethod != "TruSeq DNA PCR Free,350bp")
```

#Coverage: Most samples are generated using SureSelect V4 and the rest SureSelect V6

```
p <- ggplot(dat, aes(x=COV, color=CaptureMethod, fill = CaptureMethod)) +
  geom_histogram(alpha=0.8, position="identity", binwidth = 1)
p + scale_color_manual(values=c("#999999", "#E69F00", "#56B4E9", "#009E73")) +
```

```
  scale_fill_manual(values=c("#999999", "#E69F00", "#56B4E9", "#009E73"))
```

```
dat %>% filter(COV < 15) %>% select(LogID, case_control, COV, CaptureMethod)
```

```
dat %<>% filter(COV > 10)
table(dat$case_control)
```

```
   CASE CONTROL
    323    3655
```

#Contamination

```
p <- ggplot(dat, aes(x=contamination, color=CaptureMethod, fill = CaptureMethod)) +
  geom_histogram(alpha=0.8, position="identity", binwidth = 0.0005)
p + scale_color_manual(values=c("#999999", "#E69F00", "#56B4E9", "#009E73")) +
  scale_fill_manual(values=c("#999999", "#E69F00", "#56B4E9", "#009E73"))
```

```
summary(dat$contamination)
```

```
    Min.  1st Qu.   Median     Mean  3rd Qu.     Max.
0.000030 0.000960 0.001550 0.003074 0.002100 0.353100
```

```
dat %>% filter(contamination > 0.01) %>% select (LogID, contamination, case_control)
```

```
dat %<>% filter(COV > 10 & contamination < 0.01)
table(dat$case_control)
```

```
   CASE CONTROL
    320    3584
```

#Run peddy on QCd exome files (see merge and QC exomes)

#Remove controls with low Ti/Tv ratio (TITV < 2.45). Leaving 843 samples.

#Gender check

```
#ethnicity
s <- fread("COHORT3/cohort3.sex_check.csv")
dat <- left_join(dat, s, by=c("LogID"="sample_id"))
```

```
p <- ggplot(dat, aes(x=het_ratio, fill = predicted_sex)) +
  geom_histogram(alpha=0.9, position="identity", bins = 100)
p + scale_fill_manual(values=c("#E69F00", "#56B4E9"))
```

```
dat %>% filter(het_ratio > 0.2 & het_ratio < 0.8) %>% select (LogID, het_ratio, case_control, p
redicted_sex) %>% arrange(het_ratio)
```

```
dat %<>% filter(het_ratio < 0.2 | het_ratio > 0.8) %>% rename("het_ratio_gender" = "het_ratio")
%>% select(-error, -het_count, -hom_alt_count, -hom_ref_count, -ped_sex)
table(dat$case_control)
```

```
   CASE CONTROL
    320    3531
```

#Filter controls with ambigous gender.

```
e <- fread("COHORT3/cohort3.het_check.csv")
dat <- left_join(dat, e, by=c("LogID"="sample_id"))
summary(dat$call_rate)
```

```
   Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
 0.2973  0.9903  0.9956  0.9927  0.9979  0.9998
```

#Remove samples with low call_rate

```
dat %<>% filter(call_rate > 0.8)
```

```
p <- ggplot(dat, aes(x=median_depth, y=het_ratio, color = case_control)) + geom_point()
p + scale_color_manual(values=c( "#E69F00", "#56B4E9","#009E73", "#F0E442", "#0072B2"))
```

```
p <- ggplot(dat, aes(x = case_control,fill = `ancestry-prediction`)) + geom_bar(position="fill"
) +
  geom_bar(position="dodge") + geom_text(aes(label=..count..),stat='count',position=position_do
dge(0.9),vjust=-0.2)
p + scale_fill_manual(values=c("#999999", "#E69F00", "#56B4E9","#009E73", "#F0E442", "#0072B2")
)
```

```
dat %>% filter(case_control == "CASE" & `ancestry-prediction` != "EUR") %>% select(LogID, `Orig
inal Sample ID`, `ancestry-prediction`)
```

```
dat %<>% filter(`ancestry-prediction` == "EUR")
table(dat$case_control)
```

```
   CASE CONTROL
    300    1991
```

```
#relatedness
relat <- fread("COHORT3/cohort3.ped_check.csv")
relat %<>% filter(sample_a %in% dat$LogID & sample_b %in% dat$LogID)
ggplot(relat, aes(x=ibs0, y=rel)) + geom_point(shape = 23, size = 0.5, color = "lightblue")
```

```
ggplot(relat, aes(x=ibs0, y=ibs2)) + geom_point(shape = 23, size = 0.5, color = "lightblue")
```

```
relat %<>% filter(rel > 0.125)
relat <- left_join(relat, dat %>% select(LogID, case_control), by=c("sample_a" = "LogID"))
relat <- left_join(relat, dat %>% select(LogID, case_control), by=c("sample_b" = "LogID"))
relat %<>% select(sample_a, sample_b, rel, case_control.x, case_control.y)
relat %>% filter(case_control.x == "CASE" & case_control.y == "CASE")
```

```
rcase <- relat %>% filter(case_control.x == "CASE" & case_control.y == "CASE")
rcc <- rbind(relat %>% filter(case_control.x == "CASE" & case_control.y == "CONTROL") ,
             relat %>% filter(case_control.x == "CONTROL" & case_control.y == "CASE"))
rcc <- rbind(rcc %>% select(sample_a, case_control.x) %>% rename("sample"= "sample_a", "case_co
ntrol" = "case_control.x"),
             rcc %>% select(sample_b, case_control.y) %>% rename("sample"= "sample_b", "case_co
ntrol" = "case_control.y"))
rcc %<>% filter(case_control == "CONTROL")
rcontrol <- relat %>% filter(case_control.x == "CONTROL" & case_control.y == "CONTROL")
```

#Remove 3 cases and 833 controls.

```
dat %<>% filter(!(LogID %in% rcase$sample_b | LogID %in% rcontrol$sample_b | LogID %in% rcc$sam
ple))
table(dat$case_control)
```

```
   CASE CONTROL
    193    1262
```

```
fwrite(dat %>% select(LogID), "cohort3_samples_final.toKeep.list", col.names = F)
fwrite(dat %>% filter(case_control == "CASE") %>% select(LogID), "cohort3.cases", col.names = F
)
fwrite(dat %>% filter(case_control == "CONTROL") %>% select(LogID), "cohort3.controls", col.nam
es = F)
fwrite(dat %>% select(LogID,case_control), "cohort3.pheno", col.names = F, sep="\t")
fwrite(dat %>% mutate (ID = LogID) %>%
         mutate (FID = "0") %>%
         mutate (MID = "0") %>%
         mutate (sex = ifelse(predicted_sex == "male", "1", "2")) %>%
         select(ID, LogID, FID, MID, sex, case_control), "cohort3.ped", col.names = F, sep="\t"
)
fwrite(dat %>% mutate (ID = LogID) %>%
         mutate (FID = "0") %>%
         mutate (MID = "0") %>%
         mutate (sex = ifelse(predicted_sex == "male", "1", "2")) %>%
         select(ID, LogID, FID, MID, sex, PC1, PC2, PC3, PC4), "cohort3.covar", col.names = F,
sep="\t")
```

#Plots

```
library(qqman)
dat.rv <- fread("../results/cohort3.clean.filtered.HIGH_MODERATE.01.CMCFisherExact.assoc")
dat.rv %<>% mutate(CHR = gsub(":.*","",RANGE)) %>%
  mutate(CHR = gsub("chr","",CHR)) %>%
  mutate(BP = gsub(".*-","",RANGE)) %>%
  mutate(P = PvalueGreater) %>%
  mutate(CHR = as.numeric(CHR))  %>%
  mutate(BP = as.numeric(BP))
dat.rv %>% arrange(P) %>% head(n=20)
manhattan(dat.rv, main = "Manhattan Plot", ylim = c(0, 10), cex = 0.6,
    cex.axis = 0.9, col = c("blue4", "orange3"), suggestiveline = F, genomewideline = F,
```

```
    chrlabs = c(1:20, "P", "Q")
#qq(dat.rv$P)
library(ggplot2)
qqPlot <- function(pval) {
    pval <- pval[!is.na(pval)]
    n <- length(pval)
    x <- 1:n
    dat <- data.frame(obs=sort(pval),
                      exp=x/n,
                      upper=qbeta(0.025, x, rev(x)),
                      lower=qbeta(0.975, x, rev(x)))

    ggplot(dat, aes(-log10(exp), -log10(obs))) +
        geom_line(aes(-log10(exp), -log10(upper)), color="gray") +
        geom_line(aes(-log10(exp), -log10(lower)), color="gray") +
        geom_point() +
        geom_abline(intercept=0, slope=1, color="red") +
        xlab(expression(paste(-log[10], "(expected P)"))) +
        ylab(expression(paste(-log[10], "(observed P)"))) +
        theme_bw()
}
qqPlot(dat.rv$P)
```

4.4.4 (b) Candidate gene analysis

Gene symbol	Type of channel
ANK2	Accessory Genes/Other
BSND	Accessory Genes/Other
KCNIP1	Accessory Genes/Other
MINK1	Accessory Genes/Other
PSMD1	Accessory Genes/Other
SLC12A5	Accessory Genes/Other
TNRC15	Accessory Genes/Other
CACNA1A	Voltage-gated Calcium Channel Genes
CACNA1B	Voltage-gated Calcium Channel Genes
CACNA1C	Voltage-gated Calcium Channel Genes
CACNA1D	Voltage-gated Calcium Channel Genes
CACNA1E	Voltage-gated Calcium Channel Genes
CACNA1F	Voltage-gated Calcium Channel Genes
CACNA1G	Voltage-gated Calcium Channel Genes
CACNA1H	Voltage-gated Calcium Channel Genes
CACNA1I	Voltage-gated Calcium Channel Genes
CACNA1S	Voltage-gated Calcium Channel Genes
CACNA2D1	Voltage-gated Calcium Channel Genes
CACNA2D2	Voltage-gated Calcium Channel Genes
CACNA2D3	Voltage-gated Calcium Channel Genes
CACNA2D4	Voltage-gated Calcium Channel Genes
CACNB1	Voltage-gated Calcium Channel Genes
CACNB2	Voltage-gated Calcium Channel Genes
CACNB3	Voltage-gated Calcium Channel Genes
CACNB4	Voltage-gated Calcium Channel Genes
CACNG1	Voltage-gated Calcium Channel Genes
CACNG2	Voltage-gated Calcium Channel Genes
CACNG3	Voltage-gated Calcium Channel Genes
CACNG4	Voltage-gated Calcium Channel Genes
CACNG5	Voltage-gated Calcium Channel Genes
CACNG6	Voltage-gated Calcium Channel Genes
CACNG7	Voltage-gated Calcium Channel Genes
CACNG8	Voltage-gated Calcium Channel Genes
CHRNA1	Cholinergic Receptor Genes
CHRNA10	Cholinergic Receptor Genes
CHRNA2	Cholinergic Receptor Genes
CHRNA3	Cholinergic Receptor Genes
CHRNA4	Cholinergic Receptor Genes
CHRNA5	Cholinergic Receptor Genes
CHRNA6	Cholinergic Receptor Genes
CHRNA7	Cholinergic Receptor Genes

CHRNA9	**Cholinergic Receptor Genes**
CHRNB1	**Cholinergic Receptor Genes**
CHRNB2	**Cholinergic Receptor Genes**
CHRNB3	**Cholinergic Receptor Genes**
CHRNB4	**Cholinergic Receptor Genes**
CHRND	**Cholinergic Receptor Genes**
CHRNE	**Cholinergic Receptor Genes**
CHRNG	**Cholinergic Receptor Genes**
CLCN1	**Chloride Channel Genes**
CLCN2	**Chloride Channel Genes**
CLCN3	**Chloride Channel Genes**
CLCN4	**Chloride Channel Genes**
CLCN5	**Chloride Channel Genes**
CLCN6	**Chloride Channel Genes**
CLCN7	**Chloride Channel Genes**
CLCNKA	**Chloride Channel Genes**
CLCNKB	**Chloride Channel Genes**
DRD1	**Dopamine Receptor Genes**
DRD2	**Dopamine Receptor Genes**
DRD3	**Dopamine Receptor Genes**
DRD4	**Dopamine Receptor Genes**
DRD5	**Dopamine Receptor Genes**
GABBR1	**GABA Receptor Genes**
GABBR2	**GABA Receptor Genes**
GABRA1	**GABA Receptor Genes**
GABRA2	**GABA Receptor Genes**
GABRA3	**GABA Receptor Genes**
GABRA4	**GABA Receptor Genes**
GABRA5	**GABA Receptor Genes**
GABRA6	**GABA Receptor Genes**
GABRB1	**GABA Receptor Genes**
GABRB2	**GABA Receptor Genes**
GABRB3	**GABA Receptor Genes**
GABRD	**GABA Receptor Genes**
GABRE	**GABA Receptor Genes**
GABRG1	**GABA Receptor Genes**
GABRG2	**GABA Receptor Genes**
GABRG3	**GABA Receptor Genes**
GABRP	**GABA Receptor Genes**
GABRQ	**GABA Receptor Genes**
GABRR1	**GABA Receptor Genes**
GABRR2	**GABA Receptor Genes**
Gcom1	**Glycine Receptor Genes**
GLRA1	**Glycine Receptor Genes**
GLRA2	**Glycine Receptor Genes**

GLRA3	**Glycine Receptor Genes**
GLRB	**Glycine Receptor Genes**
GRIA1	**Glycine Receptor Genes**
GRIA2	**Glycine Receptor Genes**
GRIA3	**Glycine Receptor Genes**
GRIA4	**Glycine Receptor Genes**
GRID1	**Ionotropic Glutamate Receptor Genes**
GRID2	**Ionotropic Glutamate Receptor Genes**
GRIK1	**Ionotropic Glutamate Receptor Genes**
GRIK2	**Ionotropic Glutamate Receptor Genes**
GRIK3	**Ionotropic Glutamate Receptor Genes**
GRIK4	**Ionotropic Glutamate Receptor Genes**
GRIK5	**Ionotropic Glutamate Receptor Genes**
GRIN1	**Ionotropic Glutamate Receptor Genes**
GRIN2A	**Ionotropic Glutamate Receptor Genes**
GRIN2B	**Ionotropic Glutamate Receptor Genes**
GRIN2C	**Ionotropic Glutamate Receptor Genes**
GRIN2D	**Ionotropic Glutamate Receptor Genes**
GRIN3A	**Ionotropic Glutamate Receptor Genes**
GRINA	**Ionotropic Glutamate Receptor Genes**
GRM1	**Metabotropic Glutamate Receptor Genes**
GRM2	**Metabotropic Glutamate Receptor Genes**
GRM3	**Metabotropic Glutamate Receptor Genes**
GRM4	**Metabotropic Glutamate Receptor Genes**
GRM5	**Metabotropic Glutamate Receptor Genes**
GRM6	**Metabotropic Glutamate Receptor Genes**
GRM7	**Metabotropic Glutamate Receptor Genes**
GRM8	**Metabotropic Glutamate Receptor Genes**
HCN1	**Cyclic Nucleotide-gated Channel Genes**
HCN2	**Cyclic Nucleotide-gated Channel Genes**
HCN3	**Cyclic Nucleotide-gated Channel Genes**
HCN4	**Cyclic Nucleotide-gated Channel Genes**
HTR1A	**Serotonin Receptor Genes**
HTR1B	**Serotonin Receptor Genes**
HTR1D	**Serotonin Receptor Genes**
HTR1E	**Serotonin Receptor Genes**
HTR1F	**Serotonin Receptor Genes**
HTR2A	**Serotonin Receptor Genes**
HTR2C	**Serotonin Receptor Genes**
HTR3A	**Serotonin Receptor Genes**
HTR3B	**Serotonin Receptor Genes**
HTR3C	**Serotonin Receptor Genes**
HTR3D	**Serotonin Receptor Genes**
HTR3E	**Serotonin Receptor Genes**
HTR4	**Serotonin Receptor Genes**

HTR5A	**Serotonin Receptor Genes**
HTR6	**Serotonin Receptor Genes**
HTR7	**Serotonin Receptor Genes**
KCNA1	**Voltage-gated Potassium Channel Genes**
KCNA10	**Voltage-gated Potassium Channel Genes**
KCNA2	**Voltage-gated Potassium Channel Genes**
KCNA3	**Voltage-gated Potassium Channel Genes**
KCNA4	**Voltage-gated Potassium Channel Genes**
KCNA5	**Voltage-gated Potassium Channel Genes**
KCNA6	**Voltage-gated Potassium Channel Genes**
KCNA7	**Voltage-gated Potassium Channel Genes**
KCNAB1	**Voltage-gated Potassium Channel Genes**
KCNAB2	**Voltage-gated Potassium Channel Genes**
KCNAB3	**Voltage-gated Potassium Channel Genes**
KCNB1	**Voltage-gated Potassium Channel Genes**
KCNB2	**Voltage-gated Potassium Channel Genes**
KCNC1	**Voltage-gated Potassium Channel Genes**
KCNC2	**Voltage-gated Potassium Channel Genes**
KCNC3	**Voltage-gated Potassium Channel Genes**
KCNC4	**Voltage-gated Potassium Channel Genes**
KCND1	**Voltage-gated Potassium Channel Genes**
KCND2	**Voltage-gated Potassium Channel Genes**
KCND3	**Voltage-gated Potassium Channel Genes**
KCNE1	**Voltage-gated Potassium Channel Genes**
KCNE1L	**Voltage-gated Potassium Channel Genes**
KCNE2	**Voltage-gated Potassium Channel Genes**
KCNE3	**Voltage-gated Potassium Channel Genes**
KCNE4	**Voltage-gated Potassium Channel Genes**
KCNF1	**Voltage-gated Potassium Channel Genes**
KCNG1	**Voltage-gated Potassium Channel Genes**
KCNG2	**Voltage-gated Potassium Channel Genes**
KCNG3	**Voltage-gated Potassium Channel Genes**
KCNG4	**Voltage-gated Potassium Channel Genes**
KCNH1	**Voltage-gated Potassium Channel Genes**
KCNH2	**Voltage-gated Potassium Channel Genes**
KCNH3	**Voltage-gated Potassium Channel Genes**
KCNH4	**Voltage-gated Potassium Channel Genes**
KCNH5	**Voltage-gated Potassium Channel Genes**
KCNH6	**Voltage-gated Potassium Channel Genes**
KCNH7	**Voltage-gated Potassium Channel Genes**
KCNH8	**Voltage-gated Potassium Channel Genes**
KCNQ1	**Voltage-gated Potassium Channel Genes**
KCNQ2	**Voltage-gated Potassium Channel Genes**
KCNQ3	**Voltage-gated Potassium Channel Genes**
KCNQ4	**Voltage-gated Potassium Channel Genes**

KCNQ5	**Voltage-gated Potassium Channel Genes**
KCNRG	**Voltage-gated Potassium Channel Genes**
KCNS1	**Voltage-gated Potassium Channel Genes**
KCNS2	**Voltage-gated Potassium Channel Genes**
KCNS3	**Voltage-gated Potassium Channel Genes**
KCNT1	**Voltage-gated Potassium Channel Genes**
KCNV1	**Voltage-gated Potassium Channel Genes**
KCNV2	**Voltage-gated Potassium Channel Genes**
KCNJ1	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ10	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ11	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ12	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ14	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ15	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ16	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ2	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ3	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ4	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ5	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ6	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ8	**Potassium Inwardly Rectifiying Channel Genes**
KCNJ9	**Potassium Inwardly Rectifiying Channel Genes**
KCNK1	**Twin Pore Potassium Channel Genes**
KCNK10	**Twin Pore Potassium Channel Genes**
KCNK12	**Twin Pore Potassium Channel Genes**
KCNK13	**Twin Pore Potassium Channel Genes**
KCNK15	**Twin Pore Potassium Channel Genes**
KCNK16	**Twin Pore Potassium Channel Genes**
KCNK17	**Twin Pore Potassium Channel Genes**
KCNK2	**Twin Pore Potassium Channel Genes**
KCNK3	**Twin Pore Potassium Channel Genes**
KCNK4	**Twin Pore Potassium Channel Genes**
KCNK5	**Twin Pore Potassium Channel Genes**
KCNK6	**Twin Pore Potassium Channel Genes**
KCNK7	**Twin Pore Potassium Channel Genes**
KCNK9	**Twin Pore Potassium Channel Genes**
KCNMA1	**Calcium Activated Potassium Channel Genes**
KCNMB2	**Calcium Activated Potassium Channel Genes**
KCNMB3	**Calcium Activated Potassium Channel Genes**
KCNMB4	**Calcium Activated Potassium Channel Genes**
KCNN1	**Calcium Activated Potassium Channel Genes**
KCNN2	**Calcium Activated Potassium Channel Genes**
KCNN3	**Calcium Activated Potassium Channel Genes**
KCNN4	**Calcium Activated Potassium Channel Genes**
RYR1	**Ryanodine Receptor Genes**

RYR2	**Ryanodine Receptor Genes**
RYR3	**Ryanodine Receptor Genes**
SCN10A	**Voltage-gated Sodium Channel Genes**
SCN11A	**Voltage-gated Sodium Channel Genes**
SCN1A	**Voltage-gated Sodium Channel Genes**
SCN1B	**Voltage-gated Sodium Channel Genes**
SCN2A2	**Voltage-gated Sodium Channel Genes**
SCN2B	**Voltage-gated Sodium Channel Genes**
SCN3A	**Voltage-gated Sodium Channel Genes**
SCN3B	**Voltage-gated Sodium Channel Genes**
SCN4A	**Voltage-gated Sodium Channel Genes**
SCN4B	**Voltage-gated Sodium Channel Genes**
SCN5A	**Voltage-gated Sodium Channel Genes**
SCN7A	**Voltage-gated Sodium Channel Genes**
SCN8A	**Voltage-gated Sodium Channel Genes**
SCN9A	**Voltage-gated Sodium Channel Genes**

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