Identification of Migraine Susceptibility Genes:

Candidate Gene Studies

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Abstract

Migraine is a serious neurological disorder that affects the central nervous system causing painful attacks of headache. Attacks of head pain vary widely in intensity, frequency and duration lasting from anywhere between 4-72 hours and are often accompanied by further symptoms of nausea, vomiting, photo- and phonophobia. In 1988, a group of world leaders in the diagnosis of migraine formed the International Headache Society (IHS) and compiled and published a consensus set of diagnostic criteria known as International Classification of Headache Disorders, ICHD-I 1988. This was the first classification system and was subsequently updated in 2004, ICHD-II 2004 and more recently a 3rd Edition beta version has been released (ICHD-3rd Ed Beta Version) and is the gold standard for diagnosing headache disorders. Migraine displays two main subtypes termed migraine with or without aura (MA and MO respectively). The two forms are distinguished from each other based on the development of aura, a period of variable and diverse neurological symptoms that precede the headache phase.

The aim of this research was to examine genetic variation within candidate genes that may predispose people or in other words make them more susceptible to develop migraine using an Australian case-control population. Polymorphisms can occur in any part of the gene and affect gene function by increasing or decreasing its activity. Studying the SNPs involved within candidate genes may give us better insight into how and why specific changes in DNA sequence lead to disease in some people and not others. Researchers are trying to find answers as to why with all other factors being equal some individuals develop migraine while others do not.

In this project genes for analysis were selected using evidence from the literature that supported involvement of the glutamatergic system in migraine and RNA editing processes in the editing of neurotransmitter transcripts. This research investigated the GRIA2 and GRIA4 genes which code for subunits of the AMPA glutamate receptor. These receptors bind to the excitatory neurotransmitter glutamate and have been implicated in a number of psychiatric conditions including schizophrenia. The candidate gene approach was based on the hypothesis that malfunction of the glutamate neurotransmitter system can increase the risk of migraine and examined three markers in the GRIA2 gene at 4q32.1 and four markers in the GRIA4 gene at 11q22.3.
Numerous studies have previously implicated a role for glutamate in migraine including animal localization experiments and biochemical studies.

Despite reports of positive association by Formicola et al., 2010 of SNPs in GRIA1 and GRIA3 and Maher et al., 2013 of SNPs in GRIA3 this approach found no significant association of any of the GRIA gene markers tested with migraine in our Australian Caucasian population. The SNPs genotyped in this study were different to the SNPs genotyped by Formicola et al., 2010 and were found in intronic regions of the genes using the program Haploview to visualize the LD pattern. The Chi-square ($\chi^2$) test was performed using the Statistical Package for Social Sciences (SPSS version 21.0) and identified a negative association in the results for migraine cases and controls and also when they were subdivided into MA and MO groups and female and male subpopulations. The population utilized in the genotyping studies reported in this thesis was a case-control association population consisting of migraine cases matched for age- (± 5 years), sex and ethnicity to their controls.

In addition polymorphisms in the GLUD1 and GLUD2 genes which code for the enzyme glutamate dehydrogenase (GDH) were also investigated. The GLUD1 gene at 10q21.1-24 codes for an enzyme highly expressed in liver, brain, pancreas and kidney that plays an important role in nitrogen and glutamate metabolism. In nervous tissue where the majority of glutamate is located GDH is responsible for the reversible oxidative deamination of L-glutamate to 2-oxoglutarate which regulates energy homeostasis and ammonia detoxification. The GLUD2 gene codes for an enzyme preferentially expressed in testis and brain and is composed of only exons and originated to Xq25 via retrotransposition. Xq25 is interestingly near the Xq24-q25 region previously identified as harbouring migraine susceptibility genes in pedigrees. Both the GLUD1 and GLUD2 genes play an important role in the metabolism of glutamate and form part of the fast acting glutamatergic signalling system.

One SNP was genotyped by HRM however the combined population was not in HWE only when stratified by migraine subtype and gender, it was observed that the MO sample genotypes and the male genotypes were in Hardy Weinberg Equilibrium. The MAF of the SNP was low (G allele, 28%) but the frequency in the control group (G allele, 28.3%) was matching expected allele frequencies as determined in the Hap-Map CEU population (G allele, 28%). Chi-square ($\chi^2$) analysis indicated this SNP was not
associated with migraine. Three of the 5 SNPs genotyped by Sequenom were in HWE and were analysed. Association analysis indicated the tested polymorphisms in the GLUD1 and GLUD2 genes were not significantly associated with migraine with and without aura or gender. Moreover, these results suggest the GLUD1 and GLUD2 genes are not significantly associated with an increase in migraine risk and therefore do not play a significant role in disease susceptibility in our Australian-Caucasian population.

The candidate genes ADARB1 and ADARB2 were also investigated for association with migraine. Both genes code for RNA editing enzymes that are highly expressed in the CNS and fit criteria for migraine neuropathology. Prior work by our laboratory identified 4 SNPs forming a haplotype block in the ADARB2 gene as statistically significant in a pGWAS of the Norfolk Island population, an Australian genetic isolate whereas the ADARB1 gene was simultaneously investigated in this study due to its important function in editing neurotransmitter receptor transcripts. The ADARB2 is located on chromosome 10p15.3 and codes for a catalytically inactive protein, expressed in brain, amygdala and thalamus. The ADARB1 gene encodes an RNA editing enzyme crucial to embryonic development that catalyses a highly conserved A-I reaction and is located on chromosome 21q22.3.

Twenty-one markers were genotyped including 7 SNPs in the ADARB1 gene and 14 SNPs in the ADARB2 gene by a combination of Taqman and Sequenom genotyping platforms. Blocks of LD were visualized in the program Haploview using HapMap genotype data for each gene to pick SNPs for genotyping. Although Sequenom is a powerful genotyping method capable of multiplexing up to 36 SNPs at once there is a small percentage that drop out and have to be discarded. Among the 21 markers genotyped in total, 13 SNPs were in HWE and analysed. Chi-square (χ²) analysis of the investigated SNPs in ADARB1 and ADARB2 genes was carried out using the program PLINK. Overall chi-square (χ²) analysis indicated there was no significant association in the SNPs in the ADARB1 gene and migraine, migraine with and without aura and gender. This result indicates that this RNA editing gene does not contribute to a person’s risk of migraine. In contrast, a nominal association was detected in two SNPs in the ADARB2 gene, marker rs5015512 was positive for combined migraine, allelic p = 0.03 and MA p = 0.01 and females p = 0.02 and marker rs884861 was nominally positive for combined migraine at the allelic p = 0.04 level with significance based on p-value. The ADARB2 gene warrants further investigation in a larger population.
Finally, exome sequencing of the X-chromosome in Migraine X-linked families identified 12 mutations in 8 genes in 3 individuals belonging to 3 different pedigrees (MF47, MF14, MF6) showing X-linked inheritance. Three susceptibility loci at Xp22, Xq13 and Xq24-q28 have been previously identified by genetic studies investigating the X chromosome leading researchers to believe this maternally inherited chromosome may harbour unidentified susceptibility genes. Sanger Sequencing was performed for each of the 12 mutations to validate the results reported in the exome sequencing project. Six of 12 mutations were confirmed as existing and matching the results of the exome sequencing. The 6 mutations validated included RBMX (3), MAMLD1, FAM104B, SPANXN4, IL13RA2 and TMEM185A. The results of Sanger sequencing identified the expected genotypes from the exome sequencing in the 3 families. Therefore the Genomics Research Centre in future studies will investigate these 6 novel variants in extended pedigrees and other families by exome sequencing.

In summary, this research investigated variants within candidate genes implicated in the glutamatergic system and in RNA editing processes including: GRIA2 and GRIA4, GLUD1 and GLUD2, ADARB1 and ADARB2 using a variety of genetic analysis techniques. This is the first study that has investigated the candidate genes GLUD1 and GLUD2 and ADARB1 and ADARB2 for association with migraine. Further detailed genetic analysis of the candidate gene ADARB2 and the 12 mutations identified in the 3 individuals from 3 different pedigrees by exome sequencing is warranted to elucidate the mechanisms by which they may influence the disorder.

This project is in no way a means to an end but an open door for more in depth investigation into the molecular genetic basis of migraine. Specifically future avenues that could be investigated include investigating components of the adenosinergic system and the glutamatergic system and methylation mechanisms in migraine using novel technologies including Next Generation Sequencing (NGS), Exome Sequencing and Bioinformatics approaches. Because migraine is a polygenic disorder that manifests due to an aberrant combination of SNPs in key genes it is best investigated by taking a multi-disciplinary approach and using a variety of genetic techniques and analysis methods. Overall, the results of this study provide new directions for migraine research and build upon previous existing studies.
Statement of Originality

The material presented in this thesis has not previously been submitted for a degree or diploma in any university and to the best of my knowledge contains no material previously published or written by another person except where due acknowledgement is made in the thesis itself.

__________________________
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<th>Description</th>
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<tbody>
<tr>
<td>ABI</td>
<td>Applied Biosystems</td>
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<tr>
<td>Ado</td>
<td>Adenosine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGRF</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>AFF</td>
<td>Affected</td>
</tr>
<tr>
<td>ALL</td>
<td>All</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMM-I</td>
<td>American Migraine Study I</td>
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<tr>
<td>AMM-II</td>
<td>American Migraine Study II</td>
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<tr>
<td>AMMP</td>
<td>American Migraine Prevalence and Prevention study</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy</td>
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<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
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<tr>
<td>CM</td>
<td>Complicated Migraine</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy number variant</td>
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<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
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<td>CPU</td>
<td>Central processing unit</td>
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<td>CSD</td>
<td>Cortical Spreading Depression</td>
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<td>FM</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
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<td>Glutamine</td>
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<td>Glu</td>
<td>Glutamate</td>
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<td>GluR</td>
<td>Glutamate receptor</td>
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<td>GOM</td>
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<td>GTN</td>
<td>Glyceryl trinitrate</td>
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<td>GWAS</td>
<td>Genome-Wide Association Study</td>
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<td>Serotonin</td>
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<td>HM</td>
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<td>High Resolution Melt</td>
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<td>IHS</td>
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<td>LHON</td>
<td>Leber's hereditary optic neuropathy</td>
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<td>LOD</td>
<td>Logarithm of the odds</td>
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<td>MA</td>
<td>Migraine with Aura</td>
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<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption/Ionisation</td>
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<td>MAF</td>
<td>Minor allele frequency</td>
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<td>MgCl₂</td>
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<td>MGB</td>
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<td>mGlurRs</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<td>μL</td>
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<td>mL</td>
<td>Milli Litre</td>
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<tr>
<td>mM</td>
<td>Milli Molar</td>
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<tr>
<td>MO</td>
<td>Migraine without Aura</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MRM</td>
<td>Menstrual related migraine</td>
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</table>
MRS  Magnetic resonance spectroscopy
Mt  mitochondria
MSG  Monosodium glutamate
MS  Mass spectrometer
MZ  Monozygotic
Na  Sodium
Na-K-ATP  Sodium-potassium ATP pump
NaCl  Sodium Chloride
NE  Norepinephrine
NFQ  Non-fluorescent quencher
NGS  Next generation sequencing
NIHS  Norfolk Island Health Study
NI  Norfolk Island
NKM  Sodium-Potassium-Magnesium
ng  Nanogram
nm  Nanometre
NMH  Non-Migrainous Headache
NMDA  N-Methyl-D-aspartate
NO  Nitric Oxide
NRM  Nucleus Raphe Magnus
NTC  No template control
NSAIDs  Non-Steroidal Anti-Inflammatory drugs
OD  Optical Density
OR  Odds ratio
OMIM  Online Mendelian Inheritance in Man
OXPHOS  Oxidative phosphorylation
PACAP  Pituitary adenylate cyclase-activating peptide
PAF  Plasminogen activating factor
PCR  Polymerase Chain Reaction
PHE  L-Phenylalanine
pH  potential Hydrogen
Pi  Phosphate
PCr  Phosphocreatinine
PET  Positron emission topography
PFO  Patent Foramen Ovale
PLTS  Platelets
P-MRS  Phosphorus magnetic resonance spectroscopy
PMM  Pure menstrual migraine
Q/R  Enzymatic deamination of Glutamine with arginine
RBC  Red blood cells
RCT  Randomized controlled trial
RFLP  Restriction Fragment Length Polymorphism
RNA  Ribonucleic acid
rpm  Revolutions Per Minute
RRFs  Ragged red fibres
RR  Relative Risk
RSB  Reticulocyte standard buffer
SAP  Shrimp Alkaline Phosphatase
SBE  Single base extension
SHM  Sporadic Hemiplegic Migraine
sibpair  sibling pair
SHM  Sporadic Hemiplegic Migraine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
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<td>SP</td>
<td>Substance P</td>
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<tr>
<td>ssDNA</td>
<td>Single strand deoxyribonucleic acid</td>
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<tr>
<td>STR</td>
<td>Microsatellite</td>
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<tr>
<td>TAC</td>
<td>Trigeminal Autonomic Cephalalgias</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA buffer</td>
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<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
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<td>TDT</td>
<td>Transmission Disequilibrium Test</td>
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<td>TE</td>
<td>Tris-EDTA</td>
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<td>TEM</td>
<td>Tunneling electron microscope</td>
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<td>TGVS</td>
<td>Trigeminovascular System</td>
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<td>TMI-4</td>
<td>Transmembrane 1 to 4</td>
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<td>TOF</td>
<td>Time-of-flight</td>
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<td>TTH</td>
<td>Tension Type headache</td>
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<td>L-Tyrosine</td>
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<td>Three Dimensional</td>
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<td>United States</td>
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<td>Volts</td>
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<td>Whole-genome sequencing</td>
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<td>WGHS</td>
<td>Women’s Genome Health Study</td>
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<td>Women's Health Study</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>Aspartate aminotransferase</td>
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<td>Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1</td>
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<td>Adenosine deaminase</td>
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<td>AdK</td>
<td>Adenosine kinase</td>
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<td>Angiotensin receptor 1</td>
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<td>Apolipoprotein E</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>Aldehyde dehydrogenase</td>
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<td>AMPA</td>
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<td>ATPase, Na+K+ Transporting, Alpha-2 Polypeptide</td>
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<td>Calcium Channel, Voltage-Dependent, P/Q Type, Alpha-1a Subunit</td>
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<td>Casein kinase 1δ</td>
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<td>Catechol-O-methyl-Transferase</td>
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<td>DBH</td>
<td>Dopamine β-hydroxylase (dopamine beta-monooxygenase)</td>
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<td>DDC</td>
<td>Dopa Decarboxylase (aromatic L-amino acid decarboxylase)</td>
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<td>Estrogen Receptor 2</td>
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<td>Glutamine synthetase</td>
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<td>Glutaminase</td>
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<td>Gene Abbreviation</td>
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<td>GNMT</td>
<td>Glycine N-methyltransferase</td>
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<td>HTR2C</td>
<td>5-hydroxytryptamine (serotonin) receptor 2C</td>
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<td>IL13RA2</td>
<td>Interleukin 13 receptor, alpha 2</td>
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<td>INSR</td>
<td>Insulin receptor</td>
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</tbody>
</table>
KCNK18  Potassium channel, subfamily K, member 18
LRP1   Low density lipoprotein receptor-related protein 1
LTA    Lymphotoxin-Alpha (Tumor Necrosis Factor Beta)
MAT1A  Methionine adenosyltransferase I, alpha
MAMLD1 Mastermind-like domain containing 1
MAO-A  Monoamine oxidase A
MAO-B  Monoamine oxidase B
ME1    Cytosolic malic enzyme 1, NADP(+)-dependent
ME3    Mitochondrial malic enzyme, NADP(+)-dependent
MEF2D  Myocyte enhancer factor 2D
MTDH   Metadherin
MTHFR  Methylene tetrahydrofolate reductase
MTR    5-methyltetrahydrofolate-homocysteine methyltransferase
MTRR   5-methyltetrahydrofolate-homocysteine methyltransferase reductase
NGFR   Nerve growth factor receptor
NGS    Next Generation Sequencing
NOS    Nitric oxide synthase
NOTCH3 Notch, Drosophila, Homolog of, 3
NRIP1  Nuclear receptor interacting protein 1
PAH    Phenylalanine hydroxylase
PC     Pyruvate carboxylase
PDP1   Pyruvate dehydrogenase phosphatase catalytic subunit 1
PGR    Progesterone Receptor
PGCP   Plasma glutamate carboxypeptidase
PHACTR Phosphatase Actin Regulating Protein-1
PMAT   Plasma membrane monoamine transporter
PNMT   Phenylethanolamine N-methyltransferase
RBMX   RNA binding motif protein, X-linked
SAH    S-adenosylhomocysteine
SERT   Serotonin transporter
SLC6A3 Solute carrier family 6 (neurotransmitter transporter), member 3
SLC1A3 Solute carrier family 1 (glial high affinity glutamate transporter), member 3
SLC6A4 Solute carrier family 6 (neurotransmitter transporter), member 4
SLC29A4 Solute carrier family 29 (equilibrative nucleoside transporter), member 4
SLC1A2 Solute carrier family 1 (glial high affinity glutamate transporter), member 2
SLC1A1 Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter system Xag), member 1
SLC1A6 Solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6
SLC1A7 Solute carrier family 1 (glutamate transporter), member 7
SLC17A7 Solute carrier family 17 (vesicular glutamate transporter), member 7
SLC17A6 Solute carrier family 17 (vesicular glutamate transporter), member 6
SLC17A8 Solute carrier family 17 (vesicular glutamate transporter), member 8
SPANXN1 SPANX family, member N1
SYNE1  Synaptic nuclei expressed
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFBR2</td>
<td>Transforming growth factor β receptor 2</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TPH</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>TMEM185A</td>
<td>Transmembrane protein 185A</td>
</tr>
<tr>
<td>TNFA</td>
<td>Tumor necrosis factor -α</td>
</tr>
<tr>
<td>TNFB</td>
<td>Tumor necrosis factor -β</td>
</tr>
<tr>
<td>TPH1</td>
<td>Tryptophan hydroxylase 1</td>
</tr>
<tr>
<td>TPH2</td>
<td>Tryptophan hydroxylase 2</td>
</tr>
<tr>
<td>TRPM</td>
<td>Transient receptor potential cation channel, subfamily M, member 2</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter 2</td>
</tr>
<tr>
<td>VGLUT</td>
<td>Vesicular glutamate transporter</td>
</tr>
</tbody>
</table>
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Publications Arising from this Thesis

Journal Articles:


Conference Presentations:


Gasparini, C.F., Griffiths, L.R. An association study of Glutamatergic and RNA editing genes in migraine. The Human Genetics Society of Australasia Conference (HGSA), New Zealand, Queenstown, August 04-07, 2013. Poster presentation.

Gasparini, C.F., Griffiths, L.R. Genetic Analysis of Glutamatergic genes and the ADARB2 gene in Migraine. Gold Coast Health and Medical Research Conference, Gold Coast, Australia, November 29-30, 2012. 3min interactive poster presentation.

Gasparini, C.F., Griffiths, L.R. Candidate Genes in Migraine. The Human Genetics Society of Australasia Conference, Gold Coast, Australia, July 31-03, 2011. Poster presentation.


Gasparini, C.F., Griffiths, L. Candidate Genes in Migraine. Gold Coast Health and Medical Research Conference, Gold Coast, Australia, December 1-2, 2011. Poster presentation.
Significance

Migraine is a common disorder imparting substantial economic and psychological burdens to its sufferers. The World Health Organization has documented migraine as ranking among the top 19 leading causes of disability (WHO 2001). Migraine is a public health problem of great scope that has an impact on both the individual and society.

A high proportion of the Caucasian population suffers from migraine with an estimated prevalence of around 12% (Lipton and Bigal 2005; Lipton, Bigal et al. 2007). In addition women are three times more likely to suffer migraine than men, a statistic that has been attributed to fluctuating hormones in alignment with reproductive milestones. Migraine prevalence also varies with age affecting people during their most productive years 30-40 (Jensen and Stovner 2008). Heritability studies combined with high concordance in twin studies, and familial aggregation have confirmed a genetic component of 40-60% (Mulder, Van Baal et al. 2003). The concerted action of environmental and genetic factors brings about the migraine in genetically predisposed individuals.

Migraine attacks incur a heavy economic burden on society due to such factors as direct medical costs and indirect costs associated with lost productivity in the workplace. Migraine also imparts substantial secondary effects on friends and family members of sufferers. Migraine is among the most costly neurological disorders in the European Community costing more than €27 billion per year (Stovner and Andree 2010). The diagnosis of migraine is not always straightforward as it is based on subjective criteria complicated by co-morbidities and overlapping symptoms with other neurological disorders. In addition there is no reliable genetic marker or diagnostic test, factors which can lead to misdiagnosis and incorrect treatment.

Migraine does not follow a Mendelian mode of inheritance but is genetically heterogenous making the identification of susceptibility genes more complex. We now know that migraine is a disease of neurovascular origin with involvement of the trigeminovascular system and sensory nerves, however much work remains to be done to fully tease apart the anatomical and genetic components that increase a person’s risk of getting migraine and breach a set threshold. By genotyping and analysing candidate
genes involved in potential disease mechanisms this could lead to an improvement in diagnostic accuracy and the possibility of developing more effective pharmaceuticals tailored to treat the specific symptoms of the disorder.
Aims

The objective of this research was to investigate the molecular genetics of migraine in an Australian Caucasian cohort. The hypothesis investigated in this research is that variations within specific genes, of known biological activity increase the risk of migraine and contribute to migraine etiology. Such genes can be identified through molecular linkage and/or association studies. In this project a genetic case-control association design was used. The aim of this experimental approach is to assess the frequency of known genetic variation(s) in a group of unrelated case (i.e. migrainous) individuals and compare those frequencies to a matched group of unrelated control (i.e. non-migrainous) individuals. The “known genetic variation” refers to polymorphisms or single nucleotide changes in the DNA of affected individuals. SNPs are one of the most common types of genetic variation and their frequency can easily be measured by genotyping experiments so as to inform the relative risk of disease conferred by the possession of key SNPs.

The candidate genes we investigated are glutamate receptors GRIA2, GRIA4, glutamate dehydrogenase enzyme, GLUD1 and GLUD2 and RNA editing genes ADARB1 and ADARB2. These are an interesting class of genes to study in relation to migraine because of their involvement in neurotransmitter pathways. Evidence for the involvement of glutamate in migraine has been initially provided by Formicola et al., 2010, who found a positive association between GRIA1 and GRIA3 variants in an Italian population (Formicola, Aloia et al. 2010). Glutamate is an excitatory neurotransmitter widely involved in migraine mechanisms, and may be at the core of central hyperexcitability and trigeminovascular activation.

RNA editing genes have been linked to human cancer, viral infections and neurological diseases and also seemed good candidates due to widespread expression in the CNS (Maas, Kawahara et al. 2006). A secondary aim of this project was to expand on results of an exome sequencing project of the X chromosome to determine if other susceptibility loci could also be detected. To achieve these aims a number of lab-based genotyping techniques and statistical approaches were employed as detailed in the General Methodology of Chapter 5.
The specific aims of this research were to:

I. Investigate polymorphisms in the candidate genes *GRIA2* and *GRIA4* by genotyping an association population of migraine cases and controls to determine if the variants play a role in migraine predisposition

II. Investigate polymorphisms in the candidate genes *GLUD1* and *GLUD2* by genotyping an association population of migraine cases and controls to determine if variants play a role in migraine predisposition

III. Investigate polymorphisms in the candidate genes *ADAR1* and *ADAR2* by genotyping an association population of migraine cases and controls to determine if variants play a role in migraine predisposition

IV. Validate exome sequencing results of the X-chromosome of 3 individuals from 3 Migraine X-linked families by undertaking Sanger sequencing

The broad aims of this research were to define the molecular basis of gene dysfunction and how this relates to disease so as to use this information to develop better forms of diagnosis for genetic and lifestyle counselling to aid in disease adaptation and prevention which in turn is expected to lead to more appropriate treatments targeted to the specific genetic causes underlying individual forms of disease. Research is aimed at developing a better understanding of the gene relationships and quantifying the risk for developing migraine. Searching for genes that are linked to migraine risk and studying molecular processes to determine how migraine arises in predisposed individuals will help in the development of susceptibility screening and the identification of drug targets. Examining environmental influences that impact on migraine susceptibility and treatment is also important for improving disease management.
Structure of this Thesis

This thesis will provide a comprehensive background to the classification and diagnosis of migraine as a disorder as well as the strategies used in the analysis of complex disease. The first four chapters review the current state of molecular genetics research in migraine and have been written as three independent review articles. Chapter five outlines the general methodology used to conduct and analyse experimental results including the key lab-based and statistical techniques used in this research as well as a comparison of the different genotyping techniques.

The results of this PhD research have been divided into 4 Chapters. Firstly, genotyping results of the GRIA2 and GRIA4 genes in a migraine case-control association population are presented in Chapter 6. Chapter 7 presents the genotyping results of the GLUD1 and GLUD2 genes undertaken by HRM and Sequenom. In Chapter 8 an analysis of SNPs in ADARB1 and ADARB2 genes with migraine in an Australian case-control population is presented. Chapter 9 provides results of an exome sequencing project of the X chromosome in 5 X-linked migraine families. Finally Chapter 10 summarises the overall results and discusses future directions that may lead on from this work and implications to migraine research in the future.
CHAPTER 1  Introduction to Migraine
1.1 Migraine Historical Perspective

Migraine was researched and documented by a number of renowned physicians in the Roman era including Hippocrates, Arateus, Galen and Celsus, each making a valuable contribution to the knowledge-base of migraine pathophysiology that would help unravel the mystery behind migraine and form the foundation of current medical knowledge. Hippocrates, first described visual aura and the relief that occurs by purging in 400 BC (Abokrysha 2009). Aretaeus of Cappadocia is typically mentioned as the “discoverer” of migraine based on historical texts he wrote on classification of headache into cephalgia, cephalaea, and migraine in 100-200 AD (Koehler and van de Wiel 2001). Galen of Pergamon in approximately 200 AD introduced the Greek word hemicrania, a term that literally translates to mean “half” “skull” or “pain in half of the head” and from which the word migraine, used in today’s modern society, originates (Afridi and Kaube 2003). Celsus (215-300 AD) made the connection between migraine and certain external triggers such as wine, acidity, cold, or glaring brightness from fire or sun (Silberstein, Stiles et al. 2005). Altogether knowledge of migraine pathophysiology has continued to evolve throughout history thanks to the cumulative contributions of many individuals.

Further historical milestones include the noted association of hormone fluctuations in women and precipitation of headache by Persian physician, Bakr Mohamed Ibn Zakariya Râzi (865–925AD). Also the work of Islam scientist, Ibn Sina (Avicenna, 980–1037AD), should receive mention for his tremendous contribution to medicine and migraine in the Canon of Medicine, a medical text that became one of the first scientific encyclopaedia’s disseminated in Europe and that is thought to have hinted at a neurovascular theory of migraine (Silberstein, Stiles et al. 2005). Later in the 16th century Thomas Willis published a hypothesis linking ‘megrim’ to dilation of blood vessels and articulated a vascular theory (Abokrysha 2009). Soon enough this theory was challenged by Edward Lieving in 1873, who believed that migraine was more of a brain dysfunction caused by “nerve storms” and published a neurogenic theory of migraine (Eadie 2005). Both theories have survived into the 20th century generating some debate about the origin and cause of headache and have produced a division of scientific thought within the scientific community regarding theories of the pathogenesis of migraine.

Migraine has been the subject of much creative work in modern society with artists and writers drawing inspiration from their personal experiences and depicting their feelings of
mental dysfunction and visual disturbance into their art. Migraine has also infiltrated popular music with musicians interpreting the condition as intractable, violent, and all-consuming (Roberts and Vargas 2012). A spectrum of influential people suffered migraine, including famous MA sufferer Charles L. Dodgson, who under the pseudonym Lewis Carroll (1832-1898) authored *Alice in Wonderland* and the sequel, *Through the Looking Glass*. The adventures of Alice are thought to reflect Dodgson’s own migraine aura experiences, which included symptoms of micropsia, macropsia, negative scotomas and other visual disturbances, a condition often referred to as *Alice in Wonderland Syndrome* (Kew, Wright et al. 1998).

References to migraine are as old as 7,000 BC with remains of perforated Neolithic human skulls and historical cave paintings documenting the drastic steps taken by ancient people (Villalon, Centurion et al. 2003). Prior to modern medicine, migraine pain was ascribed to the will of evil spirits or malevolent gods (Takano and Nedergaard 2009). Some remedies prescribed by ancient people included: “drilling a hole in the skull to free evil spirits, purging and bloodletting, applying a hot iron to the site of pain, inserting a clove of garlic through an incision in the temple, incantation and by remedies such as placing a clay crocodile on one’s head to drive out the evil demons” (Villalon, Centurion et al. 2003). Such archaic remedies were based on the supernatural including witchcraft, superstition and religious beliefs of the culture (Rapoport and Edmeads 2000). These primitive concepts are now obsolete and migraine is described as a complex neurological disorder influenced by an individual’s genetic background. This chapter will define and introduce migraine and current diagnostic criteria, discuss epidemiology, social and economic impacts, known co-morbidities, the current understanding of the pathophysiology of the disease and finally the available treatments.
1.2 Definition

Migraine is derived from the Greek word *hemicrania*, a term that describes a central nervous system disorder characterised by moderate to severe headache. Migraine is defined as an episodic primary headache of debilitating nature that can last from 4 to 72 hours. The attacks are aggravated by routine physical activity and may be associated with other symptoms, including nausea and/or vomiting, photophobia and phonophobia. Attacks vary widely from patient to patient in regard to frequency, intensity and duration, all of which impact the quality of life of migraineurs. The exact cause and origin of pain remains to be fully clarified as no measurable quantitative physiological changes are apparent during an attack.

1.3 Introduction

Migraine is a highly prevalent disorder with undetermined cause which is estimated to affect 12% of the Caucasian population including 18% of adult women and 6% of adult men (Stovner and Hagen 2006). The prevalence of women suffering from migraine has been attributed to a role of ovarian hormones (Stovner, Hagen et al. 2007). The socioeconomic impact of migraine is wide-ranging and long-lasting imposing direct medical costs to individuals, families and communities and indirect costs due to lost productivity at work. The European Community has estimated migraine to cost more than €27 billion per year making it one of the most costly neurological disorders (Stovner 2006).

Migraine typically commences in puberty but has most impact on people in the 35 to 45 age bracket (Stovner, Hagen et al. 2007). Migraineurs experience a severe headache with associated symptoms of nausea, vomiting, photo- and phono-phobia (Wessman, Kaunisto et al. 2004). The pain experienced during the headache phase can localise to one side of the head or pulsate and can be worsened by physical activity. The frequency of headache attacks varies, occurring approximately once a week, or once a month to once a year, and in duration from as little as an hour to as much as three days. The variability in frequency of migraine attacks is related to both the genetic component carried by the individual and environmental triggering factors (Montagna 2008).

Migraine is diagnosed based on a patient’s recollection of their symptoms, a positive family history and the exclusion of secondary causes (Mueller 2007). Symptoms are assessed by symptom-based criteria defined by the International Headache Society (IHS), International
Classification of Headache Disorders 3rd Edition (beta version currently available at http://cep.sagepub.com/content/33/9/629.full) (IHS 2013). Two main types of migraine have been recognized: migraine with or without aura (MA and MO, respectively). Patients with MA experience an aura that precedes their headache, whilst those with MO do not, this is the distinguishing feature. The aura is experienced by about a third of patients and consists of neurological symptoms that manifest as visual hallucinations like flashing lights, sparks or lines (followed by dark spots), facial tingling or numbness as well as other sensory, motor or aphasic symptoms that usually last from five minutes to an hour (Viana, Sprenger et al. 2013). Aura has been linked to an electrophysiological event that occurs during migraine, termed cortical spreading depression (CSD) which is a wave of intense neuronal activity that gradually propagates over the cortical regions of the brain and is then followed by prolonged inhibition of neuronal activity (Dalkara, Nozari et al. 2010).

1.3.1 Migraine Classification and Diagnosis

Head pain is a rather common complaint experienced by the majority of the population at some point in their lifetime and can be a symptom of many disorders. As a result strict guidelines allowing precise diagnosis and differentiation of one disorder from the next were developed by a group of experts who formed the International Headache Society IHS. In 1988 the IHS released the first edition of the International Classification of Headache Disorders (IHS 1988). This document is a criterion for the diagnosis of headache and was revised in 2004 and is currently recognized internationally as the global standard for diagnosing headache (IHS 2004).

The ICHD-II is a criterion for headache diagnosis and classification composed of three parts that addresses: primary headaches, secondary headaches and cranial neuralgias, central and primary facial pain and other headaches. Primary headaches are distinct clinical disorders that have no apparent underlying cause, secondary headaches are attributable to an underlying disorder such as trauma, infection or psychiatric ailments, while the third category attempts to classify neuralgic pain due to disease of the cranium, neck and facial structures and subtypes of headaches described for the first time or for which insufficient information is yet available (IHS 2004). Acceptance of these criteria has facilitated modern day molecular genetic research in the absence of diagnostic tests. This criterion is somewhat limited
however by the fact that it relies on the subject’s recall of the migraine attack and the characteristics and symptoms that accompany it.

Four primary headaches are described by the diagnostic criterion;

1. Migraine (1.0.),
2. Tension-type headache (TTH) (2.0.),
3. Cluster headache (CH) and other trigeminal autonomic cephalalgias (TACs) (3.0.),
4. Other headaches (4.0.).

1.4 Migraine Subtypes

In the Classification of Primary Migraine Disorders provided by the Headache Classification Subcommittee of the International Headache Society the following subtypes of migraine are recognized:

Migraine is further divided into six categories as follows.

1.1 Migraine without Aura

1.2 Migraine with aura
   1.2.1 Typical aura with migraine headache
   1.2.2 Typical aura with non-migraine headache
   1.2.3 Typical aura without headache
   1.2.4 Familial hemiplegic migraine (FHM)
   1.2.5 Sporadic hemiplegic migraine
   1.2.6 Basilar type migraine

1.3 Childhood periodic syndromes that are common precursors of migraine
   1.3.1 Cyclical vomiting
   1.3.2 Abdominal migraine
   1.3.3 Benign paroxysmal vertigo of childhood

1.4 Retinal migraine

1.5 Complications of migraine
   1.5.1 Chronic migraine
   1.5.2 Status migrainous
   1.5.3 Persistent aura without infarction
   1.5.4 Migrainous infarction
   1.5.5 Migraine-triggered seizure
1.6 Probable migraine

1.6.1 Probable migraine without aura
1.6.2 Probable migraine with aura
1.6.3 Probable chronic migraine

The two main categories and the primary focus of this thesis are *migraine without aura* (1.1) and *migraine with aura* (1.2). The attributes listed in Table 1.1 – 1.2 are the formal (IHS) diagnostic criteria of the two main migraine subtypes; migraine with aura (MA) and migraine without aura (MO). These criteria were used for all migraine diagnosis of research participants used in this study. A description of familial hemiplegic migraine (1.2.4) was also included and a description of these three primary headache classifications follows.

1.4.1 Migraine without Aura

Migraine without aura (MO) is a headache characterised by a minimum of five headache attacks lasting 4-72 hours. These headaches must fulfill criteria B-D listed in Table 1.1 which are unilateral with a pulsating quality of moderate to severe pain intensity and are aggravated by physical activity. Symptoms of nausea and/or vomiting or photophobia and phonophobia further aid in the diagnosis of this migraine subtype.

Table 1.1 ICHD-II diagnostic criteria for migraine without aura (1.1).

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>At least 5 attacks fulfilling criteria B-D</td>
</tr>
<tr>
<td>B.</td>
<td>Headache attacks lasting 4-72 hours (untreated or unsuccessfully treated)</td>
</tr>
<tr>
<td>C.</td>
<td>Headache has at least two of the following characteristics:</td>
</tr>
<tr>
<td></td>
<td>1. unilateral location</td>
</tr>
<tr>
<td></td>
<td>2. pulsating quality</td>
</tr>
<tr>
<td></td>
<td>3. moderate or severe pain intensity</td>
</tr>
<tr>
<td></td>
<td>4. aggravation by or causing avoidance of routine physical activity (e.g., walking, climbing stairs)</td>
</tr>
<tr>
<td>D.</td>
<td>During headache at least one of the following:</td>
</tr>
<tr>
<td></td>
<td>1. nausea and/or vomiting</td>
</tr>
<tr>
<td></td>
<td>2. photophobia and phonophobia</td>
</tr>
<tr>
<td>E.</td>
<td>Not attributed to another disorder</td>
</tr>
</tbody>
</table>
1.4.2 Migraine with Aura

Migraine with aura (MA) (also referred to as classic migraine) describes a recurring headache which is associated usually preceded with other neurological (or aura) symptoms. Neurological symptoms manifest as fully reversible, visual, sensory and/or dysphasic speech disturbances (see Table 1.2). Visual symptoms may include positive symptoms such as flickering lights, spots and lines or negative symptoms such as loss or blurring of central vision. Sensory symptoms may include pins and needles (positive) or numbness (negative).

These symptoms almost certainly originate at the cerebral cortex or brain stem and tend to gradually develop over five-20 minutes and usually last less than 60 minutes. Following the neurological aura symptoms, there is a period where no pain and/or discomfort may be experienced. However, most patients develop subsequent headache, nausea or photophobia which is characteristic of the MO symptoms. The headache lasts between four and 72 hours as with MO. A minimum of two such attacks must be experienced to confirm diagnosis.

Unlike MO, MA is further clinically sub-divided. The six categories of MA are as follows:

1.2.1 Typical aura with migraine headache
1.2.2 Typical aura with non-migraine headache
1.2.3 Typical aura without headache
1.2.4 Familial hemiplegic migraine (FHM)
1.2.5 Sporadic hemiplegic migraine
1.2.6 Basilar type migraine

For the purposes of this study, the term MA will refer to any of the typical aura categories (1.2.1., 1.2.2., and 1.2.3.).
Table 1.2  ICHD-II diagnostic criteria for migraine with aura (1.2).

<table>
<thead>
<tr>
<th>Code</th>
<th>Classification/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.</td>
<td>Diagnostic criteria for migraine with aura</td>
</tr>
<tr>
<td>A.</td>
<td>At least 2 attacks fulfilling criterion B</td>
</tr>
<tr>
<td>B.</td>
<td>Migraine aura fulfilling criteria B and C for one of the sub forms 1.2.1.-1.2.6.</td>
</tr>
<tr>
<td>C.</td>
<td>Not attributed to another disorder</td>
</tr>
<tr>
<td>1.2.1.</td>
<td>Typical aura with migraine headache</td>
</tr>
<tr>
<td>A.</td>
<td>At least 2 attacks fulfilling criteria B–D</td>
</tr>
<tr>
<td>B.</td>
<td>Aura consisting of at least one of the following, but no motor weakness:</td>
</tr>
<tr>
<td></td>
<td>1. fully reversible visual symptoms including positive features (e.g., flickering lights, spots or lines) and/or negative features (i.e., loss of vision)</td>
</tr>
<tr>
<td></td>
<td>2. fully reversible sensory symptoms including positive features (i.e., pins and needles) and/or negative features (i.e., numbness)</td>
</tr>
<tr>
<td></td>
<td>3. fully reversible dysphasic speech disturbance</td>
</tr>
<tr>
<td>C.</td>
<td>At least two of the following:</td>
</tr>
<tr>
<td></td>
<td>1. homonymous visual symptoms and/or unilateral sensory symptoms</td>
</tr>
<tr>
<td></td>
<td>2. at least 1 aura symptom develops gradually over &gt;5 minutes and/or different aura symptoms occur in succession over &gt;5 minutes</td>
</tr>
<tr>
<td></td>
<td>3. each symptom lasts &gt;5 and &lt;60 minutes</td>
</tr>
<tr>
<td>D.</td>
<td>Headache fulfilling criteria B–D for 1.1. Migraine without aura begins during the aura or follows aura within 60 minutes</td>
</tr>
<tr>
<td>E.</td>
<td>Not attributed to another disorder</td>
</tr>
<tr>
<td>1.2.2.</td>
<td>Typical aura with non-migraine headache</td>
</tr>
<tr>
<td>A.</td>
<td>At least 2 attacks fulfilling criteria B–D</td>
</tr>
<tr>
<td>B.</td>
<td>As per 1.2.1. B.</td>
</tr>
<tr>
<td>C.</td>
<td>As per 1.2.1. C.</td>
</tr>
<tr>
<td>D.</td>
<td>Headache that does not fulfil criteria B–D for 1.1. Migraine without aura begins during the aura or follows aura within 60 minutes</td>
</tr>
<tr>
<td>E.</td>
<td>Not attributed to another disorder</td>
</tr>
<tr>
<td>1.2.3.</td>
<td>Typical aura without headache</td>
</tr>
<tr>
<td>A.</td>
<td>At least 2 attacks fulfilling criteria B–D</td>
</tr>
<tr>
<td>B.</td>
<td>Aura consisting of at least one of the following, with or without speech disturbance but no motor weakness:</td>
</tr>
<tr>
<td></td>
<td>1. fully reversible visual symptoms including positive features (e.g., flickering lights, spots or lines) and/or negative features (i.e., loss of vision)</td>
</tr>
<tr>
<td></td>
<td>2. fully reversible sensory symptoms including positive features (i.e., pins and needles) and/or negative features (i.e., numbness)</td>
</tr>
<tr>
<td>C.</td>
<td>At least two of the following:</td>
</tr>
<tr>
<td></td>
<td>1. homonymous visual symptoms and/or unilateral sensory symptoms</td>
</tr>
<tr>
<td></td>
<td>2. at least 1 aura symptom develops gradually over &gt;5 minutes and/or different aura symptoms occur in succession over &gt;5 minutes</td>
</tr>
<tr>
<td></td>
<td>3. each symptom lasts &gt;5 and &lt;60 minutes</td>
</tr>
<tr>
<td>D.</td>
<td>Headache that does not occur during aura nor follow aura within 60 minutes</td>
</tr>
<tr>
<td>E.</td>
<td>Not attributed to another disorder</td>
</tr>
</tbody>
</table>
1.4.3 A Severe Subtype of MA: Familial Hemiplegic Migraine

FHM is a rare, severe sub-form of MA, following strict autosomal dominant inheritance. It has a heavier genetic component and patients experience typical symptoms of aura plus motor weakness (hemiparesis) (see Table 1.3). The duration of the aura is prolonged, lasting up to 24 hours.

FHM classification requires that similar attacks are observed in at least one first- or second-degree relative. Patients who satisfy the FHM criteria but have an absence of affected first- and second-degree relatives are classed as sporadic hemiplegic migraine (SHM) (1.2.5.).

Mutations in at least three different genes have been recognized and connected to the FHM phenotype. FHM type 1 (FHM1; MIM141500) is linked to mutations in the \textit{CACNA1A} gene, 19p13, FHM type 2 (FHM2; MIM609634) linked to mutations in the \textit{ATP1A2} gene, 1q21-23, and FHM type 3 (FHM3; MIM602481) linked to mutations in the \textit{SCN1A} gene, 2q24.

Table 1.3 ICHD-II diagnostic criteria for familial hemiplegic migraine (1.2.4) (ICHBD-II 2004).

<table>
<thead>
<tr>
<th>Code</th>
<th>Classification/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>At least 2 attacks fulfilling criteria B and C</td>
</tr>
<tr>
<td>B.</td>
<td>Aura consisting of fully reversible motor weakness and at least one of the following:</td>
</tr>
<tr>
<td></td>
<td>1. fully reversible visual symptoms including positive features (e.g., flickering lights, spots or lines) and/or negative features (i.e., loss of vision)</td>
</tr>
<tr>
<td></td>
<td>2. fully reversible sensory symptoms including positive features (i.e., pins and needles) and/or negative features (i.e., numbness)</td>
</tr>
<tr>
<td></td>
<td>3. fully reversible dysphasic speech disturbance</td>
</tr>
<tr>
<td>C.</td>
<td>At least two of the following:</td>
</tr>
<tr>
<td></td>
<td>1. at least one aura symptom develops gradually over $&gt;5$ minutes and/or different aura symptoms occur in succession over $&gt;5$ minutes</td>
</tr>
<tr>
<td></td>
<td>2. each aura symptom lasts $&gt;5$ minutes and $&lt;24$ hours</td>
</tr>
<tr>
<td></td>
<td>3. headache fulfilling criteria B-D for 1.1. Migraine without aura begins during the aura or follows onset of aura within 60 minutes</td>
</tr>
<tr>
<td>D.</td>
<td>At least one first- or second-degree relative has had attacks fulfilling these criteria A-E</td>
</tr>
<tr>
<td>E.</td>
<td>Not attributed to another disorder</td>
</tr>
</tbody>
</table>
1.4.4 The Stages of a Typical Migraine Attack

A migraine episode occurs over a period of 4-72 hours and unfolds in distinct stages: the prodrome (premonitory phase), aura (for MA attacks only), the headache phase (may be absent in MA), and the postdrome (resolution). Each stage may not be experienced by all migraineurs due to the wide spectrum of phenotypes in each patient.

The prodrome phase consists of warning symptoms that precede the headache phase of the migraine attack by several hours or days, and that act as a warning signal to the migraineur for an oncoming episode (Charles 2013). Warnings symptoms during this phase can vary but typically consist of altered mood, irritability, depression or euphoria, fatigue, yawning, excessive sleepiness, craving for certain food (e.g. chocolate), stiff muscles (especially in the neck), dizziness, hot ears, constipation or diarrhea, increased or decreased urination, and other visceral symptoms (Charles 2013).

The second phase progresses to the Aura phase which overlaps with the headache phase and is experienced by approximately 30% of sufferers and that usually lasts 20 to 60 mins (Linde, Mellberg et al. 2006). Auras consist of temporary reversible visual, sensory and/or speech disturbances either before or during the attack. It is interesting to note that migraine aura symptoms are thought to have influenced some famous pieces of art and literary works.

The third phase is the headache and is generally considered to be the most debilitating and is the most easily identifiable and quantifiable feature of the headache. Its effects are not limited to the head only, but affect the entire body. During this stage migraineurs experience a moderate to severe intensity attack that lasts 4-72 hours (IHS 2013). The pain is often of a throbbing nature and is unilateral and aggravated by physical activity. Other characteristics include nausea, vomiting as well as possible photophobia and phonophobia, diarrhea and constipation. The duration of migraine headache may be related to triggering factors and age (Linde, Mellberg et al. 2006)

The migraine episode finally ends with the postdrome phase which can be thought of as a time of recovery. This is because the sufferer may feel weak or tired from the evolution of a migrainous episode and is therefore described as feeling “like a zombie” or “hungover”. A comprehensive understanding of each of the stages of a migraine attack is necessary to better
understand the pathophysiology of migraine and identify acute and preventive therapies. Ongoing quantitative clinical observations, imaging studies, electrophysiological studies, and therapeutic clinical trials continue to provide important new information regarding how a migraine starts and progresses.

1.5 Epidemiology of Migraine

More than 2000 years ago Hippocrates and others noted that the environment can influence the occurrence of disease. The idea of epidemiology was born and became an essential starting point in assessing the burden of disease in the population. Epidemiology is the study of incidence, prevalence and the distribution of disease in large populations as well as the circumstances influencing the characteristics of disease (Beaglehole, Bonita et al. 1993). The purpose of epidemiological studies is to identify risk factors, co-morbid conditions and monitor trends in disease prevalence over time so that better health care planning and financing can be applied by local governments (Stovner, Zwart et al. 2006). Prevalence is an important measure of the occurrence of disease in a population. This section focuses on current knowledge of the epidemiology of migraine.

Prevalence is defined as the number of affected individuals (in percentage units) within a defined population (at risk of the disease) measured at a specified point (or period) in time (Beaglehole, Bonita et al. 1993). Period prevalence estimates are considered more reliable than life time prevalence estimates because migraine is only diagnosed if attacks have occurred recurrently over time and also because people are only affected in certain phases of their life (Stovner and Hagen 2006). Lifetime period prevalence studies have been reported repeatedly in the literature and are considered less reliable because of the reliance on individuals’ ability to recall information from years back about their disease. For this reason researchers are now commonly reporting the 1-year period prevalence involving patient records from the year prior to the study (Stovner and Hagen 2006) as this figure indicates the proportion of the population having an active disease and is thus more useful in health economic calculations.

The most common age of onset of migraine is 25-45 with prevalence increasing with age until a peak is reached in the fourth decade of life and thereafter declines (Stovner and Hagen 2006). Prevalence varies substantially in different countries owing to differences in genetic
background, climatic, socioeconomic status, life-style, other disease spectrum and general health affecting the sourced population (Stovner, Hagen et al. 2007). In earlier studies some of this variance was attributable to differing migraine classification methods, however since the introduction of the ICHD-II 2004 most epidemiological studies have adhered to these criteria. Additional factors influencing the calculation of headache prevalence in each study include: sample size, the method of data collection, migraine case definition, how the diagnostic criteria is applied and most importantly how the screening questions are asked (Jensen and Stovner 2008). Together all these factors contribute to differences in prevalence estimates reported in each study and in different countries.

Migraine is estimated to affect approximately 12% of the Caucasian population (Stovner and Andree 2010). Ancestry specific differences are evident with people of Caucasian ancestry affected the most compared to those of African-American ancestry (Stovner et al. 2006b; Lipton et al. 2007b). On top of this statistic, an interesting and uneven gender distribution has also been noted, with 18% of females affected and only 6% of men equating to a ratio of 3:1 respectively (Stovner et al. 2006b). Migraine is highly prevalent in many European nations and parallels estimates in the United States. A study by (Stovner et al. 2006b) reports the 1-year prevalence for migraine from 8 epidemiological studies of European adults had a mean value of 13.7% with gender-specific prevalence of 16.6% in women and 7.5% in men. Migraine was the most prevalent neurological disorder, affecting some 41 million individuals.

The American Migraine study AMS-I and AMS-II are methodologically identical national surveys conducted in 1989 and 1999 which reported a US migraine prevalence of >17% in women and >5% in men (Lipton, Stewart et al. 2001). These findings were subsequently confirmed in the 2004 American Migraine Prevalence (AMPP) study (Lipton, Bigal et al. 2007). Approximately 28 million Americans are affected by migraine (Freitag 2007). Despite this figure prevalence of migraine has remained constant over the years and may increase slightly in correspondence with an increase in the population.

Migraine is predominantly investigated in high-income countries consequently there is a lack of data available for low and middle income countries (Mateen, Dua et al. 2008). However new prevalence studies from several Latin American countries, Tanzania, and Turkey
indicate that headache is a big problem also in the less affluent countries where modern pharmacologic treatment is, and will probably for years be, less accessible (Dent, Stelzhammer et al. 2011).

1.6 Social and Economic Burden of Migraine

Change has taken place over the past 15 years, revised headache classification guidelines in conjunction with genetic studies have outlined the public-health importance of headache disorders (Leonardi, Steiner et al. 2005). Health is defined not only by the absence of disease, but by the presence of physical, mental, and social well-being of people (Hazard, Munakata et al. 2009). Migraine headache significantly affects people’s health and quality of life. The socioeconomic impact of migraine is wide-ranging and long-lasting imposing a range of costs to individuals, families and communities as a whole.

Part of this economic burden is obvious and measurable, whilst part is almost impossible to quantify (Leonardi, Steiner et al. 2005). The measurable components are usually categorized as direct and indirect costs. Direct costs are related to the use of medical resources, including physician visits for diagnosis and/or treatment, emergency room (ER) visits, diagnostic tests, and mostly prescription of medication. Indirect costs are generally assessed in terms of absent days from work, reduced efficiency whilst working with headache and associated costs to employers from lost productivity (Hazard, Munakata et al. 2009). Indirect costs due to work-related disability are considered the most important determinant of the economic impact of migraine (Friedman, Feldon et al. 2009). Overall, increased awareness of migraine repercussions in the individual and on the family unit is needed to allocate resources to treatment and research in this field.

The burden of migraine on the family unit including partners and children is particularly important and is generally overlooked. The WHO defines “burden of disease to include the economic and emotional difficulties that a family experiences as a result of them, as well as the lost opportunities - the adjustments and compromises that prevent other family members from achieving their full potential in work, education and in social relationships” (Leonardi, Steiner et al. 2005). Migraine imparts substantial suffering to the family particularly as it affects more women than men and is most prevalent between the ages of 25 and 45, the years of child rearing. Migraine disturbs all aspects of social and family relationships due to angst.
about recurring future attacks which create a cycle of migraine pain and worry (Freitag 2007). In studies of migraine sufferers with a household partner, 50% to 73% of patients reported that having a headache increased family arguments (Freitag 2007). Among migraine sufferers with household partners, 32% avoided making plans for family or social activities to avoid later cancellation or interruption because of headache (Freitag 2007). The children of migraine sufferers were also affected, with 10% missing school or being late for school in the previous year because of a parent’s headache (Stovner and Andree 2008). A minority of headache sufferers choose to have fewer children than they would have had if they had not had headache.

Migraine families experience higher direct health-care costs than non-migraine families. It was found that the total medical costs of families with at least one migraineur were 70% greater than matched non-migraineur families (Stang, Crown et al. 2004). First-degree relatives of a sufferer have 25% higher total healthcare costs compared to matched non-migraine controls (Stang, Crown et al. 2004). Estimates of the economic impact of migraine often include the costs of conditions co-morbid with migraine. Co-morbid conditions increase the total direct medical costs incurred by migraineurs as a result of the patient having to fill multiple prescriptions.

In addition to the social burden on the family unit and its individual burden to the patient, migraine is costly to society. Most of the costs related to headache are indirect costs and the result of absence from work and reduced efficiency whilst working with headache (Stovner and Hagen 2006). The US migraine cost study calculate that migraine was the cause of 112 million bedridden days per year, which corresponds to 300,000 people staying in bed every 24 hours because of headache (Jensen and Stovner 2008). This study confirms the burden of migraine in the population and shows that more than 50% of all migraineurs report severe impairment or require bed rest during their headache.

The economic impact of this disorder has been evaluated in Europe and the United States. A large European cost study collected the best available epidemiological and health economical evidence for the year 2004 from a number of European countries. They report the total cost of migraine for the whole of Europe in 2004 was €27 billion, which was the highest cost among the purely neurological disorders, €1.5 billion in healthcare and €25.5 billion in
indirect cost through absenteeism and lost productivity at work (Andlin-Sobocki et al. 2005). The direct cost of a migraine patient per year was estimated at €590 (Stovner and Hagen 2006; Stovner and Andree 2008).

The estimated national direct cost burden of migraine in the US was $11 billion (Hawkins, Wang et al. 2008) consisting of:

- $4.6 billion was in prescription drugs,
- $5.2 billion in outpatient costs,
- $0.7 billion in inpatient costs,
- $0.5 billion in ER,

The direct costs measured were associated with health care utilization. Indirect costs consisted of absenteeism in the workplace and equated to $12 billion (Hawkins, Wang et al. 2007). These estimates of indirect costs were based on missed work days and impaired work performance and did not capture unemployment or underemployment because of migraine, burden experienced between attacks, lost home-worker time for chores, or lost time because of caring for family members with migraine.

Headache is among the most prevalent, burdensome, and costly neurological disorder reported globally (Jensen and Stovner 2008). The World Health Organisation has classified migraine as the 19th leading cause of disability (Leonardi et al. 2005). The social and economic burden of migraine has been evaluated in a number of different countries to assess patients’ quality of life and to determine the global medical costs to society. Those that carry that burden include patients and their families, employers, and society. The economic studies have quantified the impact of this disorder on society in terms of direct and indirect costs and clearly demonstrate that the burden to society is substantial with the greatest proportion of that burden resulting from indirect costs as a result of absence from the workplace.

1.7 Migraine Triggers

Many migraine sufferers have reported numerous and varied triggers for their migraine attacks which seem to result from the interaction of intrinsic and environmental factors in a biologically predisposed brain (Friedman and De ver Dye 2009). A migraine trigger is any
factor that on exposure or withdrawal leads to the development of a migraine attack (Hansen, Hauge et al. 2011). Many things have been labelled as triggers; however the strength and significance of these is uncertain. Common reported triggers include intrinsic factors such as hormonal cycles or Cortical Spreading Depression and external factors include environmental changes, hunger, fatigue, stress, lack of sleep, bright light, strong odours, and certain foods (Marin 1998; Martin 2009) (Table 1.4).

The complex nature of migraine arises from the interplay of genetic and environmental factors and this has led to the proposal that migraine onset may involve a threshold determined by genetic predisposition. Exposure to some previously mentioned triggers or events or circumstances that can precipitate headache may breach this threshold in the predisposed individual and trigger a migraine attack. Consequently identifying the specific factors that predispose an individual to the onset of an episode is an attractive preventive measure in clinical practice.

Approximately a quarter of patients with migraine recognize specific food as migraine triggers. Such triggers include monosodium glutamate (also known as hydrolyzed yeast extract, natural flavouring, hydrolyzed vegetable protein), often found in soups and Chinese food (Zanda, Francios.P et al. 1973; Geha, Beiser et al. 2000). Nitrites (a preservative found in lunch meats and hot dogs), tyramines (found in chocolate, garlic, nuts, raw onions, and seeds) are other potential migraine triggers. Alcohol of any kind, artificial sweeteners, citrus fruits, pickled products, and vinegars are additional likely triggers (Littlewood, Gibb et al. 1988; Martin and MacLeod 2009). Among the non-genetic factors, hormones and gender specific traits, diet and response to stress are likely to be significant.

Menses is a trigger for 60% of female migraineurs and for tension-type headache (Russell 2010). The exact mechanism through which hormones trigger a migraine episode is unclear however migraines are known to occur around menstruation. Additional, hormonal influences, including: oral contraceptive use, pregnancy, perimenopause, and menopause, also play a role (Shuster, Faubion et al. 2011). The IHS have acknowledged the relative importance of hormones in migraine by the inclusion of “pure menstrual” and “menstrually related” migraine in the IHS revised classification criteria of headache disorders (IHS 2013).
Currently many triggers have been reported by migraineurs but no common specific factor precipitating migraine onset has been identified. It appears that a combination of key factors can exacerbate an individual’s migraine experience and hence why it is important to identify them so they can be minimized and their influence avoided.

Table 1.4 Common Migraine Triggers adapted from (Marin 1998).

<table>
<thead>
<tr>
<th>Foods</th>
<th>Lifestyle</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aged Cheese</td>
<td>Fasting or skipping meals</td>
<td>Vasodilators (nitroglycerine)</td>
</tr>
<tr>
<td>Alcohol (red wine, champagne)</td>
<td>Changes in sleep pattern (jet lag)</td>
<td>Hormones (oral contraceptives)</td>
</tr>
<tr>
<td>Monosodium Glutamate (frozen dinners)</td>
<td>Unproductive worry</td>
<td>Antihypertensives (nifedipine, captopril)</td>
</tr>
<tr>
<td>Chocolate</td>
<td>Lack of exercise</td>
<td>Antibiotics (trimethoprim-sulavamethoxazole, griseofulvin)</td>
</tr>
<tr>
<td>Caffeinated Beverages</td>
<td>Letdown following stress</td>
<td>Nonsteroidal anti-inflammatory drugs (indomethacin, diclofenac, piroxicam)</td>
</tr>
<tr>
<td>Nitrites and Nitrates</td>
<td>Caffeine withdrawal</td>
<td>Histamine-2 blockers (cimetidine, ranitidine)</td>
</tr>
<tr>
<td>Nuts</td>
<td>Hormonal Cycles</td>
<td></td>
</tr>
<tr>
<td>Smoked or pickled fish or meats</td>
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<td></td>
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<tr>
<td>Aspartame (dietary sweeteners)</td>
<td></td>
<td></td>
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<tr>
<td>Yeast or protein extracts (brewer’s yeast, protein extract)</td>
<td></td>
<td></td>
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<tr>
<td>Avocado</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onions</td>
<td></td>
<td></td>
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<tr>
<td>Odours</td>
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1.8 Migraine Co-morbidity

For the purpose of studying and researching one discrete medical disorder at a given point in time it is important to distinguish between conditions that present in the same individual with identical symptoms so as to ensure accurate diagnosis and avoid false reporting. The term co-morbidity has been introduced to address this issue. Co-morbidity is defined as the occurrence of two medical disorders in the same individual at the same time at a frequency greater than pure chance (Lipton, Bigal et al. 2007). Epidemiological studies have clearly demonstrated that migraine is associated with a number of medical conditions as listed below (Lal and Singla 2010).

1. Psychiatric disorders
   a. Depression
   b. Anxiety
   c. Panic disorder
   d. Bipolar disorders

2. Neurologic disorders
   a. Epilepsy
   b. Stroke
   c. Multiple Sclerosis

3. Cardiovascular disorders
   a. Hypertension
   b. Heart disease
   c. Patent foramen ovale
   d. Stroke

4. Miscellaneous disorders
   a. Asthma/allergy
   b. Gastro-intestinal disorders
   c. Celiac disease

Co-morbidity of migraine has important implications when there are overlapping symptoms between the two co-morbid disorders. This scenario can result in uncertainty in the diagnosis and complicate treatment of the disease in question. Despite the co-occurrence of migraine with other disorders, ICHD-II criteria specify that both MA and MO must not be attributable to another disorder.
Specifically, physical and neurological examination must rule out headache attributed to:

1. Head and neck trauma
2. Cranial or cervical vascular disorder
3. Non-vascular intracranial disorder
4. A substance or its withdrawal
5. Infection
6. Disturbance of homeostasis
7. Psychiatric disorder or
8. Headache or facial pain attributed to disorders of the cranium, neck, eyes, ears, nose, sinuses, teeth, mouth or other facial or cranial structures.

Migraine attributed to any of the above disorders is classified as a secondary headache and is therefore not migraine. Identification of co-morbidity can be due to one of four possibilities: pure coincidence, a condition causes migraine, or migraine causes the other condition, migraine and another condition may co-occur due to shared genetic factors which increase the risk of the two conditions co-occurring (for example, the MTHFR C677T variant associated both with migraine and with ischemic stroke) (Silberstein, Dodick et al. 2007) and lastly independent genetic and or environmental factors predispose to both migraine and another disorder.

Understanding co-morbidities of complex diseases is an important step towards further refinement of diagnostic criteria to distinguish disorders manifesting with similar symptoms and to identify triggers and environmental risk factors common to both disease types. Recognition and classification of co-morbidities as separate entities is critical to minimizing adverse pharmacological events and reducing the burden of illness on health systems, families and work forces (Silberstein, Dodick et al. 2007). Furthermore the characterization of co-morbidities can assist to identify medications that are contraindicated in the co-morbid condition (for example triptans and ergotamines are contraindicated in cardiovascular diseases) so as to reduce potential drug interactions from treating the two disorders simultaneously. The study of co-morbidities can provide useful knowledge about disease pathophysiology and clues to uncovering causal pathways shared between migraine and its co-morbid disorders.
1.9 Migraine Pathophysiology

Migraine pathophysiology has metamorphosed migraine from a disease originally attributed to supernatural causes to a well characterised neurological disorder (Villalon, Centurion et al. 2003). Detailed documentation of patients’ symptoms and observation of patient response to the effects of various drugs have contributed to the growing knowledge of migraine pathophysiology. Migraine symptoms arise from a combination of vascular and neurological events occurring in the cranial meninges and as a result this disorder is often described as being of ‘neurovascular’ origin because of the two interacting systems (Tajti, Pardutz et al. 2011). Some key events implicated are the phenomenon of CSD and activation of the trigeminovascular system with neurogenic inflammation, leading to changes in the meningeal vasculature (Dalkara, Zervas et al. 2006). The exact sequence for these pathological events and how they interact is not clear but what is known is that genetic mutations affect an individuals’ likelihood of developing migraine and that a number of brain structures, including the trigeminal innervations of the cranial vessels, are involved (Goadsby, Charbit et al. 2009; Levy 2010). The following discussion reviews the current understanding of migraine pathophysiology and studies on the genetic basis of the disorder.

1.9.1 The Vascular and Neurogenic Theories

The exact cause of migraine headache pain is still not completely understood. Historically, two independent theories, the vascular theory and the neuronal theory, explaining the aetiology of migraine headache were proposed. The vascular theory was introduced by Thomas Willis who combined keen clinical observation and meticulous anatomic dissection to redefine thinking about "megrim" (Rapoport and Edmeads 2000). In his vascular theory he recognised that “all pain is an action violated” and argued the pain from headache is caused by vasodilation of the cerebral and meningeal arteries (Rapoport and Edmeads 2000).

In the 20\textsuperscript{th} century the work of Graham and Wolff advanced the vascular theory. Graham and Wolff were the first people to study headache in the laboratory from the (1930s-1950s) and fostered the idea that migraine is a vascular event mediated by initial intracranial vasoconstriction that is followed by rebound vasodilation (Eadie 2005). Consistent with this theory is the throbbing quality of pain and the relief patients experienced with administration of Ergotamine, a potent vasoconstrictor (Baron and Tepper 2010). Also the observed headache inducing effects of nitroglycerin, a vasodilator was taken as added proof of
migraine's vascular nature (Spierings 2009). Insights into the mechanism of action of the triptans (serotonin receptor agonists) which revolutionised the treatment of migraine, and more recently calcitonin gene-related peptide (CGRP) agonists (gepants), suggest that they may primarily relieve symptoms through cranial vasoconstriction (Humphrey 2008).

More recently in a study by Amin et al., 2013 using a high-resolution magnetic resonance angiography imaging technique during spontaneous unilateral migraine attacks has shown that although vasodilation coupled with the release of vasoactive substances is a key physiological occurrence in migraine pathophysiology, dilatation is not the cause of peripheral and central pain pathways (Amin, Asghar et al. 2013). The release of neuropeptides and proinflammatory substances from large dural vessels which accompanies vascular changes initiate the sensation of pain by sensitizing peripheral and central neurons within the trigeminovascular system (Edvinsson, Villalon et al. 2012). These vascular events cannot be ignored and are important in further understanding the full gamut of migraine processes so as to clarify how a migraine episode is initiated and sustained.

The alternative neurogenic theory centres on migraine being a disorder of the brain where vascular events are best explained by dysfunction of neuronal networks (Edvinsson, Villalon et al. 2012). Migraine is a consequence of neural events which have a strong genetic linkage however the translation of these neural events into migraine pain remains to be explained. Support for the neurogenic theory comes from the fact that certain neurological symptoms that occur during auras cannot be explained by a purely vascular model of headache (Parsons and Strijbos 2003). Also the fact that the diameter of cranial blood vessels along with other functions, are controlled by signals transmitted by nerves is further evidence to support a neurogenic theory (see Figure 1.2). This theory focuses on the cause of migraine pain and is currently linked to activation of the trigeminovascular system (Edvinsson, Villalon et al. 2012). The prime component of this system is the trigeminal nerve and its nerve fibers which innervate meningeal blood vessels and other brainstem structures (Goadsby, Charbit et al. 2009). This theory also implicates the phenomenon of cortical spreading depression (CSD) as the cause of neurological symptoms of aura (Moskowitz 2008b). Clearly neither theory can account for the entire cascade of events observed and both theories have been integrated in a neurovascular model. It is important to recognise that as new scientific evidence accumulates the knowledge is remodified and corrected.
Figure 1.1  Pathophysiology of migraine sourced from (Goadsby, Lipton et al. 2002)
1.9.2 Cortical Spreading Depression

Cortical spreading depression is an electrophysiological event that was first described in 1943 by Aristides Leão, a Brazilian neurophysiologist doing his doctoral research on epilepsy at Harvard University (Teive, Kowacs et al. 2005). Functional neuroimaging studies in human brain have clearly demonstrated changes in blood flow and brain activity in migraineurs that is indicative of CSD (Hadjikhani, Sanchez Del Rio et al. 2001). CSD is best described as an intense wave that propagates across the cerebral cortex at a rate of 2-5mm/min lasting 15-30m.ins and that causes disruption of ionic gradients (Ca\(^{2+}\), Na\(^{+}\), K\(^{+}\)) followed by a period of suppressed neural activity (Moskowitz 2007; Eikermann-Haerter and Moskowitz 2008). Similar events have been reported in stroke and brain injury (Moskowitz 2008b). CSD has been shown to cause activation and sensitization of the trigeminovascular system, initiating a series of neural, vascular and inflammatory events that result in pain (Bolay, Reuter et al. 2002). CSD is widely accepted to be the cause of visual auras based on experimental evidence from patients and animal models however at present there is no agreement that it is the cause of pain. Although CSD does occur in migraine with aura patients it does not explain the cause of the majority of headaches which occur in 70% of migraineurs. A recent study has also shown that CSD causes Pannexin1 megachannel opening in neurons which results in caspase-1 activation and HMGB1 release which initiates parenchymal inflammatory pathways that may also provide the stimulus for sustained trigeminal activation and lasting pain (Karatas, Erdener et al. 2013).

1.9.3 Imaging Studies

The use of imaging techniques has ushered in new opportunities to study functional changes in the brain during different states. The aim of imaging techniques is to study the metabolism and hemodynamics during and between acute attacks of migraine. Migraine is a disorder of the brain and given the involvement of CSD and the waves of aura imaging techniques has tried to characterise the physiological dysfunction. Not just this but also to answer the question of whether migraine is a neural or vascular (cerebral vasoconstriction causing aura through cerebral ischaemia, subsequent vasodilation producing headache) disorder.

Functional imaging methods, such as PET and functional MRI, are the most frequently used techniques in the study of primary headache disorders and have enabled scientists to study in real time the location and progression of physiologic changes that occur in the brain during
migraine. Functional MRI (fMRI) is an imaging technique that produces very clear images of the structure and biochemistry of the brain without the use of X-rays. Studies using MRI show that changes in blood flow and brain activity correlate with migraine aura and are due to cortical spreading depression (CSD), a wave of transient intense spike activity that progresses slowly along the cortex and is followed by a long-lasting neuronal suppression (Hadjikhani, Sanchez Del Rio et al. 2001; Borsook and Hargreaves 2010; Sprenger and Borsook 2012).

Activation of the brainstem has been identified in PET studies as the likely origin of spontaneous migraine attacks (Weiller, May et al. 1995). Imaging studies have suggested that activity of the spinal trigeminal nuclei and rostral pons may be primary generators or modulators of migraine episodes (Stankewitz, Aderjan et al. 2011). Additional evidence of CSD has been shown to activate the trigeminovascular system in has come from animal models as well (Bolay, Reuter et al. 2002). Studies have been conducted during the ictal (ictal - relating to or cause by stroke or seizure) and interictal (interictal - of or relating to an interval between convulsions and seizures) stages of migraine. Such studies have not only demonstrated abnormalities during the ictal state but perhaps more importantly during the interictal state in particular that complex processes within the nervous system. There is more to migraine than mere changes in blood flow instead neurological events are believed to be more responsible for the vascular processes. The findings are supported by Amin and colleagues who substantiate the idea that vasodilation also does not have a primary role in spontaneous migraine headache (Amin, Asghar et al. 2013).

To date brain imaging studies have helped better understand the anatomic and biochemical nature of migraine and have indicated abnormalities of function in certain areas (eg. Altered descending modulatory processing in the midbrain, the temporal pole, as well as in and other regions including changes occurring during spreading depression) (Hadjikhani, Sanchez Del Rio et al. 2001; Tedeschi, Russo et al. 2013a; Tedeschi, Russo et al. 2013b). Imaging has clearly demonstrated that there are differences between the migraine brain and the healthy control and that CSD is a real phenomenon of migraine aura. Specific areas of the brain are overactive or show an increase in metabolic functions. The interpretation of the biological significance of these various changes however remains incomplete.
Imaging studies have contributed to the conclusion that migraine is a neurovascular disorder where any changes in vascular function are not the primary cause of pain. Neuroimaging techniques can help us understand the physiological dysfunction that characterizes migraine and primary headache syndromes and inform clinical practice. The consensus has shifted from a purely vascular model to a more neurovascular theory.

1.9.4 The Trigeminovascular System

Although numerous theories and models emerge frequently, the trigeminovascular system (TGVS) has been consistently implicated and well-studied anatomically and physiologically. The trigeminovascular system (TGVS) consists of the trigeminal nerve and nerve fibers which innervate the network of extra- and intra- cranial meningeal blood vessels and the brain stem (May and Goadsby 1999). This system is thought to play an integral role in regulating vascular tone and in the transmission of pain signals.

Activation of this system during the pain phase of migraine is thought to initiate a cascade of chemical activity from trigeminal sensory nerve endings (Arulmani, Maassenvandenbrink et al. 2004; Goadsby, Charbit et al. 2009). Precisely the peripheral terminations of the TGVS are located in correspondence of the extracranial soft tissues, such as muscles, eye, ear, skin, subcutaneous tissue, nasal cavities, arteries, periosteum, and also of intracranial structures, or venous sinuses, vagus and glossopharyngeal nerves (Edvinsson, Villalon et al. 2012).

Component trigeminal sensory nerves of this system store several vasoactive neuropeptides including: substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A, nitric oxide (NO) and pituitary adenylate cyclase-activating peptide (PACAP) that upon being released lead to inflammation and dilation of blood vessels aggravating the pain (Samsam, Covenas et al. 2010). CGRP is the most potent vasodilating neuropeptide and is richly localized in perivascular nerves in large cerebral arteries as well as in meningeal arteries (Arulmani et al. 2004; Goadsby et al. 2009). Increased plasma levels of CGRP have been noted during spontaneous migraine attacks and also when induced by nitroglycerin (Goadsby, Edvinsson et al. 1990; Juhasz, Zsombok et al. 2003b). Neuropeptides are important molecules that cause vasodilation and increase blood flow leading to edema in the meningeal vasculature as well as an inflammatory response around vascular structures in the meninges which is believed to be responsible for head pain (Moskowitz 2008a).
1.10 Migraine Treatments

Migraine treatment has been described over many centuries with the earliest concepts based upon primitive methods and superstitions. Some of the treatments prescribed included: drilling a hole in the skull to free "evil spirits", purging and bloodletting, applying a hot iron to the site of pain, inserting a clove of garlic through an incision in the temple, incantation and by remedies such as placing a clay crocodile on one’s head to drive out the evil demons and spirits (Villalon, Centurion et al. 2003). Significant treatment milestones have occurred within the past 50 years – superstition and crude "medical" procedures have given way to advances in medical science. Treatment strategies for migraine fall into two broad categories: symptomatic (acute) and preventive (prophylactic). Symptomatic treatment aims to alleviate, abort, or reduce pain and accompanying symptoms. Preventive treatment is used to decrease the frequency, intensity, and duration of attacks and includes identifying potential triggers. Prescription of individual treatment is dependent on the level of pain intensity, disability, associated symptoms, presence of co-morbid illness and the patient’s previous history of response to headache medication. Although a variety of medications are available, they affect a broad range of pharmacological targets and therefore produce a widely variable inter-patient response.

1.10.1 Pharmacological Treatments

Drugs for the management of migraine, primarily affect neurovascular structures of the brain. They either; modify the release of transmitters involved in pain transmission, block sensitization of nerve fibers or affect inflammatory mechanisms involving the blood vessel wall (Silberstein 2009). The actions are numerous and varied. The following section reviews approaches to migraine treatment.

Ergots were the first documented anti-migraine drug and their first recorded use in treating migraine appeared in Italy in 1862 (Baron and Tepper 2010). Ergots are alkaloid products found in the purple spur or sclerotium produced by the growing fungus Claviceps purpurea. The potent action of Ergots was indirectly discovered when masses of people, in the Middle Ages, ate bread contaminated with the fungus. Additional references noting the effect of ergots date back to 400BC by the Parsees who described “grasses that cause pregnant women to drop the womb and die in childbirth,” and to 600BC where the Assyrians described the “noxious pustule in the ear of grain” (Baron and Tepper 2010). There is an extensive history
of ergotism plagues (from eating contaminated rye and wheat) in Europe and the rest of the world which led to their use in migraine therapy (Baron and Tepper 2010).

Ergots have a broad spectrum of receptor actions with low specificity (affinities for serotonin, dopamine and noradrenalin receptors) but generally constrict blood vessels and cause the muscle of the uterus to contract. This is problematic for people that are at an increased risk of stroke and cardiovascular disease and must avoid medications that constrict arteries because of the unwanted side effects produced in the heart and uterus (Tfelt-Hansen, Saxena et al. 2000). Better drugs with more specific actions have since evolved. However initially, given that the ergots were the only known compounds to be therapeutic against migraine made them widely used and studied, providing the substrate for later development of the triptans and contributing to formulating theories of migraine pathophysiology. The ergots were soon followed by non-steroidal anti-inflammatory agents (NSAIDs), which target inflammation (Pfaffenrath and Scherzer 1995). The anti-hypertensive calcium channel blockers and β-adrenergic-receptor agonists were the next generation anti-migraine treatments (Limmroth and Michel 2001).

The newer generation of drugs on the market for treatment of migraine headaches are the triptans (Humphrey 2008). The triptans are currently the most commonly used acute medication in migraine and are thought to alleviate migraine symptoms through their action on Serotonin (5-HT₁b, d and f) receptors (Humphrey 2008). They are well characterised with high receptor-specificity and efficacy for acute migraine attacks. Serotonin receptors are located in cranial blood vessels and nerve endings and upon chemical interaction with triptans leads to vasoconstriction which inhibits the release of peptides, including CGRP and substance P (see Figure 1.3) (Shields and Goadsby 2006; Sokolov, Lyubashina et al. 2011). The combination of these processes reduces inflammation and stops the headache. Serotonin-related genes have been investigated in numerous migraine association studies because of the known effects of triptans on serotonin receptors (Juhasz, Zsombok et al. 2003a; Kusumi, Araki et al. 2004; Racchi, Leone et al. 2004; Marziniak, Mossner et al. 2005).

Clinical trials of triptans in migraine treatment are continuing to assess their efficacy (Ferrari, Spaccapelo et al. 2010). Currently, there are 7 unique triptans from which to choose
(sumatriptan, zolmitriptan, rizatriptan, naratriptan, almotriptan, eletriptan and frovatriptan) while these are pharmacologically similar the efficacy and tolerability differs among patients (Johnson, Fernandez et al. 2007). The triptan with the longest history of use is sumatriptan. Triptans act by binding to serotonin 5-HT_{1b, d and f} receptors (Sokolov, Lyubashina et al. 2011). Agonism at 5HT_{1b} receptors constricts the pain producing intracranial, extracerebral blood vessels in the meninges. Agonism at 5HT_{1d} receptors presynaptically inhibits trigeminal peptide release and interferes with central trigeminal nucleus caudalis nociceptive transduction and processing (Shields and Goadsby 2006).

![Figure 1.2 Possible sites of action of triptans in the trigeminovascular system sourced from (Goadsby, Lipton et al. 2002).](image-url)
1.10.2 Non-pharmacological treatments

The best outcome for the patient is obtained by a careful and comprehensive consideration of the aims of the patient based on his or her attack frequency, duration and other symptoms. Non-pharmacological options include a combination of: (i) education and reassurance; (ii) preventing attacks by avoiding triggers; (iii) the use of non-pharmacological treatments such as relaxation, massage, biofeedback, and life style regulation, such as maintaining a regular schedule, diet changes, getting adequate sleep and exercise (iii) periodic reassessment and reconsideration of the treatment plan (Sandor and Afra 2005). Although there is some scientific data to support the use of non-pharmacological treatments they need more thorough evaluation. No single treatment is effective for everyone or even for a given person with every migrainous attack and therefore it is important to make patients aware of the existence of non-pharmacological treatments and that such techniques may work for some.

Nutrigenomics approaches to the treatment of migraine are composed of vitamins and supplements (eg., magnesium, riboflavin, coenzyme Q10, α-lipoic acid and folic acid) and herbal preparations (eg., Feverfew) and are gaining acceptance and moving the treatment of migraine away from the traditional pharmaceutical drugs which are prone to with side effects (Sun-Edelstein and Mauskop 2011). Nutritional supplements acting on mitochondrial metabolism, such as magnesium, coenzyme Q10, and riboflavin were shown to be effective in small, randomized, controlled trials and may be beneficial due to the related energy deficiency state migraineurs exhibit (Chiu, Tsai et al. 2013). Magnesium is particularly important for energy production and is necessary in numerous cellular functions and enzymatic reactions that affect the health of nerve, muscle and cell membranes. Although a few studies have shown positive effects of magnesium (Facchinetti, Sances et al. 1991; Taubert 1994; Peikert, Wilimzig et al. 1996; Pfaffenrath, Wessely et al. 1996; Koseoglu, Talashoglu et al. 2008), its use has not been convincingly demonstrated to be superior to placebo and therefore more controlled studies of magnesium need to be done in the future to determine its utility in treating migraine symptoms.

Coenzyme Q10 is a naturally occurring substance and essential element of the mitochondrial electron transport chain (Sandor and Afra 2005). Coenzyme Q10 has been the most extensively studied agent for the treatment of mitochondrial disorders and has been shown to have almost no identifiable side-effects in humans. Coenzyme Q10 is normally produced by
the human body, although deficiency may occur in patients with impaired CoQ10 biosynthesis due to severe metabolic or mitochondrial disorders. Daily consumption of CoQ10 was demonstrated in a few different trials to decrease frequency of migraine (Rozen, Oshinsky et al. 2002; Sandor, Di Clemente et al. 2005; Slater, Nelson et al. 2011). Riboflavin, or vitamin B₂, is a necessary co-factor in oxidation and reduction reactions in the mitochondria and is therefore vital to the generation of energy. Riboflavin has been shown to be effective in children with mitochondrial disorders with mild side effects due to the potential to improve mitochondrial function (O'Brien and Hershey 2010). Trials with this supplement in migraine have been conducted, in less than 100 participants, and have shown some interesting results (Schoenen, Jacquy et al. 1998; Yee 1999; MacLennan, Wade et al. 2008; Bruijn, Duivenvoorden et al. 2010). However, at present there is no consensus on nutraceutical treatment in migraine and larger RCTrials using larger doses or longer follow-up are needed to ascertain the potential of many of these nutraceutical products.

However the most encouraging nutraceutical treatment for migraine sufferers is supplementation with B vitamins. A pilot trial supplementing migraineurs with a B vitamin complex ameliorated the frequency and severity of migraine symptoms in clinical trial participants (Lea, Colson et al. 2009). Results of this trial have been promising and prompted a larger trial in which 206 migraineurs were recruited and stratified into MTHFR C667T and MTRR A66G genotypes. Similar positive findings were obtained and in addition the influence of specific genotypes on treatment efficacy was demonstrated with C allele carriers of MTHFR C667T and A allele carriers of MTRR A66G genotypes responding the best (Menon, Lea et al. 2012). More recently a further phase III trial to identify specific vitamin supplement dosages tailored to the genetic complement of individual migraine sufferers is being pursued. Researchers are expecting to develop a nutraceutical (nutrition-based product) in the near future that will reduce the burden of migraine in a large proportion of those affected.

1.10.3 Current Research

Recently, calcitonin gene related peptides (CGRPs) have been found to play a role in the pathogenesis of the pain associated with migraine, as triptans also decrease its release and action. CGRP receptor antagonists, such as olcegepant and telcagepant, are being investigated both in vitro and in clinical studies for the treatment of migraine (Ho, Ferrari et
al. 2008; Connor, Shapiro et al. 2009). Currently Merck Corp is developing a new drug, Telcagepant, intended to relieve pain without causing vasoconstriction (narrowing of blood vessels) as current medications such as triptans do. Telcagepant could be a safe therapy for migraineurs that have risk factors for cardiovascular disease. The most active area in migraine drug research in 2013 was CGRP-related agents with at least 6 novel CGRP-related agents in clinical development (Peroutka 2014). Arteaus, Alder, and Amgen are conducting Phase 2 efficacy trials for anti-CGRP antibodies with data expected in 2014. Other targets for migraine treatments include ion channels, PACAP receptor antagonists, nitric oxide synthase (NOS) and glutamate receptors (Magis and Schoenen 2011; Olesen and Ashina 2011). In 2010, scientists identified a genetic defect in the protein TRESK, a potassium channel to be linked to migraine which could also provide a target for new drug treatments (Lafreniere, Cader et al. 2010).
CHAPTER 2  Studies on the Pathophysiology and Genetic Basis of Migraine

2.1 Abstract

Migraine is a neurological disorder that affects the central nervous system causing painful attacks of headache. A genetic vulnerability and exposure to environmental triggers can influence the migraine phenotype. Migraine interferes in many facets of people’s daily life including employment commitments and their ability to look after their families resulting in a reduced quality of life. Identification of the biological processes that underlie this relatively common affliction has been difficult because migraine does not have any clearly identifiable pathology or structural lesion detectable by current medical technology. Theories to explain the symptoms of migraine have focused on the physiological mechanisms involved in the various phases of headache and include the vascular and neurogenic theories. In relation to migraine pathophysiology the trigeminovascular system and cortical spreading depression have also been implicated with supporting evidence from imaging studies and animal models. The objective of current research is to better understand the pathways and mechanisms involved in causing pain and headache to be able to target interventions. Genetic studies of a rare migraine subtype, familial hemiplegic migraine, have identified some causal genes in ion channels and have provided hypotheses applicable to common migraine. The genetic nature of migraine is complex and it is thought that many genetic variants act synergistically to contribute to the migraine phenotype. It is well known that susceptibility to migraine is caused partly by defective genes, but the number and types of genes responsible is still not clearly understood. The genetic component of migraine has been teased apart using linkage studies and both candidate gene and genome-wide association studies, in family and case-control cohorts. Genomic regions that increase individual risk to migraine have been identified in neurological, vascular and hormonal pathways.
2.2 Population Cohorts

Population genetics is the quantitative study of genetic variation in populations and how allele and genotype frequencies are influenced and change over time (Nussbaum and Peltonen 2007). A population is a set of organisms in which any pair of members can breed together. This implies that all members belong to the same species and live near each other. Allele frequencies, and the frequency of disease-causing mutations, vary between populations, reflecting their genetic origins. Over time evolutionary forces including: natural selection, genetic drift, mutation and gene flow inevitably change the allele frequency distribution of the population.

Cohorts for studying the genetics of a disease are typically organized into case-control populations, family pedigrees, and pedigrees of genetically isolated populations. Case-control cohorts consist of a collection of unrelated individuals suffering from a disease that are matched for variables of gender ethnicity and age to an independent collection of healthy unrelated individuals. Such outbred cohorts are advantageous for the following reasons: large numbers of affected individuals to study, availability of large sample sizes, genetic markers are highly polymorphic and greater opportunity for replication. On the other hand, family pedigrees consist of a cohort of related individuals within a family. A family tree is constructed indicating the presence or absence of the trait in question for each member of each generation. Family pedigrees are useful in linkage studies where they allow geneticists to ascertain the mode of inheritance and to quantify familial aggregation among family members (Klug 2012). Together genetic isolates and outbred cohorts make the study of population genetics powerful.

In population genetics, isolated populations are a unique resource to study Mendelian and complex human traits compared to outbred cohorts as genetic and environmental variances can, to some extent be controlled. Many population isolates with diverse demographic histories exist worldwide from the Adriatic Islands of Croatia (Rudan, Campbell et al. 1999; Vitart, Biloglav et al. 2006) to countries such as Finland, located on the European continent (Norio 2003). Isolated populations arise when a small number of founding individuals become separated geographically from a larger parent population (Bellis, Hughes et al. 2005). Prolonged geographical isolation combined with limited immigration exposes individuals to a shared lifestyle (e.g. diet, exercise, sanitation) which together contribute to high levels of
inbreeding forging more similar genetic profiles leading to an overall reduction in the genetic diversity of the population. This makes an isolated population unique to study from geneticists’ point of view as the prevalence of many genetic diseases is much higher making the identification of susceptibility genes easier.

The Norfolk Island population is one example of an isolated cohort in which to study complex disorders. Current inhabitants of Norfolk Island can trace their family history back to a group of 9 (Caucasian) ‘Bounty mutineers’ and 6 Tahitian (Polynesian) women, and 2 European Whalers (Male) who initially settled Pitcairn Island (then uninhabited) in 1790 and later relocated to Norfolk Island, a little volcanic island in the South Pacific Ocean, when the population grew too big (Macgregor, Bellis et al. 2010). The heritability of migraine in this pedigree is (H2 = 0.53) and the prevalence of migraine is 25.5%, a two-fold increase compared to outbred populations (Cox, Lea et al. 2012b). Cardiovascular disease (CVD), particularly hypertension, obesity and hyperlipidemia are also increased in the Norfolk Island community making it a genetic treasure for investigating the genetic make-up of complex disorders.

2.3 Evidence of Genetic Susceptibility

2.3.1 Heritability

The causation of some diseases can be linked primarily to genetic factors, but is more commonly the result of an interaction between genetic and environmental triggers. Heritability is a measure of the total genetic variation in a trait ie. that is transmissible from parent to offspring. In the case of migraine, heritability studies have shown that both genetic and environmental factors play a role in manifesting the migraine phenotype. Heritability is measured on a scale from 0 to 1. A value of 1 indicates complete regulation by genetic factors; this is typical of Mendelian disorders like Cystic Fibrosis where one gene accounts for the disorder completely. Migraine is a little bit more complex. It is genetically heterogeneous with different gene loci producing the migraine phenotype. At the population level heritability is estimated at 0.40-0.60 (Mulder, Van Baal et al. 2003) with the residual heritability reflecting the environmental component influencing the disorder. Heritability estimates as high as 0.96 for MA, 0.77 for MO and 0.56 for all migraine have been reported in a Dutch genetic isolate (Stam, de Vries et al. 2010). In the Norfolk Island population, an
Australian genetic isolate, heritability is 0.53 and the prevalence of migraine is even higher at 25.5% this is twice as high as the 12% reported in outbred Caucasian populations (Cox, Lea et al. 2012b).

2.3.2 Familial Aggregation

There is robust evidence indicating genetics plays a significant part in migraine expression. Migraine is partly heritable and partly attributed to environmental triggers and as a result is described as a heterogeneous disorder. Migraine is phenotypically and genetically heterogeneous and no single variant can explain the entire underlying genetic component across different families and populations. The common form of migraine does not follow a Mendelian mode of inheritance making the identification of susceptibility genes more complex. However, there is robust evidence from epidemiological studies in twins, families and unrelated cases of migraine indicating genetics plays a significant part in migraine expression.

The increased occurrence of migraine in close relatives of an affected individual is known as familial aggregation. Familial aggregation is measured as the disease relative risk RR. Reports state that first degree relatives of migraineurs are RR=1.88 times more likely to suffer migraine than first degree relatives of non-migraineurs (Stewart, Bigal et al. 2006). RR is also higher in families of probands who have more severe migraine as evidenced by a self-reported pain rating (Stewart, Bigal et al. 2006). Also noteworthy is the fact that familial aggregation was found to be elevated among probands with an early age of onset. This has been documented in epidemiological studies which yield important insight into the genetic aetiology of complex disorders (Stewart, Bigal et al. 2006).

Estimates of familial aggregation can vary from study to study and be potentially biased by the method used to determine migraine status of family members. This is the result of differences in migraine case definition. A poorly specified definition will lead to an underestimate of the relative risk (Stewart, Staffa et al. 1997). In contrast, including a positive family history of migraine as a diagnostic criterion for the proband will lead to an overestimate of the relative risk (Stewart, Staffa et al. 1997).
Uncertainty in diagnostic methods can create problems when replicating results in independent populations maintained by external groups. Therefore the diagnostic strategies used for genetic studies should be well documented and adopted systematically. In particular the method of collection of phenotypic data (i.e. survey, interview, questionnaire) on which diagnosis is based should be carefully considered so as to minimize error (Stewart, Bigal et al. 2006).

The variable phenotypic presentation of migraine and the results of many genetic studies to date suggest that common migraine is polygenic. The growing body of evidence from population-based family studies and twin studies indicates that genetic factors play a significant but incomplete role in migraine aetiology.

2.3.3 Twin Studies

Twins are a valuable resource to study in population genetics due to a shared family environment and similar genotypes. Twins have been used to dissect out the relative contribution of environmental and genetic influences on individual traits and behaviours. More specifically, monozygotic (MZ) or "identical" twins are 100% genetically identical and consequently will be concordant for any genetically determined character. Concordance is whether one or both members of a twin pair is affected. Monozygotic twins have approximately twice the concordance rate of migraine compared to dizygotic twins (Gervil, Ulrich et al. 1999a). This means that identical twins suffer from migraine together twice as often as fraternal dyzygotic twins. This is expected because MZ twins are genetically identical and suggests migraine to have a genetic basic. A study by Urlich et al., 1999 of MA found pair wise concordance rates to be significantly higher in MZ (34%) versus DZ twin pairs (12%), providing strong evidence of a genetic component to MA (Ulrich, Gervil et al. 1999). However MZ twin pairs do not both suffer migraine 100% of the time and therefore this finding indicates that environmental factors must play an important part in migraine aetiology.

Dizygotic (DZ) or "fraternal" twins share only about 50% of their polymorphisms and are still useful to study because they share many aspects of their environment (e.g., uterine environment, parenting style, education, wealth, culture, community) by virtue of being born in the same time and place. In a study called the GenomeEUtwin project migraine prevalence
and heritability was assessed across 6 European countries and included a total number of 29,717 twin pairs. Heritability ranged from 34% to 57% with lowest estimates in Australia, and highest estimates in the older cohort of Finland, the Netherlands, and Denmark. Migraine was found to be most prevalent in Danish and Dutch females (32% and 34%, respectively) whereas the lowest prevalence was found in the younger and older Finnish cohorts (13% and 10% respectively) (Mulder, Van Baal et al. 2003).

2.4 Molecular Genetic Studies of Migraine

Insights into the genetic basis of migraine have come from a number of different angles. Linkage studies in family pedigrees in which inheritance of migraine is apparent have been used to identify genomic regions, and in some cases the genes, which are responsible for susceptibility. Knowledge gained from studies of migraine pathophysiology has led to investigation of candidate genes in migraine case-control populations and more recently genome-wide association studies in large case-control cohorts have been used to identify genes potentially involved in migraine with no a priori assumptions.

2.4.1 Studies on Familial Hemiplegic Migraine

Familial hemiplegic migraine (FHM), a rare subtype of MA first described by Clarke (1910) in a UK family of 4 generations, has been the object of much interest by geneticists researching migraine (Ophoff, Terwindt et al. 1996). FHM is inherited in an autosomal dominant fashion and some symptoms overlap with those of migraine with aura. FHM symptoms typically include hemiparesis (weakness of half the body) during the aura phase and the aura is generally more prolonged and consists of temporary visual changes such as blind spots (scotomas), flashing lights, zig-zagging lines, and double vision (Carrera, Stenirri et al. 2001). Familial diagnosis requires that at least one first- or second-degree relative be affected. In cases of identical symptomatology and the absence of affected first- or second-degree relatives, the disorder is classed as sporadic hemiplegic migraine (SHM) (IHS 2013). The stronger genetic component and phenotypic overlap of FHM with MA has made FHM a favourable model to study the mechanisms of headache and aura (Thomsen, Kirchmann et al. 2007).
FHM was the first primary headache disorder connected to genetic mutations (Ophoff, Terwindt et al. 1996; De Fusco, Marconi et al. 2003; Dichgans, Freilinger et al. 2005). Three causative genes have been identified via linkage in FHM families and include: CACNA1A, ATP1A2 and SCN1A (Table 2.1). The proteins produced by these genes form channels that regulate the flow of ions across neuronal and glial cell membranes. The common theme that ties these three genes together is ‘ion transport’.

The familial forms of hemiplegic migraine identified are referred to as FHM1, FHM2, and FHM3, respectively. Each type of FHM is caused by mutations in a different gene.

The first FHM region (FHM1; MIM141500) was localized to 19p13 and the defective gene CACNA1A (MIM601011) was identified in five unrelated FHM families (Table 2.5) in 1996 (Ophoff, Terwindt et al. 1996). This gene covers 300kb is composed of 47 exons and was identified whilst studying families segregating with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (MIM125310) a disorder that causes stroke and vascular impairments which can also cause migraine (Liem, Oberstein et al. 2010). The CACNA1A gene encodes the α1 subunit of the voltage-dependent P/Q-type Ca\textsuperscript{2+} channel protein (see Figure 3.1) (Pietrobon 2010b). This channel is expressed in neuronal tissue where it regulates the flow of calcium ions Ca\textsuperscript{2+} into excitable cells (Diriong, Lory et al. 1995). Mutations in this gene contribute to cerebellar ataxia and epilepsy and can cause 2 other neurological disorders with autosomal dominant inheritance, episodic ataxia type 2 (EA2; MIM108500), and spinocerebellar ataxia type 6 (SCA6; MIM183086) (Pietrobon 2010b).

The second type of familial hemiplegic migraine, FHM2 (FHM2; MIM602481), is caused by mutations in the gene ATP1A2 (ATP1A2; MIM182340) (De Fusco, Marconi et al. 2003). The ATP1A2 gene encodes the α2 isoform of the major subunit of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase pump (see Figure 3.2). Astrocytes are the main cells expressing this type of channel and when mutated ion pumps have higher resting potentials (Russell and Ducros 2011). The third FHM locus (FHM3; MIM609634) is at 2q24, and the implicated gene SCN1A (SCN1A; MIM182389) encodes the α1 subunit of the neuronal voltage-gated Na\textsuperscript{+} channel (see Figure 3.3) (Dichgans, Freilinger et al. 2005). This channel is important for action potential generation in neurons. Mutations in SCN1A were first observed to cause the epilepsy syndrome, generalised epilepsy with febrile seizures (GEFS+; MIM604233) and severe
myoclonic epilepsy of infancy (SMEI, MIM607208) (Meisler and Kearney 2005; Mulley, Scheffer et al. 2005).

Variants in the three FHM genes, however do not account for 100% of FHM cases and it has been proposed that there are additional mutations at other locations that could potentially contribute to the FHM phenotype. *SLC1A3* (encoding the glial glutamate transporter *EAAT1*) and *SLC4A4* (encoding the electrogenic sodium bicarbonate cotransporter NBCe1) have been proposed as potential fourth and fifth genes (FHM4 and FHM5) responsible for pure hemiplegic migraine (see Table 2.5) (de Vries, Stam et al. 2009; Russell and Ducros 2011). Functional studies of mutations at each FHM locus in animal and model cells have shown that various missense mutations, large and small scale deletions exist and greatly affect the conductive properties of the channels upsetting the balance of ions in neurons (Kahlig, Rhodes et al. 2008). The flow of ions is critical for normal physiological functioning and any disruption can make people more susceptible to developing these severe headaches.

| Table 2.1 Familial hemiplegic migraine and defective genes identified. |
|---|---|---|
| Gene | Chromosome location | Protein |
| FHM1 (MIM141500) | *CACNA1A* | 19p13 | Pore-forming α1 subunit of neuronal Ca2.1(P/Q type) voltage-gated calcium channels |
| FHM2 (MIM609634) | *ATP1A2* | 1q23 | Catalytic α2 subunit of a glial and neuronal sodium-potassium pump |
| FHM3 (MIM602481) | *SCN1A* | 2q24 | Pore forming α1 subunit of neuronal Na1.1 voltage-gated sodium channels |
Figure 2.1 Topology of the α1 subunit of the voltage-dependent P/Q-type Ca\(^{2+}\) channel protein encoded by the FHM1 *CACNA1A* gene showing positions of known FHM mutations.

The protein forms a transmembrane channel located in the neuronal membrane which is composed of four repeated domains. Each domain includes six membrane-spanning segments (S1 to S6) and a so-called P loop between S5 and S6. The four S4 segments form the voltage sensor part of the channel, the four S5 and S6 segments form the inner part of the pore, and the four P loops line the inside of this pore. Symbols: Circle with solid line = FHM, circle with dotted line = SHM. Asterisk = Mutations for which also SHM was reported, black circles mutation was tested for functional consequences, white circle mutation was not tested for functional consequences sourced from (de Vries, Frants et al. 2009).
Figure 2.2  Topology of the α2 isoform of the Na⁺/K⁺-ATPase pump protein encoded by the FHM2 ATP1A2 showing positions of known FHM mutations.

Symbols: Circle with solid line = FHM, circle with dotted line = SHM, circle with horizontal striped pattern = basilar-type migraine, circle with vertical striped pattern = common migraine. Asterisk = Mutations for which also SHM was reported, black circles = mutation was tested for functional consequences, white circle = mutation was not tested for functional consequences sourced from (de Vries, Frants et al. 2009).
Figure 2.3  Topology of the the α1 subunit of the neuronal voltage-gated Na⁺ channel encoded by the FHM3 SCNA1A gene showing positions of known FHM mutations.

Symbols: Black circles = mutations was tested for function consequences, white circle = mutation was not tested for functional consequences sourced from (de Vries, Frants et al. 2009).
2.4.2 Mouse Models of FHM

Genetically modified mice have been engineered based on mutations in the three specific genes responsible for FHM with the expectation that they may by extrapolation reflect on the pathophysiology of common migraine subtypes and also due to the fact that no other genes of strong effect are available and amenable to study common migraine (Eikermann-Haerter and Moskowitz 2008). Despite the large number of mutations characterized at each FHM locus, only a small fraction of mutations that produce very extreme phenotypes have been studied in genetically altered mice given the expense and time involved in generating modified animals (Garza-Lopez, Sandoval et al. 2012).

Two common FHM1 mutations in the \textit{CACNA1A} gene have been introduced in knock-in mice: R192Q (van den Maagdenberg, Pietrobon et al. 2004; Tottene, Conti et al. 2009) and S218L (Kaja, Van de Ven et al. 2010; van den Maagdenberg, Pizzorusso et al. 2010). Both mutations change the amino acid sequence of the protein and affect the 3D structure and function of the \(\alpha 1\) subunit of the voltage-dependent P/Q-type \(\mathrm{Ca}^{2+}\) channel. The channel mediates the influx of calcium ions \(\mathrm{Ca}^{2+}\) and changing the amino acid sequence has important consequences to normal physiological functions which can be observed \textit{in vivo}. The R192Q mutation is located in the fourth transmembrane domain and causes a gain-of-function effect. This mutation contributes to excess release of glutamate in the cortex and has been suggested to increase susceptibility to CSD, the physiological event responsible for the aura in migraine (Pietrobon 2005). The phenotype of S218L mutation is more extreme than R192Q and mice exhibit symptoms of cerebellar ataxia, seizure and head trauma and in particular increased sensitivity to CSD (van den Maagdenberg, Pizzorusso et al. 2010). The S218L mutation is located in the second intracellular loop of the protein and mice with this mutation exhibit repetitive CSD events after a single stimulus with an increase in \(\mathrm{Ca}^{2+}\) influx (van den Maagdenberg, Pizzorusso et al. 2010).

A knock-in mouse carrying the FHM2 mutation W887R in the human \textit{ATP1A2} gene has also been generated. This mutation is located in the fourth extracellular loop between transmembrane domains M7 and M8 of the subunit that codes for the \(\mathrm{Na}^+\)-\(\mathrm{K}^+\)-ATP pump involved in ion transport in the brain and causes the protein to be non-functional and unable to pump ions. Mice homozygous for this mutation do not survive past birth due to neurological dysfunctions, while in heterozygotes CSD occurs more easily and quickly than
in wild type mice (Leo, Gherardini et al. 2011). Thus FHM1 and FHM2 mouse models have contributed to better understanding the functional properties of Ca\textsuperscript{2+} channels and suggest that relevant mutations reduce the threshold for CSD induction and propagation, supporting a role of CSD in triggering migraine (Leo, Gherardini et al. 2011).

### 2.4.3 Animal Models of Migraine

Animal models have been used for many diseases to study the pathological mechanisms and to test the efficacy of drugs. In migraine the broad clinical phenotype and the fact that multiple genetic and environmental factors play a role in the disorder has made the development of a reliable animal model difficult. Nevertheless a variety of animal models of migraine have been developed to better study the pathophysiology of migraine including activation of the trigeminovascular system, the pain-producing cranial structures, the large vessels, dura mater and electrical stimulation of the trigeminal ganglion to illustrate pain pathways and model migraine symptoms such as allodynia (Bergerot, Holland et al. 2006). Many animal models also try to replicate the phenomenon of CSD however none of them come close to replicating all facets of the migraine syndrome (Andreou, Summ et al. 2010). Animals have also contributed to the study of migraine mainly by simulating the effects of headache using different pharmacological compounds (James, Smith et al. 1999; Fabricius, Fuhr et al. 2006). A well validated human migraine model is based on the administration of glyceryl trinitrate (GTN) to simulate the effects of headache (Olesen and Jansen-Olesen 2012). Also structures like in vitro blood vessels have served the testing of drugs like triptans and for probing the location of ‘triptan receptors’ in vascular tissue (Eikermann-Haerter and Moskowitz 2008). Additionally Melo-Carrillo et al., 2013 describe a murine model of chronic meningeal nociception that mimics migraine clinical features and propose their technique as a new animal model to study migraine (Melo-Carrillo and Lopez-Avila 2013). This model demonstrates an increase in nociceptive behaviours related to headache: rest, facial grooming and freezing as a result of meningeal infusion of inflammatory soup (López-Avila, Melo et al. 2009). These models allow the study of migraine in vivo and are good tools with which to screen potential pharmaceutical compounds.
2.5 Candidate Gene Studies

The goal of molecular genetic studies is to find a genetic abnormality that may predispose an individual to a specific disease. This problem is approached by identifying the physiological mechanism causing disease and then looking for a disrupted biochemical product at the protein level that can be exploited pharmacologically or diagnostically (King, Rotter J. I. et al. 2002). Genetic efforts have focussed on candidate genes involved in pathological pathways of the disease and have examined genes involved in neurological, vascular, hormonal and more recently mitochondrial functions.

2.5.1 Migraine Linkage Studies

To date several linkage studies have been performed utilizing families of different ethnic origin and have successfully identified migraine susceptibility loci on a range of chromosomes demonstrating that migraine is polygenic. The majority of linkage studies have used the ‘migraine end diagnosis’ definition to diagnose migraine patients as either MA or MO. This has worked well for the most part however many loci have not been replicated in populations of different ethnicity. It has been suggested by some that the reason for this occurrence is the presence of rare high-impact family specific markers. Other reasons to explain lack of replication include inaccurate migraine diagnosis and phenotyping of migraine cases leading to heterogeneity of cohorts.

Two alternative phenotyping strategies to the end diagnosis have been introduced in an attempt to identify novel regions which are more specifically related to migraine biological processes. One strategy utilizes latent classes (LCA) whilst the other examines trait components (TCA). The LCA and TCA strategies introduced in studies by Nyholt, Anttila and colleagues make better use of the questionnaire-based information (Nyholt, Gillespie et al. 2004; Anttila, Kallela et al. 2006). Employment of strategies like LCA and TCA have shown that certain chromosomal regions to specific migraine symptoms like pulsating pain, phono-/photophobia, nausea, and age at onset of migraine. One example is the identification of the 5q21 region in an Australian study and the 17p13 region in a Finnish study associated with pulsating head pain through the application of the LCA method by Nyholt et al., 2005 (Lea, Nyholt et al. 2005; Nyholt, Morley et al. 2005). Also the LCA method identified linkage at 18p11 (Lea, Nyholt et al. 2005). These two methods have added somewhat to
standard ICHD-II classification criteria in identifying linkage regions with specific migraine symptoms.

Out of all the linkage studies undertaken the most consistent locus resides on chromosome 4. This is an interesting candidate region with two independent linkage studies of 50 Finnish MA families showing linkage to 4q24 (Wessman, Kallela et al. 2002; Anttila, Kallela et al. 2006). Another study using 103 Icelandic families identified an overlapping locus at 4q21 using MO patients only (Bjornsson, Gudmundsson et al. 2003). In a recent study by Oedegaard, et al., 2010 (Oedegaard, Greenwood et al. 2010) performed in 31 families with bipolar disorder co-morbid with migraine they replicated the 4q24 region implicating this locus as potentially containing a gene predisposing to MA and MO (Oedegaard, Greenwood et al. 2010).

The power of linkage is evident in the study by Lafreniere et al., 2010 who identified the first causal typical migraine gene, a potassium channel in a multigenerational family with dominant, fully penetrant typical MA (Lafreniere, Cader et al. 2010). The gene encoding the two-pore domain potassium (K2P) channel is KCNK18 (KCNK18; MIM613655) at chromosome band 10q25.3 and TRESK is the protein produced. TRESK is one of many potassium channels in the CNS activated by Ca\textsuperscript{2+} that passes outwardly rectifying K+ currents essential for signalling in excitable tissues and is particularly relevant to neurological diseases. K+ conductance in excitable cells is particularly important and TRESK activity is regulated by the calcium-dependent phosphatase calcineurin. \textit{In situ} hybridization in the mouse embryo detected expression in the trigeminal ganglion, dorsal root ganglia and autonomic nervous system ganglia implicating TRESK in neuronal excitability (Lafreniere, Cader et al. 2010).

Mutations in the KCNK18 gene were identified by Sanger sequencing the coding region of 110 unrelated migraineurs and 80 controls. Among the different variants identified the most interesting variant was a frameshift (F139WfsX24) mutation (a 2-bp deletion causing frameshift and truncation of TRESK protein in the second transmembrane segment) which prematurely truncates the protein and results in a total loss of channel function and may contribute to migraine by some mechanism yet to be determined (Lafreniere, Cader et al. 2010). In 16 members of the family in which the gene was sequenced, eight members carried the F139WfsX24 frameshift mutation and migraine headache whilst the TRESK mutation
was absent from the eight unaffected individuals (Lafreniere, Cader et al. 2010). Further gene sequencing in an Australian cohort (511 migraine, 505 controls) identified nine additional variants in this gene however not all variants have a negative impact on channel function. TRESK, G339R functional knockout mice lead to enhanced DRG excitability (Dobler, Springauf et al. 2007). Down-regulation of TRESK activity has been connected with a neuropathic pain model (Tulleuda, Cokic et al. 2011) and changes in channel expression have been reported in inflammation (Marsh, Acosta et al. 2012). The results of these studies indicate mutations in the KCNK18 gene producing loss or diminished function of TRESK causing down-regulation of the wild-type channel may be involved in MA pathogenesis by lowering the threshold for CSD and enhance the propensity of a migraine episode (Andres-Enguix, Shang et al. 2012).

Functional studies have characterized the physical location of missense variants in the TRESK channel pore using a homology model and have studied the physiological effect of different missense variants by recording whole-cell basal currents from Xenopus oocytes expressing wild-type and mutant TRESK subunits (Andres-Enguix, Shang et al. 2012). More than one missense variant is needed to cause disruption to TRESK channel activity and to manifest in a migraine phenotype as found in the genetic data obtained by Lafreniere et al., 2010, not all variants identified affect the function of the channel. Crystal structures are now available of K2P channels showing that the two P domain sequences of these channels assemble as dimers (Brohawn, del Marmol et al. 2012; Miller and Long 2012). Volatile anesthetics activate TRESK activity (Yoo, Liu et al. 2009).

Altogether the results of genetic and functional studies suggest that mutations within the KCNK18 gene can cause deregulation of potassium homeostasis and this may be a mechanism that leads to neuronal hyperexcitability and increases a person’s predisposition to migraine. In light of the role of TRESK in migraine it is rational to investigate additional genes encoding potassium channels, as well as those within pathways regulating TRESK activity for potential roles in migraine onset. The fact that TRESK codes for an ion channel strengthens the hypothesis of neuronal hyperexcitability and provides a novel target for the pharmaceutical industry to prevent or alleviate symptoms of migraine.
2.5.2 Neurological Pathways

Although D’Onofrio et al., 2009 (D’Onofrio, Ambrosini et al. 2009) found that a combination of two SNPs in the \textit{CACNA1A} gene may contribute to migraine susceptibility, polymorphisms in FHM genes have not been generally found to be associated with common migraine (Maher and Griffiths 2011) and references therein). Neurological candidate genes of the serotonergic and dopaminergic systems have predominantly been investigated in migraine, given that it is a neurological disease with autonomic nervous system symptoms. Receptors, transporters and enzymes involved in neurotransmitter synthesis have been targeted. Evidence for the involvement of serotonin stems from biochemical studies in the 1960s which demonstrated altered levels of circulating 5-HT levels in the urine and platelets of migraineurs during their attacks (Sicuteri, Anselmi et al. 1961). Also the fact that serotonin and other neurotransmitters can trigger or affect vascular dysfunction/tone stimulated scientific interest in genes of these neurotransmitter systems as targets to investigate. A fine balance in and across neurotransmitter systems is necessary to maintain normal physiological processes and low function in one system can in turn affect the activity of the other in a cumulative manner.

Various serotonin receptors have been investigated however the most interesting finding was association in the human serotonin transporter \textit{SLC6A4} gene located on chromosome 17q11.2 and migraine (Hamel 2007). The \textit{SLC6A4} serotonin transporter codes for an integral membrane protein that clears serotonin at the synapse and recycles it back into neurons and blood platelets. Two main polymorphisms have been identified in this gene and investigated in association studies. One is a 44bp insertion/deletion functional polymorphism in the promoter region, termed 5-HTTLPR with two common allelic forms, the short variant (S) has 14 repeats of a sequence and the long variant (L) has 16 repeats (Schurks, Rist et al. 2010a). Initial evidence of association with the promoter 5-HTTLPR polymorphism was correlated with possession of the short S allele (Ogilvie, Russell et al. 1998). Possession of the S allele results in slower clearing of serotonin from the synaptic cleft due to down-regulation in gene expression meaning that migraineurs express only half the number of serotonin transporters. Some studies identified an association between the S allele in the promoter 5-HTTLPR polymorphism and migraine (Yilmaz, Erdal et al. 2001; Juhasz, Zsombok et al. 2003a; Borroni, Brambilla et al. 2005; Marziniak, Mossner et al. 2005), while others found no evidence of association(Todt, Freudenberg et al. 2006) (Corominas, Sobrido et al. 2010).
Schurks et al., 2010 (Schurks, Rist et al. 2010a) reviewed the literature and performed a meta-analysis of 10 studies to determine if any association existed between the 5-HTTLPR polymorphism and migraine and their results indicate no overall association.

The second polymorphism investigated in the \textit{SLC6A4} gene consists of a 17bp variable number of tandem repeats known as (STin2 VNTR) in intron 2 with 2 common alleles STin2.10 and STin2.12 composed of 10 or 12 repeat units (Schurks, Rist et al. 2010a). Schurks et al., 2010 (Schurks, Rist et al. 2010d) conducted a meta-analysis of 5 studies considering this polymorphism and found that 5-HTT VNTR STin2 12/12 genotype is associated with an increased susceptibility to migraine especially among populations of European descent. In an attempt to resolve discrepancies in results of association studies Liu et al., 2011 reviewed 15 studies for meta-analysis and found that the 5-HTT VNTR STin2 12/12 genotype confers an increased risk for migraine in the general population (Liu, Liu et al. 2011). The two polymorphisms 5-HTTLPR and VNTR in the serotonin transporter \textit{SLC6A4} gene have been associated with slower removal of 5-HT at the synapse.

The dopaminergic system has also been investigated in migraine because the interaction of Dopamine (DA) with its receptors is known to mediate certain prodromal symptoms experienced by migraineurs. Dopamine receptor antagonists are effective at relieving migraine and DA receptors have been localised in the trigeminovascular system an integral component of migraine pain mechanisms (Bergerot, Storer et al. 2007). Also reports in the literature exist that migraineurs have an increased density of dopamine receptors DRD3 and DRD4 on lymphocytes and that DA agonists can bring about migraine (Barbanti, Bronzetti et al. 1996; Barbanti, Fabbrini et al. 2000). Genetic studies have thus focused on candidate genes encoding proteins of the dopaminergic system, including DA receptors, DA transporter proteins and enzymes involved in the synthesis and metabolism of DA.

Interest in the role of dopamine in migraine was motivated after Peroutka et al. 1997 identified a susceptibility polymorphism at rs61689984 in the DRD2 gene with increased frequency of the C allele in migraine with aura (0.84) compared to migraine without aura (0.70) and controls (0.71) (Peroutka, Wilhoit et al. 1997). Del Zompo et al., 1998 subsequently analysed a number of candidate genes \textit{DRD2}, \textit{DRD3} and \textit{DRD4} in a Sardinian population but found no association (Del Zompo, Cherchi et al. 1998). Todt et al. 2009 investigated a total of 53 SNPs in 10 genes from the dopaminergic system, including \textit{COMT},
DBH, DDC, DRD1, DRD2, DRD3, DRD4, DRD5 SLC6A3 and TH, in a large German case-control cohort of migraine with aura and from this study the dopamine transporter SLC6A3 5p15 and DBH 9q34 enzyme emerged as significant (Todt, Netzer et al. 2009). SLC6A3 is an important transporter of dopamine that maintains a low concentration of DA in the extracellular space. However it should be noted that this gene, also named DAT1, showed no association in other studies (Mochi, Cevoli et al. 2003; McCallum, Fernandez et al. 2007; Karwautz, Campos de Sousa et al. 2008).

The DBH locus 9q34 encodes the Dopamine Beta Hydroxylase gene an enzyme involved in synthesis of noradrenaline from the substrate dopamine. There have been some reports of elevated serum levels of DBH enzyme in migraineurs during an attack (Weinshilboum 1978). Polymorphisms that reduce the plasma enzymatic activity of this gene and lead to an increase in DA have been investigated as shown in Figure 2.4. The functional DBH insertion/deletion polymorphism 19bp indel (-4784-4803) was found to associate with migraine in an Australian population (Fernandez, Lea et al. 2006). This study resulted in a patent for an invention that provides a diagnostic kit for detecting a -1021C→ T SNP associated with migraine which may facilitate selection of individuals for migraine therapy which targets the dopaminergic system. In particular migraine risk increases in males with the homozygous del/del genotype up to three times (Fernandez, Lea et al. 2006). Also a promoter functional polymorphism (-1021C→ T, rs1611115) in DBH which reduces plasma enzyme activity by up to 52% was found associated with migraine aura in an Australian population (Fernandez, Colson et al. 2009).

The level of individual DBH activity has a strong genetic background. The study by Todt et al. 2009, showed significant association for another SNP rs2097629 in a German MA population which is not in LD with the promoter polymorphisms (Todt, Netzer et al. 2009). Another group Corominas et al. 2009 analysed 50 tag SNPs in 8 genes from the dopaminergic system: DRD1, DRD2, DRD3, DRD5, DBH, COMT, SLC6A3 and TH in two case control populations of Spanish origin and found no evidence of robust genetic association of any of these genes with migraine (Corominas, Ribases et al. 2009a). Investigations in dopamine genes have shown some relationship to migraine but results have not always replicated. The DBH locus and the SLC6A3 dopamine transporter seem to correlate with more dopamine at the synapse and support a hypersensitivity to DA hypothesis (Charbit, Akerman et al. 2010).
Figure 2.4 DBH gene structure and polymorphisms sourced from (Fernandez, Colson et al. 2009).
The glutamatergic system has not been studied as extensively as that of serotonin and dopamine however the biological constituents including enzymes, glutamate receptors and transporters which mediate excitatory signals via ionotropic and metabotropic receptors have been proposed as candidates to investigate in light of recent genetic studies implicating glutamate in migraine (Anttila, Stefansson et al. 2010; Formicola, Aloia et al. 2010; Maher, Lea et al. 2013). Further research into the glutamatergic system is necessary to confirm its role in migraine susceptibility and pathophysiology.

The relationship between migraine and sleep is one that has been recognized for some time due to the fact that both sleep deprivation and excessive sleep are associated with migraine and is a topic open to discussion given that research on the subject in sparse (Dodick, Eross et al. 2003; Kelman and Rains 2005). Sleep and circadian rhythms are neuronally regulated functions and influenced by melatonin secretion, and serotonin kinetics (Brzezinski 1997). Importantly, because melatonin regulates calcium entry into cells, a reduction in melatonin might alter the tone or vasoreactivity of cerebral blood vessels. Brennan et al., 2013 found two distinct missense mutations in the gene casein kinase I δ (CKIδ) which is known to affect sleep to also be causing migraine. The CKIδ protein is a serine threonine kinase that plays an important role in the function of the biological clock that influences the circadian rhythm by phosphorylating the circadian clock protein Per2 and many other proteins involved in brain signalling (Brennan, Bates et al. 2013). The loss-of-function mutations (T44A and H46R) identified in two independent families with MA and familial advanced sleep phase syndrome occur in the conserved catalytic domain of CKId and reduce enzyme activity (Brennan, Bates et al. 2013). Similarly CKId-T44A mice have a reduced threshold for CSD and greater arterial dilatation, a phenotype consistent with the neuronal hyperexcitability of migraine (Brennan, Bates et al. 2013). Identifying protein targets of CKIδ may also enable the identification of new therapeutic drug targets.
2.5.3 Vascular Pathways

The link between migraine and the vascular system was encapsulated in the vascular theory proposed by Graham and Wolff in the 20th Century when they hypothesized that pain arises due to dilated blood vessels. Current revised theories of migraine pathophysiology suggest an interaction between cranial blood vessels and the brain’s neural circuitry. The role of the vasculature in migraine pathophysiology is certainly well established given the effects of vasoactive drugs and the observation that migraine is co-morbid with vascular conditions of stroke, Patent Foramen Ovale (PFO) and hypertension (Dalkara, Nozari et al. 2010). Susceptibility loci on different chromosomes that affect vascular endothelial function have been investigated. These include variants such as the renowned and most cited C677T polymorphism in the homocysteine metabolism gene, \textit{MTHFR} to nitric oxide synthase (NOS), calcitonin gene-related peptide (CGRP), angiotensin I-converting enzyme (ACE), and the NOTCH3 gene.

There is a well-known relationship between migraine aura and cardiovascular disease however no causal link has been established (Schurks, Buring et al. 2010; Pierangeli, Giannini et al. 2012). The NOTCH3 gene is a gene that was first identified in a subset of families segregating with a disorder called CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; MIM125310) in which migraine like symptoms occur (Liem, Oberstein et al. 2010). CADASIL is an inherited genetic vasculopathy that causes stroke and other impairments. The underlying pathology of CADASIL is progressive loss of vascular smooth muscle cells in the tunica media of small arteries, and accumulation of granular osmiophilic material (GOM) that is detected by electron microscopy (Mosca, Marazzi et al. 2011). This damage reduces blood flow to various tissues in the body resulting in ischemia. This blood vessel damage can interestingly cause migraine and other impairments of normal brain function. The NOTCH3 gene implicated in this disorder and in migraine pathogenesis encodes a transmembrane receptor composed of 34 tendem epidermal growth factor-like (ELF-like) repeats and is involved in cellular differentiation and cell cycle regulation (Liem, Oberstein et al. 2010; Mosca, Marazzi et al. 2011). Approximately 65% of all CADASIL mutations are found in exons 3 and 4 of the 33 exon gene and are known to be mutation hotspots (Joutel, Vahedi et al. 1997; Federico, Bianchi et al. 2005).
Studies of polymorphisms at the MTHFR locus are not unique to migraine as it is a gene which has been implicated in a multitude of diseases due to its central role in the homocysteine (Hcy) pathway. Diseases that have detected a correlation with this gene include neural tube defects, heart disease, stroke, high blood pressure (hypertension), high blood pressure during pregnancy (preeclampsia), an eye disorder called glaucoma, psychiatric disorders, and certain types of cancer (Rubino, Ferrero et al. 2009). Some of these conditions are co-morbid with migraine. The MTHFR gene located on chromosome 1p36.3 codes for an important enzyme methylenetetrahydrofolate reductase in the Hcy pathway, a pathway that is pivotal to many functions throughout the body including DNA methylation, immune function, muscle metabolism, and regulation of cardiovascular and central nervous system health and removal of toxins (Matthews 2002). This enzyme is responsible for reducing 5,10-methylenetetrahydrofolate to active 5-methyltetrahydrofolate, the substrate required for the conversion of homocysteine to methionine in the Hcy pathway (Matthews 2002). When the MTHFR protein or its levels are compromised Hcy accumulates in the bloodstream and the production of methionine is reduced.

This situation can arise because of genetic alterations in MTHFR. Genetic defects in MTHFR have been well characterized with more than 40 different polymorphisms identified in the MTHFR gene that reduce the activity of the enzyme and lead to an excess of Hcy in the bloodstream (Garilli 2012). In migraine the C to T single nucleotide polymorphism (SNP) at codon 677 of the MTHFR gene has been the most extensively studied functional polymorphism for a common migraine type. The MTHFR C677T variant changes an alanine to a valine within the catalytic domain of the enzyme, affecting the quaternary structure of the protein and reducing its enzymatic activity by up to 50% (Frosst, Blom et al. 1995). This reduced enzyme activity leads to higher levels of homocysteine in the blood which contributes to a condition known as mild hyperhomocysteineimia (hHcy) which is a risk factor for migraine and a number of cardiovascular diseases (Taylor, DeFrang et al. 1991; Cortese and Motti 2001; Graham and O’Callaghan 2002). Although deficiency in MTHFR functionality can produce mild hHcy in humans, this condition can also occur due to environmental factors such as insufficient dietary intake of folate B9, vitamin B12, and vitamin B6.

A few studies indicate that excess Hcy in the blood can cause remodelling of vascular tissue via pathological mechanisms that stimulate the growth of smooth muscle cells, cause
endothelial dysfunction by down-regulating expression of endothelial NO synthase and release of inflammatory mediators (Smilde, van den Berkmortel et al. 1998; Voutilainen, Alfthan et al. 1998; Neves, Endemann et al. 2004; Juo, Liao et al. 2008; Chiang, Sung et al. 2011; Munjal, Givvimani et al. 2011). The vascular changes produced by Hcy are detrimental to arteriolar integrity and can make people more susceptible to vascular inflammation and atherogenesis, which in turn can result in ischemic injury. This has been suggested as a mechanism contributing to migraine on a vascular or ischemic basis.

Although numerous studies have investigated the genetic association of this polymorphism in different migraine populations the status of this gene remains controversial with positive and negative results obtained by independent groups. A couple of meta-analyses have however shed some light on the hypothesised role of the MTHFR gene in migraine. Rubino et al., 2009 pooled results from 8 published articles and identified a significant association for the MTHFR C677T, TT genotype polymorphism but in MA only (Rubino, Ferrero et al. 2009). A recent meta-analysis conducted by Shurks et al., 2010 pooling results from 13 studies investigating the association of MTHFR C677T variant and migraine identified a positive association for the TT genotype in the MA only group (Schurks, Rist et al. 2010b). This study demonstrates that migraine sufferers in particular MA carry the MTHFR 677TT genotype. The TT genotype was associated in both meta-analyses with an increased risk for migraine with aura. It has been suggested that there may be genetic differences which are specific to each of the migraine subtypes. People who have inherited the MTHFR TT genotype have a less active enzyme and are said to be genetically slower homocysteine metabolizers.

The biochemical properties of wild type and mutant MTHFR enzymes reveal that mutations decreasing the activity of MTHFR can contribute to high circulating plasma homocysteine and mild hyperhomocysteineimia, a condition which may contribute to migraine (Leclerc, Sibani et al. 2000). MTHFR knockout mice show elevated levels of plasma and total homocysteine in comparison to wild-type and suffer other abnormalities, including hypomethylation of their DNA, developmental delay, and thromboses of the arteries and cerebral veins (Matthews 2002). Biochemical and animal studies have been useful in demonstrating that these abnormalities are due to non-functional MTHFR (Matthews 2002).
A genetic defect in MTHFR activity can be bypassed by increasing dietary intake of B vitamins as shown in a pilot study (Lea, Colson et al. 2009) of migraineurs supplemented with folate B9 and vitamins B12 and B6 over a six month period. Study participants reported an improvement in migraine symptoms, this improved response was correlated with a reduction in homocysteine levels from the supplementation with B vitamins and with carriage of at least one C allele (Lea, Colson et al. 2009). This study shows that supplementation with B vitamins can help restore balance in this pathway in individuals with this mutation and is an effective Nutrigenomics measure.

Clinical trials examining the role of B vitamins in vascular disorders have also implicated MTHFR as the culprit of disease and demonstrated that dietary supplementation with B vitamins can reduce the risk of disease (Huang, Chen et al. 2012; Menon, Lea et al. 2012; Nachum-Biala and Troen 2012; Wien, Pike et al. 2012; Marti-Carvajal, Sola et al. 2013; Spence 2013; Trimmer 2013). Trials of folate or vitamin supplementation have been conducted with apparent beneficial outcomes, particularly in migraineurs with the MTHFR C allele (Lea, Colson et al. 2009). In the latest study migraineurs were stratified into MTHFR C667T, demonstrating the influence of specific genotypes on treatment efficacy. Larger trials are now underway to study the effect of gene expression and DNA methylation changes in migraineurs following folic acid and vitamin B treatment as well as the impact of different dosages of B vitamins (unpublished) and hence explore the pharmacogenetic relationship with a view to personalize treatment. Testing different vitamin dosages has implications for migraineurs with differing MTHFR genotypes, for example C allele carriers respond better to treatment compared with TT genotypes in terms of reduction in homocysteine levels and migraine symptom severity.

2.5.4 Hormonal Pathways

The influence of female sex hormones in migraine is evident from the noticeably distorted gender ratio (3:1 female to male) clearly observed in migraine prevalence studies (Lipton, Bigal et al. 2007). Accordingly women are 3 times more likely to suffer migraine than men, a fact attributed to fluctuating hormones due to reproductive milestones: menstruation, pregnancy and menopause (Maggioni, Alessi et al. 1997; MacGregor 2009). Genetic studies of hormonal genes have focused on the estrogen receptor (ESRI) and the progesterone
receptor ($PGR$) genes, a logical choice based on fluctuating hormones of the ovarian cycle which many women recognize as triggers for their migraine.

The observation that migraine prevalence varies with hormonal transitions including pregnancy and menopause has stimulated scientific interest into the impact that endogenous and exogenous hormones have on the frequency and severity of migraine attacks in women. The general trend observed is that migraine affects boys and girls equally at puberty and thereafter increases in adulthood with women predominantly affected (Sacco, Ricci et al. 2012). During pregnancy oestrogen is constantly high whilst during menopause hormones fluctuate for a while but then level out and are constantly low post menopause, it is at these times that migraine attacks diminish (Shuster, Faubion et al. 2011). Thus lack of hormonal balance, i.e. rapid rise and fall in the circulation is thought to contribute to migraine and may be a trigger particularly in menstruating women as this is the time when hormones are likely to fluctuate (Martin and Behbehani 2006).

The ovarian hormones estrogen and progesterone are steroid hormones capable of modulating many biological functions in either a genomic (transcription dependent) or nongenomic (non–transcription dependent) mechanism via their cognate receptors (Martin and Behbehani 2006). The estrogen and progesterone receptors are among the most studied genes in relation to hormonal pathways in migraine and besides their obvious role in sexual development and reproductive function they also affect functioning of the cardiovascular and nervous systems as well as growth and maintenance of the skeleton (Wang, Liu et al. 2008). The effect of exogenous estrogen and progesterone in the form of oral contraception or hormone replacement therapy have shown that some womens migraine symptoms can improve because of stabilizing hormonal fluctuations but at the same time can also worsen migraine in certain people (Wang, Liu et al. 2008). Consequently hormone administration as a therapy in the treatment of migraine is not indicated.

Polymorphisms in $ESR1$ and $PGR$ have been studied with respect to migraine susceptibility: some studies have shown that independent polymorphisms in $ESR1$ and $PGR$ are associated with increased migraine risk whilst other studies have detected no association whatsoever. Initial positive findings were detected for $ESR1$ by Colson et al., 2004 (Colson, Lea et al. 2004) for the G594A SNP rs2228480 in exon 8 in two independent Australian case-control populations (population 1, $p<$0.008, population 2, $p<$4x10$^{-5}$). Variation in this gene is
particularly interesting because estrogen receptors have been implicated in disease processes in breast cancer, endometrial cancer, and osteoporosis and the *ESR1* SNP rs2228480 genotyped in migraine was chosen based on the known association with breast cancer (Lipphardt, Deryal et al. 2013). *ESR1* is localized to chromosome 6q25.1 and is expressed in many areas of the brain regulating many functions including regulating gene expression through cell signalling affecting glutamate and serotonin synthesis and CGRP and can regulate vascular tone by stimulating release of NO (Lewis and Winner 2006; Gupta, Mehrotra et al. 2007).

The association of this SNP 594G>A (exon 8) with migraine however was not replicated in 3 subsequent studies (Kaunisto, Kallela et al. 2006; Oterino, Pascual et al. 2006; Corominas, Ribases et al. 2009b). A further 3 SNPs were investigated in *ESR1* namely, 325C>G (exon 4), T/C *Pvu* II SNP (intron 1) and T30C. For SNP 325C>G (exon 4), 5 studies reported no association and 2 studies reported a positive association in a Caucasian population. The T/C *Pvu* II SNP (intron 1) was interrogated in two studies only one reporting a positive association in an Indian population and the T30C was only investigated in a Spanish study and no association detected (Joshi, Pradhan et al. 2010). In summary the synonymous polymorphisms 325C>G (exon 4) and 594G>A (exon 8) have been investigated the most for *ESR1* and migraine. Although they are located in exonic regions of the gene their functional implication in disease is unknown and some have indicated they may be in LD with a nearby causal variant yet to be identified. The ethnicity of the populations used in these studies included, Caucasian, Indian, Finnish and Spanish.

Subsequent to the estrogen receptor, the PROGINS variant (a 306 base pair insertion within intron 7) in *PGR* located on chromosome 11q22, was next investigated by Colson et al., 2005 in the same Caucasian population. Colson et al., 2005 found a positive association again only to be replicated by Lee et al., 2007 in patients with migraine-associated vertigo and Joshi et al., 2010 in a north Indian population (Lee, Sininger et al. 2007). Interestingly when the original authors analyzed the interaction of both hormonal genes together (*ESR1* 594A allele and PROGINS variant) they identified a synergistic effect whereby migraine risk was increased 3.2 times (Colson, Lea et al. 2005).

Oterino, et al., 2008 approached the relationship of hormone receptors and migraine from a multigenic, gene-gene interactions perspective (Oterino, Toriello et al. 2008). They analysed
5 estrogen related genes: oestrogen receptor 1 gene (ESR1), oestrogen receptor 2 gene (ESR2), follicle stimulating hormone receptor gene (FSHR), CYP19 aromatase gene polypeptide A1 (CYP19A1) and nuclear receptor interacting protein 1 (NRIP1) (Oterino, Toriello et al. 2008) in case-control cohorts and in pedigrees. The ESR1 C325G locus was genotyped and the ESR1 gene emerged as the strongest candidate associated with migraine, a result which is consistent with previous association studies. The other four genes were tested in migraine because they had not been previously included and they are steroid hormone genes which converge in the estrogenic pathway. FSHR and ESR2 showed less significant association in comparison to ESR1 (p<0.05) higher in the MA phenotype when analysed singularly. Not convinced by this result the authors analysed the interaction of ESR2-ESR1-FSHR loci together. The best genetic model indicated an interaction in these loci expressed as a risk Haplotype. Two haplotypes were identified only one of which nearly doubled the risk (OR=1.97) for migraine more so in MA and was replicated in family studies. Studying the interaction of genes together is a more powerful approach to understanding the mechanism of gene interactions in disease.

The inconsistencies in association results of hormone receptors were addressed in a review and meta-analysis by Schurks, et al., 2010 (Schurks, Rist et al. 2010c). This study included 8 published articles following criteria for reviewing genetic association studies and identified the ESR1 G594A and C325G SNPs as contributors to migraine with pooled Odds Ratios yielding greater statistical significance. The variants increase migraine risk by 40-60% (Schurks, Rist et al. 2010c). In contrast the PGR PROGINS variant was not associated. Polymorphisms in ESR1 and PGR may increase migraine prevalence only in some populations demonstrating an ethnic-specific effect.

Association of markers in the ESR1 and PGR genes and migraine susceptibility has more recently been demonstrated in a study by Rodriguez-Acevedo et al., 2013 examining a large pedigree of 285 related individuals from the Norfolk Island population (135 males; 150 females) (Rodriguez-Acevedo, Maher et al. 2013). Although no association was detected in PGR, ten markers in the ESR1 gene showed association with migraine (p<0.05) and haplotype analysis revealed three haplotypes in ESR1 to be associated with migraine (p<0.004, 0.03, 0.005) (Rodriguez-Acevedo, Maher et al. 2013). In addition a couple of SNPs showed marginal association with the SYNE1 gene (a neighbour gene to ESR1). SYNE1 encodes a spectrin repeat-containing protein expressed in skeletal and smooth muscle, and
peripheral blood lymphocytes that localises to the nuclear membrane and in some other vascular tissues (Rodriguez-Acevedo, Maher et al. 2013). This gene may be worth investigating further given that it has been associated with bipolar disorder, recurrent major depression (Green, Grozeva et al. 2013) and ovarian cancer (Doherty, Rossing et al. 2010) and that mutations in this gene have been associated with autosomal recessive spinocerebellar ataxia 8, also referred to as autosomal recessive cerebellar ataxia type 1 or recessive ataxia of Beauce (Gros-Louis, Dupre et al. 2007; Noreau, Bourassa et al. 2013).

A less studied gene is the Aromatase gene, \textit{CYP19A1} which codes for a monooxygenase enzyme that catalyzes numerous reactions in drug metabolism and synthesis of cholesterol, steroids and other lipids and importantly the last steps of estrogen biosynthesis. Ghosh, et al., 2012 recently studied polymorphisms in the Aromatase gene, \textit{CYP19A1} (rs10046 and rs4646); estrogen receptors, \textit{ESR1} (rs2234693, rs1801132, rs2228480 and rs9340799) and \textit{ESR2} (rs1271572 and rs1256049) polymorphisms (Ghosh, Joshi et al. 2012). From this study the authors concluded that polymorphisms in \textit{CYP19A1} show stronger involvement in migraine susceptibility instead of polymorphisms in estrogen receptors. This is contrary to the majority of studies which support the involvement of estrogen receptors in migraine in genetic isolates and out-bred populations and therefore cannot be taken seriously. Due to aromatase’s role in estrogen biosynthesis it is plausible that aromatase dysfunction caused by gene mutation could lead to a decrease in estrogen synthesis, this hypothesis is worth investigating in future studies in different ethnic groups to produce a clearer picture of the role of aromatase and the hormone estrogen in the pathobiology of migraine. Particularly as estrogen influences serotonergic tone in women and serotonergic and glutamatergic systems.

In trying to understand the effect of hormones in migraine some researchers have focussed on migraine subtypes occurring around menstruation. The International Headache Society (IHS) now recognizes and has included ‘candidate’ criteria for pure menstrual migraine without aura (PMM) and menstrually related migraine without aura (MRM) in the appendix of their International Classification of Headache Disorders, 3rd Edition (IHS 2013). This classification modality specifically requires attacks of migraine to occur within a 5-day menstrual window (Loder 2005). In the case of PMM, attacks must occur only around the time of the month in at least two out of three menstrual cycles to establish a pattern that is greater than by chance alone whereas in MRM attacks can additionally occur at other times outside of menstruation (IHS 2013).
Focussing on an “enriched” study group of patients with “menstrual migraine” as highlighted by Colson et al., 2010, whose migraine closely coincides with the hormonal cycle may be the way to go for the future to interpret the relationship between migraine and hormones (Colson, Fernandez et al. 2010). Hershey et al., 2012 investigated gene expression in menstrual-related migraine patients (MRM), non-MRM and controls using blood and an affimetrix human exon ST 1.0 array (Hershey, Horn et al. 2012). This study identified 279 genes differentially expressed for MRM: many of the genes were functionally related having known immune or mitochondrial functions, but only a few were hormone-related. The study supports the notion that although overlaps do exist, MRM patients possess a different genomic expression profile to non MRM individuals, however further validation in other subject groups is required (Hershey, Horn et al. 2012). Although hormones clearly play a role in migraine how they contribute at a physiological level to migraine mechanisms is not well understood and complicated by the complex interactions they have with a number of physiological systems.

2.5.5 Mitochondrial Pathways

Migraine research has recently taken a new direction by investigating genes of the mitochondrial genome. The evidence supporting the idea of mitochondrial involvement in migraine has accumulated from a variety of biochemical, imaging, morphological and genetic studies. Together the data have raised an interesting hypothesis that a dysfunction of mitochondrial energy metabolism could account for the pathogenesis of some subtypes of migraine. The argument put forth is that decreased energy production brought about by defects in oxidative phosphorylation (OXPHOS) may contribute to an energy imbalance in migraine via a decrease in the threshold for cortical spreading depression.

OXPHOS is an important metabolic pathway for the production of energy in the form of adenosine tri-phosphate (ATP) that occurs in the mitochondria (Scholte 1988). Although ATP is normally produced through oxidative phosphorylation it can also be produced via the creatine kinase reaction by transfer of inorganic phosphate (Pi) from phosphocreatine (PCr) to adenosine diphosphate (ADP) (Reyngoudt, Achten et al. 2012). This metabolic shift occurs when mitochondrial enzymes are saturated or during anaerobic conditions and is considered a measure of mitochondrial efficiency (Reyngoudt, Paemeleire et al. 2011a).
Cells highly dependent on ATP production have the most mitochondria these include neurons and muscle cells and as a result these tissues are the first to be affected in mitochondrial disorders (Scholte 1988).

Mitochondria are strictly maternally inherited due to the fact that the mammalian egg at fertilization contributes about 100,000 of its mitochondria from its large cytoplasm to the burgeoning zygote (Wallace, Brown et al. 1999). The inner folds (crystae) of mitochondria organelles are rich in proteins/enzymes due to their important function in ATP production. More than 1,000 nuclear encoded genes affect mitochondrial function and are currently being investigated as potential candidates in migraine. The majority of proteins in the mitochondria are actually encoded and produced by the nuclear genome and have to be imported via membrane receptor proteins. A small minority of proteins are encoded by the mitochondrial genome and are essential subunits of the mitochondrial energy-generating enzymes of oxidative phosphorylation (Wallace, Brown et al. 1999). The mitochondrial genome is comparatively smaller than the nuclear genome measuring only 16.6 kilobases (kb) of DNA that codes for a total of 37 genes (Wallace, Brown et al. 1999). This circular genome codes for ~13 proteins of the OXPHOS enzyme complexes, 22 tRNA genes and 2 rRNA genes (Saxena, de Bakker et al. 2006). The mitochondrial genome is also fascinating to study from a population history point of view where it has been used as a blue print for the reconstruction of ancient lineages of women and the migration of human populations from different continents.

The brain in particular has a high energy demand and is sensitive to reduced energy production. Pathogenic mutations affecting mitochondrial function can impair energy metabolism and ion homeostasis in neurons resulting in a range of downstream abnormalities, the full extent of which is not yet characterized or fully understood. Certain mitochondrial encephalopathies show clinical resemblance to migraine these include lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres (MERRF) and Kearns Sayre syndrome and the Leber's hereditary optic neuropathy (LHON) (Tanji, Kunimatsu et al. 2001). The majority of these mitochondrial disorders lead to an “energy deficiency” state and some manifest with migraine headache (Saxena, de Bakker et al. 2006).

Morphological and biochemical studies have showed a reduction in the metabolism of cellular energy in different tissues, including brain. Specific morphologic changes in the
mitochondria have been detected; these include ragged red fibres (RRFs) with an abnormal number of sarcolemmal mitochondria and cytochrome c oxidase (COX) negative fibres with a higher fat content in the skeletal muscle of some patients with migraine with prolonged aura and FHM patients (Montagna, Sacquegna et al. 1988; Bresolin, Martinelli et al. 1991; Uncini, Lodi et al. 1995). Biochemical evidence has shown levels of lactic acid in blood and CSF to be higher in migraine patients (Sparaco, Feleppa et al. 2006). Lactic acid in blood is used as a biomarker of metabolic dysfunction and is typically elevated in mitochondrial disorders. Additionally evidence of reduced activity of several mitochondrial enzymes in the mitochondria of platelets of migraineurs supports the involvement of mitochondria in migraine (Littlewood, Glover et al. 1984; Sangiorgi, Mochi et al. 1994; D’Andrea and Leon 2010). The consequence of these findings has led to the therapeutic administration of Nutraceuticals acting on mitochondrial metabolism. Magnesium, co-enzyme Q10 and riboflavin which are enzyme co-factors known to affect components of the respiratory chain have shown some promise (Sandor and Afra 2005; Sandor, Di Clemente et al. 2005; Parikh, Saneto et al. 2009; Pringsheim, Davenport et al. 2012). Additionally supplementation with vitamins B6, B12 and folate B9 have shown a decrease in severity and frequency of MA attacks (Lea, Colson et al. 2009; O’Brien and Hershey 2010).

More recently non-invasive imaging techniques, such as magnetic resonance spectroscopy (MRS) have been employed in several studies to quantify in vivo energy metabolism and cerebral metabolites in brain and muscle of migraine patients (Bresolin, Martinelli et al. 1991; Uncini, Lodi et al. 1995; Reyngoudt, Paemeleire et al. 2011a; Reyngoudt, Paemeleire et al. 2011b). Phosphorus-MRS (P-MRS) measurements of phosphorylated metabolites including Pi, PCr, low-energy phosphates (ADP) and high energy phosphates adenosine triphosphate (ATP) from migraine patients reveal a lower ratio of PCr/Pi correlating with less available energy in neurons (Trabka-Janik, Kulaga et al. 2010; Reyngoudt, Paemeleire et al. 2011a; Reyngoudt, Achten et al. 2012). A further marker characteristic of a disturbance in energy metabolism is the accumulation of lactate (Lac) during anaerobic glycolysis due to inefficient pyruvate metabolism resulting in lactic acidosis a condition characterized by low pH in body tissues and blood and proton 1H MRS (H-MRS) studies have detected high levels of lactate in patients with migraine (Watanabe, Kuwabara et al. 1996). Therefore, MRS studies have revealed a lower energy metabolism in brain and muscle of migraine patients which is possibly linked to mitochondrial defects. These studies hint at a mitochondrial component in migraine however it is unclear at this stage if these differences are due to a
primary mitochondrial dysfunction or secondary to alterations in brain excitability and thus further studies utilizing consistent methodology in larger homogenous populations are needed (Reyngoudt, Achten et al. 2012).

Mutations associated with mitochondrial encephalopathies have been investigated for association in migraine susceptibility however so far no specific mt polymorphisms have been associated with migraine. The most interesting study by Zaki et al., 2009 found two polymorphisms in the mitochondrial genome C16519T and G3010A associated with migraine and cyclic vomiting syndrome CVS patients (Zaki, Freilinger et al. 2009). Only a small number of genetic studies have examined the influence of mitochondrial DNA variants in migraine susceptibility and to date little has been concluded from these studies due to small sample size. A significant drawback of investigating mitochondrial variants in the population is in the recruitment of sufficient numbers of affected people and also the fact that they are often rare with low allele frequency makes it difficult to obtain statistically meaningful results. To overcome some of these obstacles it may be more fruitful to invest in full mitochondrial genome sequencing rather than funding small scale underpowered projects.

Current genetic studies are investigating possible mitochondrial dysfunction in migraine pathogenesis in the genetic isolate of Norfolk Island. Full mitochondrial genome sequencing using the Ion Torrent platform and employing statistical modelling methods are expected to reveal haplogroups and if mtDNA variants are associated with heritable migraine traits. Complete mitochondrial genomes are becoming more available in databases such as Mitomap, Mitoproteome, mtDBA, and Phylotree, in the last few years and are important for tracing human migration and settlements of populations. In summary a thorough examination of mtDNA and nuclear mitochondrial genes and epistatic interactions between the mitochondrial and nuclear genomes would help to address the question of whether a mitochondrial mechanism in migraine is at play.

2.5.6 Migraine Genome Wide Association Studies

Genome wide association studies (GWAS) allow scanning of the genome for gene regions associated with a disease without a priori assumptions about disease aetiology. GWAS has emerged as a powerful genotyping technique capable of genotyping millions of SNPs across the entire genome and involves screening for potential associations between SNPs and
complex diseases using DNA from people affected with a disease (cases) and healthy individuals (controls). GWAS studies have been possible thanks to the completion of the Human Genome Project, International HapMap Project, the development of dense genotyping chips and the availability of biobanks, repositories of human genetic material (Lewis, Nsoesie et al. 2011). A GWAS is conducted using SNP arrays, the most popular platforms currently being Illumina and Affimex (Lindquist, Jorgenson et al. 2013).

The first migraine GWAS was of European migraineurs (Anttila, Stefansson et al. 2010) and identified one SNP that reached genome wide significance, this was susceptibility variant rs1835740 at locus 8q22.1 (Anttila, Stefansson et al. 2010) (Table 3). This marker rs1835740 is located between the PGCP and MTDH genes, both of which affect the accumulation of glutamate at the synapse. The authors tested the marker’s effect on gene expression by using fibroblasts, primary T cells, and LCLs from umbilical cords in an attempt to propose a mechanism to explain its role in migraine. They showed the risk allele A of this marker to be associated with higher levels of expression of MTDH. MTDH in turn down-regulates EAAT2 gene: a protein responsible for removing glutamate from synapses in the brain. Consequently rs1835740 may contribute to migraine through its effect on MTDH and EAAT2. Reduced activity of EAAT2 may lead to too much extracellular glutamate which may increase susceptibility to CSD.

The second migraine GWAS conducted a meta-analysis of six European cohorts. The most interesting finding from this study is the SNP rs9908234 in the nerve growth factor receptor (NGFR) gene with (P-value 8.00x10^-8). The association of this SNP was not replicated in 3 cohorts in the Netherlands and Australia suggesting that population genetics is more complex than expected and hence the need for replication cohorts to validate potential associations (Ligthart, de Vries et al. 2011). The reason provided by the authors for lack of replication of this SNP is insufficient power due to the nature of the population-based cohorts employed. The cohort used in the study was heterogeneous consisting of patients with variable severity of migraine and a smaller genetic component which the authors argue may prohibit replication. This study also identified modest support for association with the MTDH gene (astrocyte elevated gene 1 or AEG-1) previously associated in the Antilla, et al., 2010 study.

The third GWAS was conducted in a cohort of European women from the Women’s Genome Health Study (WGHS) including 5,122 migraineurs and 18,108 controls (Chasman, Schurks
et al. 2011). This is the largest migraine GWAS undertaken with a sample size of 23,230. Three new loci were identified, rs2651899 (1p36.32, PRDM16), rs10166942 (23q37.1, TRPM8) rs11172113 (12q13.3, LRP1) (Chasman, Schurks et al. 2011). Little is known about the function of PRDM16 and how it relates to migraine. TRPM8 belongs to the TRP super family of channels in nerves innervating the skin and mouth and that are activated by sensation of cold temperatures and of chemical substances that evoke a cold sensation, such as menthol or eucalyptol and involved in pain pathways (Janssens and Voets 2011). (Julius 2013). LRP1 is a lipoprotein expressed in brain and vasculature that is co-localized with glutamate receptors in neurons and lends some support to the involvement of the neurotransmitter glutamate in migraine pathophysiology. In conclusion this study has identified 3 unique SNPs in different pathways, although how they each contribute to migraine remains to be determined.

The most recent GWAS was conducted in people of German and Dutch origin using the migraine without aura phenotype only (Freilinger, Anttila et al. 2012). The previously identified loci TRPM8 and LRP1 were replicated in this GWAS and two new loci MEF2D (myocyte enhancer factor 2D), a transcription factor that regulates neuronal differentiation and TGFBR2 (encoding transforming growth factor β receptor 2) were identified. Minor associations in two other genes PHACTR (Phosphatase Actin Regulating Protein-1) and ASTN2 were also reported. The PHACTR1 locus encodes a protein (a member of the PHACTR/scapinin family) highly expressed in brain which controls PP1 activity and F-actin remodelling and plays a role in the control of tube formation and endothelial cell survival (Jarray, Allain et al. 2011). It is unclear at this point what role this gene plays in migraine pathogenesis it could be vascular through endothelial dysfunction, or neuronal through aberrant synaptic transmission. The ASTN2 locus, encodes a neuronal protein that is expressed in the brain and functions in glial-guided migration and appears important for the development of the laminar architecture of cortical regions of the brain (Wilson, Fryer et al. 2010). Overall, replication and functional studies are needed to better understand the involvement and significance of these genes in migraine aetiology.

Although the contribution of GWAS findings to current knowledge of migraine has been modest with a total of six genomic regions found to be significantly and reproducibly implicated, further studies with larger sample groups will increase the power and help to reveal further genes that contribute to migraine. Importantly the genes that have been
identified via GWAS with respect to migraine to date are novel and offer new avenues to pursue. Although GWAS does not identify the causal variant involved at the detected locus or address gene function it can create new valid hypotheses (novel candidate genes) to direct research to study genes or molecular pathways with unexpected involvement in disease.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Variants Identified</th>
<th>CASES/CONTROLS</th>
<th>Origin of Samples</th>
<th>Replication Samples</th>
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<tbody>
<tr>
<td>Anttila et al. 2010</td>
<td>rs1835740 (between MTDH and PGCP)</td>
<td>2,731/10,747</td>
<td>Clinic-based; Finland, Germany, The Netherlands</td>
<td>$1.69 \times 10^{-11}$, OR 1.18, (1.13-1.24)</td>
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<tr>
<td>Ligthart et al. 2011</td>
<td>rs9908234 (NGFR)</td>
<td>2,446/8,534</td>
<td>Population-based; The Netherlands, Iceland</td>
<td>$8.00 \times 10^{-8}$</td>
</tr>
<tr>
<td>Chasman et al. 2011</td>
<td>rs2651899 (PRDM16) rs10166942 (TRPM8) rs11172113 (LRP1)</td>
<td>5,122/18,108</td>
<td>Population-based; US, European descent</td>
<td>$3.8 \times 10^{-9}$, OR 1.11, (1.07-1.15) $5.5 \times 10^{-12}$, OR 0.85, (0.82-0.89) $4.3 \times 10^{-9}$, OR 0.90, (0.87-0.93)</td>
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<tr>
<td>Freilinger et al. 2012</td>
<td>MEF2D     Rs1050316 Rs 2274316 Rs 1925950 Rs 3790455 Rs 3790459 Rs 12136856 TGFBR2 Rs 7640543 PHACTR1 Rs 9349379 ASTN2 Rs 6478241 TRPM8 Rs 10166942</td>
<td>2,326/4,580</td>
<td>German-Dutch individuals</td>
<td>$7.06 \times 10^{-11}$, OR 1.2, (1.14-1.27)</td>
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<td>$1.17 \times 10^{-9}$, OR 1.19, (1.13-1.26)</td>
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<td>$3.20 \times 10^{-8}$, OR 0.86, (0.81-0.91)</td>
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<td>$3.86 \times 10^{-8}$, OR 1.16, (1.10-1.23)</td>
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<td></td>
<td>$9.83 \times 10^{-13}$, OR 0.78, (0.73-0.84)</td>
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<tr>
<td>Rs 17862920</td>
<td>LRP1</td>
<td>Rs 11172113</td>
<td>2.97x10^8, OR 0.86, (0.81-0.91)</td>
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</table>
2.6 Conclusion and Future Directions

Migraine is a well-studied disorder that results from the interaction of multiple genes with environmental triggers. Physiological studies using new and non-invasive imaging techniques have led to a clearer understanding of migraine pathophysiology providing insights into the basic functioning of the neuronal and vascular systems and how they interact to produce the full spectrum of migraine symptoms. However the origin of the pain experienced during migraine episodes is still widely debated. Similarly genetic studies have produced a large list of genes implicated in migraine, but how they actually participate in migraine processes is still poorly understood for the majority of them leaving many gaps in the jigsaw to be filled. High throughput genotyping and next generation sequencing techniques will greatly enhance the study of migraine genetics in the near future. In particular exome and full genomic sequencing will make detecting mutations involved in migraine that show strong familial inheritance an attainable goal. Future GWAS conducted with larger numbers of samples, will most likely lead to the discovery of more genes of small effect that potentially contribute to the risk of developing migraine and identify genes specific to migraine subgroups, such as MO, MA and MRM. Although there are some effective anti-migraine therapies available and more being developed, their mechanisms of action are not completely known and it is therefore not understood why they do not work for some individuals. More complete understanding of the molecular pathways involved and the relevant genomic profile of migraineurs will aid in the development of new anti-migraine drugs and treatments, and or enable those currently available to be better targeted to suit individuals. Finally, because migraine is a complex disorder with no identifiable pathology the best way to move forward is with a multidisciplinary approach incorporating results from emerging imaging techniques, biochemical, pharmacologic and genetic studies in order to better understand the genetic basis of this debilitating disease.
CHAPTER 3  The Biology of the Glutamatergic System and Potential role in Migraine

3.1 Abstract

Migraine is a common genetically linked neurovascular disorder. Approximately ~12% of the Caucasian population are affected including 18% of adult women and 6% of adult men. A notable female bias is observed in migraine prevalence studies with females affected ~3 times more than males and is credited to differences in hormone levels arising from reproductive achievements. Migraine is extremely debilitating with wide-ranging socioeconomic impact significantly affecting people’s health and quality of life. Genetic research has implicated the neurotransmitters serotonin and dopamine in migraine. The biology of the Glutamatergic system in migraine is the least studied however there is mounting evidence that its constituents could contribute to migraine. Glutamate is an abundant neurotransmitter that mediates excitatory signals in the mammalian central nervous system and plays a part in cognition, memory and learning. The availability of antagonists that selectively block glutamate receptors has facilitated studies on the physiologic role of glutamate, on one hand, and simultaneously provided avenues to pursue the potential therapeutic applications of glutamate receptor antagonists in diverse neurologic diseases. In this brief review, we discuss the biology of the Glutamatergic system in migraine outlining recent findings that support a role for altered Glutamatergic neurotransmission from genetic studies in the manifestation of migraine and the implications of this on migraine treatment.
3.2 Introduction

Migraine is a complex debilitating neurovascular disorder, characterized by recurrent attacks of headache that differ in intensity, frequency and duration. The headache is often accompanied by an assortment of symptoms which can include nausea, emesis, photophobia, phonophobia, and occasionally, visual sensory disturbances. Migraine is estimated to affect approximately 12% of the Caucasian population (Lipton, Bigal et al. 2007) and shows a marked female preponderance (~3:1) attributed to the influence of hormones. Migraine imparts significant mental, physical and social health burden to sufferers and their families.

Most migraineurs possess a number of genes that together contribute to susceptibility. Thus far genetic linkage and association studies have implicated some causative mutations that are of significant clinical relevance. However not all migraine genes have been uncovered and further research is necessary to determine the definitive molecular genetics of migraine. Numerous theories regarding the causes and underlying mechanisms that result in migraine symptoms have also been proposed.

The pathophysiology of this disorder implicates both neurological and vascular mechanisms. Current research suggests that the trigeminovascular system plays a significant role in migraine (Parsons and Strijbos 2003; Lambert and Zagami 2008; Messlinger 2009) due to its critical interaction with the meningeal vasculature and because various neurotransmitters, peptides, receptors and transporters are located in this system. The main neurotransmitters implicated in migraine pathogenesis are: serotonin, dopamine and glutamate. An alteration in the balance of any of these neurological systems may lead to a higher susceptibility to migraine. Serotonin and dopamine remain the most studied neurotransmitter circuits in case-control association studies investigating polymorphisms in receptors, transporters, and enzymes of these systems (Akerman and Goadsby 2007; Hamel 2007).

Migraine has a strong inherited component, and a large GWAS study (Anttila, Stefansson et al. 2010) suggests the involvement of glutamate pathways in migraine pathogenesis. Glutamate is implicated in elements of the pathophysiology of the disorder, including trigeminovascular activation, central sensitization and cortical spreading depression. Biochemical and pharmacological studies also support the involvement of glutamate in

3.3 Glutamatergic Biology

The pioneering work of Hayashi in 1954 established the physiological significance of glutamate as an excitatory neurotransmitter (Hayashi 1954). Hayashi demonstrated glutamate’s role as a neurotransmitter in the central nervous system (CNS) in experiments in dogs, monkeys and men, where injection of monosodium glutamate into the grey matter of the cortex was found to produce clonic convulsions (Hayashi 1954).

The brain contains large amounts of glutamate a common amino acid that cannot cross the blood-brain barrier but that is cycled from an exogenous to an endogenous environment at the synapse and plays a key role in cellular metabolism (Vikelis and Mitsikostas 2007). The majority of glutamate is found intracellularly and readily synthesized in neurons from the precursor glutamine by the mitochondrial enzyme phosphate-activated glutaminase (McKenna 2007). The precursor glutamine is synthesized in astrocytes (a type of glial cell), released into the extracellular space and taken up by neurons for synthesis back into glutamate (Purves 2001). This continuous cycling of glutamate which occurs between neurons and astrocytes of the CNS synaptic circuitry is described as the glutamate-glutamine cycle (McKenna 2007). The glutamate-glutamine cycle interfaces freely with many other metabolic pathways and as a result different precursors besides glutamine arise that can be used in the synthesis of glutamate (see Figure 3.1).

In the brain glutamate has a number of metabolic fates, including oxidation via the tricarboxylic acid (TCA) cycle for energy, incorporation into proteins and formation of glutamine, γ-aminobutyric acid (GABA) and glutathione (McKenna 2007). Glutamate is found in neurons of structures related to migraine pathophysiology, including the trigeminal ganglion, trigeminocervical complex and the thalamus (Kaikai and Howe 1991). The neuronal-glial cell interface where glutamate cycling occurs contains a number of enzymes, glutamate receptors and transporters (see Table 3.1, 3.2, 3.3). Together these components form the biological constituents of the Glutamatergic system which enable fast excitatory synaptic transmission. The Glutamatergic system of the brain is one of the two major amino acid systems, GABA being the other. A deficiency in Glutamatergic
neurotransmission due to genetic mutation in the biological constituents of the Glutamatergic system may result in a disturbed balance of synaptic activity and may play a key role in the pathophysiology of migraine.

Table 3.1   Enzymes involved in glutamate cycling.

<table>
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<th>Enzymes</th>
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<tr>
<td>PDH, pyruvate dehydrogenase</td>
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<tr>
<td>PAG, phosphate activated glutaminase</td>
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<tr>
<td>mME, mitochondrial malic enzyme</td>
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<tr>
<td>GAD, glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GS, glutamine synthetase</td>
</tr>
<tr>
<td>PC, pyruvate carboxylase</td>
</tr>
<tr>
<td>cME, cytosolic malic enzyme</td>
</tr>
<tr>
<td>AAT, aspartate aminotransferase</td>
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<tr>
<td>GDH, glutamate dehydrogenase</td>
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</table>
Figure 3.1 A simplified scheme of the metabolism of glutamate and glutamine in astrocytes and neurons in brain sourced from (McKenna 2007).
3.4 Glutamate Toxicity and Neurodegeneration

Following the pioneering work of Hayashi further experiments driven by Lucas and Newhouse in 1957 (Lucas and Newhouse 1957) described the toxic properties of glutamate by injecting monosodium glutamate into the neurons in the inner layers of the retina of newborn mice. The term ‘glutamate excitotoxicity’ was introduced by Olney (Olney 1969) about ten years later to describe the neurotoxic properties of glutamate to the CNS and the incurred neuronal cell death.

Excess levels of glutamate can be neurotoxic and result in excitotoxicity a pathological process by which glutamate and related compounds destroy neurons by prolonged overstimulation of glutamate receptors and is considered a normal physiologic response to CNS insult (Danysz, Parsons et al. 1995). The excessive activation of neuronal glutamate receptors can literally excite neurons to death and lead to neurodegeneration.

The mechanism of this excitotoxicity is thought to be due to the entry of high levels of Ca$^{2+}$ ions into the cell which trigger a chain of events that directly activate catabolic enzymes, including phospholipases, endonucleases, and proteases such as calpain which in turn can damage cell structures such as components of the cytoskeleton, membrane and DNA (Farooqui and Horrocks 2004) due to over stimulation with glutamate (Choi 1985).

These events may be responsible for neuronal injury and neurodegenerative diseases such as Multiple sclerosis, Alzheimer's disease, Amyotrophic lateral sclerosis (ALS), Fibromyalgia, Parkinson's disease, and Huntington's disease (Bittigau and Ikonomidou 1997; Lau and Tymianski 2010). Excitotoxic mechanisms have also been shown to be involved in other acute forms of neuronal insult, including hypoglycemia, trauma, and repeated intense seizures (called status epilepticus). There are important implications to understanding the mechanism of excitotoxicity for treating a variety of neurological disorders.
3.5 Glutamate Receptors

Glutamate is a plentiful excitatory amino acid neurotransmitter in the brain. The transmission of Glutamatergic signals within CNS circuits is dependent upon two main subtypes of membrane receptors, ionotropic and metabotropic. The family of ionotropic receptors was the first characterized and is classified into three groups, referred to as N-methyl-D-aspartate (NMDA) receptors, \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, and kainate (KA) receptors on the basis of DNA sequence similarity and their activation by different pharmacologic agonists (Table 3.2) (Vikelis and Mitsikostas 2007). The ionotropic glutamate receptor (iGluR) subtypes, NMDA, KA, and AMPA form ligand-gated ion channels, which are activated on a fast time scale (msec) and are permeable to (Gasic and Hollmann 1992). Each subunit possesses four transmembrane hydrophobic domains (TM1-4) within the central portion of the sequence.

The family of metabotropic receptors (mGluRs) consist of at least eight receptor types also divided into three groups (see Table 3.2). These receptors differ from ionotropic receptors in that they are coupled to G-proteins which activate intracellular biochemical cascades, leading to the modification of other proteins, as for example ion channels and operate on a time scale of several hundred milliseconds to seconds (Gasic and Hollmann 1992). Metabotropic receptors are composed of seven transmembrane domains that span the cell membrane (Julio-Pieper, Flor et al. 2011).

X-ray crystal structures have produced detailed 3D images of glutamate receptors revealing the structural basis of glutamate receptors in particular which domains are involved in binding to agonists and antagonists so as to study the effect of genetic mutations on protein conformation to better understand the structure-function relationship and how this relates to neurological disease. So far a number of structures have been described for a membrane-spanning tetrameric glutamate receptor as well as in complex with various agonists, antagonists, and modulators (Tsuchiya, Kunishima et al. 2002; Mayer and Armstrong 2004; Holm, Lunn et al. 2005; Kinarsky, Feng et al. 2005; Mayer 2005; Jin, Singh et al. 2009; Sobolevsky, Rosconi et al. 2009). These experiments have been possible due to advances in molecular modelling and structural biology techniques. These data, along with functional and biochemical experiments, have begun to define the molecular relationship between
receptors and their binding sites and have contributed to understanding the neurotransmitter binding mechanisms at the synapse.

Dysfunction of glutamatergic pathways has been implicated in several CNS disorders such as epilepsy, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis, lathyism, AIDS encephalopathy and dementia complex, and Alzheimer's disease (Gasic and Hollmann 1992). Although linkage with a known genetic disorder has not been established, Glutamatergic receptors are principal candidates for maintaining if not initiating several of these disease processes and it is interesting to note that a number of neurological disorders are accompanied by the appearance of antibodies to glutamate receptor subunits or autoantigen activity (e.g. GluR3 in Rasmussen's encephalitis) (Dingledine, Borges et al. 1999).

Table 3.2 Ionotropic glutamate receptors.

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPA</td>
<td>GRIA1</td>
<td>5q33</td>
</tr>
<tr>
<td>AMPA</td>
<td>GRIA2</td>
<td>4q32-33</td>
</tr>
<tr>
<td>AMPA</td>
<td>GRIA3</td>
<td>Xq25-26</td>
</tr>
<tr>
<td>AMPA</td>
<td>GRIA4</td>
<td>11q22-23</td>
</tr>
<tr>
<td>Kainate</td>
<td>GRIK1</td>
<td>21q21.1-22.1</td>
</tr>
<tr>
<td>Kainate</td>
<td>GRIK2</td>
<td>6q16.3-q21</td>
</tr>
<tr>
<td>Kainate</td>
<td>GRIK3</td>
<td>1p34-p33</td>
</tr>
<tr>
<td>Kainate</td>
<td>GRIK4</td>
<td>11q22.3</td>
</tr>
<tr>
<td>Kainate</td>
<td>GRIK5</td>
<td>19q34.3</td>
</tr>
<tr>
<td>NMDA</td>
<td>GRIN1</td>
<td>9q34.3</td>
</tr>
<tr>
<td>NMDA</td>
<td>GRIN2A</td>
<td>16p13.2</td>
</tr>
<tr>
<td>NMDA</td>
<td>GRIN2B</td>
<td>12p12</td>
</tr>
<tr>
<td>NMDA</td>
<td>GRIN2C</td>
<td>17q24-q25</td>
</tr>
<tr>
<td>NMDA</td>
<td>GRIN2D</td>
<td>19q13.1qter</td>
</tr>
<tr>
<td>NMDA</td>
<td>GRIN3A</td>
<td>9q31.1</td>
</tr>
<tr>
<td>NMDA</td>
<td>GRIN3B</td>
<td>19p13.3</td>
</tr>
<tr>
<td>Orphan</td>
<td>GRID1</td>
<td>10q22</td>
</tr>
<tr>
<td>Orphan</td>
<td>GRID2</td>
<td>4q22</td>
</tr>
</tbody>
</table>
### Table 3.3  Metabotropic glutamate receptors.

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGluR1</td>
<td>GRM1</td>
<td>6q24</td>
</tr>
<tr>
<td>mGluR5</td>
<td>GRM5</td>
<td>11q14.3</td>
</tr>
<tr>
<td>mGluR2</td>
<td>GRM2</td>
<td>3p21.2</td>
</tr>
<tr>
<td>mGluR3</td>
<td>GRM3</td>
<td>7q21.1-q21.2</td>
</tr>
<tr>
<td>mGluR4</td>
<td>GRM4</td>
<td>6p21.3</td>
</tr>
<tr>
<td>mGluR6</td>
<td>GRM6</td>
<td>5q35</td>
</tr>
<tr>
<td>mGluR7</td>
<td>GRM7</td>
<td>3p26-p25</td>
</tr>
<tr>
<td>mGluR8</td>
<td>GRM8</td>
<td>7q31.3-q32.1</td>
</tr>
</tbody>
</table>

### 3.6 RNA Editing of Glutamate Receptors

Some neurotransmitter receptors undergo post-transcriptional editing prior to being fully expressed. The genetic mechanism known to do this is called RNA editing. This is relevant to the GluR2 subunit (encoded by the GRIA2 gene) of the AMPA receptor which is edited at a specific point that affects the Ca$^{2+}$ permeability of the channel (Maas, Kawahara et al. 2006). The position that regulates the Ca$^{2+}$ permeability is the Q/R site and this is edited by the ADARB1 enzyme. ADARB1 is an RNA editing enzyme that performs the RNA editing function necessary for the maturation of glutamate and serotonin receptor transcripts and therefore plays an important role in the regulation and fine tuning of Glutamatergic neurotransmission (Maas, Rich et al. 2003). ADARB1 belongs to the family of ADAR enzymes which are generated in humans by three independent RNA editing genes ADAR1, 1q21.3, ADAR2, 21q22.3 and ADAR3 10p15 (Hogg, Paro et al. 2011). The enzymatic activity of ADARB1 leads to the chemical modification of adenosine residues to inosines in specific coding regions which are translated as if it were guanosine by the cell’s translational machinery (Nishikura 2010).

RNA editing is a physiologically important process that affects several features of neurotransmitter receptors, including kinetics, subunit assembly and cell-surface expression. If RNA editing is prevented the channels become increasingly permeable to Ca$^{2+}$ causing neuronal cell death (Maas, Kawahara et al. 2006; Hogg, Paro et al. 2011). Genes affected by A-to-I RNA editing include neurotransmitter transcripts of the glutamate receptor subunits GluR-2, -3, -4, and GluR5 and GluR6 (Kohler, Burnashev et al. 1993; Lomeli, Mosbacher et al. 1994; Barbon and Barlati 2011) and the serotonin receptor subtype 5-HT$_{2C}$ where editing is known to regulate G-protein coupling functions of the receptor and the human K(V)1.1
potassium channel where editing regulates channel inactivation (Bhalla, Rosenthal et al. 2004; Maas, Kawahara et al. 2006).

RNA editing genes have been suggested as candidate genes for complex neurological disorders such as epilepsy, depression and schizophrenia and amyotrophic lateral sclerosis (ALS). Knockout mice in RNA editing genes are lethal implying that this mechanism is essential for survival and that deregulation could potentially affect Glutamatergic function. The role of RNA editing of glutamate and serotonin receptor transcripts is further exemplified by the disorder, amyotrophic lateral sclerosis (ALS) (MIM 105400). In amyotrophic lateral sclerosis (ALS) editing of mRNA encoding the GluR2 subunit of glutamate AMPA receptors in spinal motor neurons is defective and interferes with normal functioning of the glutamate receptors. Genes involved in RNA editing (ADAR-1-3) are candidate genes to investigate due to the impact that this mechanism has on neurotransmitter transcripts.

### 3.7 Glutamate Transport

The concentration of glutamate in both the extracellular and intracellular space under normal conditions is kept low and is tightly regulated by neurotransmitter transporters located around the synapse in the plasma membranes of astrocytes and to a lesser degree, neurons. Glutamate transporters are divided into two superfamilies according to their structure and site of action: the plasma membrane transporters (EEATs) and the vesicular transporters (VGLUTs) (Table 3.4) (Liguz-Lecznar and Skangiel-Kramska 2007). These two transporter families differ in many of their functional properties as will be discussed.

EAATs are located in the plasma membrane of both nerve endings and surrounding astrocytes and are primarily responsible for the termination of the neurotransmitter action of glutamate and the prevention of neuronal damage due to excessive activation of glutamate receptors (Minami, Matsumura et al. 2001). EAATs import extracellular glutamate into cells at the synapse by a high-affinity sodium-dependent process (Tremolizzo, DiFrancesco et al. 2006). The re-uptake of excess glutamate at the synaptic cleft by EAATs is essential to recycling the amino acid pool and replenishing the supply of neurotransmitter in Glutamatergic terminals (Seal and Amara 1999). Five different ‘high-affinity’ glutamate
transporters located in the plasma membrane have been identified by cloning including EAAT1, EAAT2, EAAT3, EAAT4 and EAAT5 (Seal and Amara 1999).

Alongside the EAATs are the vesicular glutamate transporters (VGLUTs) whose job is the translocation of glutamate into the lumen of pre-synaptic vesicles for intracellular storage (Liguz-Lecznar and Skangiel-Kramska 2007). Three VGLUT isoforms: VGLUT1, VGLUT2, VGLUT3 have been molecularly identified and functionally characterized to date (Table 3.4) (Benarroch 2010).

These two molecular transport systems are effective at protecting neurons from the accumulation of glutamate and its neurotoxic effect. Release of excess glutamate due to defective glutamate transport can cause hyperexcitability in post-synaptic neurons and cell death due to excitotoxicity (Foran and Trotti 2009). Genetic mutations in receptors, transporters and enzymes involved in glutamate homeostasis could contribute to derailed function of the Glutamatergic system and therefore these are also key candidates to target for future genetic studies.

Table 3.4  Glutamate transporters.

<table>
<thead>
<tr>
<th>Transporter Type</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAT1</td>
<td>SLC1A3</td>
<td>5p13</td>
</tr>
<tr>
<td>EAAT2</td>
<td>SLC1A2</td>
<td>11p13</td>
</tr>
<tr>
<td>EAAT3</td>
<td>SLC1A1</td>
<td>9p24</td>
</tr>
<tr>
<td>EAAT4</td>
<td>SLC1A6</td>
<td>19p13.12</td>
</tr>
<tr>
<td>EAAT5</td>
<td>SLC1A7</td>
<td>1p32.3</td>
</tr>
<tr>
<td>VGLUT1</td>
<td>SCL17A7</td>
<td>9q13.33</td>
</tr>
<tr>
<td>VGLUT2</td>
<td>SCL17A6</td>
<td>11p14.3</td>
</tr>
<tr>
<td>VGLUT3</td>
<td>SCL17A8</td>
<td>12q23.1</td>
</tr>
</tbody>
</table>
3.8 Migraine Molecular Genetics

Our understanding of ‘migraine genetics’ is an evolving subject due to its relative novelty in this disease system. The recent identification of a functional mutation in the TRESK gene is one example of the success of current genomic efforts. The TRESK gene codes for a potassium channel that is part of the subfamily K member 2 (K2P) channels, which are expressed throughout the central nervous system, including the trigeminal ganglion neurons (Lafreniere and Rouleau 2011). A number of mutations in this gene were identified using a candidate gene approach and functional analysis in a large cohort of both case-control individuals and multi-generational families by directly Sanger sequencing the DNA of a panel of 110 unrelated migraine probands (Lafreniere, Cader et al. 2010). An important frame shift mutation (F139WfsX24), which segregated perfectly in a family affected with typical MA and that completely inactivates the channel was the most interesting variant identified. The TRESK gene is involved in pain pathways and regulates neuronal excitability and is an exciting discovery because it is the first genetic mutation to be linked to common migraine (Wood 2010).

The identification of mutations in three ion channel genes that independently cause familial hemiplegic migraine (FHM) – \textit{CACNA1A}, \textit{ATP1A2} and \textit{SCN1A} has improved our knowledge of the mechanistic and genetic basis of headache development (Ophoff, Terwindt et al. 1996; De Fusco, Marconi et al. 2003; Dichgans, Freilinger et al. 2005). Familial Hemiplegic migraine (FHM) is a very severe, rare monogenic subtype of MA that is distinguished from typical migraine by its association with reversible hemiparesis, prolonged aura symptoms and a clear autosomal dominant mode of inheritance (Carrera, Stenirri et al. 2001). Transcription of the three FHM causal genes results in protein products that assemble to make heteromeric ion channels in the plasma membranes of cells. The mechanism by which mutations in FHM genes contribute to disease is thought to be via an increase of neuronal excitability and a reduction in the threshold for cortical spreading depression, the event known to cause aura symptoms in sufferers (Iizuka, Takahashi et al. 2012). Ion channel genes play a critical role in regulating the normal functioning of the central nervous system where they control important biological functions including the release of neurotransmitters, hormones and muscle contraction (D'Andrea and Leon 2010).
Ion channels maintain electrical currents across excitable membranes. Minute alterations in the amino acid sequence or expression of channel protein due to genetic mutations can result in changes affecting the biophysical properties of the channel such as permeation and gating (Pietrobon 2010a). Dysfunctional conductance of ion channels due to genetic mutation may result in a disturbed balance of synaptic activity which could pose significant consequences to normal physiological functioning in vivo and play a key role in the pathophysiology of many diseases. This is of significant consequence to neurological, retinal, cardiac, and muscular tissues that rely on fast signal transmission and gross pathological changes can lead to serious chronic disorders (D'Andrea and Leon 2010). Inherited mutations in ion channel genes give rise to a wide range of disorders broadly classified as ‘channelopathies’. Channelopathies is the term used to describe disorders caused by genetic mutations in ion channel genes, currently more than 40 different channelopathies exist including for example migraines, epilepsies and cardiac dysfunction all of which affect electrically excitable tissues including: brain, peripheral nerve, skeletal muscle, smooth muscle and heart (Cannon and D'Alessandro 2006; Cannon 2007). FHM is listed as a channelopathy because two causative genes encode voltage-gated ion channels and as a result has added evidence to support migraine being a disorder of ‘neuronal excitability’.

### 3.9 Glutamate Genetic Studies

Genetic association studies of migraine have mostly investigated variants in serotonin and dopamine receptor genes. Fewer studies have been done in relation to the genetics of the Glutamatergic system in migraine. Disease occurs when there are changes to the gating and conducting properties of a channel or transport processes become defective. High levels of extracellular glutamate are detrimental and may be linked to migraine through activation of cortical spreading depression the proposed phenomenon responsible for migraine aura (Sanchez-Del-Rio, Reuter et al. 2006; van den Maagdenberg, Pizzorusso et al. 2010). Therefore pharmacological manipulations aim to either enhance or inhibit these processes. These changes may manifest as mutations in the DNA sequence of the gene which change the amino acid sequence and consequently the 3D structure and conformation of the protein or may affect the expression and regulation of the protein. It is important therefore to characterize the genetic and expression profile of the molecular components of the glutamatergic system in future studies so that this information can be used to make structure-based drug design more targeted.
Formicola et al., 2010 provide the first genetic evidence of a link between migraine and the Glutamatergic system. In this study they genotyped SNPs in subunits of the ionotropic AMPA receptor (Formicola, Aloia et al. 2010). The ionotropic AMPA receptor is assembled from 4 subunits coded by the (GRIA1-4) genes at chromosomal loci 5q33, 4q32, Xq24 and 11q24 respectively (Vikelis and Mitsikostas 2007). The AMPA receptor forms a ligand-gated ion channel in the plasma membrane of nerve cells that is permeable to Na⁺, K⁺ and Ca²⁺ (Traynelis, Wollmuth et al. 2010). Formicola et al., 2010 found a positive association in two SNPs in GRIA1 (rs548294 MO allelic P=0.008, rs2195450 MA allelic P=0.0005) and one SNP in GRIA3 with MA (rs3761555 MA Females allelic P=0.003) (Table 3.5) (Formicola, Aloia et al. 2010).

The GRIA3 gene is located in a previously identified migraine susceptibility locus Xq24-28 by our laboratory in two large, multigenerational, independent, Australian Caucasian families (Nyholt, Dawkins et al. 1998; Nyholt, Curtain et al. 2000). Association of the GRIA3 promoter polymorphism (rs3761555) in the MA subgroup of migraineurs was replicated by Maher et al., 2013 in an Australian case-control cohort (Maher, Lea et al. 2013). In contrast Cargnin et al, 2013 were unable to replicate a positive association in the rs548294 polymorphism in the GRIA1 subunit reported by Formicola et al., 2010 and migraine without aura (Cargnin, Viana et al. 2013). More recently a study by Gasparini et al., 2013 genotyped SNPs in glutamate receptor subunits GRIA2 and GRIA4 and reported a negative association.

GWAS evidence by Anttila et al., 2010 has indirectly implicated the Glutamatergic system in migraine by identifying a marker localised to 8q22.1 (Anttila, Stefansson et al. 2010). The genetic risk variant (rs1835740) is located between the PGCP (Plasma Glutamate Carboxypeptidase) and MTDH (Astrocyte Elevated Gene 1, also known as AEG-1) genes, both of which affect the accumulation of glutamate at the synapse. The variant rs1835740 may contribute to migraine by down-regulating EAAT2 and thereby increasing levels of extracellular glutamate. Meta-analysis of GWAS results by Ligthart, et al., 2011 replicated the association with the MTDH gene (astrocyte elevated gene 1 or AEG-1) (Ligthart, de Vries et al. 2011). A further GWAS by Chasman et al., 2011 identified three distinct loci PRDM16, LRP1 and TRPM8 in the population-based Women’s Genome Health Study (WGHS) of migraine (Chasman, Schurks et al. 2011). LRP1 is a lipoprotein expressed in brain and vasculature that is co-localized with glutamate receptors in neurons and lends some
support to the involvement of the neurotransmitter glutamate in migraine pathophysiology. Together these studies provide support for further investigation of glutamate related genes.

In addition to glutamate receptors, glutamate transporters can contribute to neurologic dysfunction and could be useful molecular targets for treatment. EAATs transporters play a key role in the regulation of extracellular glutamate levels in the central nervous system where they protect neurons from excitotoxic damage (glutamate neurotoxicity). A number of studies have implicated EAATs in the pathophysiology of stroke, epilepsy, amyotrophic lateral sclerosis (ALS), Huntington Disease, HIV-associated dementia, malignant glioma, and other neurologic disorders (Benarroch 2010). EAAT1 or EAAT2 knockout mice demonstrate that lead to an accumulation of extracellular glutamate levels, neurodegeneration characterized by excitotoxicity, and progressive paralysis in rats (Foran and Trotti 2009). These models highlight the importance of glutamate transport processes in the CNS in maintaining homeostasis.

A de novo missense mutation P290R mutation in the SLC1A3 gene that codes the glutamate transporter; Excitatory Amino Acid Transporter (EAAT1) was identified in a single patient with episodes of ataxia, migraine, hemiplegia and seizures (Table 3.6) (Jen, Wan et al. 2005). This mutation in the EAAT1 transporter reduces transporter function and results in a decrease in glutamate uptake potentially contributing to neuronal hyperexcitability and resulting in the hemiplegia and other neurological disturbances described in the patient (Jen, Wan et al. 2005). De Vries et al., 2009 identified a novel pathogenic mutation C186S in the EAAT1 gene in one patient with Episodic Ataxia (EA). The mutated EAAT1 protein showed severely reduced uptake of glutamate and transporter dysfunction correlated with the severity of EA symptoms (de Vries, Mamsa et al. 2009). The syndrome was designated EA6 and shares overlapping clinical features with EA2, which is caused by mutations in CACNA1A the FHM locus.

An association study by Shin et al., 2011 evaluated the contribution of polymorphisms in the EAAT2 transporter and found no direct association between this genetic factor and migraine (Shin, Han et al. 2011). The EAAT2 transporter has been investigated in association with a number of other disorders given it is responsible for up to 90% of all glutamate transport in adult tissue (Jackson, Steers et al. 1999; Sander, Berlin et al. 2000; Pampliega, Domercq et al. 2008). A study by Mallolas and colleagues, 2006 has found a novel and highly prevalent
polymorphism in the promoter of the EAAT2 glutamate transporter gene (Mallolas, Hurtado et al. 2006). This polymorphism was associated with higher and maintained plasma glutamate concentrations as well as with higher frequency of neurological deterioration in patients with acute hemispheric stroke. In conclusion, this study has revealed a novel functional polymorphism in the EAAT2 promoter region and a pattern of regulation that decreases promoter activity in patients with stroke. This is of significance in migraine due to the co-morbidity of the two disorders.

The remaining subfamilies of kainate (KA) and N-methyl-D-aspartate (NMDA) and metabotropic receptors are yet to be investigated in migraine association studies. It is noteworthy that genes of the Glutamatergic system have also been investigated in association studies of other neurological disorders like schizophrenia given their neuronal role (Carter 2006; Magri, Gardella et al. 2008; Chaki and Hikichi 2011). Future research may focus on a broader range of genes associated with Glutamatergic biology for example enzymes involved in cycling glutamate to ascertain its potential role in migraine susceptibility and aetiology.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Reference</th>
<th>Ethnicity</th>
<th>Controls/Cases</th>
<th># SNPs</th>
<th>Associated SNPs</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRIA1</td>
<td>5q31.1</td>
<td>(Formicola, Aloia et al. 2010)</td>
<td>Italian</td>
<td>260/250</td>
<td>6</td>
<td>rs2195450, rs548294</td>
<td>$P=0.00002$ (MA) $P=0.0003$ (MO)</td>
</tr>
<tr>
<td>GRIA2</td>
<td>4q32.1</td>
<td>(Formicola, Aloia et al. 2010)</td>
<td>Italian</td>
<td>260/250</td>
<td>1</td>
<td></td>
<td>$P=0.003$</td>
</tr>
<tr>
<td>GRIA3</td>
<td>Xq25</td>
<td>(Formicola, Aloia et al. 2010)</td>
<td>Italian</td>
<td>260/250</td>
<td>8</td>
<td>rs3761555</td>
<td>$P=0.0001$ (MA)</td>
</tr>
<tr>
<td>GRIA3</td>
<td>Xq25</td>
<td>(Maher, Lea et al. 2013)</td>
<td>Australian</td>
<td>472/472</td>
<td>3</td>
<td>Associated rs3761555</td>
<td>$P=0.008$ (MA)</td>
</tr>
<tr>
<td>GRIA2</td>
<td>4q32.1</td>
<td>(Gasparini, Sutherland et al. 2014)</td>
<td>Australian</td>
<td>284/284</td>
<td>3</td>
<td>Not associated</td>
<td></td>
</tr>
<tr>
<td>GRIA4</td>
<td>11q22.3</td>
<td>(Gasparini, Sutherland et al. 2014)</td>
<td>Australian</td>
<td>284/284</td>
<td>4</td>
<td>Not associated</td>
<td></td>
</tr>
<tr>
<td>GRIA1</td>
<td>5q31.1</td>
<td>(Cargnin, Viana et al. 2013)</td>
<td>Italian</td>
<td>186/312</td>
<td>2</td>
<td>Not associated</td>
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</table>
Table 3.6 Genetic Studies: Glutamate Transporter genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Reference</th>
<th>Population/patient</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAT1</td>
<td>5p13</td>
<td>(Jen, Wan et al. 2005)</td>
<td>Episodic Ataxia (EA), migraine, hemiplegia and seizures.</td>
<td>Mutation P290R</td>
</tr>
<tr>
<td>EAAT1</td>
<td>5p13</td>
<td>(de Vries, Mamsa et al. 2009)</td>
<td>Episodic Ataxia (EA), reduction in glutamate uptake.</td>
<td>Mutation C186S</td>
</tr>
<tr>
<td>EAAT2</td>
<td>11q13.-13</td>
<td>(Shin, Han et al. 2011)</td>
<td>Korean</td>
<td>Not associated</td>
</tr>
<tr>
<td>EAAT2</td>
<td>11q13.-13</td>
<td>(Mallolas, Hurtado et al. 2006)</td>
<td>Patient with acute ischemic stroke.</td>
<td>Mutation A181C</td>
</tr>
</tbody>
</table>
3.10 Glutamatergic System and Clinical Implications

Modulation of ionotropic and metabotropic glutamate receptors in migraine is a novel route to investigate that may contribute further understanding to the pathophysiology of migraine and its treatment. Interest in investigating the glutamatergic pathway in migraine has been fuelled by studies implicating glutamate in CSD (D'Andrea and Leon 2010) development manifesting in the aura and as well as playing a role in activation of the trigeminovascular system. Glutamate receptors are reported to modulate capsaicin-induced c-fos expression in the trigeminal nucleus caudalis, an established model of migraine (Sang, Hostetter et al. 1998). Inhibition of glutamate release culminates in an anti-nociceptive effect and could be an effective approach to reduce migraine pain (Neeb, Meents et al. 2010).

Modulating nerve cells that disturb the normal pattern of brain circuit activity by blocking the cell surface receptors that bind glutamate may help reduce the state of hyperexcitability. The advantage of targeting the glutamatergic system is that it offers a non-vasoactive approach and a novel alternative in the repertoire of migraine treatment. Many therapies for the acute treatment of migraine attacks have potent vasoconstrictor properties on the uterus and human coronary arteries in the heart and are therefore problematic for people that are at an increased risk of stroke and cardiovascular disease (Tfelt-Hansen, Saxena et al. 2000). Therefore modulating the glutamatergic system could be a novel strategy to eliminate the vascular side effects that currently plague acute anti-migraine therapies. It is proposed that these agents may be advantageous to subgroups of migraineurs who may not tolerate or gain adequate relief from existing agents.

Pharmacological compounds capable of modulating glutamate receptors have helped untangle the functional role of glutamate receptor family members and present promising targets for the treatment of migraine. Considerable scope however remains for the development of novel ligands that will encompass the family of glutamate receptors. Currently the most promising compounds reported in the literature include: topiramate, ketamine and memantine. Several types of drugs, like generic beta blockers, calcium channel blockers, tricyclic antidepressants and anti-epileptic drugs are given to prevent migraine, these are not always effective in all patients.
Topiramate is a derivative of the naturally occurring monosaccharide D-fructose that was originally developed as an anticonvulsant and is recognized as an effective medication for migraine prevention (Andreou and Goadsby 2011). Topiramate is a glutamate receptor antagonist within the trigeminothalamic pathway. Topiramate has several actions which are relevant, including the blockade of Na\(^+\) and Ca\(^{2+}\) channels, enhancement of GABA activity, and blockade of ionotropic glutamate receptors (Andreou and Goadsby 2011).

A few other compounds Memantine, Ketamine and ADX10059 are drugs that act on glutamate signalling through NMDA receptors (Bigal, Rapoport et al. 2008; Marin and Goadsby 2010). Memantine is a moderate-affinity noncompetitive antagonist at glutamatergic N-methyl-D-aspartate (NMDA) receptors (Bigal, Rapoport et al. 2008). Preclinical experiments with modulators of Glu receptors in migraine have helped progress this pharmacological angle and support the hypothesis that blocking any one of these ionotropic glutamate receptor subtypes is a potential logical approach. A prerequisite of clinically acceptable modulators is that they block excessive activation of receptors while preserving normal function to avoid any side effects.

Well-designed clinical studies using larger and stratified migraine cohorts will further our current understanding of the role of modulators of the glutamatergic system in migraine and aid in the development of therapies to prevent and/or abort migraine attacks. It is foreseen that targeting components of the glutamatergic system may be a fruitful target for new therapies. Although there are some toxicity hurdles with realizing the full potential of these modulators as they also block normal neuronal function, chemical techniques and structure-based drug design approaches may make it possible to bypass some of these difficulties in the future.
3.11 Conclusion

Migraine is a disabling costly brain disorder, with hypothesised involvement of neurotransmitters. The major excitatory neurotransmitter of the brain, glutamate and the receptors and transporters upon which it acts are intimately involved in migraine processes including cortical spreading depression, trigeminovascular activation and central sensitization. The efficacy of glutamate antagonists in the treatment of migraine is added evidence of a role for glutamate in migraine. Given the important involvement of glutamate in biological processes in the brain, genes of this system remain candidates for further investigation. Genetic characterization of migraine as a disorder is making steady progress with an increasing number of genomic susceptibility loci now identified. The data and ideas presented above have lent some support implicating Glutamatergic biology in migraine pathophysiology at the turn of the 21st century. However the genetic studies are small and more data is needed to draw any solid conclusion about potential involvement of Glutamatergic genes in migraine. Further research will elucidate the mechanism through which Glutamatergic genes may contribute to migraine susceptibility and to determine if other unknown mutations in components of this system may be contributing to the migraine phenotype. Nonetheless the genetic evidence is growing with results from association, linkage and GWAS studies bringing to light new variants and genomic regions. The identification of these migraine specific loci remains an important objective given that genetic variation greatly affects patient response to treatment and can therefore contribute to more individualized treatments leading to better tolerability in migraine patients.
CHAPTER 4 Biochemical studies of the neurotransmitters serotonin, dopamine and glutamate in Migraine

4.1 Abstract

Migraine is a brain disorder characterized by a piercing headache which affects one side of the head, located mainly at the temples and in the area around the eye. Migraine imparts substantial suffering to the family particularly as it affects more women than men and is most prevalent between the ages of 25 and 45, the years of child rearing. Migraine typically occurs in individuals with a genetic predisposition and is aggravated by specific environmental triggers. The disorder varies in severity and in symptoms, which can include: persistent throbbing, nausea, vomiting, and light and sound sensitivity. At present there is no biochemical marker or simple diagnostic test for the disorder. Attempts to study the biochemistry of migraine began as early as the 1960s and were primarily directed at serotonin metabolism after Sicuteri observed an increase of 5-HIAA, the main metabolite of serotonin in the urine of migraineurs. Additional neurotransmitters implicated in migraine pathogenesis include: dopamine and glutamate and catecholamines. Other compounds besides neurotransmitters have also been considered, including histamine, free fatty acids, selected peptides and hormones. The aim of studies has been the identification of differences in biochemical values in the systemic circulation of migraine patients versus controls to give insight into mechanisms of pathophysiology and allow monitoring and/or selection of specific treatment. In this review we discuss the evidence available from biochemical studies of the serotonin, dopamine and glutamate neurotransmitters in platelets, plasma, CSF and urine that support a dysfunction of neurotransmitters in migraine susceptibility.
4.2 Introduction

Migraine is a painful neurological disorder imparting significant burden to sufferers and their families. Migraine pain is difficult to ignore and is disconcerting manifesting as throbbing and localized to one side of the head and may be accompanied by nausea, vomiting, and hypersensitivity to lights, sounds and/or smells (IHS 2013). Higher rates of occurrence are observed in females ~3 times more than males and in Caucasian populations affecting ~12% of people (Stovner and Hagen 2006).

Migraine and headache related disorders are diagnosed according to international standard criteria set by the International Headache Society (IHS) (beta version currently available at http://cep.sagepub.com/content/33/9/629.full) (IHS 2013). Acceptance of these criteria has facilitated modern day molecular genetic research in the absence of any clearly identifiable pathology, biomarker and diagnostic tests. Migraine is a complex disorder involving multiple chromosomal loci and substantial heterogeneity, which makes it difficult to track down the relevant genomic risk factors. This is why reliable classification criteria are required in the study of complex genetic disorders involving various subtypes and are a prerequisite in order to achieve consistency and for results to be replicated by independent groups.

The International Headache Society (IHS) classifies migraine into two phenotypes migraine with aura (MA) and migraine without aura (MO) (IHS 2013). A major characteristic that distinguishes the MA sufferer is the presence of visual aura prior to or during the headache that is typified by neurological symptoms (Spierings 2004). Only about 30% of migraineurs experience aura symptoms which include reversible numbness and tingling, speech disturbances, or positive or negative visual impairment (Viana, Sprenger et al. 2013).

One pathophysiological description of this disorder is based on the interaction of vascular and neurological physiology. Additional events that describe migraine and integrate with current views of the pathogenesis of migraine include triggering of the trigeminovascular system and Cortical Spreading Depression (CSD) a slow wave of cortical depression initiated in the occipital lobe that gradually spreads across the cortex and has been linked to visual aura (Hadjikhani, Sanchez Del Rio et al. 2001; Dalkara, Zervas et al. 2006; Akerman, Holland et al. 2009). Migraine is believed to be primarily driven by neural events which result in dilation of blood vessels aggravating the pain and resulting in further nerve activation leading
to the release of inflammatory molecules. The neurophysiological basis of migraine is a work in progress; however, biochemical and pharmacological findings support the involvement of the neurotransmitters serotonin, dopamine and glutamate in migraine aetiology (Figure 4.1). The aim of this review is to discuss biochemical changes in specific neurotransmitters including serotonin, dopamine and glutamate in platelets, plasma, CSF, and urine of migraine patients.
Figure 4.1  Neurotransmitter Systems implicated in Migraine.
4.3 Serotonergic Biology

Biochemical, genetic and pharmacological studies have investigated potential dysfunction of neurotransmitters in migraine susceptibility. The first evidence of a biochemical deviation in headache was reported by Federigo Sicuteri in 1961 (Sicuteri, Anselmi et al. 1961). In this study Sicuteri observed an increase of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of serotonin, in the urine of 15 patients with typical migraine during their attacks (Sicuteri, Anselmi et al. 1961). Curran et al and Curzon have confirmed the 5HIAA excretion is usually increased at the time of a migraine attack (Curran, Hinterberger et al. 1965). This was the first indication of an anomaly of serotonin metabolism in migraine and since then most scientific research studies into the biochemical changes that occur in migraineurs have focused on the biological constituents of the serotonergic system comprising of receptors, transporters and enzymes (Table 4.1). Although polymorphisms (and the STin2 VNTR) in the serotonin transporter gene (SERT, SLC6A4) have showed altered allelic distribution in migraine populations there has been, to date, insufficient evidence to confirm a specific serotonin receptor gene being directly associated with the disorder (Ogilvie, Russell et al. 1998).

The Serotonergic system consists of an extensive network of nerve routes originating in two midbrain areas, the dorsal raphe and the median raphe that innervate blood vessel ramifications, the ependymal lining of the ventricular system and the pial surface (Azmitia and Whitaker-Azmitia 2000). This system is wired by Serotonin (5-hydroxytryptamine, 5-HT) a neurotransmitter synthesized inside serotonergic neurons from the amino acid precursor tryptophan in a short metabolic pathway consisting of two enzymes: tryptophan hydroxylase (TPH) and amino acid decarboxylase (DDC) (Marcus 1993). Just as important as is the synthesis of serotonin so is its breakdown for which the enzyme monoamine oxidase (MAO) is responsible for metabolizing serotonin in the liver by oxidation to an aldehyde which undergoes further oxidation by aldehyde dehydrogenase (ALDH) to 5-HIAA, the indole acetic acid derivative (Browning 2004) (Table 4.1).
Table 4.1 The biological and gene constituents of the Serotonergic System.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH1, Tryptophan hydroxylase 1</td>
<td>TPH1</td>
<td>11p15.3-p14</td>
</tr>
<tr>
<td>TPH2, Tryptophan hydroxylase 2</td>
<td>TPH2</td>
<td>12q21.1</td>
</tr>
<tr>
<td>DDC, Amino acid decarboxylase</td>
<td>DDC</td>
<td>7p12.2</td>
</tr>
<tr>
<td>MAO-A, Monoamine oxidase</td>
<td>MAO-A</td>
<td>Xp11.3</td>
</tr>
<tr>
<td>MAO-B, Monoamine oxidase</td>
<td>MAO-B</td>
<td>Xp11.23</td>
</tr>
<tr>
<td>ALDH2, Aldehyde dehydrogenase 2</td>
<td>ALDH2</td>
<td>12q24.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Receptor Protein</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HT1</td>
<td>HTR1A</td>
<td>5q11.2-q13</td>
</tr>
<tr>
<td></td>
<td>HTR1B</td>
<td>6q13</td>
</tr>
<tr>
<td></td>
<td>HTR1D</td>
<td>1p36.3-p34.3</td>
</tr>
<tr>
<td></td>
<td>HTR1E</td>
<td>6q14-q15</td>
</tr>
<tr>
<td></td>
<td>HTR1F</td>
<td>3p12</td>
</tr>
<tr>
<td>5HT2</td>
<td>HTR2A</td>
<td>13q14-q21</td>
</tr>
<tr>
<td></td>
<td>HTR2B</td>
<td>2q36.3-q37.1</td>
</tr>
<tr>
<td></td>
<td>HTR2C</td>
<td>Xq24</td>
</tr>
<tr>
<td>5HT3</td>
<td>HTR3A</td>
<td>11q23.1</td>
</tr>
<tr>
<td></td>
<td>HTR3B</td>
<td>11q23.1</td>
</tr>
<tr>
<td></td>
<td>HTR3C</td>
<td>3q27.1</td>
</tr>
<tr>
<td></td>
<td>HTR3D</td>
<td>3q27.1</td>
</tr>
<tr>
<td></td>
<td>HTR3E</td>
<td>3q27.1</td>
</tr>
<tr>
<td>5HT4</td>
<td>HTR4A</td>
<td>5q31-q33</td>
</tr>
<tr>
<td>5HT5</td>
<td>HTR5A</td>
<td>7q36.1</td>
</tr>
<tr>
<td></td>
<td>HTR5B</td>
<td>2q14.1</td>
</tr>
<tr>
<td>5HT6</td>
<td>HTR6A</td>
<td>1p36-p35</td>
</tr>
<tr>
<td>5HT7</td>
<td>HTR7A</td>
<td>10q21-q24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transporter Protein</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERT, Serotonin transporter</td>
<td>SLC6A4</td>
<td>17q11.2</td>
</tr>
<tr>
<td>PMAT, Plasma membrane monoamine transporter</td>
<td>SLC629A4</td>
<td>7p22.1</td>
</tr>
</tbody>
</table>

The majority of total serotonin about 90% is found in the enterochromaffin cells in the gastrointestinal tract where it regulates intestinal movements (Izzati-Zade 2008). The remaining 10% of serotonin is located in the brain and thrombocytes (Izzati-Zade 2008). Serotonin functions via a specific set of receptors and transporters in neuronal and several non-neuronal tissues of the cardiovascular system, gastrointestinal, renal systems and the blood (Hoyer, Hannon et al. 2002). The large family of Serotonin receptor classes and their subtypes is evidence of the numerous and varied physiological functions involving serotonin within the CNS (Sokolov, Lyubashina et al. 2011). Serotonin controls a myriad of activities in the CNS including regulating mood, cognition, circadian rhythm, anxiety, appetite, pain, sleep, aggression and sexual behaviour and is therefore an important player not just in the aetiology of migraine but of several neurological and psychiatric disorders (Berger, Gray et al. 2009).
The serotonergic system is very much involved in inhibition by decreasing behaviours while dopamine stimulates behaviours. There is an opponent partnership between the serotonin system and the dopamine system as indicated by anatomical and pharmacological evidence (Daw, Kakade et al. 2002). Serotonin is very widespread modulating the activity of glutamate and dopamine and many other neurotransmitters such as GABA, epinephrine/norepinephrine, acetylcholine, as well as many hormones, including oxytocin, prolactin, vasopressin, cortisol, corticotropin, and substance P, among others (Daw, Kakade et al. 2002). Each neurotransmitter does not work alone in CNS circuits but overlaps and interacts with other neurotransmitters in a finely coordinated manner in order to transmit the complex signals that maintain homeostasis. Although each neurotransmitter system is responsible for different functions, uses a different set of receptors and is implicated in different disorders functional interactions exist between neurotransmitter systems so that deficiency in one system can in turn affect the function of the other system in a synergistic fashion (Ciranna 2006).

The metabolism and excretion of neurotransmitters in various biological fluids makes their detection a potentially useful tool for disease diagnosis and to monitor treatment efficacy. Metabolic changes due to disease can manifest as a change in the biochemical profile of a specific body fluid and therefore analysis of metabolites in body fluids can help identify biochemical markers that can give insight into the changing biochemical milieu. More complete understanding of metabolic variations arising from genetic variants in predisposed migraine individuals could enable future monitoring of the migrainous disorder similar to blood pressure or serum glucose measurements in hypersensitive or diabetic individuals. The hypothesis investigated in this review is that disturbances in circulating levels of neurotransmitters or in the function of its receptors due to an aberrant combination of genes may express the biochemical phenotype of migraine. Research into the biochemistry of migraine although not as defined as genetic research is just as important and necessary to advance knowledge of the pathophysiology of migraine.
4.3.1 Serotonin Biochemical Studies

In the study of neurological disorders such as migraine, a recognizable limitation when investigating *in vivo* the biochemistry of CNS functioning is access to neuronal brain tissue of affected patients for tissue specific gene expression studies (Stahl 1977). These practical difficulties have forced researchers to turn to alternative peripheral models such as platelets and urine to study neurotransmitter function. Platelets have been proposed as a suitable model to study migraine processes due to the association and co-morbidity of migraine with several hereditary and acquired cerebrovascular disorders, including arterial dissection, ischemic stroke, and cardiovascular disease and the fact that platelets may reflect biochemical dysfunction in the CNS (Kurth 2013). The cerebral circulation contains vasoactive biochemical substances that when altered can contribute to vascular changes that impact the endothelium and may be responsible for the changes in cerebral hemodynamics in migraine.

The vascular endothelium is very important as it can produce a number of potent local vasoactive agents that can change the circulation and the coagulant properties of blood and produce alterations in neurovascular function. Heritable differences in genes affecting vascular endothelial function have shown an association with migraine and include the homocysteine metabolism gene, methylenetetrahydrofolate reductase (MTHFR), nitric oxide synthase (NOS), calcitonin gene-related peptide (CGRP), angiotensin I-converting enzyme (ACE), the NOTCH3 gene, tumor necrosis factor -α and -β (TNFA and TNFB) and lymphotoxin-α (LTA), angiotensin receptor 1 (AGTR1), angiotensinogen (AGT).

Biochemical studies of serotonin content in platelets and urine of migraine patients have identified some abnormalities relative to controls. Platelets are small blood cells that run through veins, arteries and capillaries and develop by budding from megakaryocytes in the bone marrow and whose primary function is to stop bleeding (Sarchielli and Gallai 2001). Although platelets do not synthesize serotonin de novo they actively uptake circulating serotonin in the plasma via a serotonin transporter and store the neurotransmitter in dense granules and release it upon being activated (Jernej, Vladic et al. 2002). Serotonin stored in platelets has important vasoconstrictive properties and is utilized to regulate hemostasis and blood clotting.
The majority of studies looking at platelet function in migraine focus on measuring physiological parameters such as agonist induced aggregation and secretion, which increase during and in between attacks. Alterations in platelet function, leucocyte function, and intercellular communication between these cells in the bloodstream could contribute to migraine and impact the cerebral circulation by an inflammatory mechanism via tethering to the endothelium. Morphological differences in platelets of migraineurs have been noted and include 1) contain more ADP, 2) have more dense granules, and 3) show some qualitative differences in their release reaction, an increase in spontaneous clumping together and reduction of serotonin release (Hanington 1989).

Somerville observed a drop in platelet-bound 5-HT and plasma-free levels in both jugular and forearm blood in 5 patients at the height of their migraine symptoms and this finding was also extended by Curran (Somerville 1976). Numerous subsequent studies have found an association between migraine and a dysfunction in platelets (Dalsgaard-Nielsen and Genefke 1974; Dvilansky, Rishpon et al. 1976; Coppen, Swade et al. 1979; Malmgren, Olsson et al. 1980; Rolf, Wiele et al. 1981; Gawel and Rose 1982; Oxman, Hitzemann et al. 1982; Takeshima, Shimomura et al. 1987; Leira, Castillo et al. 1993; Jarman, Pattichis et al. 1996). Abnormality in platelet 5-HT metabolism has also been described in tension-type headache and in patients with analgesic induced headache (Shukla, Shanker et al. 1987; Srikiatkhachorn and Anthony 2002; Ayzenberg, Obermann et al. 2008). For the most part investigations report increased platelet activation and it is argued that these changes could reflect parallel biochemical changes in the CNS.
Table 4.2  Biochemical studies of platelet Serotonin levels in migraine patients and controls.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Dalsgaard-Nielsen and Genefke 1974)</td>
<td>Platelets from migraineurs gave higher values of aggregation show increased uptake of 5-HT by platelets and increase endogenous content of platelets</td>
</tr>
<tr>
<td>(Dvilansky, Rishpon et al. 1976)</td>
<td>Plasma during attacks released significantly more 5-HT</td>
</tr>
<tr>
<td>(Oxman, Hitzemann et al. 1982)</td>
<td>Platelet membranes are sensitive</td>
</tr>
<tr>
<td>(Shukla, Shanker et al. 1987)</td>
<td>5-HT is implicated in tension headache.</td>
</tr>
<tr>
<td>(D’Andrea, Welch et al. 1987)</td>
<td>Decreased 5-HT turnover</td>
</tr>
<tr>
<td>(Joseph, Welch et al. 1988)</td>
<td>Abnormal sensitivity to PAF</td>
</tr>
<tr>
<td>(D’Andrea, Welch et al. 1989b)</td>
<td>Increase in the content of serotonin, not evident in MA</td>
</tr>
<tr>
<td>(D’Andrea, Welch et al. 1989a)</td>
<td>High NE levels and low 5-HT/NE ratio in platelets of patients with MA</td>
</tr>
<tr>
<td>(Joseph, Welch et al. 1989)</td>
<td>Increased number of dense bodies; altered coupling of 5-HT secretion from dense bodies and ionised calcium; decreased serotonin secretion</td>
</tr>
<tr>
<td>(Takeshima, Takao et al. 1989)</td>
<td>Muscle contraction headache and migraine. Platelet activation and plasma norepinephrine during the cold pressor test</td>
</tr>
<tr>
<td>(Riddle, D’Andrea et al. 1989)</td>
<td>Increase in the number of dense bodies</td>
</tr>
<tr>
<td>(D’Andrea, Hasselmark et al. 1994)</td>
<td>Increased basal platelet 5-HT and increased 5-HT secretion induced by both collagen and PAF (not evident in MA)</td>
</tr>
<tr>
<td>(D’Andrea, Hasselmark et al. 1995)</td>
<td>Plasma and platelet 5-HT peak in MM in ovulatory phase; 5-HT peak evident in follicular phase in TTH and controls</td>
</tr>
<tr>
<td>(Fioroni, Andrea et al. 1996)</td>
<td>Reduced 5-HT and increased 5HIAA in luteal phase, suggesting a greater susceptibility to attacks in this period</td>
</tr>
<tr>
<td>(Jarman, Pattichis et al. 1996)</td>
<td>Red wine-induced release of [14C]5-hydroxytryptamine from platelets of migraine patients and controls</td>
</tr>
<tr>
<td>(Allais, Facco et al. 1997)</td>
<td>Patterns of platelet aggregation in menstrual migraine</td>
</tr>
<tr>
<td>(Pukhal’skaya, Kolosova et al. 1998)</td>
<td>Serotonin-transport system plays an important role in disease pathology.</td>
</tr>
<tr>
<td>(Zeller, Lindner et al. 2005)</td>
<td>Patients suffering from migraine without aura had a significantly increased platelet activation and leucocyte–platelet aggregation compared with the control group, unlike the migraine patients with aura.</td>
</tr>
</tbody>
</table>
4.3.2 Serotonin Studies from Urine

In addition biochemical studies investigating plasma serotonin levels (Anthony, Hinterberger et al. 1967; Anthony 1968; Anthony and Lance 1971; Anthony and Lance 1989) report that a fall in plasma 5-HT is followed by enhanced urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA). 5-HT level in the MA patients was significantly lower than that in the controls and MO patients (Nagata, Shibata et al. 2006). A surplus of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of serotonin degradation in the urine of migraine patients was initially noted by Sicuteri (Sicuteri, Anselmi et al. 1961). Later studies also observed urinary excretion of the main metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA), to be increased in association with migraine attacks in a number of independent studies (Sicuteri, Anselmi et al. 1961; Curran, Hinterberger et al. 1965; Curzon, Theaker et al. 1966; Kangasni, Sonninen et al. 1972; Deanovic, Iskric et al. 1975) see Table 4.2.

Although these studies were undertaken in the early 60s and 70s using outdated classification criteria they were fundamental in highlighting serotonin’s involvement in headache and drive the pharmacological development of Tryptans. These early studies sought to find an explanation as to why urinary excretion of serotonin might be more elevated in migraine sufferers. One explanation is that an increase of the metabolite 5-HIAA in the urine during a migraine might be due to increased activity of monoamine oxidase (MAO) an enzyme responsible for metabolizing serotonin which is finally excreted in urine as 5-HIAA. Monoamine oxidase inhibitors have rationally been used in the treatment of migraine because they promote the accumulation of monoamines and reduce uncontrolled vasodilation, which is beneficial in a migraine headache, however they have not generally been adopted for the treatment of migraine due to complications (Anthony and Lance 1969).

In contrast Bousser et al., 1986 provide evidence of lowered urinary 5-HIAA excretion in 44 young adult female migraine patients between attacks and in 33 healthy controls (Bousser, Elghozi et al. 1986). They report that 5-HIAA excretion was significantly decreased in female migraine patients when compared with their controls this result is also matched by Milovanovic et al., 1999 (Milovanovic, Majkic-Sing et al. 1999). Some contradictory findings are to be expected and most likely due to any number of factors including: drug therapy, diet, age, sex, the nature and status of migraine participants collected in each study.
all of which can interfere with the measurement of 5-HIAA levels and lead to biological variation.

There is debate namely in two reviews regarding the clinical value of urinary neurotransmitter testing for the identification of biomarkers. Hinz et al., 2010 argue that urinary neurotransmitter testing can vary greatly from day to day and that results are not reproducible, being essentially random (Hinz, Stein et al. 2010). The urine is sensitive to stress chemistry and diet related especially pH changes. Whereas Marc et al. 2010 on the other hand support urinary assessments as being “non-invasive, with the added advantage of enhanced stability compared to CSF or blood and that they are an objective means to assess nervous system function” (Marc, Ailts et al. 2011). There are advantages and disadvantages to using each type of biological fluid as an indicator of disease status however at this stage more studies are needed to make a definitive conclusion.

Apart from platelets and urine other studies have considered the content of serotonin in other body regions. Chugani et al., 1999 reported a trend of elevated serotonin synthesis capacity in MO female patients with PET using the tracer $\alpha$-$[^{11}C]$methyl-L-tryptophan before and also after prophylactic treatment with beta-adrenergic antagonists (Chugani, Niimura et al. 1999). The authors caution that this is a preliminary finding because it is based upon only three patients with migraine aura (Chugani, Niimura et al. 1999). Higher levels of serotonin have also been reported in lymphoblasts of MA patients in a study by Nagata et al., 2007 (Nagata, Hamada et al. 2007). Finally a small number of studies have evaluated serotonin levels in CSF and found no direct correlation with migraine. The serotonin concentration of the cerebrospinal fluid does not seem to be increased during the attacks or at present there is insufficient data to draw a conclusion (Barrie and Jowett 1967).
### Table 4.3  Biochemical studies of Serotonin levels in the urine of migraine patients and controls.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Migraine Classification</th>
<th>Controls/Cases</th>
<th>Sample tested</th>
<th>Levels</th>
<th>Sample tested</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Sicuteri, Anselmi et al. 1961)</td>
<td>Not reported</td>
<td><strong>Controls: 15</strong></td>
<td>Urine, 5-HIAA</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Curran, Hinterberger et al. 1965)</td>
<td>Not reported</td>
<td><strong>Controls: 10</strong></td>
<td>Urine, 5-HIAA</td>
<td>Controls: 21 Plasma, 5-HT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Curzon, Theaker et al. 1966)</td>
<td>Not reported</td>
<td><strong>Controls: 4</strong></td>
<td>Urine, 5-HIAA</td>
<td>M: 11 ↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Kangasni, Sonninen et al. 1972)</td>
<td>Not reported</td>
<td><strong>Controls: 6</strong></td>
<td>Urine, 5-HIAA</td>
<td>M: 11 ↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Deanovic, Iskric et al. 1975)</td>
<td>(Headache 1962)</td>
<td><strong>Controls: 4</strong></td>
<td>Urine, 5-HIAA</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Bousser, Elghozi et al. 1986)</td>
<td>(Headache 1970)</td>
<td><strong>Controls: 33</strong></td>
<td>Urine, 5-HIAA</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Milovanovic, Majkic-Sing et al. 1999)</td>
<td>(IHS 1988)</td>
<td><strong>Controls: 11</strong></td>
<td>Urine, 5-HIAA</td>
<td>Plasma, 5-HT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MA=migraine, TH=Tension Headache, ↑ Increase, ↓ Decrease, = Unchanged
4.3.3 Serotonin Pharmacotherapy

Individual 5-HT receptor-subtypes have been exploited in depth by the pharmaceutical industry due to serotonin’s pervasive involvement in several neurological and psychiatric disorders. Injectable serotonin was used in the 60s to study its effect in precipitating a migraine attack. Kimball and Friedman demonstrated that infusion of serotonin systemically (Kimball, Friedman et al. 1960; Friedman 1963) produced typical unilateral headaches six hours after injection and symptoms of nausea, abdominal cramp, increased peristaltic sounds, diarrhoea, and tingling in the extremities (Curzon, Theaker et al. 1966). Likewise infusion of serotonin into experimental animals causes an increase in intestinal activity (Curran, Hinterberger et al. 1965). Serotonin however has not been used in migraine treatment due to side effects of dyspnea, tightness of the chest, dizziness and nausea instead serotonin like compounds have been tried (Lance 1990).

Sicuteri helped develop methysergide, a serotonin antagonist at 5-HT$_2$ receptors and agonist at 5-HT$_{1B/D}$ receptors for the prophylactic treatment of migraine and cluster headache that is more stable with fewer side effects than using serotonin and has been used since 1959 sold under the name Sansert (Graham 1964; Curran, Hinterberger et al. 1965). Methysergide was the first drug developed for migraine prevention and although its usefulness is limited by reports of some side effects and adverse reactions the clinical value of methysergide is still recognized today, though how it relieves migraine is not entirely known but its use was instrumental in further advancing the development of tryptans (Ferrari, Odink et al. 1989). Cyproheptadine, also a serotonin antagonist chemically unrelated to methysergide, is effective in the prevention of migraine which is thought to simulate the action of serotonin, i.e. occupying the same receptor sites in the manner of competitive antagonists (Curran, Hinterberger et al. 1965).

The substance reserpine and fenfluramine has been used in the prophylactic treatment of migraine, it is a catecholamine depleting agent that has been shown to induce typical migraine headache (Nappi, Savoldi et al. 1979; Panconesi and Sicuteri 1997). Reserpine works by depleting the body of serotonin from its major storage sites intestine, platelets and brain (Curran, Hinterberger et al. 1965). This type of pharmacologic depletion of serotonin can induce a migraine attack. Reserpine has been shown to induce typical migraine headache.
Serotonin, its agonists and antagonists have provided the substrate for development of the triptans and contributed to the interpretation of migraine pathophysiology. Triptans are currently the newer generation of drugs on the market for the treatment of migraine that are the most effective (Humphrey 2008). Triptans alleviate migraine symptoms by binding to serotonin 5-HT<sub>1b, d and f</sub> receptors (Sokolov, Lyubashina et al. 2011). Agonism at 5HT<sub>1b</sub> receptors constricts the pain producing intracranial, extracerebral blood vessels in the meninges. Agonism at 5HT<sub>1d</sub> receptors presynaptically inhibits trigeminal vasoactive peptide release and stops inflammation interferes with central trigeminal nucleus caudalis nociceptive transduction and processing (Shields and Goadsby 2006). Clinical trials of triptans in migraine treatment are continuing to assess their efficacy (Ferrari, Spaccapelo et al. 2010).

4.4 Dopaminergic Biology

In addition to serotonin there has been ongoing interest in the involvement of dopaminergic pathways in migraine. Initial evidence for this idea was published by the same researcher Sicuteri in 1977 (Sicuteri 1977). Sicuteri observed that migraine patients are hypersensitive to dopamine agonists and proposed that overstimulation of dopamine receptors could account for some of the symptoms of migraine, such as hyperactivity, irritability, nausea, vomiting, yawning and hypotension which are mediated by dopamine (Sicuteri 1977). Hence the Dopamine hypersensitivity hypothesis arose and postulates that the dopaminergic system may play a role in migraine either due to defective synthesis of mediator or agonist deficiency in the synaptic cleft and synergistic to serotonergic impairment. Lance, 1981 also acknowledged that since nausea usually precedes the headache of migraine, then alterations in brainstem dopaminergic neurotransmission must be part of the attack (Lance 1981).

The neurotransmitter system operated by dopamine originates in the substantia nigra pars compacta, ventral tegmental area (VTA), and hypothalamus and activates dopamine receptors to mediate its physiological effects in the body (Grace, Lodge et al. 2009). The input of dopamine influences many neuronal functions as it is part of the brain’s system of motivation involving behaviour and cognition, voluntary movement, punishment and reward, inhibition of prolactin production (involved in lactation and sexual
gratification), sleep, mood, attention, working memory, and learning (Akerman and Goadsby 2007).

DA receptors are the molecular mediators through which DA acts and are found in the trigeminovascular system (Bergerot, Storer et al. 2007). Although smaller in number in comparison to serotonin receptors they are divided into two major groups: the D1 and D2 classes of dopamine receptors and are coupled to G protein-mediated signalling (see Table 4.3) (Beaulieu and Gainetdinov 2011). Administration of dopamine agonists inhibits neuronal firing and consequent nociceptive transmission and have been studied for potential therapeutic effects in migraine (Bergerot, Storer et al. 2007).

Table 4.4 The biological and gene constituents of the Dopaminergic System.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH, Phenylalanine hydroxylase</td>
<td>PAH</td>
<td>12q22-q24.2</td>
</tr>
<tr>
<td>TH, Tyrosine hydroxylase</td>
<td>TH</td>
<td>11p15.5</td>
</tr>
<tr>
<td>DDC, Dopa decarboxylase</td>
<td>DDC</td>
<td>7p12.2</td>
</tr>
<tr>
<td>DBH, Dopamine β-hydroxylase</td>
<td>DBH</td>
<td>9q34</td>
</tr>
<tr>
<td>PNMT, Phenylethanolamine N-methyltransferase</td>
<td>PNMT</td>
<td>17q</td>
</tr>
<tr>
<td>COMT, Catechol-O-methyl-Transferase</td>
<td>COMT</td>
<td>22q11.21</td>
</tr>
<tr>
<td>MAO-A, Monoamine oxidase A</td>
<td>MAO-A</td>
<td>Xp11.3</td>
</tr>
<tr>
<td>MAO-B, Monoamine oxidase B</td>
<td>MAO-B</td>
<td>Xp11.23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Receptor Protein</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1-like</td>
<td>DRD1</td>
<td>5q35.1</td>
</tr>
<tr>
<td></td>
<td>DRD5</td>
<td>4p16.1</td>
</tr>
<tr>
<td>D2-like</td>
<td>DRD2</td>
<td>11q23</td>
</tr>
<tr>
<td></td>
<td>DRD3</td>
<td>3q13.3</td>
</tr>
<tr>
<td></td>
<td>DRD4</td>
<td>11p15.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transporter Protein</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAT, Dopamine transporter</td>
<td>SLC6A3</td>
<td>5p15.3</td>
</tr>
<tr>
<td>PMAT, Plasma membrane monoamine transporter</td>
<td>SLC629A4</td>
<td>7p22.1</td>
</tr>
<tr>
<td>VMAT2, Vesicular monoamine transporter 2</td>
<td>SLC18A2</td>
<td>10q25</td>
</tr>
</tbody>
</table>

Note: PMAT (Plasma membrane monoamine transporter) is involved in the transport of both serotonin and dopamine.

Dopamine (DA) is a monoamine neurotransmitter in the catecholamine family which includes epinephrine and norepinephrine and is synthesized from three amino acids precursors L-Phenylalanine (PHE), L-Tyrosine (L-4-hydroxyphenylalanine; TYR), L-DOPA (L-3,4-dihydroxyphenylalanine; DOPA) using the enzyme listed in Table 4.3 (Vallone, Picetti et al. 2000).
Table 4.5 The metabolic pathway for Dopamine synthesis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Co-factors</th>
<th>Product</th>
<th>Co-products</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH:</td>
<td>L-Phenylalanine</td>
<td>THB + O₂ + Fe²⁺</td>
<td>L-Tyrosine</td>
<td>DHB + H₂O + Fe²⁺</td>
</tr>
<tr>
<td>TH:</td>
<td>L-Tyrosine</td>
<td>THB + O₂ + Fe²⁺</td>
<td>L-DOPA</td>
<td>DHFA + H₂O + Fe²⁺</td>
</tr>
<tr>
<td>DDC:</td>
<td>L-DOPA</td>
<td>PLP</td>
<td>Dopamine</td>
<td>PLP + CO₂</td>
</tr>
<tr>
<td>DBH:</td>
<td>Dopamine</td>
<td>Ascorbic Acid + O₂</td>
<td>Norepinephrine</td>
<td>DHA + H₂O</td>
</tr>
<tr>
<td>PNMT:</td>
<td>Norepinephrine</td>
<td>SAMe</td>
<td>Epinephrine</td>
<td>Homocysteine</td>
</tr>
</tbody>
</table>

Dopamine and other neurotransmitters, are metabolized by various enzymes such as monoamine oxidase (MAO), catechol-O-methyl transferase (COMT), and dopamine beta hydroxylase (DBH) into various substances based on the body’s metabolic requirements. A balanced dopaminergic system also contains DA transporters that traffic dopamine from the synapse into neurons, these include Dopamine transporter (DAT), Plasma membrane monoamine transporter (PMAT), and Vesicular monoamine transporter 2 (VMAT2) (Table 4.3) (Fornai, Chen et al. 1999).

Interest in the role of dopamine in migraine, particularly at the molecular genetic level was propelled after Peroutka et al in 1997 reported an allelic association between the dopamine receptor gene DRD2 and migraine with aura (Peroutka, Wilhoit et al. 1997). Not surprisingly, variants in other dopamine metabolism genes, DBH (Fernandez, Colson et al. 2009), DDC (Corominas, Sobrido et al. 2010), MAOA (Corominas, Sobrido et al. 2010) and SLC6A3 (Todt, Netzer et al. 2009), have been reported to impact migraine risk. The emerging consensus is that whilst some clashing results are reported in different populations, considerable genetic evidence exists implicating genes involved in neurotransmitter pathways, particularly ion channel function and transport, as good migraine candidates particularly as dopamine antagonists have proven useful in the treatment of migraine.

4.4.1 Dopamine Biochemical Studies

Biochemical studies of DA content in body fluids of migraine patients are sparse and less reliable because DA is rapidly metabolized into norepinephrine by the enzyme dopamine beta hydroxylase (DBH) (Cerbo, Barbanti et al. 1997). Pradalier et al. reported an increase of non-conjugated DA in plasma during attacks, accompanied by a decrease in platelets in a small number of migraine without aura patients (Pradalier, Launay et al. 1987). D’Andrea and colleagues have found that platelet levels of dopamine are increased in MO (but not MA) and cluster headaches (D'Andrea, Granella et al. 2006). In contrast Nagel-Leiby et al found
plasma levels of DA in women with MO were highest during menses (Nagelleiby, Welch et al. 1990). Oestrogens influence the activity of several neurotransmitter systems (Fanciullacci, Alessandri et al. 2000). The increase in DA during menses might relate to estrogen fluctuation throughout the menstrual cycle.

The enzyme DBH is localized in human cerebral blood vessels, in circulating blood and peripheral sympathetic nerves (Gallai, Gaiti et al. 1992). DBH activity is genetically controlled and polymorphisms have been identified in this gene that reduce the plasma enzymatic activity and lead to an increase in DA to associate with migraine Fernandez, 2006, 2009. Studies investigating DBH enzyme activity have reported reduced levels of this enzyme in migraineurs and in tension-type headache (Gotoh, Kanda et al. 1976; Gallai, Gaiti et al. 1992; Fernandez, Colson et al. 2009). DBH may serve as an index of sympathetic activity. More biochemical studies are needed to work out what the role of DA is in the migraine attack and to explain the observed variation in DBH enzyme levels. These results must be reconfirmed with greater numbers of migraine patients.

Some studies have reported a greater density of dopamine receptors DRD3, DRD4, DRD5 on peripheral blood lymphocytes of migraineurs (Barbanti, Bronzetti et al. 1996; Barbanti, Fabbrini et al. 2000). The sample size in the Barbanti et al., 1996 study was small DRD5 consisting of only 11 migraine patients and in the Barbanti et al., 2000 study 25 migraine patients participated. The authors are unsure as to why these differences in expression of DA receptors on lymphocytes occur in migraineurs and mention that it could be some peripheral adaptive response to central dopaminergic alterations (Barbanti, Fabbrini et al. 2000). The expression of dopamine D5 receptor was also noticeably increased in peripheral blood lymphocytes of eleven migraine patients and of ten healthy control subjects using a radioligand binding technique with [3H]SCH 23390 as a ligand (Barbanti, Bronzetti et al. 1996). A significant decrease in the equilibrium disassociation constant of DRD2 binding platelets of migraineurs was reported (Shukla, Khanna et al. 2009). Studies of CSF are few, one study by Castillo et al., 1996 measuring DOPAC (3,4-dihydroxyphenylacetic acid), a DA metabolite, in the cerebrospinal fluid of migraineurs during attacks correlated an increase of its levels well with the severity of pain (Castillo, Martinez et al. 1996). Table 4.5 shows a summary of DA biochemical studies.
### Table 4.6 Biochemical studies of Dopamine levels in migraine patients and controls.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Main Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Gotoh, Kanda et al. 1976)</td>
<td>DBH activity was significantly higher in the migraine patients than in the controls.</td>
</tr>
<tr>
<td>(Sicuteri 1977)</td>
<td>Severe attack-related symptoms such as nausea, vomiting and blood pressure changes are mediated by the dopaminergic system.</td>
</tr>
<tr>
<td>(Pradalier, Launay et al. 1987)</td>
<td>Increase of non-conjugated DA in plasma and decrease in platelets.</td>
</tr>
<tr>
<td>(Nagelleiby, Welch et al. 1990)</td>
<td>Plasma levels of DA are higher in women with MO during the menses than in control subjects.</td>
</tr>
<tr>
<td>(Gallai, Gaiti et al. 1992)</td>
<td>DBH activity is altered in migraine and tension-type headache.</td>
</tr>
<tr>
<td>(Castillo, Martinez et al. 1996)</td>
<td>An increase in 3,4-dihydroxyphenylacetic acid in cerebrospinal fluid correlates the severity of pain.</td>
</tr>
<tr>
<td>(Barbanti, Bronzetti et al. 1996)</td>
<td>Increase D5 receptor expression in peripheral blood lymphocytes.</td>
</tr>
<tr>
<td>(Cerbo, Barbanti et al. 1997)</td>
<td>Migraineurs have a hypersensitized dopaminergic system resulting in an increased dopamine receptor density on T-cells.</td>
</tr>
<tr>
<td>(Barbanti, Fabbrini et al. 2000)</td>
<td>Increased density of dopamine DRD3 and DRD4 receptors on lymphocytes of migraineurs.</td>
</tr>
<tr>
<td>(Fanciullacci, Alessandri et al. 2000)</td>
<td>The alteration in the dopaminergic control of prolactin secretion in female migraineurs.</td>
</tr>
<tr>
<td>(D'Andrea, Granella et al. 2006)</td>
<td>Platelet levels of dopamine are increased in migraine and cluster headache.</td>
</tr>
<tr>
<td>(Shukla, Khanna et al. 2009)</td>
<td>Decrease in the equilibrium disassociation constant of DRD2 binding platelets of migraineurs.</td>
</tr>
<tr>
<td>(Fernandez, Colson et al. 2009)</td>
<td>Increase in DBH enzyme activity has been noted in migraineurs during the headache-free interval.</td>
</tr>
</tbody>
</table>
4.4.2 Dopamine Pharmacotherapy

Like the serotonergic system the dopaminergic system has been the target of much effort by geneticists and the pharmaceutical industry due to its role in muscle contraction, motivation, energy levels, cognition and memory and especially in psychiatric diseases (Beaulieu and Gainetdinov 2011). D2-like DA receptor antagonists like prochlorpromazine, metoclopramide, droperidol, haloperidol and domperidone which block the action of DA are a valuable group of drugs that cannot be discounted and are used in the treatment of acute migraine. They have been used successfully to treat the premonitory symptoms of migraine, such as yawning, irritability, nausea, vomiting and gastrokinetic dysfunction (Charbit, Akerman et al. 2010; Marmura 2012) and to alleviate the headache component to some extent however long-term adverse events limit their use in clinical practice.

Given the broad phenotypic expressivity of migraine and variable patient response and presence of co-morbidities, a pharamacological repertoire is necessary to target the multitude of symptoms exhibited by different patients. In contrast migraine sufferers are hypersensitive to DA receptor agonists such as apomorphine, L-dopa and bromocriptine which mimic the action of DA a selective D1 and D2 receptor agonist and induce yawning and drowsiness in healthy volunteers (Blin, Azulay et al. 1991; Cerbo, Barbanti et al. 1997; Honkaniemi, Liimatainen et al. 2006).

4.5 Glutamatergic Biology

Glutamate like serotonin and dopamine is an important neurotransmitter in the CNS that mediates fast excitatory synaptic transmission via ionotropic and metabotropic receptors (McKenna 2007). The brain contains large amounts of the neurotransmitter glutamate a common excitatory amino acid that cannot cross the blood-brain barrier but that is cycled from an exogenous to an endogenous environment at the synapse and plays a key role in cellular metabolism (McKenna 2007). The neuronal-glial cell interface where glutamate cycling occurs contains a number of enzymes, glutamate receptors and transporters (see Table 4.6). Together these components form the biological constituents of the Glutamatergic system which enable fast excitatory synaptic transmission.
Table 4.7 The biological and gene constituents of the Glutamatergic System.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDH, Pyruvate dehydrogenase</td>
<td>PDP1</td>
<td>8q22.1</td>
</tr>
<tr>
<td>PAG, Phosphate activated glutaminase</td>
<td>GLS2</td>
<td>12q13.3</td>
</tr>
<tr>
<td>mME, Mitochondrial malic enzyme</td>
<td>ME3</td>
<td>11cen-q22.3</td>
</tr>
<tr>
<td>GAD, Glutamic acid decarboxylase</td>
<td>GAD1</td>
<td>2q31</td>
</tr>
<tr>
<td>GS, Glutamine synthetase</td>
<td>GLUL</td>
<td>1q31</td>
</tr>
<tr>
<td>PC, Pyruvate carboxylase</td>
<td>PC</td>
<td>11q13.4-q13.5</td>
</tr>
<tr>
<td>cME, Cytosolic malic enzyme</td>
<td>ME1</td>
<td>6q12</td>
</tr>
<tr>
<td>AAT, Aspartate aminotransferase</td>
<td>GOT1</td>
<td>10q24.1-q25.1</td>
</tr>
<tr>
<td>GDH, Glutamate dehydrogenase</td>
<td>GLUD1</td>
<td>10q23.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ionotropic glutamate receptors (iGluRs)</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPA</td>
<td>GRIA1</td>
<td>5q33</td>
</tr>
<tr>
<td></td>
<td>GRIA2</td>
<td>4q32-33</td>
</tr>
<tr>
<td></td>
<td>GRIA3</td>
<td>Xq25-26</td>
</tr>
<tr>
<td></td>
<td>GRIA4</td>
<td>11q22-23</td>
</tr>
<tr>
<td>Kainate</td>
<td>GRIK1</td>
<td>21q21.1-22.1</td>
</tr>
<tr>
<td></td>
<td>GRIK2</td>
<td>6q16.3-q21</td>
</tr>
<tr>
<td></td>
<td>GRIK3</td>
<td>1p34-p33</td>
</tr>
<tr>
<td></td>
<td>GRIK4</td>
<td>11q22.3</td>
</tr>
<tr>
<td></td>
<td>GRIK5</td>
<td>19q34.3</td>
</tr>
<tr>
<td>NMDA</td>
<td>GRIN1</td>
<td>9q34.3</td>
</tr>
<tr>
<td></td>
<td>GRIN2A</td>
<td>16p13.2</td>
</tr>
<tr>
<td></td>
<td>GRIN2B</td>
<td>12p12</td>
</tr>
<tr>
<td></td>
<td>GRIN2C</td>
<td>17q24-q25</td>
</tr>
<tr>
<td></td>
<td>GRIN2D</td>
<td>19q13.1qter</td>
</tr>
<tr>
<td></td>
<td>GRIN3A</td>
<td>9q31.1</td>
</tr>
<tr>
<td></td>
<td>GRIN3B</td>
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</tr>
<tr>
<td>Orphan</td>
<td>GRID1</td>
<td>10q</td>
</tr>
<tr>
<td></td>
<td>GRID2</td>
<td>4q22</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Metabotropic glutamate receptors (mGluRs)</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGluR1</td>
<td>GRM1</td>
<td>6q24</td>
</tr>
<tr>
<td>mGluR2</td>
<td>GRM2</td>
<td>3p21.2</td>
</tr>
<tr>
<td>mGluR3</td>
<td>GRM3</td>
<td>7q21.1-q21.2</td>
</tr>
<tr>
<td>mGluR4</td>
<td>GRM4</td>
<td>6p21.3</td>
</tr>
<tr>
<td>mGluR5</td>
<td>GRM5</td>
<td>11q14.3</td>
</tr>
<tr>
<td>mGluR6</td>
<td>GRM6</td>
<td>5q35</td>
</tr>
<tr>
<td>mGluR7</td>
<td>GRM7</td>
<td>3p26-p25</td>
</tr>
<tr>
<td>mGluR8</td>
<td>GRM8</td>
<td>7q31.3-q32.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transporter Type</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAT1</td>
<td>SLC1A3</td>
<td>5p13</td>
</tr>
<tr>
<td>EAAT2</td>
<td>SLC1A2</td>
<td>11p13</td>
</tr>
<tr>
<td>EAAT3</td>
<td>SLC1A1</td>
<td>9p24</td>
</tr>
<tr>
<td>EAAT4</td>
<td>SLC1A6</td>
<td>19p13.12</td>
</tr>
<tr>
<td>EAAT5</td>
<td>SLC1A7</td>
<td>1p32.3</td>
</tr>
<tr>
<td>VGLUT1</td>
<td>SCL17A7</td>
<td>9q13.33</td>
</tr>
<tr>
<td>VGLUT2</td>
<td>SCL17A6</td>
<td>11p14.3</td>
</tr>
<tr>
<td>VGLUT3</td>
<td>SCL17A8</td>
<td>12q23.1</td>
</tr>
</tbody>
</table>

Note: Some enzymes are composed from individual subunits that assemble to form larger multimeric complexes and therefore the same enzyme name will appear to have multiple chromosomal locations in the databases.
4.5.1 Glutamate Biochemical Studies

Glutamate produced outside of the human body is found as a sodium salt of glutamic acid known as monosodium glutamate (MSG) (Jinap and Hajeb 2010). Although MSG is a well-known food additive and flavour enhancer that has been used for more than 100 years the natural form of glutamate used by the body, L-glutamic acid is different to that found in MSG and in processed food which consists of a mixture of L-glutamic acid and impurities of D-glutamic acid and pyroglutamic acid, mono and dichloro propanols, and heterocyclic amines (Freeman 2006). MSG, tyramine and aspartame have all been shown to be migraine triggers in susceptible individuals (Scopp 1991).

There is some discussion in the literature about the existence of a so-called Chinese restaurant syndrome, which may be brought on by ingestion of MSG the relationship between processed (manufactured) free glutamic acid (MSG) and migraine headache has not been proven (Schaumbu, Byck et al. 1969; Morselli and Garattin.S 1970; Zanda, Francios.P et al. 1973; Geha, Beiser et al. 2000). One study by Baad-Hansen investigated the influence of oral administration of MSG to healthy young male volunteers on: the occurrence of headache, sensitivity to pressure pain in masseter and temporalis muscles, blood pressure, heart rate and the occurrence of side-effects (Baad-Hansen, Cairns et al. 2009). This study concluded there was a significant increase in reports of headache and subjectively reported pericranial muscle tenderness after ingestion of MSG. Systemic administration of MSG has also been shown to elevate intramuscular glutamate levels in the rat (Cairns, Dong et al. 2007). More recently Shimada et al., 2013 (Shimada, Cairns et al. 2013) conducted a double-blind, placebo-controlled, crossover study to look at the effect of MSG intake on spontaneous pain and concluded that people consuming MSG are more likely to suffer from headaches and have masseter muscle sensitivity.

Biochemical studies of glutamate, undertaken in the last 30 years have shown that the levels of glutamate are significantly more elevated in plasma, platelets and CSF of migraine patients, particularly those with aura. So far 7 studies including those of (Ferrari, Odink et al. 1990; Martinez, Castillo et al. 1993; Cananzi, Dandrea et al. 1995; Deufemia, Finocchiaro et al. 1997; Alam, Coombes et al. 1998; Vaccaro, Riva et al. 2007; Vieira, Naffah-Mazzacoratti et al. 2007; Ferrari, Spaccapelo et al. 2009) have evaluated levels of Glu in plasma in migraine patients between and during attacks (Table 4.2). Two out of seven studies were
contradictory reporting lower levels of Glu these included a study by Martinez et al., 1993 (Martinez, Castillo et al. 1993) and a study by Deufemia et al., 1997 (Deufemia, Finocchiaro et al. 1997). Differences in the results may be explained by the nature of the control group which was composed of patients under stress in the Martinez study and a group of paediatric migraine patients in the Deufemia study. It has been suggested that plasma glutamate level monitoring in migraine patients might serve as a biomarker of response to treatment and as an objective measure of disease status however more studies utilizing the latest diagnostic criteria are needed to make a more solid conclusion.

Platelets have been considered a useful model for studying Glutamatergic dysfunction because they possess glutamate transport processes similar to Glutamatergic neurons (Mangano and Schwarcz 1981). Three studies have evaluated Glu in PLTS, a study by (Dandrea, Cananzi et al. 1991; Cananzi, Dandrea et al. 1995; Vaccaro, Riva et al. 2007). All studies reported higher levels of glutamate in platelets more so in the MA group. Some authors have suggested there may be a Glu metabolic profile specific to a migraine phenotype that may explain the observed differences in MA and MO. There is one study to date that has evaluated free amino acids in saliva of patients with migraine and found these to be elevated relative to controls (Rajda, Tajti et al. 1999). Saliva has been suggested as a fluid for investigation because it is stable, easy to obtain and is closely related to the nerve terminals of the trigeminal branches. More studies using saliva as a sample are needed to ascertain its potential as a fluid to detect metabolic abnormalities.

Cerebrospinal fluid (CSF) has been proposed as a source for biomarkers because it is within the blood-brain barrier. The earliest study investigating CSF as a source of biomarkers in migraine was by Kovacs in 1989 (Kovacs, Bors et al. 1989). Since then five independent studies by (Martinez, Castillo et al. 1993; Zukerman 1993; Gallai, Alberti et al. 2003; Peres, Zukerman et al. 2004; Vieira, Naffah-Mazzacoratti et al. 2007) have investigated CSF glutamate levels in migraine patients with results showing a significant increase in CSF glutamate levels in migraine patients compared to controls. The two studies by (Gallai, Alberti et al. 2003) and (Peres, Zukerman et al. 2004) used Silberstein classification criteria and chronic daily headache patients. The study by Peres differed to that of Gallai as they included migraine patients with and without fibromyalgia a muscular condition co-morbid with migraine. Fibromyalgia (FM) is a common syndrome of musculoskeletal pain and fatigue, which occurs mostly among middle-age women. They demonstrated CSF glutamate
to be significantly higher in migraine patients with fibromyalgia compared to those without fibromyalgia.

The study by (Gallai, Alberti et al. 2003) and (Vieira, Naffah-Mazzacoratti et al. 2007) considered the influence of medication on the levels of Glu in CSF. In both these studies patients were divided into groups either using or not using medication. The study by Vieira considered the following groups: Group 1 comprised patients overusing analgesics such as NSAIDs; Group 2 comprised CM patients without overuse of medications; and Group 3 comprised patients overusing Triptans. They found that CM patients from all groups showed higher CSF glutamate levels when compared with values found in control subjects. However patients overusing Triptans had lower Glu levels in CSF when compared to nonoveruse patients. The authors suggest Tryptans may be implicated in triptran response mechanisms and may work in part by reducing extracellular glutamate levels in the brain. Also worth mentioning is a study by (Rothrock, Mar et al. 1995) who found the levels of taurine, glycine and glutamine in CSF significantly higher in migraine patients, without significant differences among three migraine subgroups coined as infrequent migraine, frequent migraine and transformed migraine.

More recently a study by (Ragginer, Lechner et al. 2012) evaluated urinary glutamate levels of female migraineurs and reported a significant decrease with respect to the control group. This is the first study to utilize urine as a sample. The majority of the biochemical evidence suggests that glutamate levels are more elevated in migraineurs as detected in a range of biological fluids. Although the studies involved a small number of subjects they are concordant and appear to have some solid basis and are somewhat supported by recent genetic findings. Studies with larger numbers of subjects are needed to more thoroughly establish the potential involvement of glutamate and other biochemical compounds in migraine. Collectively, these studies indicate that there may be a biochemical abnormality of glutamate and therefore treatments based on this idea have been investigated.
Table 4.8   Biochemical studies of Glutamate levels in migraine patients and controls.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Migraine Classification</th>
<th>Controls/Cases</th>
<th>Sample tested</th>
<th>Levels</th>
<th>Sample tested</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ferrari, Odink et al. 1990)</td>
<td>(IHS 1988)</td>
<td>Controls: 9</td>
<td>Plasma</td>
<td>↑</td>
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<td></td>
<td></td>
<td>MA: 10</td>
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<td></td>
<td></td>
<td>MO: 21</td>
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<td></td>
<td>TH: 9</td>
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<td>(Dandrea, Cananzi et al. 1991)</td>
<td>(IHS 1988)</td>
<td>Controls: 17</td>
<td>PLTS</td>
<td>↑</td>
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<td></td>
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<td>MO: 22</td>
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<td>TH: 15</td>
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<td>CH: 37</td>
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<td>MO: 19</td>
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<td></td>
<td></td>
<td>TH: 14</td>
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<tr>
<td>(Gallai, Alberti et al. 2003)</td>
<td>(Silberstein 1996)</td>
<td>Controls: 20</td>
<td>CSF</td>
<td>↑</td>
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<td></td>
<td></td>
<td>CDH: 10</td>
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<td>(Peres, Zukerman et al. 2004)</td>
<td>(Silberstein 1996)</td>
<td>Controls: 20</td>
<td>CSF</td>
<td>↑</td>
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<td></td>
<td></td>
<td>CMF: 12</td>
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<td>↑</td>
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<tr>
<td>Study</td>
<td>Year</td>
<td>Diagnosis</td>
<td>Control Group</td>
<td>Sample Size</td>
<td>Outcome</td>
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<tr>
<td>Vaccaro, Riva et al. 2007</td>
<td>2007</td>
<td>CM</td>
<td>Controls: 20</td>
<td>Plasma</td>
<td>PLTS</td>
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<td></td>
<td></td>
<td>MA: 25</td>
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<td></td>
<td></td>
<td>MO: 25</td>
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<tr>
<td>Vieira, Naffah-Mazzacoratti et al. 2007</td>
<td>2007</td>
<td>CM</td>
<td>Controls: 19</td>
<td>CSF</td>
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<td></td>
<td></td>
<td>CM: 5</td>
<td></td>
<td>↓</td>
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<td></td>
<td></td>
<td>CM overuse NSAIDS: 8</td>
<td></td>
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<td></td>
<td></td>
<td>CM overuse Tryptans: 6</td>
<td></td>
<td>↓</td>
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</tr>
<tr>
<td>Ferrari, Spaccapelo et al. 2009</td>
<td>2009</td>
<td>CM</td>
<td>Controls: 24</td>
<td>Plasma</td>
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<td>MO: 24</td>
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<td>↑</td>
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<tr>
<td>Ragginer, Lechner et al. 2012</td>
<td>2012</td>
<td>CM</td>
<td>Controls: 48</td>
<td>Urine</td>
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<tr>
<td></td>
<td></td>
<td>MA: 48</td>
<td></td>
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</tbody>
</table>

MA=migraine with aura, MO=migraine without aura, TH=Tension Headache, CH=Cluster Headache, CM=Chronic Migraine, CMF=Chronic Migraine with Fibromyalgia, CDH=Chronic Daily Headache, PLTS=Platelets, CSF=Cerebrospinal fluid, RBC=Red blood cells, ↑ Increase, ↓ Decrease, = Unchanged.
4.5.2 Glutamate Pharmacotherapy

Treatment options for migraine include a variety of prophylactic and acute therapies which modulate a broad range of pharmacological targets and thus produce a widely variable inter-patient response. Targeting receptors of the glutamatergic system using specific agonists and antagonists could be a valuable non-vasoactive neurologic alternative to explore given the variable efficacy of existing drugs. Animal and human models of pain have shown that the N-methyl D-aspartate (NMDA) antagonists can prevent the induction of central sensitization and reverse existing central sensitization which are events implicated in migraine (Hewitt 2000; Block and Habermeyer 2003; Latremoliere and Woolf 2009). Therefore, blockade of NMDA and other glutamatergic receptors may help to reduce the transmission of afferent signals that turn into a migraine. Glutamate receptor antagonists that act on glutamate signalling through glutamate receptors are currently being trialled in the treatment of migraine and include ADX10059, a mGluR5 modulator (Sprenger and Goadsby 2009), ketamine and memantine (Bigal, Rapoport et al. 2008; Marin and Goadsby 2010). Such compounds may benefit the patient in situations where other regimens have been unsuccessful.

Memantine is a moderate-affinity noncompetitive antagonist of glutamatergic NMDA receptors that blocks excessive NMDA receptor activity (Bigal, Rapoport et al. 2008). Memantine has also been used in the treatment of Alzheimer's disease and in dementia and its ability to affect pain behaviors has been demonstrated in animal models and in human clinical pain states (Carlton, Rees et al. 1998; Medvedev, Malyshkin et al. 2004; Zhang, Min et al. 2004). Ketamine has been shown to reduce the severity and duration of aura symptoms in humans and block cortical spreading depression (CSD) in animals (Afridi, Giffin et al. 2013). An important feature of CSD is that it is associated with an increase in glutamate, which drugs such as anticonvulsants can prevent.

Anticonvulsants such as topiramate, valproate, gapapenti, and lamotrigine which decrease glutamate levels and enhance (GABA) decrease abnormal excitability in the brain and have shown effectiveness in the treatment of migraine and support the hypothesis of a defective glutamatergic mechanism in migraine (Silberstein 2010). Topiramate is a derivative of the naturally occurring monosaccharide D-fructose and is an interesting example as it was originally developed as an anticonvulsant in epilepsy treatment but is also recognized as an
effective medication for migraine. In summary, components of the Glutamatergic system may be a fruitful target for new therapies.

4.6 Conclusion

Migraine can be considered a neurobiological phenomenon tied to transient changes in the functioning of nerve cells. The evidence so far points to migraine as a biochemically complex disorder involving several neurotransmitter systems (serotonin, dopamine, glutamate) which converge in their synaptic pathways. Although biochemical and physiological approaches to migraine have been extensively investigated, at present migraine has no clearly identifiable pathology and no evident biochemical marker or diagnostic test is available. The general trend observed in regards to neurotransmitters is that alterations do exist in various biological fluids of migraineurs however the interpretation of the biological significance of these various peripheral changes remains incomplete. Also the inevitable conclusion is reached that in most probably a multiplicity of factors, rather than any single one, must play a role. Therefore further studies utilizing larger sample sizes and current IHS classification criteria may produce more informative results. Biochemical approaches interwoven with studies in other disciplines are necessary and fundamental in illuminating the causes of disease and to further aid in the diagnosis, treatment and management of migraine. Although the underlying causes of migraine are still elusive and to be clarified, the biochemical results presented here indicate migraineurs possess an abnormal biochemical milieu characterized by higher circulating levels of neurotransmitters and underscore the need for further investigation to better understand the biochemical profile of migraineurs. The multifactorial character created by the interaction of a predisposed individual’s genotype with the environment has made it difficult to treat all patients with equal success. Although significant treatment milestones have occurred in the past 50 years quenching the unmet need in the community and providing necessary relief to many sufferers there is still much need for better and more effective medications. In conclusion the entirety of migraine is best investigated by incorporating results from a number of sources including biochemical, genetic and pharmacologic studies so that a more thorough and realistic picture of migraine identity can be presented.
CHAPTER 5  General Methodology
5.1 General Methodology

The research described in this thesis utilises both phenotypic and genetic data (DNA) collected from a group of migraineurs, in the year 1996 by the Genomics Research Centre (GRC), Griffith University, Gold Coast, Australia. The population used in this study consisted of individuals unrelated and of Australian Caucasian origin that were drawn from three large age and sex, matched unrelated case/control populations that were previously recruited by the GRC from the east coast of Australia.

The primary objective of this research was to investigate the role of selected candidate genes in migraine predisposition with a view to develop personalised treatments for migraineurs based on migraine candidate gene information. To achieve these goals, DNA and phenotypic data for each participant was collected. DNA was obtained from a sample of whole blood and phenotypic data were obtained via a comprehensive medical questionnaire that included a section specific to migraine. Detailed questions regarding family history, migraine symptoms, age of onset, frequency, severity and medication, and details of hormonal cycle and triggers were also obtained.

This chapter describes the general materials and methods undertaken to investigate the genetic basis of complex disease in subjects from the Migraine population. The following results chapters present more thorough detail of the application of specific techniques. Several methods were used to accomplish these aims which have been described in the sections that follow.

5.2 Ethical Approval

Ethics approval from Griffith University’s Human Research Ethics Committee (HREC) was granted for this PhD project under the current approval number (App. No. MSC/09/05/HREC). Participants were informed of the aims of the project, signed a consent form and donated a blood sample for genetic analysis.
5.3 Acquisition of participants

One thousand participants, for this study were recruited from people residing in the South East Queensland Region and that were of Australian Caucasian origin. The cohort consisted of a collection of unrelated individuals suffering from one of two types of migraine (MO or MA) and an independent collection of healthy unrelated individuals without migraine headache. Whilst the two subtypes have significant symptomatic overlap, individuals with MA experience a distinct phase of neurological disturbance known as “aura”, that usually precedes the headache phase of an attack and are therefore part of a separate group within migraine. Migraine Diagnosis was established by two clinical neurologists, Dr Peter Brimage and Dr John MacMillan in accordance with current IHS guidelines (IHS 2004). A higher proportion of female migraineurs have been included in the population for consistency with the female preponderance of the disorder (3:1). Venous whole blood samples were collected and genomic DNA was extracted using a standard salting out procedure as previously described (Miller et al. 1988).

These samples were separated into three independent association cohorts denoted as Migraine Association Population MAPI, MAPII and MAPIII. All cases of migraine were matched for gender, age (+- 5 years) and ethnicity (Caucasian ancestry) to the controls. The matched control samples were obtained via the Genomics Research Centre Clinic, Southport. This population is comprised of healthy age/sex/ethnicity (Caucasian) matched controls certified as having no family history of migraine and individuals who did not meet these criteria were excluded from the study.

5.4 Preparation of Migraine Samples

While the majority of DNA samples were collected and prepared in 1996, the continuation of the MAPI, II, III population has required DNA stocks to be replenished from original blood samples stored at the Genomics Research Centre during the course of this experimentation. Originally, DNA was extracted from whole venous blood using a modified version of a standard salting-out procedure described by Miller et al., 1988 (Miller S.A, Dykes D.D et al. 1988). DNA for each of the MAPI, II and III populations was available as TE stocks, these required clean-up (ethanol precipitation) and quantitation steps outlined below.
5.4.1 Materials

Autoclaved or filter sterilised deionised water from a Milli-Q Water Purification System was utilised in all aqueous solutions, dilutions and reactions. Aerosol filtered barrier tips were used in all DNA work to prevent contamination. DNA was extracted from venous blood using a variety of reagents. All reagents were of analytical grade and were supplied by Sigma-Aldrich (St Louis, CA), Astral Scientific (Gymea, Australia), and Gibco (Rockville, MD) unless otherwise stated. Quantum Scientific Pty Ltd (Milton, QLD) supplied all disposable laboratory equipment. A NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) was used to quantitate all genomic DNA and oligonucleotides.

5.4.2 Isolation of DNA

The DNA isolation stage required the removal of red blood cells, followed by overnight incubation to ensure complete lymphocyte lysis and the release of DNA into solution. Blood samples were removed from -80°C storage, completely thawed and transferred to a 50mL centrifuge tube. The original storage vile was rinsed with sodium-potassium-magnesium (NKM) buffer (0.14M NaCl, 30mM KCl, 3mM MgCl2) to ensure the entire blood specimen was removed and transferred to the centrifuge tube. Each sample was brought to a final volume of 25mL with NKM buffer and shaken vigorously. Samples were centrifuged at 4°C for 25min at 4,800 revolutions per minute (rpm), using a Sigma 4K15 centrifuge. The supernatant was discarded and each sample was brought to a final volume of 25mL with RSB buffer and centrifuged at 4°C for 15mins at 4000rpm (with a Sigma 4K15 centrifuge). The supernatant was discarded. The pellet was resuspended in 1mL of RSB buffer, prior to the addition of 4mL of lympholysis solution and 250μL of Proteinase K. Samples were placed in a 37°C shaking water bath overnight.

Once complete lymphocyte lysis was ensured, a salting out procedure was employed to precipitate DNA from the solution. Samples were removed from the water bath. 2mL of saturated sodium chloride (NaCl) solution was added to each specimen. Samples were mixed for 15s by inversion, prior to centrifugation at 4°C for 15min at 2,500rpm using a Sigma 4K15 centrifuge. This step ensures the removal of proteins from the lymphocytes. The DNA-containing supernatant was collected and transferred to a 15mL centrifuge tube. This
crifugation step was repeated to ensure maximum removal of proteins from solution. The supernatant was collected and transferred to a new 50mL centrifuge tube.

The volume of each sample was approximated and 2 volumes of room temperature absolute ethanol were added to each sample. Tubes were gently swirled to precipitate the DNA strands. DNA was removed using an inoculation loop and transferred into a new tube containing 2mL of Tris-EDTA (TE) (10mM Tris-Cl-EDTA) buffer at pH 8. DNA was dissolved in TE by incubation at 37°C for 2h, mixing at regular intervals. DNA can be stored for infinite amounts of time at 4°C in TE buffer.

For experiments samples were diluted to a concentration of 20ng/µL in sterile H₂O for PCR use as quantitated by the Nanodrop TM Spectrophotometer and working trays of these samples put together for experimentation. Additionally DNA has been extracted from junk DNA samples from saliva to be used as positive controls to validate the HRM technique.

The extraction procedure required the following reagents: NKM Buffer (0.14M NaCl-30mM KCl-3mM MgCl₂), RSB Buffer (10mM Tris pH 7.5-10mM NaCl-3mM MgCl₂), Proteinase K, Lympholysis Buffer, 6M NaCl, 1x TE Buffer, 100% (absolute) ethanol.

5.4.3 Ethanol Precipitation of DNA Stocks

Long term storage of DNA stocks is necessary for ongoing research. This is achieved by storing DNA in TE buffer. However for conducting PCR based experiments working stocks of DNA need to be in sterile water because the presence of ethylenediaminetetraacetic acid (EDTA) and/or any residual tri-phosphates can inhibit downstream PCR reactions. To remove these contaminants a simple clean up step is performed known as ethanol precipitation. This procedure required the following reagents: Sodium Acetate, 100% Ethanol,70% Ethanol, H₂O, Ice. To perform an ethanol precipitation a 100µL aliquot of stock DNA in TE buffer is added to a 1.5mL Eppendorf tube containing 300µL chilled 100% Ethanol and 10µL of sodium acetate solution (pH 5.5). The contents of the tube were then vortexed and centrifuged at 4°C for 25mins at 10,000rpm. The supernatant was discarded and 100µl chilled 70% ethanol added to the remaining DNA pellet. Samples were again vortexed and centrifuged at 4°C for 25 mins at 10,000rpm. As much supernatant as possible was removed with care not to remove the DNA pellet which was then air dried prior to
resuspension of the DNA in 100μl H\textsubscript{2}O. Samples were then incubated at 37°C to ensure resuspension.

### 5.4.4 Quantitation of DNA

After DNA from the migraine population was extracted and resuspended in H\textsubscript{2}O the samples were quantitated to determine the concentration and purity of DNA obtained from the extraction and clean-up process. Double-stranded DNA was quantitated using the Thermo Scientific NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) (see Figure 5.1). This instrument uses fiber optics and surface tension to accurately measure DNA concentration and quality (purity).

![Image of Nanodrop ND-1000 Spectrophotometer](image)

**Figure 5.1 Nanodrop ND-1000 Spectrophotometer Apparatus.**

Only 0.5 µl to 2.0µL of DNA sample is required for quantitation. The purity of the DNA from every stock sample from MAPI was measured by the ratio of absorbance at 260nm and 280nm. Pure double-stranded DNA gives a ratio of 1.8, hence a value that deviates from this indicates contamination by proteins, phenols or other nucleic acids. Upon determination of the DNA concentration of each DNA stock, working trays were made and these were used in all subsequent genotyping experiments. DNA samples were diluted to a working concentration of 20ng/µL in water ready for PCR. The dilution was carried out in accordance with equation 1.
Equation 1: \[ V_1 = \frac{C_2 \times V_2}{C_1} \]

V1 is the volume of undiluted sample required, C1 is the concentration of undiluted sample (acquired spectrophotometrically), V2 is the total volume of diluted sample and C2 is the concentration of diluted sample. For example, an initial DNA concentration (C1) of 200ng/µL to be diluted to 40ng/µL (C2) requires 10µL of undiluted sample (V1) plus 40µL of dH₂O in a total volume of 50µL (V2). Figure 5.2 shows what a normal uncontaminated spectrum of DNA looks like.

![Image of DNA spectrum](image)

Figure 5.2 Typical spectral pattern for Nucleic Acid.
5.5 Genotyping Methods

The 21st century has seen an avalanche of high-throughput, cost-effective genotyping and sequencing technologies emerge in the field of molecular genetics. This technological wave has been sparked by the completion of the first DNA reference sequence for Homo Sapiens (Human Genome Sequencing 2004) and the establishment of a human haplotype map detailing the distribution and frequency of SNPs across diverse human populations (The International HapMap Consortium 2005). The fruits of these projects have unlocked the door to unfathomable opportunity to delve beneath the surface and learn about the intricate molecular architecture of the double helix and the most fundamental functions regulated by our genes. The implications of this genetic achievement are: in depth understanding of the causes of disease attributable to genetic factors with identification of the responsible genes. The most common diseases in the developed world, such as coronary artery disease, hypertension, and diabetes to name a few occur due to a combination of both genetic and environmental factors and their interactions. Single nucleotide polymorphisms (SNPs) account for most of this genetic variability across human populations. They make excellent landmarks for navigating the genome due to their abundance and wide distribution and are important markers for disease diagnosis, prevention, and treatment. In this chapter the advantages and disadvantages of four commonly used genotyping methods (RFLP, HRM, TaqMan and MassARRAY platform) are discussed as well as which is the most cost-effective and time efficient method to genotype DNA.

5.5.1 Genetic Markers

The burgeoning genomics era is moving at a voracious pace and the public and the scientific community are both struggling to maintain pace with the growing body of knowledge generated about our genes, proteins and alternative splicing mechanisms. The success of this genomic era has been the result of coordinated international efforts that have resulted in, first, the complete sequencing of the human genome and second, its public availability to researchers globally. This has been a key milestone in the development of science that has enabled incredible progress in the area of gene mapping of complex disease. This information has allowed the development of individualized health assessment, orientated away from expensive hospital-based care but towards prevention and health promotion equipped with tools to identify people at risk in families and in the population at large (King, Rotter J. I. et al. 2002). Common diseases (diabetes, cancer, hypertension) which are the
product of the environment interacting with the genome have greatly benefited. Specific knowledge of which genes are involved in biochemical reactions and disease processes and how the products of defective genes control phenotype and function is integral to greater understanding the mechanism of how a disease occurs and progresses.

The human genome has been found to consist of ~22,000 genes with individuals identical for ~99.5% of their sequence (Pettersson, Anderson et al. 2009). The remaining 0.5% portion is variable from one person to the next and identifies and distinguishes people from one another. These small differences have been identified as single nucleotide polymorphisms (SNP) which are single base-pair changes that occur ubiquitously across the genome, in coding, non-coding and untranslated regions and are the most common contributor of variation comprising ~90% of all human variation within the human genome (Pettersson, Anderson et al. 2009). This genetic variation is called a polymorphism if it occurs with a frequency > 1% in the population and a mutation if it occurs less frequently (Hirschhorn, Lohmueller et al. 2002). This research is particularly concerned with single nucleotide polymorphisms.

The different variations that a polymorphism can have at a particular locus are termed alleles. For SNPs, single base pair changes occur predominantly within the two classes of nucleotides, between purines (A G) and pyrimidines (C T), which means that most SNPs will only have two alleles in a population. The specific combination of these alleles in an individual counting across the two relevant chromosomes is referred to as a genotype. In contrast, the combination of consecutive alleles on a single chromosome is termed a haplotype (Hirschhorn, Lohmueller et al. 2002). SNPs are evolutionarily conserved as a result they have been proposed as markers for use in quantitative trait loci analysis and in association studies in place of microsatellites. SNPs can also provide a genetic fingerprint for use in identity testing. SNPs have a high abundance and low mutation rate making them useful probes in the study of the structure and history of our genome. The majority of people possess a combination of SNPs in more than one gene which contributes to the manifestation of a disease phenotype. SNPs provide a measure of the likelihood that an individual will develop a particular disease and in explaining differences in genetic susceptibility. They are useful markers to track the inheritance of disease genes within families and for use in GWAS studies that utilize large cohorts of affected and unaffected people. Comparisons of variation between diseased (cases) and healthy (control) individuals from the same population may
give insight which may elucidate which genetic pathways are involved in disease onset and progression (Hirschhorn, Lohmueller et al. 2002).

There are a number of approaches to the identification of SNPs within candidate genes for genotyping experiments. One approach is to locate SNPs in the exonic sequence of the gene as this may lead to a change in composition and/or function of the protein that it encodes and can therefore contribute to disease. These are called functional SNPs. Another approach is to look for SNPs that may affect the regulation or expression levels of the candidate gene which could affect protein availability. One way to do this is to identify functional elements within the gene such as regulatory regions, promoters, enhancers which are essential for controlling the extent to which the gene is transcribed. Alternatively intronic SNPs can be chosen and more often than not these are the only ones available with frequency information reported and still regarded informative of variation within a gene. The International HapMap Project has detailed information on genetic variation across the genome. An important use of these data is to help identify genetic determinants of human disease (Xu, Kaplan et al. 2007).

There are a variety of methods to determine the genetic variant that an individual may have inherited at a specific locus. The process that determines the “genotype” or the complement of alleles an individual has inherited from their parents by examining their DNA sequence is called genotyping. All genotyping assays rely on polymerase chain reaction (PCR) as an initial step, which amplifies the specific DNA fragments to be analysed. Common methods of genotyping available include: Restriction Fragment Length Polymorphism (RFLP), High Resolution Melt Analysis (HRM), TaqMan analysis, MassARRAY platform and Sanger Sequencing, this is not an exhaustive list. Genotyping techniques have evolved greatly over time into highly sophisticated technologies no longer restricted to a single genotype per reaction but are now able to multiplex many SNPs at once. The aim of the work reported in this thesis was to optimize, test and perform different genotyping techniques, the following section describes some of these methods of genotyping in detail as well as the technical aspects of performing them.
5.5.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a technique for selectively and repeatedly replicating defined sequences of DNA (Lo and Chan 2006). PCR may be regarded as a simplified version of the DNA replication process that intricately occurs inside a cell during cell division (Lo and Chan 2006). PCR is the equivalent of this process only carried out in vitro. The versatility of PCR has made it one of the most widely used methods in molecular biology. The number of PCR-based applications has continued to increase rapidly and has impacted oncology genetics and pathogen detection. All aspects of this research requiring analysis of specific variants within specific genes utilised PCR technology.

In PCR, the reaction mixture contains the DNA sequence to be amplified, two primers complementary to the DNA sequence of interest, a special DNA polymerase, Taq Polymerase which extends the primers, and various buffers to optimise the reaction conditions (Lo and Chan 2006). PCR consists of three steps: thermal denaturation of double stranded DNA, annealing of primers and extension of the annealed primers by the Taq Polymerase in the 5’-3’ direction (see Figure 5.3). This three-step cycle is repeated ~30-40 times over so that the reaction occurs exponentially with each cycle theoretically doubling the amount of DNA produced until dNTPs and other reagents are exhausted (Baumforth, Nelson et al. 1999). PCR amplifies minute quantities of DNA a million fold quickly and reliably.

Primer design is aimed at obtaining a balance between two goals: specificity and efficiency of amplification (Dieffenbach, Lowe et al. 1993). Primers are designed to flank the sequence of interest and are usually between 18 and 30 bases long, with a GC content of about 50%. Complementarity at the 3’ ends of the primers should be avoided to decrease the likelihood of forming hairpin structures and primer-dimers all of which are detrimental and limit the efficiency of the PCR reaction.
Figure 5.3  Polymerase Chain Reaction sourced from (Maher 2011).
5.5.3 Agarose Gel Electrophoresis

Once the region of interest has been amplified the DNA segment can be visualised in an agarose gel stained with ethidium bromide to check for successful amplification. This method is called agarose gel electrophoresis and is used to separate nucleic acids based on size and charge. The process of electrophoresis refers to the passage of an electrical current through a slab of agarose gel (see Figure 5.4). In a laboratory this process takes place inside specialized equipment known as gel electrophoresis tank.

The agarose gel is made by mixing chemical grade agarose powder with TAE buffer and heating in a microwave to dissolve. When the solution cools to 60°C, it is poured into a casting tray containing a sample comb to form sample wells and allowed to solidify. The gel is stained with Ethidium bromide, a dye that intercalates in the grooves of DNA. This dye is added to the gel (final concentration 0.5µg/mL) to visualize DNA bands.

Agarose is a polysaccharide extracted from certain seaweeds that polymerizes to form a porous lattice through which negatively charged DNA molecules migrate. The molecules migrate from the negative to the positive terminal and are separated based on their molecular weight. Gel loading dye is added to sink DNA into the wells and allow visual monitoring of the progress of the electrophoretic experiment.

A DNA ladder is concurrently loaded to help estimate the size of DNA molecules in each sample. This is a mixture of DNA amplicons of specified sizes (e.g. 100bp, 200bp, 300bp, 400bp 500bp 1000bp etc) (see Figure 5.5). The size of the PCR product can thus be checked against this banding pattern to ensure the amplicon is the expected size and that primers are amplifying the correct sequence (Baumforth, Nelson et al. 1999). After electrophoresis the gel is visualized on a U.V. trans-illuminator connected to a digital camera so that an image can be recorded. Once a region of interest has been successfully amplified the sequence of that amplicon can be genotyped for variants associated with disease.
Figure 5.4  Agarose Gel Electrophoresis adapted from (Brown 2002). Samples are loaded into wells and electric current is applied. Samples migrate in the gel from the negative to the positive terminal and separate according to molecular weight. Bands are visualized under UV light.
Figure 5.5  Gel electrophoresis of PCR product sourced from (Maher 2011).
5.5.4 Restriction Fragment Length Polymorphism

The RFLP method of genotyping evolved from the discovery of a group of enzymes that recognise and cut specific sequences of DNA (Meselson and Yuan 1968). These were termed restriction endonucleases, now more commonly known as restriction enzymes. Restriction enzymes can cut double stranded or single stranded DNA into fragments upon recognition of a specific DNA restriction site. They are derived from bacteria and were initially discovered in *E. coli* which appeared to be restricting the infection of specific bacteriophages ("viruses" of bacteria) (Station. 2012). These enzymes are thought to have evolved by bacteria as a host defense mechanism to eliminate viral infections.

Nevertheless they have found widespread application in the field of molecular biology where they are regularly used to investigate genetic relationships within and between specific human ethnicities. The term RFLP arose to describe the genotyping technique but also refers to a polymorphism (SNP) whose alleles can be differentiated by digestion with a restriction enzyme whereby one allele is preserved while the other is cleaved resulting in differently-sized DNA fragments (Twyman 2005).

This method was initially described in the mid-1980s and is one of the oldest and most widely used genotyping techniques in many science laboratories as it was the first technique employed in the genotyping process. The principle of this method is to firstly amplify sample DNA in a standard PCR reaction and to then digest the DNA in a second step with a restriction enzyme. If the amplicon contains the polymorphism then one allele will result in enzyme recognition and cleavage (see Figure 5.6). The resulting restriction fragments produced by the unique DNA sample can then be separated according to their lengths by gel electrophoresis to determine the genotype of the individual.

RFLP on the one hand is easy to set up provided a restriction enzyme is available for the polymorphism of interest. The advantage of this method is that a 96-well plate format and a multi-channel pipette can be used for dispensing reagents. RFLP is now considered a slow and cumbersome technique in comparison to the more efficient high-throughput techniques available today. RFLP is labour intensive requiring careful optimization of the PCR and the restriction enzyme digest reaction. The disadvantage is that RFLP is difficult to adapt to high-throughput genotyping and requires making agarose gels, the use of restriction enzymes.
followed by electrophoresis which could take up to a month to complete. The use of a restriction enzyme is what increases the cost most dramatically. However the upside is that RFLP requires minimal investment in instrumentation, has flexibility in amplicon design and although more steps are involved, it develops laboratory skill and expertise in the novice student.

**Figure 5.6  Schematic Diagram of RFLP for a C > G SNP change.**

The C allele contains the restriction enzyme recognition site and is therefore cut producing two separate fragments of 88bp and 58bp. The G allele is not digested as it does not contain the restriction enzyme recognition site it appears as one fragment of 146bp.
5.5.5 High-Resolution Melt Analysis

The key difference between RFLP and HRM is in the use of a fluorescent dye to detect the alleles. High-resolution melting (HRM) analysis has been around for 16 years since it was first introduced in 1997 by Ririe et al. (Ririe, Rasmussen et al. 1997) and is a real time PCR method that can visualize the melting behaviour of DNA product through a fluorescent dye. Like RFLP, HRM starts with PCR amplification of the target sequence with two primers but with one difference, the inclusion of a fluorescent dye such as SYTO®9 into the reaction mixture. The dyes used for HRM are known as intercalating dyes and have the unique property whereby they bind specifically to double-stranded DNA during PCR amplification and when they are bound they fluoresce brightly (see Figure 5.7). This unique property allows monitoring of the change in fluorescence with temperature during DNA melting.

![Schematic of 2nd and 3rd Generation DNA Intercalation Dyes in HRM sourced from (Research 2006).](image)

**Figure 5.7** Schematic of 2nd and 3rd Generation DNA Intercalation Dyes in HRM sourced from (Research 2006).
HRM begins with PCR amplification of a target sequence in the presence of a dsDNA intercalating fluorescent dye. After PCR has finished HRM begins with gradual heating of the DNA sample through a range of temperatures. As the temperature increases dsDNA dissociates (or melts) into single strands and the fluorescent dye that attached to dsDNA during amplification is released resulting in a decrease in fluorescence. The shift in fluorescence as DNA is gradually melted is used to measure the accumulation of DNA product during a pre-HRM amplification reaction and then to differentiate DNA samples with different genotypes according to their dissociation behaviour as they “melt” from double stranded DNA to single stranded DNA (ssDNA) with increasing temperature. The extent of melting is measured by the change in fluorescence of the DNA sample which is captured and plotted in a graph that is displayed as a melt curve profile characteristic of the polymorphism in the amplicon (see Figure 5.8). Three distinct melt curves are obtained allowing discrimination of three genotypes (see Figure 5.9). Different alleles for a single nucleotide polymorphism will therefore melt at a slightly different temperature reflecting the chemistry of the nucleotide base pair present.

Figure 5.8 Typical High Resolution Melt plot sourced from (Research 2006).
The melt curve in green shows the transition from high fluorescence of the initial pre-melt phase and then the sharp decrease in fluorescence when double-stranded DNA melts whilst it is being heated into its single-stranded form and the characteristic melt curve obtained. Fluorescence minimizes as DNA intercalating dye is released from double-stranded DNA as it dissociates (melts) into single strands. The midpoint of the melt phase shown by the black vertical line, at which the rate of change in fluorescence is maximum, corresponds to the melting temperature (Tm) of the specific DNA sequence under investigation (Research 2006).

![Melt Curve Diagram](image)

**Figure 5.9** The end result of an HRM experiment showing aligned melt curves of three DNA samples followed by DNA sequencing sourced from (Biosystems 2010).

The advantages of using HRM are clear; one does not have to use expensive restriction enzymes, pour agarose gels and use hazardous chemicals (ethidium–bromide), melting is faster than electrophoresis occurring all in one instrument, and data analysis can be performed automatically with user friendly software that allows genotype data to be exported into an Excel spreadsheet (Vossen, Aten et al. 2009). As a technique HRM is fiddly, the technician must use their fingers to gently apply the lids to the tubes which can be cumbersome for people with big hands. This method can be labour intensive because the technician is restricted to using a single channel pipette due to the spacing of the small HRM strip tubes. The most expensive element is acquisition of the sophisticated instrument, the
Corbett Rotor-gene 6000 that performs the thermal cycles required for the PCR as well as the DNA melting.

Pipetting technique is the most crucial variable in this method, accurate and consistent pipetting when preparing reactions is needed to ensure uniform reagent concentrations for each sample to produce good genotyping calls. Typing DNA samples in duplicate with positive controls of known genotypes and one no template control in every assay run can minimize variation in genotype calls. The fact that each sample is run in duplicate means that twice as much DNA is used in the experiment. The maximum number of samples that can be genotyped in each HRM experiment is 32 samples run in duplicate with three positive controls and one no template control (72-well rotor). Each run takes roughly 90mins to complete.

The HRM method has surpassed RFLP and catapulted into a powerful tool for genotyping polymorphisms and detection of mutations. Developments in specialized instrumentation capable of high-resolution amplicon melting combined with third generation fluorescent intercalating dyes such as SYTO®9 have made HRM a popular genotyping method due to its speed and simplicity. The HRM method is simple and flexible, has minimal requirements for optimization and for these reasons is a method of choice.

5.5.6 TaqMan® Analysis

TaqMan is another method of genotyping that is similar to the HRM genotyping method in that it uses fluorescent dyes. The difference however is that in TaqMan allelic discrimination is achieved using two distinct fluorescent dyes, VIC® and FAM™, for the detection of different alleles and genotypes. The fluorescent dyes, VIC® and FAM™ are attached to the 5’ end of allele-specific probes (short oligonucleotide sequences). Each fluorescent dye either VIC® or FAM™ is specific for only one allele. The 3’ end of the probe is labelled with a non-fluorescent quencher (NFQ) which prevents the dye from fluorescing while in the vicinity of the quencher, and a minor-groove binder (MGB), which stabilises the binding of the MGB-probe/template DNA complex (Biosystems 2011).

Purchased TaqMan genotyping assays include a Universal PCR master mix containing buffer, MgCl₂, dNTPs, and AmpliTaq Gold (a high quality polymerase with exonuclease activity)
and the assay mix containing a pair of forward and reverse primers and two fluorescently-
labelled MGB probes. During normal PCR amplification the forward or reverse primer as well as the MGB-probe both binds to the target DNA if complementary to the allele, Taq
polymerase extends from where the primer for each strand has bound until it reaches the 5’
end of the probe (Figure 5.10). At this point the exonuclease activity of the Taq polymerase
cleaves the reporter dye (either VIC® or FAM™) from the 5’ end of the MGB probe which
fluoresces as it moves away from the bound probe.

In each PCR cycle continuous cleavage of one or both allele-specific reporter dyes from the
probes produces an exponentially increasing fluorescent signal corresponding to one or both alleles that directly correlates with the amount of PCR product that was synthesized in the
PCR reaction. A fluorescent signal is generated when the intact probe, which is hybridized to
the target allele, is cleaved by the 5’ exonuclease activity of AmpliTaq Gold DNA
Polymerase during each cycle of the PCR reaction. The type of fluorescence (either VIC or
FAM or a combination of both is what determines whether a sample is heterozygote or
homozygote. At the end of the PCR, a laser collects a fluorescence spectrum a linear
combination of the fluorescence emissions from the component dyes and bioinformatic
algorithms within the software extract the contribution of each component dye to the
observed spectrum to produce a scatter diagram plotting the allele-specific components of
each reaction for interpretation of the genotype (see Figures 5.11-5.13).

The TaqMan method offers a number of advantages when compared to other methods,
genotyping is achieved in a single enzymatic step with no post-PCR processing; the plate
remains sealed, thus reducing the chance of sample mix-up, sample loss and contamination.
TaqMan genotyping assays are quality control tested and run under universal reagent
concentrations and thermal cycling conditions with minimal optimization of assay conditions
needed. The standardized reagents and conditions employed in TaqMan assays as well as the
use of the ovation multi-channel pipette make genotyping a breeze. The pre-made Universal
PCR master mix creates a very easy workflow for the technician by reducing pipetting errors
and pipetting time ensuring a smooth and more consistent experiment. The assays are quite
robust giving the same genotype regardless of time of day and problems generally only arise
when using poorly extracted DNA containing PCR inhibitors and/or contaminants. TaqMan
assays become cost-effective when genotyping large sample sets. Applied Biosystems also
offers a service for the design of custom TaqMan SNP genotyping assays for which specific markers may not be available in their catalogue.

The most time consuming part involved is dispensing DNA accurately into the 384-well plate and adding master mix and remembering to spin the plate for 2mins prior to loading into the 7900 instrument. Despite the fact that this method is only suitable for genotyping one polymorphism at a time it is accurate and time saving and of considerable scale-up in throughput over the RFLP and HRM methods (McGuigan and Ralston 2002). One individual can easily genotype 5 384-well plates per day if not more (2304 samples). The TaqMan method is a major improvement over the original RFLP method for the medium-sized laboratory performing association studies (McGuigan and Ralston 2002). The cost of the probes becomes less of an issue when many hundreds of DNA samples are analyzed in a significantly reduced reaction volume.

Figure 5.10  Schematic of TaqMan Chemistry sourced from (Biosystems 2011).
Figure 5.11 Multicomponent Plot for rs2271275 P01 showing heterozygous (CT) genotype.

Figure 5.12 Multicomponent Plot for rs2271275 P01 showing homozygous (CC) genotype.
5.5.7 Sequenom MassARRAY MALDI-TOF MS platform

The iPLEX Gold technology Sequenom relies upon a primer SBE (single base extension) reaction for allelic discrimination (Bouakaze, Keyser et al. 2011). The MassARRAY system differentiates genotypes based on differences in the mass of a single nucleotide (Ghebranious, Ivacic et al. 2005). This method began with the analysis of proteins and peptides and later found its way with DNA. The first experimental step in this method is to perform PCR reactions with standard forward and reverse primers to amplify a region that includes the SNP polymorphic site (Figure 5.14). Sequenom’s MassARRAY Designer software is used for designing PCR primers and extension primers for each SNP to be included in the multiplex assay. Multiplex PCR reactions are set up with forward and reverse primer pairs designed to amplify each SNP all in one multiplexed assay pool. The PCR reactions are then treated with Shrimp Alkaline Phosphatase (SAP) enzyme. SAP treatment of the reaction mixture containing the amplicon and unincorporated dNTPs effectively deactivates residual dNTPs preventing them from participating in and thus interfering with subsequent base extension reactions (Farkas, Miltgen et al. 2010). The SAP enzyme does this by dephosphorylating dNTPs to dNDPs (deoxyribonucleoside diphosphates) making them unusable by the polymerase for DNA chain elongation.

After this step, the purified amplicons are used as templates for the primer extension reaction and are thermocycled again in a cocktail containing buffer, the extension primer, ddNTPs and
the polymerase. In this primer-extension reaction the extension primer is elongated by only one base thus generating allele-specific products with distinct masses. The extension primer is specifically designed to anneal exactly one bp upstream to the SNP and once hybridized is extended by the polymerase which adds a mass-modified base complementary to the nucleotide of the SNP allele. The extension reaction uses modified ddNTPs as terminating substrates. Each ddNTP (e.g., ddATP, ddCTP, ddTTP, ddGTP) has a different mass varying by at least 16 Da (Oeth, Beaulieu et al. 2005). The difference in the mass of the extension products generated for each allele of a DNA sample allows the data analysis software to discriminate two alleles of a SNP and assign a genotype. Mass spectrometry enables mass determination of the products and the interpretation of homozygotes and heterozygotes genotypes (Figure 5.15). Finally, prior to being analysed, the extension products are desalted with resin to remove salts such as Na\(^{+}\), K\(^{+}\), and Mg\(^{2+}\) which may give high background noise in the mass spectra (Gabriel, Ziaugra et al. 2001). Throughout the entire process Sequenom iPLEX Gold chemistry is used for Sequenom genotyping. Genotyping with Sequenom enables up to 36 SNPs to be assayed simultaneously in one multiplex with standardized conditions.

After desalting, the extension reaction products are ready to be dispensed onto Sequenom's silicon based SpectroCHIP with the nanodispenser portion of the instrument. The SpectroCHIP is pre-spotted with a specially formulated MALDI matrix for facile incorporation of oligonucleotides in a small volume (25nL) directly arrayed onto existing matrix spots of the silica chip available in one of two formats, a 96-element chip or a 384-element chip (Gabriel, Ziaugra et al. 2001). The SpectroCHIP coated with the extension reaction products is placed inside the mass spectrometer (MS) and each spot is irradiated with a laser under vacuum (Gabriel, 2009). The laser energizes and vaporises the matrix generating a particle cloud that carries the analyte into the vacuum flying through a one-meter long path to an ion detector based on its mass (Gabriel, Ziaugra et al. 2001). Although there are different ionization methods that can be used with different mass analyzers most commonly, MALDI (Matrix-Assisted Laser Desorption/Ionisation) is coupled to a Time-of-Flight (TOF) analyser (Pusch and Kostrzewa 2005).

The time-of-flight is the time required for the analyte to traverse the distance between the ion source and detector and is proportional to the mass of the molecule (in this case the extended primer) (Jackson, Scholl et al. 2000). Smaller DNA fragments will travel faster than larger
ones thus by measuring the time-of-flight of each extension primer the mass can be
determined and this information can be converted into a genotype. By comparing the
molecular weight of each sample to expected molecular weights based on known sequence
the genotype of a sample can be determined (Ross, Hall et al. 1998; Kwok 2001). Automated
allele calling is carried out in real-time using the SpectroTYPEr software which calculates
the mass of the fragments and translates the mass of the extension products into a genotype
for each reaction which is displayed in a cluster plot seen in Figure 5.16.

Experimentally all the steps needed for PCR amplification, SAP treatment, primer extension
and desalting of the extension products are performed in a single tube format during the
complete experiment in the same well using standard 96-well plates like in RFLP. This setup
has the advantage of using very small reaction volumes, only 10ng/μL of DNA are required
in the experiment and the potential for contamination and potential errors associated with
transferring reaction products is eliminated. Overall, this method stands out due to the high
multiplexing capability which reduces the time to genotype many SNPs at once and because
the detection of analytes is based on their molecular weights there is no need for labeling or
any separation steps again facilitating the experimental setup (Ghebranious, Ivacic et al.
2005). The combination of many genotyping reactions in one assay means that this technique
is cost effective and high-throughput and suitable for mutation analysis in diagnostic
research.
Figure 5.14  Overview of Sequenom genotyping workflow sourced from (Ghebranious, Ivacic et al. 2005).
Figure 5.15  Example of spectra MALDI-TOF detection sourced from (Ghebranious, Ivacic et al. 2005).
Figure 5.16  Example of Cluster plot showing 3 distinct genotypes.
5.5.8 Automated Sanger Sequencing

Sequencing is considered the gold standard for performing direct genotyping or validating other genotyping methods. DNA sequencing is the process of determining the order of chemical building blocks (called bases or nucleotides A, T, C, and G) that make up a DNA molecule. The classical chain-termination method of sequencing developed by Frederick Sanger in 1977 consists of multiple steps which have to be completed before the products to be sequenced can be loaded onto the sequencing analyser (Figure 5.17) (Kircher and Kelso 2010). The important difference in Sanger sequencing is in the use of modified dideoxynucleotides triphosphates (ddNTPs) as substrates used to terminate the growing DNA strand during the sequencing reaction. These dideoxynucleotides differ from normal nucleotides used in PCR reactions in that they are labelled with a unique fluorescent dye that emits light at a different wavelength.

Firstly amplified PCR product is mixed with Exo-SAP-IT reagent to remove unconsumed dNTPs and primers. Exo-SAP-IT utilizes two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase which together degrade and hydrolyse any unwanted dNTPs and primers from the reaction leaving only double stranded PCR product. Exo-SAP-IT is inactivated by heating to 80°C for 15mins. This step prepares the PCR product for the sequencing reaction.

The sequencing reaction is performed using a commercially available BigDye Terminator v3.1 Cycle Sequencing Kit. The kit contains the required reagent components for the sequencing reaction in a pre-mixed format. The PCR product is added to a mixture of normal dNTPs with the addition of fluorescently labelled terminator dideoxyribonucleotides (ddNTPs) and uses a separate reaction for each of the forward and reverse primers. The cycle process is generally the same as a standard PCR however when a ddNTP is incorporated into the amplicon during the elongation step the DNA synthesis is terminated, creating amplicons of varying lengths with a fluorescently labelled ddNTP at each end. Lastly, an Ethanol precipitation step is required to clean the DNA or else the samples will not run well due to salts or other interfering reagents. Moreover, there could be large dye blobs of unincorporated Dye Terminators which can obscure the base calls prior to loading on the genetic Analyser.
The fragments are then size-separated by capillary electrophoresis and the wavelength of each amplicon is measured over time allowing the sequence of the amplicon to be read. The terminated fluorescent fragments are separated by length from longest to shortest. Each of the four dideoxy nucleotides is fluorescent at different wavelengths when illuminated by a laser beam. The fluorescent tails of each amplicon are then read by the automated genetic analyser. Sequencing is usually reserved for confirmation of positive controls or for mutation scanning due to the expense however it is becoming more affordable with the advent of next generation sequencing machines and will likely be applied in future projects.

![Sequencing reaction and analysis](source-image)

**Figure 5.17  Sequencing reaction and analysis sourced from (Maher 2011).**
5.6 Discussion

The next generation of high-throughput genotyping platforms is constantly changing due in part to the development of advanced instrumentation and dye-based chemistries and also the inundation of precise genetic variation data from international genome projects. The demand for genotyping large cohorts to achieve statistical significance is shaping the development of genotyping technology. Namely assay development has to be cheap, fast and reliable. Second, the cost of the assay from instrumentation to reagents must come at a price that people can pay and third, the assay must have the capacity to multiplex several SNPs in a single reaction. Currently at least 20 different SNP genotyping methods exist from which to choose. This can seem a daunting number to the eager laboratory manager browsing glossy catalogues. However, such an armamentarium of diverse genotyping techniques enables scientists to tackle difficult regions of the genome thus providing flexibility in project design. The study design and number of samples to be genotyped in a project is a key factor that determines the choice of genotyping technique.

In our study, we have compared four SNP genotyping methods for laboratories undertaking candidate gene approaches to enable researchers to choose the most convenient method based on their requirements and the availability of each instrument. The following three genotyping methods, RFLP, HRM and TaqMan and MassArray were used to genotype DNA samples from an Australian Caucasian population. These are traditional methods in common practice today and used in our laboratory. The aim of this study was to compare each method on the basis of ease of use/technical aspects, accuracy, time, cost, and throughput capabilities. Each method has a number of advantages and disadvantages which must be carefully considered when designing a genotyping study in order to achieve a balance between time and cost that will give the best result. At the end of the day with proper optimization accurate results can be obtained regardless of the genotyping method employed. However, when reflecting upon the technical aspects of executing each method and setting up the reaction in the laboratory at the bench considerable technical differences surface which can make or break the experiment if successfully tackled.

In discussing the genotyping throughput of each method the MassARRAY platform and TaqMan method surpass RFLP and HRM by a mile. This is clearly evidenced in the ability to genotype a maximum of 36 SNPs at once with the MassARRAY platform and our entire
case-control population with TaqMan in one day versus weeks for RFLP and HRM. Naturally this varies with the speed or the inclination of the technician and how many assays per day they are able to set up which is often dictated by the availability of equipment. In terms of cost, MassARRAY and TaqMan are expensive due to the specialised equipment that must be purchased to get started and the sophisticated nature of the reagents. Our results showed that overall when considering factors of speed and efficiency the MassARRAY and TaqMan platforms stand out as being the most fast and efficient to genotype DNA.

5.7 Genetic Mapping Techniques

Two basic genetic study approaches used to study the inheritance of disease are family-based linkage studies and case-control association studies. Both of these methods aim to determine the location of risk genes associated with disease. These methods have advantages and limitations and have been used throughout history to track down genes involved in causing disease. Linkage studies ask the question, is a trait of interest segregating in families? Whereas association studies ask if a specific allele of a genetic marker is found with increased frequency in individuals with the disease compared to the frequency of that marker in individuals without the disease.

5.7.1 Association studies

Association studies are a popular design for mapping risk genes in complex disease. This is because they do not rely on familial patterns of inheritance but rather a strict comparison of disease allele frequencies between affected and unaffected individuals (Figure 5.18). The case and control groups differ only by the disease state and are independent and unrelated to one another. One requirement of this study is that the groups are matched for age, sex and ethnicity as failure to do so can lead to false positive/negative associations between the genetic marker and disease.

Typically genes for such studies are selected based on prior known biological function or location in a region implicated in a previous linkage or association study (Hirschhorn, Lohmueller et al. 2002). Alternatively genes may be considered from knowledge of physiological pathways and because effective treatments target the pathway in which the gene interacts (Gardner 2006). The types of genetic association studies conducted are commonly divided into candidate gene and genome-wide association studies. Both
approaches involve genotyping SNPs in large collections of cases and controls (Pettersson, Anderson et al. 2009). These tests can result in the identification of part of the genetic component of a disease and may lead to significant implications for public health authorities by offering advances in diagnostics and treatment options.

Positive association of a marker and disease can indicate a number of possibilities; the variant directly causes the disease; the variant is indirectly associated with the disease due to association with a nearby variant (linkage disequilibrium); or the association is the result of population substructure. An allele (or genotype) is said to be associated with the disease if the frequency of the variant genotyped is significantly different in the affected group compared to controls. The magnitude of this deviation is measured using standard non-parametric Chi-Square analysis, logistic regression and odds ratios.

Some limitations in the case-control design are that genotype and haplotype frequencies vary between ethnic or geographic populations independent of effects of disease. If the populations are not well matched for ethnicity then false positive associations can occur because of the confounding effects of population stratification. Population stratification can arise if the total population is derived from a combination of two or more sub-populations leading to ethnic admixture (Hoggart et al. 2003). To overcome this problem and ensure results are interpreted correctly the family-based transmission disequilibrium test is applied. Finally, associations can be real but may not be reproducible if the underlying genetic effect is weak. Consequently association studies may be underpowered to reliably detect weak effects and, therefore fail to achieve statistical significance. Increasing sample size is the best method of improving power and overcoming limitations of study design.
Figure 5.18  Association study design sourced from (Maher 2011).
5.7.2 Linkage analysis

Linkage is a method that aims to identify chromosomal regions likely to contain a risk gene by studying families affected with a disease. By genotyping DNA markers and studying their segregation through large multigenerational pedigrees, it is possible to infer the position of disease causing regions. Loci harbouring disease can be identified through the use of linkage analysis and this method has proven useful for the identification of genes in single gene disorders such as Cystic Fibrosis and variants of complex traits following simple Mendelian inheritance such as familial hemiplegic migraine.

Linkage analysis is based on the fact that during meiosis recombination occurs randomly and that each parental allele has the same chance of being transmitted to the offspring at every locus. Alleles that reside close to each other on the same chromosome are said to be ‘genetically linked’ because they will be transmitted together during meiosis more often than loci that are on different chromosomes (Teare and Barrett 2005). Two loci are linked if, during meiosis, recombination occurs between them with a probability of less than 50% (Teare and Barrett 2005).

There are two statistical models of linkage analysis which are applied depending on how the disease in question is inherited, these are parametric and non-parametric. Parametric or model-based linkage analysis requires a defined disease inheritance model and uses a logarithm of the odds (LOD) score to determine if a trait is linked to the disease and the strength of the linkage. This score is a function of the recombination fraction (θ) or chromosomal position measured in cM and was first proposed by Morton in 1955 (Teare and Barrett 2005). This method is typically used for single gene disorders which have a straightforward Mendelian mode of transmission, in other words a specific mutation in a single gene is responsible for the disorder.

The disease is studied using multigenerational families (pedigrees) that include both affected and unaffected family members and genotyping DNA markers which can be mutations in a single base pair (Single Nucleotide Polymorphisms (SNPs) or microsatellites that act as reference points for identifying stretches of DNA co-inherited with a certain trait or disease (Vink and Boomsma 2002). DNA regions that are co-inherited with a disease locus are generally close to one another and will be in linkage disequilibrium (see Figure 5.10).
Significant evidence of linkage (or co-segregation) in Autosomal markers is indicated by large positive LOD scores equal to or greater than $\geq 3$ ($P=0.0001$), while suggestive significance is generally taken at LOD $\geq 2$ ($P=0.001$) whereas negative scores are evidence against linkage.

In contrast when investigating multifactorial diseases, where several genes and environmental factors and their interactions contribute to the risk of a disease and there is no clear mode of inheritance a non-parametric approach is used. This model-free approach is most suited for complex diseases like migraine which have an undefined genetic model. Non-parametric analysis evaluates the portion of alleles shared at a particular genetic locus in affected relatives without any assumed parameters; it is model free. Linkage to the trait is reported when there is excess sharing of alleles at a genetic locus between affected relatives. This method considers the information on alleles shared identical-by-descent (IBD) in affected relatives and assumes that in the presence of a disease they will exhibit excess sharing of haplotypes. This form of analysis is also referred to as allele-sharing methods. Similar to parametric methods, linkage is reported as a likelihood ratio using natural logarithms. This score can be converted to a traditional LOD score by dividing by 4.6 (i.e. $2\times\log_{10}$) (Teare et al. 2005).
Figure 5.19  Example of linkage between a trait and a marker sourced from (Maher 2011).
5.7.3 Migraine GWAS studies

Genome wide association studies are an additional technique for the identification of genetic variations associated with disease and that have the advantage of being non-candidate-driven in contrast to gene-specific candidate-driven studies (Lewis, Nsoesie et al. 2011). This means that this approach is hypothesis free and that no prior information and/or assumptions about disease aetiology is necessary and has the potential to discover unknown pathways.

In a GWAS large case-control cohorts are used and up to a million SNPs are genotyped covering the entire genome with commercially available SNP arrays (Zhang, Liu et al. 2007). The cases are composed of people affected with a disease whilst the controls consist of healthy individuals. The SNPs tested in a GWAS are common having a minor allele frequency of >5%. Commercial SNP arrays used in GWAS use a tag-SNP approach, tagging SNPs are representative SNPs that take into account patterns of LD (linkage disequilibrium) making the investigation more targeted. The allele frequencies of the tested SNPs in the two groups, disease and control groups, are compared and tested for association. When statistically significant differences in allele or genotype frequencies are identified between cases and controls the marker is said to be associated with the disease. This finding however does not mean that the genotyped variants are causal but may simply be in linkage disequilibrium with the causal variant and therefore the loci require further studies to fine-map the actual causal variants and affected genes.

Although GWAS cannot on their own specify which genes are causal they do provide an alternative route by which to assess the genome in an unbiased manner and derive information concerning pathways with potential disease involvement. Accurate well-defined phenotypes are important in a GWAS and do affect the overall strength and quality of the association obtained. If for example only a subset of the cases contain the culprit variant in the gene the association will be weaker due to the effect of genetic heterogeneity of the cohorts utilized. Often in GWAS the majority of common alleles found to be associated with disease contribute weakly to overall disease risk and susceptibility, the cumulative effect of all associations are generally not responsible for the main genetic component of a disease and account for only a small proportion of the predicted genetic variation. The ‘missing heritability’ may be due to rare variants with a low allele frequency which are expected to contribute a larger effect size in complex diseases.
GWAS poses many computational challenges due to the massive amounts of GWAS data obtained and consequently biostatistical methods for quality control, imputation and analysis including multiple testing have been developed to address this issue. Another factor to be considered is the power of the study; quite often the sample sets used in genetic studies are too small to detect risk loci of small effect. This is a well-recognised limitation that can only be overcome with the use of well-defined case material and continued collaborations utilising large datasets.

The odds ratio reports the ratio between two proportions, the cases and the controls. If the allele frequency in the case group is much higher than in the control group, the odds ratio will be greater than 1 and indicate a SNP is associated with disease and further analysis can be undertaken. The objective of a GWAS is to find SNPs with odds ratios greater than 1. Additionally, a P-value for the significance of the odds ratio is calculated using a simple chi-squared test (Zhang, Liu et al. 2007). After an association has been detected the next step is to replicate it in independent populations of similar characteristics and carry out functional studies to further characterize the function of the identified genes in vivo. Also after a GWAS additional analysis can be performed such as haplotype analysis, interaction analysis, or pathway analysis to identify biological mechanisms of disease. Pathway analysis can help to clarify GWAS results because it looks for moderately associated genes (as part of a larger pathway), and it relates these genes to the underlying pathophysiology thus helping to build a picture of disease pathology.

GWAS has been possible due to the availability of large biobanks, repositories of human genetic material and also the completion of the International HapMap Project in 2003 from which a majority of the common SNPs are interrogated in a GWAS and the development of high-density genotyping SNP arrays (Moore, Asselbergs et al. 2010). In conclusion GWAS is a robust method that can help uncover variants with unexpected involvement in disease and that can provide direction of investigations of genetic pathways.
5.8 Statistical Analysis

5.8.1 Chi-Square Analysis

The chi-square statistic is a nonparametric statistical test commonly used to determine if a distribution of observed frequencies differs from the theoretical expected frequencies according to a specific hypothesis. The chi-square test is always testing the null hypothesis, which states that there is no significant difference between the expected and the observed result. The value of the chi-square statistic is given by the formula in equation 2:

Equation 2: $\chi^2 = \sum \left( \frac{(O - E)^2}{E} \right)$

That is, chi-square is the sum of the squared difference between observed ($O$) and the expected ($E$) data (or the deviation, $d$), divided by the expected data in all possible categories.

This test aims to determine the goodness of fit between the observed and expected ratios of two groups as seen in contingency Table 5.1 and is useful for analysing the association between two categorical variables. The contingency Table 5.1 in the example below is used for analysing the relationship between disease and exposure. $n$ is the number of individuals in a particular group, with a particular exposure to a risk factor, and row1 refers to the total number of individuals in row1 (with exposure 1), and so on.

Table 5.1 Generalized Contingency Table for Analysing Disease-Exposure risk (Ogino and Wilson 2004).

<table>
<thead>
<tr>
<th></th>
<th>Group1</th>
<th>Group2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure 1</td>
<td>$n_{1,1}$</td>
<td>$n_{2,1}$</td>
<td>row1</td>
</tr>
<tr>
<td>Exposure 2</td>
<td>$n_{1,2}$</td>
<td>$n_{2,2}$</td>
<td>row2</td>
</tr>
<tr>
<td>Total</td>
<td>column1</td>
<td>column2</td>
<td>$\Sigma$ (all n)</td>
</tr>
</tbody>
</table>

The two groups that are compared in a contingency table for analysis of genetic risk factors involved in disease susceptibility are cases and controls, and either genotypes (2x3 table) or alleles (2x2) as the exposures. The purpose of the chi-square statistic is to compare the
observed count in each cell of the contingency table versus the count which would be expected under the assumption of no association between the row and column classifications. It measures the deviation of observed values from expected values. Chi-square tests were performed to determine if significant differences existed in the genotype and allele frequencies between the case and control samples examined with significance threshold set at $p \leq 0.05$.

5.8.2 The Hardy-Weinberg Equilibrium

The Hardy-Weinberg equilibrium is an important law that describes the distribution of genotypes in a population from one generation to the next according to Mendelian genetics in the context of populations of diploid, sexually reproducing individuals. In the absence of certain disturbing influences such as non-random mating, mutations, selection, limited population size, "overlapping generations", random genetic drift, gene flow and meiotic drive allele and genotype frequencies in a population are expected to remain constant and in equilibrium and to conform to HWE (Salanti, Amountza et al. 2005).

The reality however is that outside of the laboratory, one or more of these "disturbing influences" are always at play. Therefore genetic equilibrium is an ideal state only and exists when the allele frequencies in a population are not changing. The population is evolving when the genetic equilibrium is disrupted. In the simplest case of a single locus with two alleles: HWE assumes the presence of a dominant and recessive allele, with both maintaining equilibrium throughout each generation. The dominant allele is denoted $A$ and the recessive allele $a$ and their frequencies are denoted by $p$ and $q$ in the following formula:

\[
\text{Equation 3: } p^2 + 2pq + q^2 = 1
\]

If the population is in equilibrium the frequency of each genotype is $AA = p^2$, $aa = q^2$, $Aa = 2pq$ for the heterozygotes. Results of genetic association studies are typically checked for HWE. Violation of HWE is indicative of problems with the population, samples or the genotyping process or that one of the disturbing forces mentioned above is in effect (Salanti et al. 2005). The samples used in this project were selected from unrelated people, from a
Caucasian background, random mating and collected from a similar geographic area. The genotypes obtained from this study were tested for conformity with HWE.
CHAPTER 6  Genetic analysis of *GRIA2* and *GRIA4* genes in Migraine

6.1 Abstract

Background:
Migraine is a brain disorder affecting ~12% of the Caucasian population. Genes involved in neurological, vascular and hormonal pathways have all been implicated to play a role in predisposing individuals to developing migraine. The migraineur presents with disabling head pain and varying symptoms of nausea, emesis, photophobia, phonophobia and occasionally visual sensory disturbances. Biochemical and genetic studies have demonstrated dysfunction of neurotransmitters: serotonin, dopamine and glutamate in migraine susceptibility. Glutamate mediates the transmission of excitatory signals in the mammalian central nervous system which affect normal brain function including cognition, memory and learning. The aim of this study was to investigate polymorphisms in the \textit{GRIA2} and \textit{GRIA4} genes which encode subunits of the ionotropic AMPA receptor for association in an Australian Caucasian population.

Methods:
Genotypes for each polymorphism were determined using High Resolution Melt analysis and the RFLP method.

Results:
Statistical analysis showed no association between migraine and the \textit{GRIA2} and \textit{GRIA4} polymorphisms investigated.

Conclusions:
Although the results of this study showed no significant association between the tested GRIA gene variants and migraine in our Australian Caucasian population further investigation of other components of the glutamatergic system may help to elucidate if there is a relationship between glutamatergic dysfunction and migraine.
6.2 Introduction

Migraine is a painful disorder of neurovascular origin with a substantial genetic involvement. Global prevalence estimates indicate ~12% (6% males; 18% females) of Caucasian individuals suffer migraine throughout their lifetime (Stovner and Hagen 2006; Bigal and Lipton 2009). In middle adulthood migraine affects women three times more than men and is thought to be due to changes in ovarian hormones. Migraine is a distressing disorder that disables the sufferer and interferes with social relationships creating a cycle of worry, fear and anxiety that has repercussions on family life.

Migraine presents as a severe headache that can last 4 to 72 hours and can include symptoms of nausea, vomiting, photophobia and phonophobia and sometimes visual sensory disturbances (IHS 2013). Diagnosis is established by fulfilment of symptom-based criteria defined by the International Headache Society (IHS) (IHS 2013). Two main types of migraine are classified by the IHS, migraine with aura MA and migraine without aura MO. MA patients are less common representing 30% of the total migraine population. These patients in addition to experiencing a throbbing headache, also experience an aura before their headache that lasts from 5 minutes to less than 1 hour and consists of fully reversible visual, sensory or language symptoms (Silberstein 2009).

The onset of migraine with and without aura is shaped by both genetic and environmental variables. Family pedigrees and twin studies have estimated hereditary factors to contribute between 34% and 57% to the aetiology of migraine this figure may vary in different populations (Mulder, Van Baal et al. 2003). Migraine is triggered by a complex cascade of chemical changes occurring between neurological and vascular tissues. The innervation of the meninges by the trigeminovascular system and its nerve fibers has been implicated in the development of headache (Parsons and Strijbos 2003; Lambert, Hoskin et al. 2004; Messlinger, Lennerz et al. 2012). CSD is an electrophysiological event that has also been implicated in headache particularly migraine with aura (Dalkara, Zervas et al. 2006). Imbalances in neurotransmitters including, serotonin, dopamine and glutamate, have been identified and hypothesised to contribute to the cellular hyperexcitable state exhibited by migraineurs (D'Andrea and Leon 2010).
The biological components of these neurotransmitter systems including receptors and transporters have been studied at a genetic level in migraine pathogenesis with the aim of identifying genetic defects that could potentially lead to a higher susceptibility to migraine. Recently genetic studies have indicated that excess glutamate in the brain could play a role in the development of migraine (Anttila, Stefansson et al. 2010; Formicola, Aloia et al. 2010; Lighhart, de Vries et al. 2011; Maher, Lea et al. 2013). Excess glutamate at the synapse is thought to contribute to the neuronal hyper-excitability state a mechanism possibly implicated in common migraine. Glutamate has been linked to physiological processes including trigeminovascular activation, central sensitization, allodynia and cortical spreading depression (Ramadan 2003). Glutamate is an important neurotransmitter that relays excitatory signals along the trigeminothalamic pathway (Andreou and Goadsby 2011).

Glutamatergic neurotransmission occurs via the interaction of glutamate with metabotropic and ionotropic receptors including Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionin acid (AMPA), kainate receptors (KA), and N-methyl-D-aspartate (NMDA) receptors (Vikelis and Mitsikostas 2007). The ionotropic AMPA receptors are constructed from four types of subunits coded by four separate genes designated as GluR1 (GRIA1), GluR2 (GRIA2), GluR3 (GRIA3), and GluR4 (GRIA4) at chromosomal loci 5q33, 4q32, Xq24 and 11q24 respectively (Vikelis and Mitsikostas 2007). The GRIA subunits assemble to form ligand-gated ion channels in the plasma membrane permeable to Na+, K+ and Ca2+ quickly opening and closing in response to ligand (e.g. glutamate) binding. AMPA receptors are expressed in CNS glial cells and neurons where they relay fast excitatory signals mediated by their interaction with glutamate (Traynelis, Wollmuth et al. 2010). AMPA receptors have been among the most studied in regard to a variety of neurological diseases including schizophrenia (Carter 2006; Magri, Gardella et al. 2008; Chaki and Hikichi 2011). Dysfunctional conductance of these ion channels due to genetic mutation may result in a disturbed balance of synaptic activity which could pose significant consequences to normal physiological functioning in vivo and play a key role in the pathophysiology of migraine. Ion channel genes have been implicated in the rare autosomal inherited FHM providing good impetus for pursuing these variants (Pietrobon 2010c).

Biochemical studies undertaken in the last 20 years have demonstrated differences in the levels of glutamate in a range of biological fluids of migraine patients when compared to controls particularly in those with aura (Peres, Zukerman et al. 2004; Vieira, Naffah-
Mazzacoratti et al. 2007; D'Andrea and Leon 2010). Animal experiments have shown that glutamate can affect Spreading Depression and that consumption of glutamate-rich food can provoke migraine attacks in predisposed subjects (D'Andrea and Leon 2010). Recently Gonzalez de la Aleja et al., 2013 quantified the metabolites glutamate and glutamine by proton magnetic resonance spectroscopy in neurons and astrocytes in the occipital lobe of a group of women with migraine (Gonzalez de la Aleja, Ramos et al. 2013). Glutamate and glutamine are important in neurotransmission and energy metabolism. In this study a more elevated Glu/Gln ratio was found in the brains of migraineurs during the pain-free interval which the authors suggest could facilitate CSD in the occipital lobe of migraineurs (Gonzalez de la Aleja, Ramos et al. 2013). They caution the study is limited by a small sample size (27 migraineurs and 19 controls) but that a statistical difference in the Glu/Gln ratio between groups was detected (Gonzalez de la Aleja, Ramos et al. 2013). Although linkage with a known genetic disorder has not been established, low function of glutamate in neurons is associated with schizophrenia (Carter 2006; Magri, Gardella et al. 2008; Chaki and Hikichi 2011) and some neurological disorders are accompanied by the appearance of antibodies or autoantigen activity to glutamate receptor subunits (e.g. GluR3 in Rasmussen's encephalitis) (Dingledine, Borges et al. 1999).

More research investigating the components of the glutamatergic system is necessary to confirm or rule out a role in either causing or predisposing to migraine when mutated. In a previous study Formicola et al., 2010 investigated subunits of the AMPA receptor GRIA1-4 and identified a positive association with 2 SNPs in GRIA1 (rs548294 MO allelic P=0.008, rs2195450 MA allelic P=0.0005) and 1 SNP in the GRIA3 promoter (rs3761555 MA Females allelic P=0.003) in an Italian population (Formicola, Aloia et al. 2010). In this study we investigated if polymorphisms, different to those tested by Formicola and Colleagues, in the GRIA2 and GRIA4 genes contribute to migraine susceptibility in an Australian case-control cohort.
6.3 Materials and methods

6.3.1 Study Population

This research was approved by Griffith University's Ethics Committee for experimentation on human subjects. The study population was composed of 284 migraine cases and 284 age- (± 5 years), sex and ethnicity matched healthy controls. Each study participant gave informed consent and provided a sample of whole blood from which genomic DNA was extracted. The migraine population was recruited from the Gold Coast Hospital, Southport and all samples were obtained from patients residing in the South East Queensland Region of Australia. Migraine diagnosis was overseen by an experienced clinical neurologist based on responses provided in a questionnaire in accordance with the International Headache Society (IHS) criteria (IHS 2013). Migraineurs were diagnosed as having either migraine with aura or migraine without aura. The matched control group was obtained via the Genomics Research Centre Clinic, Southport, and comprised of healthy Caucasian controls certified as having no history of personal or familial migraine.

6.3.2 Genotyping Methods

DNA was extracted from white blood cells following a salting out method as described by Miller et al., 1988 (Miller S.A, Dykes D.D et al. 1988), DNA was then quantified to a concentration of 20ng/µL for genotyping experiments. Three polymorphisms in \textit{GRIA2} and four polymorphisms in \textit{GRIA4} were genotyped for all cases and controls. Detailed information regarding polymorphisms genotyped in the \textit{GRIA2} and \textit{GRIA4} genes and a summary of assay conditions and primer sequences for each polymorphism are listed in Table 1. PCR buffers, MgCl₂, Go-\textit{Taq} polymerase (Promega Corp., Madison, WI, USA), dNTPs, restriction enzymes, and enzyme buffers were from (New England Biolabs, Ipswich, MA, USA), SYTO9 dye was from (Invitrogen, Melbourne, Australia). Specific protocol and assays for each polymorphism are described in detail below. Primers were obtained from IDT (Integrated DNA Technologies) in sequencing quality at a concentration of 100µM in liquid form. Aliquots for each set of primers were made at a concentration of 5µM in H₂O for use in PCR optimization experiments. PCRs were run on a combination of Applied Biosystems Veriti® Thermal Cyclers, Corbett Robotics Palm Cyclers, Qiagen RotorGene PCR instrument (QIAGEN, Doncaster, VIC, Australia).
6.3.3 Polymorphism Selection

Selection of SNPs was undertaken in the program Haploview v4.2 (Barrett 2009). HapMap release #28 CEU SNP genotype data was downloaded for each candidate gene and analysed for LD. The aim of this analysis was to exclude SNPs that were in LD with each other but to select SNPs that would cover different LD blocks and still be informative across each tested gene. SNPs chosen were sampled in a European-Caucasian population and were located in intronic regions of the gene.

6.3.4 Genotyping GRIA2

The human GRIA2 gene codes for one of four subunits of the AMPA receptor and maps to chromosome 4q32.1 and spans a genomic region of about 143 kb. The following polymorphisms: rs4131622, rs9307961, rs4403097 were genotyped by PCR followed by HRM analysis. The PCR protocol was as follows: 1X PCR buffer, 2.0mM MgCl2, 0.2mM dNTPs, 0.2µM forward primer, 0.2µM reverse primer, 1.5µM SYTO9, 0.35U GoTaq in a 15µl reaction. The primer sequences were designed manually for each marker and checked for specificity using NCBI Primer-BLAST to ensure the DNA sequence of each primer set was unique and amplified the correct region of the gene (http://www.ncbi.nlm.nih.gov/BLAST/).

The PCR followed by HRM analysis was conducted on a Qiagen Rotor-Q and the thermocycling conditions for rs4403097 consisted of: 95°C for 5mins, then 95°C for 5 seconds and 60°C for 10 seconds for 45 cycles. PCR products were melted from 65°C to 75°C at 0.1°C increments every 2 seconds. Amplicon Tm occurred at 70°C and three separate melt curves were obtained corresponding to the three genotypes CC, CT, and TT.

Thermocycling conditions for rs9307961 consisted of: 95°C for 5mins, then 95°C for 5 seconds and 45°C for 10 seconds for 45 cycles. PCR products were melted from 68°C to 78°C at 0.1°C increments every 2 seconds. Amplicon Tm occurred at 73°C and three separate melt curves were obtained corresponding to the three genotypes GG, GT, and TT.

Thermocycling conditions for rs4131622 consisted of: 95°C for 5mins, then 95°C for 5 seconds and 50°C for 10 seconds for 45 cycles. PCR products were melted from 70°C to
80°C at 0.1°C increments every 2 seconds. Amplicon Tm occurred at 80°C and three separate melt curves were obtained corresponding to the three genotypes AA, AC, and CC.

6.3.5 Genotyping GRIA4

The human GRIA4 gene codes for one of four subunits of the AMPA receptor and maps to chromosome 11q22.3 and spans a genomic region of about 370kb. The GRIA4 rs10895856, GC polymorphism was genotyped by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis. The PCR thermocycling conditions were as follows: 95°C for 3mins, then 94°C for 40 seconds, 45°C for 40 seconds, and 72°C for 40 seconds for 30 cycles, followed by a final extension step of 72°C for 5 minutes. The 124bp PCR products were electrophoresed on a 2% agarose gel containing stained with ethidium bromide for 30mins at 90V, and then visualised under ultraviolet light. 10 µL of PCR product was then digested with 0.8U of HpyCH4V and 1X NEB Buffer 4 at 37°C for 16 hrs. Restriction digest products were electrophoresed in a 4 % agarose gel for 50 min at 70 V, and visualised under ultraviolet light. HpyCH4V digestion of fragments containing the G allele produced two fragments of 78bp and 46bp while fragments containing the C allele remained undigested 124bp by HpyCH4V.

The following polymorphisms: rs1938966, rs10128540, rs10502059 were genotyped by PCR followed by HRM analysis. The PCR followed by high resolution melting analysis was conducted on a Qiagen Rotor-Q (Qiagen, Doncaster, VIC, Australia). Thermocycling conditions for rs10128540 consisted of: 95°C for 5mins, then 95°C for 5 seconds and 56°C for 10 seconds for 45 cycles. PCR products were melted from 70°C to 80°C at 0.1°C increments every 2 seconds. Amplicon Tm occurred at 73°C and three separate melt curves were obtained corresponding to the three genotypes CC, CT, and TT.

Thermocycling conditions for rs10502059 consisted of: 95°C for 5mins, then 95°C for 5 seconds and 58°C for 10 seconds for 45 cycles. PCR products were melted from 72°C to 82°C at 0.1°C increments every 2 seconds. Amplicon Tm occurred at 80°C and three separate melt curves were obtained corresponding to the three genotypes AA, AG, and GG.

Thermocycling conditions for rs1938966 consisted of: 95°C for 5mins, then 95°C for 5 seconds and 58°C for 10 seconds for 45 cycles. PCR products were melted from 74°C to
84°C at 0.1°C increments every 2 seconds. Amplicon Tm occurred at 70°C and three separate melt curves were obtained corresponding to the three genotypes AA, AG, and GG. Positive controls of DNA samples with known genotypes were genotyped on the ABI-3130 Genetic Analyser (Applied Biosystems) to validate the genotypes obtained by the HRM method. Sequence electrophoretograms were analyzed in Chromas software (version 2.33, Technelysium Pty, South Brisbane, QLD, Australia).
Table 6.1 SNP and assay information.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>rs number</th>
<th>SNP</th>
<th>Assay</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>Enzyme</th>
<th>Digest fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRIA2</td>
<td>4q32.1</td>
<td>rs4131622</td>
<td>A/C</td>
<td>HRM</td>
<td>F: 5’ TGCAGAGCAAATGCTATTACAAG 3’ R: 5’ GCTGCCATTAACATTTCATCA 3’</td>
<td>150bp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>GRIA2</td>
<td>4q32.1</td>
<td>rs9307961</td>
<td>T/G</td>
<td>HRM</td>
<td>F: 5’ TAAGAGGCATGACATGAGCT 3’ R: 5’ AATCTTGTATTACATCTAAAGG 3’</td>
<td>120bp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>GRIA2</td>
<td>4q32.1</td>
<td>rs4403097</td>
<td>C/T</td>
<td>HRM</td>
<td>F: 5’ TGTAATCTTGCCTTTAAATGCTTTTTGTG 3’</td>
<td>106bp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>GRIA4</td>
<td>11q22.3</td>
<td>rs10895856</td>
<td>G/C</td>
<td>PCR-RFLP</td>
<td>F: 5’ CAGAACCTCGGAACCCCAATAC 3’ R: 5’ GTATACATCATAGGTTCAAGCATATGG 3’</td>
<td>124bp</td>
<td>HpyCH4V</td>
<td>C-124bp, G-78bp, 46bp</td>
</tr>
<tr>
<td>GRIA4</td>
<td>11q22.3</td>
<td>rs1938966</td>
<td>G/A</td>
<td>HRM</td>
<td>F: 5’ TTGGAGAAGGCGACCTAACA 3’ R: 5’ ACCCCACCCTAGCACCCTTC 3’</td>
<td>107bp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>GRIA4</td>
<td>11q22.3</td>
<td>rs10128540</td>
<td>T/C</td>
<td>HRM</td>
<td>F: 5’ CCTCTGTCCCTAGAAATCCCAGG 3’ R: 5’ ACAACTGAAACTGAAATACCACAG 3’</td>
<td>125bp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>GRIA4</td>
<td>11q22.3</td>
<td>rs10502059</td>
<td>G/A</td>
<td>HRM</td>
<td>F: 5’ TAAGGACGAGACTTTGTACTGG 3’ R: 5’ TGACCCTATTAGCTTTACCAGAA 3’</td>
<td>102bp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; HRM: high resolution melt.
6.3.6 Statistical Analysis

The genotypes for migraine cases and controls were in HWE for each of the SNPs examined (p>0.05). Genotype and allele frequencies were compared between case and control groups for each marker using the Chi-square ($\chi^2$) test, with two and one degrees of freedom, respectively. The Statistical Package for Social Sciences (SPSS version 21.0) was used for statistical analyses. The results were also subdivided into MA and MO groups and female and male subpopulations.

6.4 Results

The aim of this study was to determine if seven polymorphisms in the \textit{GRIA2} and \textit{GRIA4} genes contribute to migraine susceptibility in an Australian case-control cohort. The study population consisted of 284 migraine cases and 284 age- (± 5 years), sex and ethnicity matched healthy controls. This migraine population had a higher proportion of MA cases compared to MO which typically accounts for a third of total migraineurs. We chose to investigate polymorphisms in subunits of the AMPA receptor as some SNPs within subunits of this receptor were previously found associated in a study by Formicola et al., 2010 (Formicola, Aloia et al. 2010).

SNP selection was conducted in Haploview for the European-Caucasian population to select SNPs to genotype for each gene. The genotypes for six SNPs were determined by HRM whilst one SNP was determined by the RFLP method of genotyping. Genotypes were determined by interpreting shifts in the amplification profiles and product melt curves of the genotyped samples using the Rotor-Gene software (version 1.7, Corbett Life Sciences, Cambridge, UK). The raw genotype data was individually checked against baseline genotypes from positive controls at a confidence threshold of 70% and any ambiguous samples were identified and where possible these individuals were re-genotyped or genotypes were excluded from the analysis as errors. Three distinct melt curves were obtained allowing discrimination of three genotypes.

Chi-square ($\chi^2$) analysis was performed to determine if significant differences existed in the genotype and allele frequencies in the migraine population versus controls as well as the subgroups of migraineurs with aura versus controls and migraineurs without aura versus
controls examined with significance threshold set at \( p \leq 0.05 \). Genotypic and allelic frequencies for polymorphisms investigated in the GRIA2 gene are shown in Table 6.2 and GRIA4 in Table 6.3.

### 6.4.1 GRIA2 gene

Genotypic and allelic frequencies obtained for these polymorphisms are shown in Table 6.2. A total of 470 individuals were successfully genotyped for the GRIA2 intronic SNP rs4403097, including 239 controls and 231 migraine cases, of which 146 were diagnosed with MA and 85 with MO. Genotype frequencies between cases and controls were not significantly different \( (p=0.39) \), MA and controls \( (p=0.57) \), nor MO and controls \( (p=0.42) \). There was also no significant difference in the allele frequencies between cases and controls \( (p=0.29) \), MA and controls \( (p=0.54) \), nor MO and controls \( (p=0.23) \). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls \( (p=0.60 \text{ and } p=0.30, \text{ respectively}) \). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes \( (p=0.29) \) or alleles \( (p=0.27) \).

A total of 479 individuals were successfully genotyped for the GRIA2 intronic SNP, rs9307961, including 248 controls and 231 migraine cases, of which 141 were diagnosed with MA and 90 with MO. Genotype frequencies between cases and controls were not significantly different \( (p=0.39) \), MA and controls \( (p=0.57) \), nor MO and controls \( (p=0.42) \). There was also no significant difference in the allele frequencies between cases and controls \( (p=0.29) \), MA and controls \( (p=0.54) \), nor MO and controls \( (p=0.23) \). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls \( (p=0.43 \text{ and } p=0.63, \text{ respectively}) \). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes \( (p=0.29) \) or alleles \( (p=0.27) \).

A total of 489 individuals were successfully genotyped for the GRIA2 intronic SNP, rs4131622, including 251 controls and 238 migraine cases, of which 146 were diagnosed with MA and 92 with MO. Genotype frequencies between cases and controls were not significantly different \( (p=0.85) \), MA and controls \( (p=0.23) \), nor MO and controls \( (p=0.38) \). There was also no significant difference in the allele frequencies between cases and controls.
(p=0.84), MA and controls (p=0.93), nor MO and controls (p=0.62). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (p=0.43 and p=0.63, respectively). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes (p=0.42) or alleles (p=0.31).

Table 6.2  Genotyping results of GRIA2.

<table>
<thead>
<tr>
<th>GRIA2</th>
<th>Genotypes</th>
<th>Alleles</th>
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<th>Controls</th>
<th>MA</th>
<th>MO</th>
<th>Total</th>
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</tr>
<tr>
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<td>52 (52.0%)</td>
<td>118 (53.4%)</td>
<td>69 (43.6%)</td>
<td>239</td>
<td>222 (46.4%)</td>
<td>256 (53.5%)</td>
<td>478</td>
</tr>
<tr>
<td>CT</td>
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<td>37 (56.9%)</td>
<td>19 (29.2%)</td>
<td>65</td>
<td>55 (42.3%)</td>
<td>75 (57.6%)</td>
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<td>CC</td>
<td>43 (24.7%)</td>
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<td>50 (28.7%)</td>
<td>174</td>
<td>167 (47.9%)</td>
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<tr>
<td>Male</td>
<td>48 (48.0%)</td>
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<td>80 (53.7%)</td>
<td>231</td>
<td>199 (43.0%)</td>
<td>263 (56.9%)</td>
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</tr>
<tr>
<td>MA</td>
<td>32 (21.9%)</td>
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<td>49 (33.6%)</td>
<td>146</td>
<td>129 (44.1%)</td>
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<tr>
<td>MO</td>
<td>16 (18.8%)</td>
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<td>157 (33.9%)</td>
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<td>Female</td>
<td>23 (13.3%)</td>
<td>71 (41.0%)</td>
<td>79 (45.7%)</td>
<td>173</td>
<td>117 (33.8%)</td>
<td>229 (66.1%)</td>
<td>346</td>
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<td>31 (34.4%)</td>
<td>90</td>
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<td>112 (62.2%)</td>
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<td>χ² = 2.7, P = 0.1</td>
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<td>125 (34.3%)</td>
<td>239 (65.6%)</td>
<td>364</td>
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<tr>
<td>Total Migraine</td>
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<td>χ² = 0.0, p = 0.8</td>
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<td></td>
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<tr>
<td>Male</td>
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<td>238</td>
<td>312 (65.5%)</td>
<td>164 (34.4%)</td>
<td>476</td>
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<tr>
<td>Female</td>
<td>10 (15.3%)</td>
<td>30 (46.1%)</td>
<td>25 (38.4%)</td>
<td>65</td>
<td>50 (38.5%)</td>
<td>80 (61.5%)</td>
<td>130</td>
</tr>
<tr>
<td>Female</td>
<td>22 (12.7%)</td>
<td>70 (40.4%)</td>
<td>81 (46.8%)</td>
<td>173</td>
<td>232 (67.1%)</td>
<td>114 (32.9%)</td>
<td>346</td>
</tr>
<tr>
<td>Total MA</td>
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<td>χ² = 0.0, p = 0.9</td>
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<td></td>
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</tr>
<tr>
<td>MA</td>
<td>23 (15.7%)</td>
<td>52 (36.6%)</td>
<td>71 (48.6%)</td>
<td>146</td>
<td>194 (66.4%)</td>
<td>98 (33.5%)</td>
<td>292</td>
</tr>
<tr>
<td>MO</td>
<td>9 (0.9%)</td>
<td>48 (52.1%)</td>
<td>35 (38.0%)</td>
<td>92</td>
<td>118 (64.1%)</td>
<td>66 (35.8%)</td>
<td>184</td>
</tr>
<tr>
<td>Total MO</td>
<td>χ² = 1.9, p = 0.4</td>
<td>χ² = 0.2, p = 0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MA — migraine with aura, MO — migraine without aura.

p-values were calculated by χ²-analysis, significance was taken at p≤0.05.
6.4.2  *GRIA4* gene

Rs1938966, rs10128540, rs10502059, rs10895856 are SNPs that fall in different introns of the *GRIA4* gene and results obtained for these are shown in Table 6.3. A total of 505 individuals were successfully genotyped for the *GRIA4* intronic SNP, rs10128540, including 252 controls and 253 migraine cases, of which 153 were diagnosed with MA and 100 with MO. Genotype frequencies between cases and controls were not significantly different (p=0.90), MA and controls (p=0.33), nor MO and controls (p=0.49). There was also no significant difference in the allele frequencies between cases and controls (p=0.95), MA and controls (p=0.56), nor MO and controls (p=0.60). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (p=0.34 and p=0.94, respectively). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes (p=0.90) or alleles (p=0.99).

A total of 498 individuals were successfully genotyped for the *GRIA4* intronic SNP, rs10895856, including 248 controls and 250 migraine cases, of which 154 were diagnosed with MA and 96 with MO. Genotype frequencies between cases and controls were not significantly different (p=0.51), MA and controls (p=0.74), nor MO and controls (p=0.55). There was also no significant difference in the allele frequencies between cases and controls (p=0.57), MA and controls (p=0.58), nor MO and controls (p=0.72). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (p=0.99 and p=0.99, respectively). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes (p=0.51) or alleles (p=0.25).

A total of 501 individuals were successfully genotyped for the *GRIA4* intronic SNP, rs10502059, including 254 controls and 247 migraine cases, of which 149 were diagnosed with MA and 98 with MO. Genotype frequencies between cases and controls were not significantly different (p=0.31), MA and controls (p=0.24), nor MO and controls (p=0.39). There was also no significant difference in the allele frequencies between cases and controls (p=0.17), MA and controls (p=0.10), nor MO and controls (p=0.59). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (p=0.54 and p=0.30, respectively). Similarly for the male
sub-population, there was no significant difference between migraineurs and controls for either genotypes (p=0.50) or alleles (p=0.35).

A total of 501 individuals were successfully genotyped for the *GRIA4* intronic SNP, rs1938966 including 252 controls and 249 migraine cases, of which 151 were diagnosed with MA and 198 with MO. Genotype frequencies between cases and controls were not significantly different (p=0.70), MA and controls (p=0.84), nor MO and controls (p=0.62). There was also no significant difference in the allele frequencies between cases and controls (p=0.53), MA and controls (p=0.57), nor MO and controls (p=0.66). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (p=0.72 and p=0.70, respectively). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes (p=0.85) or alleles (p=0.57).
### Table 6.3 Genotyping results of GRIA4.

<table>
<thead>
<tr>
<th>GRIA4 rs10128540</th>
<th>Genotypes</th>
<th>Alleles</th>
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<th>G</th>
<th>C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>CT</td>
<td>CC</td>
<td>Total</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Controls</td>
<td>57 (46.7%)</td>
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<td>64 (46.7%)</td>
<td>252</td>
<td>245 (47.6%)</td>
<td>259 (51.3%)</td>
</tr>
<tr>
<td>Male</td>
<td>16 (28.1%)</td>
<td>32 (24.4%)</td>
<td>19 (29.7%)</td>
<td>67</td>
<td>64 (47.7%)</td>
<td>70 (52.2%)</td>
</tr>
<tr>
<td>Female</td>
<td>41 (71.9%)</td>
<td>99 (75.6%)</td>
<td>45 (70.3%)</td>
<td>185</td>
<td>181 (48.9%)</td>
<td>189 (51%)</td>
</tr>
<tr>
<td>Cases</td>
<td>65 (53.3%)</td>
<td>115 (46.7%)</td>
<td>73 (53.3%)</td>
<td>253</td>
<td>245 (48.4%)</td>
<td>261 (51.5%)</td>
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<td>17 (26.2%)</td>
<td>29 (25.2%)</td>
<td>20 (27.4%)</td>
<td>66</td>
<td>149 (68.3%)</td>
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<tr>
<td>Female</td>
<td>48 (73.8%)</td>
<td>86 (74.8%)</td>
<td>53 (72.6%)</td>
<td>187</td>
<td>182 (48.6%)</td>
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<td>$\chi^2 = 0.0, p = 0.9$</td>
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<tr>
<td>MA</td>
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<tr>
<td>MO</td>
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<td>46 (33.6%)</td>
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<td>163 (53.2%)</td>
</tr>
<tr>
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<td>$\chi^2 = 0.3, p = 0.6$</td>
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<table>
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<td>GC</td>
<td>GG</td>
<td>Total</td>
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</tr>
<tr>
<td>Controls</td>
<td>66 (26.6%)</td>
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<td>58 (32.3%)</td>
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<td>256 (51.6%)</td>
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<td>32 (47.0%)</td>
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<td>181</td>
<td>187 (51.6%)</td>
<td>175 (48.3%)</td>
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<td>47 (25.9%)</td>
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<td>41 (22.6%)</td>
<td>68</td>
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<tr>
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<td>123 (49.2%)</td>
<td>55 (22.0%)</td>
<td>250</td>
<td>267 (68.4%)</td>
<td>233 (31.5%)</td>
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<tr>
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<td>28 (43.0%)</td>
<td>13 (20.0%)</td>
<td>185</td>
<td>191 (51.6%)</td>
<td>179 (48.3%)</td>
</tr>
<tr>
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<td>48 (25.9%)</td>
<td>95 (51.3%)</td>
<td>42 (22.7%)</td>
<td>65</td>
<td>76 (58.4%)</td>
<td>54 (41.5%)</td>
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<td>$\chi^2 = 0.3, p = 0.6$</td>
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<tr>
<td>MA</td>
<td>42 (27.2%)</td>
<td>81 (52.5%)</td>
<td>31 (20.1%)</td>
<td>154</td>
<td>102 (51.0%)</td>
<td>98 (49.0%)</td>
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<tr>
<td>MO</td>
<td>30 (31.2%)</td>
<td>42 (43.7%)</td>
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<td>96</td>
<td>143 (46.7%)</td>
<td>163 (53.2%)</td>
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<td>133 (52.3%)</td>
<td>60 (25.6%)</td>
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<td>253 (49.8%)</td>
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<td>36 (52.1%)</td>
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<td>97 (52.4%)</td>
<td>41 (22.1%)</td>
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<td>53 (21.4%)</td>
<td>121 (48.9%)</td>
<td>73 (29.5%)</td>
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<td>267 (54%)</td>
<td>227 (45.9%)</td>
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<td>12 (18.4%)</td>
<td>29 (44.6%)</td>
<td>24 (36.9%)</td>
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<td>77 (59.2%)</td>
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<td>41 (22.5%)</td>
<td>92 (50.5%)</td>
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<td>182</td>
<td>190 (52.1%)</td>
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<td>Total Migraine</td>
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<td>MA</td>
<td>27 (18.1%)</td>
<td>77 (51.6%)</td>
<td>44 (29.5%)</td>
<td>149</td>
<td>165 (55.7%)</td>
<td>131 (44.2%)</td>
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<tr>
<td>MO</td>
<td>25 (25.5%)</td>
<td>44 (44.8%)</td>
<td>29 (29.5%)</td>
<td>98</td>
<td>102 (52%)</td>
<td>94 (47.9%)</td>
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<td>Total MO</td>
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<td>$\chi^2 = 0.3, p = 0.6$</td>
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<td>GA</td>
<td>GG</td>
<td>Total</td>
<td>G</td>
<td>A</td>
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<tr>
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<td>73 (51.0%)</td>
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<td>51 (46.8%)</td>
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<td>230 (45.6%)</td>
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<td>32 (25%)</td>
<td>14 (27.5%)</td>
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<tr>
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<td>37 (72.5%)</td>
<td>183</td>
<td>196 (53.5%)</td>
<td>170 (46.4%)</td>
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<tr>
<td>Cases</td>
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<td>121 (48.6%)</td>
<td>58 (53.2%)</td>
<td>249</td>
<td>261 (52.4%)</td>
<td>237 (47.5%)</td>
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<tr>
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<td>31 (74.4%)</td>
<td>15 (74.1%)</td>
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<td>69 (53.0%)</td>
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<tr>
<td>Female</td>
<td>51 (72.9%)</td>
<td>90 (74.4%)</td>
<td>43 (74.1%)</td>
<td>184</td>
<td>192 (52.1%)</td>
<td>176 (47.8%)</td>
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<tr>
<td>Total Migraine</td>
<td>$\chi^2 = 0.7, P = 0.7$</td>
<td>$\chi^2 = 0.4, P = 0.5$</td>
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<tr>
<td>MA</td>
<td>41 (28.7%)</td>
<td>76 (30.5%)</td>
<td>34 (31.2%)</td>
<td>151</td>
<td>158 (52.3%)</td>
<td>144 (47.6%)</td>
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<tr>
<td>Total MA</td>
<td>$\chi^2 = 0.4, P = 0.8$</td>
<td>$\chi^2 = 0.3, P = 0.6$</td>
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<tr>
<td>MO</td>
<td>29 (20.3%)</td>
<td>45 (18.1%)</td>
<td>24 (22%)</td>
<td>98</td>
<td>103 (52.5%)</td>
<td>93 (47.4%)</td>
</tr>
</tbody>
</table>
MA — migraine with aura, MO — migraine without aura.

p-values were calculated by $\chi^2$-analysis, significance was taken at $p \leq 0.05$

### 6.5 Discussion

The major excitatory neurotransmitter of the brain, glutamate and the receptors and transporters upon which it acts are intimately involved in migraine processes including cortical spreading depression, trigeminovascular activation and central sensitization (Ramadan 2003). X-ray crystal structures of glutamate receptors have defined structure-function relationships by revealing which domains are involved in binding to agonists and antagonists providing insight into the mechanism of action of glutamate receptor antagonists (Mayer 2005; Jin, Singh et al. 2009; Sobolevsky, Rosconi et al. 2009). Targeting the glutamatergic system may be an alternative non-vasoactive approach to the future treatment of migraine given the efficacy of glutamate receptor antagonists and that the multitude of currently prescribed drugs often produce variable patient responses (Andreou and Goadsby 2009; Formicola, Aloia et al. 2010). There is indirect evidence of glutamate involvement in large genome-wide association studies utilizing large population cohorts. A few genes affecting glutamatergic homeostasis ($PGCP, MTDH14$ and $LRP115$) and a cation channel ($TRPM8$) have stood out as statistically significant (Anttila, Stefansson et al. 2010; Chasman, Schurks et al. 2011).

In light of the positive findings reported by Formicola et al., 2010 whereby some SNPs in the $GRIA1$ and $GRIA3$ genes which encode subunits of the AMPA receptor were found associated with migraine in an Italian population of 250 migraineurs and 260 controls we decided to explore further variation in the $GRIA2$ and $GRIA4$ subunits (Formicola, Aloia et al. 2010). In the Formicola et al., 2010 study the association reported in the $GRIA3$ gene is interesting as this locus was also identified in two large Australian Caucasian families by our research group and is within a migraine susceptibility region Xq24-q28 (Nyholt, Dawkins et al. 1998; Nyholt, Curtain et al. 2000). The SNPs studied by Formicola et al., 2010 were located in regulative regions of each gene where they may affect gene expression of the AMPA receptor. $GRIA3$ has also been identified as a susceptibility gene in female patients with schizophrenia (Magri, Gardella et al. 2008). A recent study by Maher et al., 2013 replicated the Formicola et al., 2010 result of a positive association in the $GRIA3$ promoter.
polymorphism (rs3761555) in an Australian migraine case-control cohort (Maher, Lea et al. 2013). This replication study was performed in 454 controls and 452 cases and identified a significant association in the MA subtype (Allele P = 0.008) (Maher, Lea et al. 2013). Confirmation of the GRIA3 locus by an independent group in a bigger and ethnically different population adds evidence to the hypothesis that dysfunction of glutamate homeostasis may be at the heart of susceptibility to migraine. Also because of the genetic heterogeneity of migraine in people of different cultures replication of positive results in populations of different ethnicity is needed to determine if the association is indeed valid and ancestry specific or common.

In the present association study we utilized an age (+/- 5yrs), gender and ethnicity matched Australian Caucasian population composed of 284 migraine cases diagnosed with one of two migraine subtypes, MA or MO matched to 284 healthy controls. Study participants were recruited from the South East Queensland Region of Australia and our study population consisted of a slightly higher proportion of MA cases. In this study, we investigated polymorphisms in the GRIA2 and GRIA4 genes of the ionotropic AMPA receptor to explore their potential role in susceptibility to migraine.

Statistical analysis of the GRIA markers revealed no significant difference between genotyped migraineurs and the matched control group with regard to genotype frequencies and allele frequencies (Table 6.2 and Table 6.3). Furthermore, no significant difference resulted when the migraine population was subdivided by gender into male and female groups (data not shown) and disease subtypes of MA and MO. While an association between migraine and the tested GRIA gene variants was not detected in this population given the importance of the glutamatergic system and its involvement in biological processes in the brain it is likely that perhaps other genes coding for other components of this system could play a role. However, as there are no studies examining the effect of other components of the glutamatergic system including kainate (KA) and N-methyl-D-aspartate (NMDA) and metabotropic receptors as well as EAAT transporters and enzymes that cycle glutamate we cannot exclude the possibility that these could be contributing to migraine aetiology and this is an avenue that future studies could address.
6.6 Conclusion

In summary, although the results of this study did not provide significant association to the GRIA2 and GRIA4 genes with migraine risk in our migraine population additional variants in genes regulating glutamate pathways in the CNS are worthy of analysis. Future studies will continue to build a gene profile of the complex common migraine disorder and help to provide new drug targets to research. The identification of migraine susceptibility genes remains an important task in this population that could contribute to understanding the molecular basis of gene dysfunction in migraine and the consequences to treatment response.
CHAPTER 7  Association study of \textit{GLUD1} and \textit{GLUD2} polymorphisms and migraine
7.1 Abstract

Migraine is an episodic and complex neurovascular brain disorder characterised by attacks of headache and often accompanied by symptoms of nausea, vomiting and neurological disturbances. Migraine is caused by both genetic and environmental factors and it is listed in the top 10 health disabilities by The World Health Organisation. Migraine undeniably has a major impact on the wellbeing and quality of life of sufferers. Genetic and pharmacological studies have implicated the glutamate pathway in migraine pathophysiology. Glutamate is a major excitatory neurotransmitter in the central nervous system (CNS) synthesized from the amino acid glutamine by the mitochondrial enzyme glutaminase, and stored in vesicles at glutamatergic synapses. Previous studies have shown an association between glutamate receptor genes, namely, GRIA1 and GRIA3 with migraine. In this study, we investigated whether variants in the GLUD1 and GLUD2 genes that encode the enzyme Glutamate Dehydrogenase are associated with migraine pathogenesis and susceptibility. The single nucleotide polymorphism rs4934292 in the GLUD1 gene was genotyped using the HRM method, chi-square ($\chi^2$) analysis indicated this SNP is not associated with migraine. Three of the 5 SNPs genotyped by Sequenom were in HWE and were analysed. Association analysis indicated the tested polymorphisms in the GLUD1 and GLUD2 genes were not significantly associated with migraine with and without aura or gender. Moreover, these results suggest the GLUD1 and GLUD2 genes are not significantly associated with an increase in migraine risk and therefore do not play a significant role in disease susceptibility in our Australian-Caucasian population. Although we find no significant association of any of the SNPs tested in the GLUD1 and GLUD2 genes with migraine, many SNPs in other genes regulating glutamate homeostasis remain to be studied in future migraine genetic studies.
7.2 Introduction

The International Headache Society (IHS) defines migraine as a recurrent headache disorder typified by attacks lasting 4-72 hours accompanied by nausea, vomiting, neurological disturbance and sensory hypersensitivity (IHS 2013). Migraine severity, duration and symptoms vary within and among individuals. Classification criteria recognize two types of migraine, migraine with aura MA and migraine without aura MO. The difference between the two is that in migraine with aura patients in addition to experiencing a painful headache they also experience an aura just prior to their headache which is accompanied by neurological symptoms that manifest as fully reversible, visual, sensory and/or dysphasic speech disturbances (Viana, Sprenger et al. 2013).

Migraine has a very significant impact on both people and society at large affecting the majority of individuals during their most productive years and leading to a loss of productivity in their work and reduced proficiency and remuneration which also contribute to significant economic consequences. The impact of migraine on the emotional and physical well-being of sufferers and their family is difficult to interpret in monetary terms but is still a consequence suffered by migraineurs and their families.

In Australia migraine affects 12% of the Caucasian population showing up more often in females a statistic correlated with instability of ovarian hormones (Stovner and Hagen 2006). Several studies suggest that genetic and environmental factors are both involved in developing migraine. The number and types of genes involved is yet to be elucidated, but until now, the search for migraine susceptibility genes has focussed on neurotransmitter-related pathways, as these pathways are considered to play a significant role in the migraine process. There is evidence from genetic, biochemical and pharmacological studies to indicate that perturbed glutamate homeostasis can contribute to migraine.

Glutamate is an abundant excitatory neurotransmitter in the mammalian CNS which is involved in mechanisms of synaptic plasticity, memory, and neuronal or glial cell death (Frigerio, Karaca et al. 2012). Glutamate is a substrate for various enzymes at glutamatergic synapses and is at a crossroads of metabolic pathways (Frigerio, Karaca et al. 2012). Glutamate dehydrogenase 1, GDH1 is an enzyme encoded by the \textit{GLUD1} gene which is about 45kb long and is located on chromosome 10q21.1-24.3 (Plaitakis and Zaganas 2001).
A second related gene GLUD2 originated via retrotransposition to the human X-chromosome (Xq25). This gene is different to GLUD1 in that it lacks introns and is solely composed of exons and is preferentially expressed in testis and brain (Plaitakis, Latsoudis et al. 2011). It is interesting that this gene is located in the Xq24-q25 region which is within Xq24-q28 a region previously identified as harbouring migraine susceptibility genes in pedigrees. GDH1 alongside other enzymes maintains the chemical balance at Glutamatergic synapses in the CNS and is an important contributor to cellular processes, such as the Krebs cycle, ammonia management and energy production (Plaitakis, Latsoudis et al. 2011).

GLUD1 is a mitochondrial enzyme expressed in all tissues, including brain which in combination with cofactors catalyzes reversible oxidative deamination of L-glutamate to 2-oxoglutarate (Plaitakis, Latsoudis et al. 2011). Mutations in GLUD1 have been linked to congenital hyperinsulinism–hyperammonaemia (HI/HA) syndrome a condition characterized by recurrent hypoglycemia due to an inappropriate insulin secretion by the pancreatic islet beta cells (Arnoux, de Lonlay et al. 2010). Certain mutations at the GTP binding sites correlate with the development of epilepsy a disorder known to be co-morbid with migraine.

Disruption of the GLUD1 gene in murine models results in increased glutamate release and neuronal damages (Bao, Pal et al. 2009). Significant alterations in GLUD enzymatic activity have been associated with certain neurodegenerative human disorders such as Parkinson’s disease, epilepsy, schizophrenia, and Alzheimer’s disease (Michaelidis, Tzimagiorgis et al. 1993; Dobrek and Thor 2011; Marmiroli and Cavaletti 2012). This study investigated if genetic variations in the GLUD1 and GLUD2 genes are implicated in migraine pathophysiology in an Australian case-control population.

7.3 Materials and Methods

7.3.1 Study Population

A case-control association population was used in this study, involving 291 cases of migraine and 314 control individuals. Migraineurs and controls were matched for gender, age and ethnicity. The study participants were Caucasian from Australia and provided informed consent. Migraineurs were diagnosed by a clinical neurologist as having either MA or MO based on strict International Headache Society (IHS) criteria. The study was approved by the
Griffith University Ethics Committee for Experimentation on Human Subjects. The extraction of genomic DNA was realized from blood samples from each participant by the salting out method. Samples used did not have a family history of migraine for the genotyping study.

7.3.2 Genotyping Methods

Genomic DNA was extracted from peripheral blood samples using a salting out method as described by Miller et al., 1988 (Miller S.A, Dykes D.D et al. 1988). DNA was quantified and normalised to a concentration of 20ng/µL for genotyping experiments. Four polymorphisms in GLUD1 gene were genotyped for all cases and controls using high resolution melt and sequenom. Detailed information regarding polymorphisms and a summary of assay conditions and primer sequences for each polymorphism are listed in Table 7.1. PCR buffers, MgCl₂, Go-Taq polymerase (Promega Corp., Madison, WI, USA), dNTPs, restriction enzymes, and enzyme buffers were from (New England Biolabs, Ipswich, MA, USA), SYTO9 dye was from (Invitrogen, Melbourne, Australia). Specific protocol and assays for each polymorphism are described in detail below. Primers were obtained from IDT (Integrated DNA Technologies) in sequencing quality at a concentration of 100µM in liquid form. Aliquots for each set of primers were made at a concentration of 5µM in H₂O for use in PCR optimization experiments. PCRs were run on a combination of Applied Biosystems Veriti® Thermal Cyclers, Corbett Robotics Palm Cyclers, Qiagen RotorGene PCR instrument (QIAGEN, Doncaster, VIC, Australia).
Table 7.1 SNP and assay information.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>rs number</th>
<th>SNP</th>
<th>HapMap CEU MAF</th>
<th>Assay</th>
<th>Primer sequence</th>
</tr>
</thead>
</table>
| GLUD1| 10q23.3  | rs1923934 | C/T | A=0.40         | HRM, 112bp | F: 5’ ACTGTGGTTTGAGGCCTTAAGT 3’  
|      |          |           |     |                |           | R: 5’ AGCTCCATGTGCCACTATGTT 3’ |
| GLUD1| 10q23.3  | rs4934292 | C/G | G=0.28         | HRM, 137bp | F: 5’ GTGTATGAATCCATTAGCAAGTT 3’  
|      |          |           |     |                |           | R: 5’ ATATTGGTGCTTAGATCAAT 3’   |
| GLUD1| 10q23.3  | rs493326  | A/G | G=0.02         | Sequenom  | F: 5’  
|      |          |           |     |                |           | R: 5’  |
| GLUD1| 10q23.3  | rs17343630| C/T | T=0.24         | Sequenom  | F: 5’  
|      |          |           |     |                |           | R: 5’  |
| GLUD2| Xq24-q25 | rs191769566| G/A | A=0.06         | Sequenom  | F: 5’  
|      |          |           |     |                |           | R: 5’  |
| GLUD2| Xq24-q25 | rs5956265 | G/T | T=0.48         | Sequenom  | F: 5’  
|      |          |           |     |                |           | R: 5’  |
| GLUD2| Xq24-q25 | rs9697983 | T/G | G=0.02         | Sequenom  | F: 5’  
|      |          |           |     |                |           | R: 5’  |

7.3.3 Polymorphism selection

Selection of SNPs was undertaken in the program Haplovie v4.2 (Barrett 2009). HapMap release #28 CEU SNP genotype data was downloaded for the GLUD1 and GLUD2 genes and analysed for LD. The aim of this analysis was to exclude SNPs that were in LD with each other but to select SNPs that would cover different LD blocks and still be informative across each tested gene. SNPs chosen were sampled in a European-Caucasian population and were located in intronic regions of the gene.

7.3.4 PCR Optimization

Primers were needed to be optimized with 4 junk DNA samples extracted from a batch of salivary samples to check their good working. First of all, optimization of PCR protocol was performed with a gradient of MgCl₂ with concentrations ranging from 1.7mM to 2.5mM (Figure 7.1). It was determined that bands were brighter and distinct when the samples had a higher concentration of 2.0 MgCl₂. Figures 7.1 and 7.2 are examples of visualization of PCR products on agarose gel under UV light.
Figure 7.1  Agarose gel electrophoresis of PCR products for rs4934292.

Figure 7.1 above demonstrates the expected agarose gel after PCR amplification of a region of the GLUD1 gene containing the C/G single nucleotide polymorphism (SNP). Lane 1 contains a 100bp ladder to allow determination of the band size of DNA in the other lanes. PCR amplicons measure 137bp, two unique DNA specimens were used in replicate for each MgCl₂ gradient, as loaded from left to right. Lane 2-5 contain a concentration of MgCl₂ of 1.7mM, lane 7-10 MgCl₂ of 2.0mM, lane 12-15 MgCl₂ of 2.5mM. Lanes 6, 11 and 16 contain 3 negative controls (NTC) for each concentration to show that there is no contamination. The lanes containing negative reaction controls show no bands, indicating there is no PCR product present. A higher MgCl₂ concentration worked better as indicated by the brighter and thicker bands seen in lanes 12-15.
Subsequent to the MgCl₂ gradient a temperature gradient was used to confirm the best annealing temperature. This temperature allows primers to bind to template DNA and varies in the PCR reactions with the aim of producing highly specific PCR product. Figure 7.2 shows the reaction used to find the optimal primer annealing temperature for further PCR reactions. The temperature gradient covered temperatures from (54°C, 56, 58, 60, 62, 64°C) for rs4934292 and from (50°C, 52, 54, 56, 58, 60°C) for rs1923934 (Figure 7.2).

Figure 7.2 Agarose gel electrophoresis of PCR products for rs1923934.

Figure 7.2 above demonstrates the expected agarose gel after PCR amplification of a region of the GLUD1 gene containing the C/G single nucleotide polymorphism (SNP). Lane 1 and 9 contains a 100bp ladder to allow determination of the band size of DNA in the other lanes. PCR amplicons measure 112bp, two unique DNA specimens were used in replicate for the temperature gradient, as loaded from left to right. Lane 2-3, contain two different samples of junk DNA at 50°C; lane 4-5 at 52°C; lane 6-7 at 54°C; lane 8-10 at 56°C; lane 11-12 at 58°C; lane 13-14 at 60°C. Lane 15 contains a negative control (NTC) and shows no bands, indicating there is no PCR product present. A temperature of 58°C-60°C worked better.
7.3.5 Sequencing

Sequencing was used to determine the genotypes of DNA to get positive controls for the experiments to use to confirm the results obtained in HRM. The method of sequencing used is performed in 3 steps. The first step starts by mixing PCR product obtained by HRM with Exo-Sap-IT reagent to remove unconsumed dNTPs and primers and water to have a total volume of 10 μL to do a PCR following thermal conditions specific such as: 1 cycle of 37°C for 15 min and 1 cycle for 80°C for 15 min. Subsequently, for each primer in a 20 μl reaction the following were combined: 20ng/μl of the Exo-SAP-IT product, 650 nM of each primer (one reaction per primer), 3.5μl of BDTv3.1 Ready Reaction Mix (100RR) and 1× Sequencing Buffer. The following cycling conditions were used: 1 cycle of 96°C for 1min, 30 cycles of 96°C for 10s, 50°C for 5s and 60°C for 4 min, 1 cycle of 4°C for 5 min and 1 cycle of 10°C for 5 min. A standard ethanol precipitation was performed on the products before they were loaded onto the ABI-3130 Genetic Analyser.

7.3.6 Statistical analysis

To see the deviation from the normal genotype distribution in the population, the genotype frequencies of each SNP were tested by checking adherence to Hardy-Weinberg equilibrium. Chi-square analysis ($\chi^2$) of genotypic and allelic results was undertaken for each SNP to test for significant differences in genotype and allele frequencies in migraineurs MA, MO and combined migraine groups versus control results to detect any association with migraine. The Statistical Package for Social Sciences (SPSS version 21.0) was used for all computations.

7.3.7 Genotyping GLUD1 and GLUD2 genes

The following polymorphisms: rs1923934, rs4934292 were genotyped by PCR followed by HRM analysis. The PCR protocol was as follows: 1X PCR buffer, 2.0mM MgCl$_2$, 0.2mM dNTPs, 0.2μM forward primer, 0.2μM reverse primer, 1.5μM SYTO9, 0.35U GoTaq in a 15μl reaction. The primer sequences were designed with Primer-BLAST for each marker and checked for specificity using NCBI nucleotide-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) then DINAMELT (http://dinamelt.bioinfo.rpi.edu) was used to predict whether primers will form hairpins or primer-dimer structures.
The PCR followed by high resolution melting analysis was conducted on a Qiagen Rotor-Q (Qiagen, Doncaster, VIC, Australia) and the thermocycling conditions for rs1923934 were as follows: 95°C for 5 mins, then 95°C for 5 seconds and 60°C for 10 seconds for 40 cycles. PCR products were melted from 65°C to 75°C at 0.1°C increments every 2 seconds. Amplicon Tm occurred at 70°C and three separate melt curves were obtained corresponding to the three genotypes CC, CT, and TT.

Thermocycling conditions for rs4934292 were: 95°C for 5 mins, then 95°C for 5 seconds and 56°C for 10 seconds for 40 cycles. PCR products were melted from 70°C to 80°C at 0.1°C increments every 2 seconds. Amplicon Tm occurred at 73°C and three separate melt curves were obtained corresponding to the three genotypes CC, CG, and GG.

The following polymorphisms: rs493326, rs17343630, in the GLUD1 gene and rs191769566, rs5956265, rs9697983 in the GLUD2 gene were genotyped by Sequenom MassArray, refer to Chapter 8 for detailed experimental protocol.

7.4 Results

The two SNPs rs4934292 and rs1923934 in GLUD1 were genotyped using the HRM method. The results of genotyping the two SNPs by HRM were analysed with the Rotor Gene 6000 Software and genotypes were determined by variation in melting profiles as demonstrated in Figure 7.3. The raw genotype data was individually checked against baseline genotypes from positive controls at a confidence threshold of 70% and any ambiguous samples were identified and where possible these individuals were re-genotyped or genotypes were excluded from the analysis as errors.

A total of 491 individuals consisting of (233 migraineurs cases; including 184 MA and 49 MO, and 259 controls) were genotyped for rs4934292 in the GLUD1 gene (Table 7.1). The study population consisted of 291 migraine cases and 314 age- (± 5 years), sex and ethnicity matched controls. This migraine population consisted of a higher proportion of MA cases compared to MO which typically accounts for a third of migraineurs.

The genotyping of the Australian Caucasian population of the SNP rs1923934 in the GLUD1 gene was unfortunately terminated due to difficulty in distinguishing the homozygous TT and
homozygous CC in the first HRM results, despite a second melting of HRM products carried out thereafter. It was also observed that the HapMap genotype frequencies of rs1923934 were not well established. Therefore the rs1923934 SNP was not genotyped in the Australian Caucasian population.

**Figure 7.3 Example of normalized melt curve for rs4934292.**

Figure 7.3 shows the variation in the melting profile of each genotype of the rs4934292 single nucleotide polymorphism (SNP) investigated in this study. Genotypes can be determined by examining variation in the shape of the melting curve produced following normalization of fluorescence.

The GC genotype was more commonly observed in both the control and the case population whereas the GG genotype was very rare. The genotype and allele frequencies for the rs4934292 SNP were not in Hardy Weinberg Equilibrium (HWE) in the tested population. When the results were stratified by migraine subtype and gender, it was observed that the MO sample genotypes and the male migraineurs genotypes were in Hardy Weinberg Equilibrium. Chi-square ($\chi^2$) analysis was performed to determine if significant differences existed in the genotype and allele frequencies in the migraine population versus controls as well as the subgroups of migraineurs with aura versus controls and migraineurs without aura versus
controls examined with significance threshold set at p≤0.05 and the results are shown in Table 7.2.

### Table 7.2 Genotyping results for the GLUD1 SNP rs4934292 genotyped by HRM.

<table>
<thead>
<tr>
<th>GLUD1 rs4934292</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>GC</td>
<td>GG</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>122 (47.3)</td>
<td>126 (48.8%)</td>
<td>10 (3.9%)</td>
<td>258</td>
</tr>
<tr>
<td>Male</td>
<td>30 (46.2%)</td>
<td>32 (49.2%)</td>
<td>3 (4.6%)</td>
<td>65</td>
</tr>
<tr>
<td>Female</td>
<td>92 (47.7%)</td>
<td>94 (48.7%)</td>
<td>7 (3.6%)</td>
<td>193</td>
</tr>
<tr>
<td>Cases</td>
<td>106 (45.5%)</td>
<td>118 (50.6%)</td>
<td>9 (3.9%)</td>
<td>233</td>
</tr>
<tr>
<td>Male</td>
<td>22 (40.0%)</td>
<td>30 (54.5%)</td>
<td>3 (5.5%)</td>
<td>55</td>
</tr>
<tr>
<td>Female</td>
<td>84 (47.2%)</td>
<td>88 (49.4%)</td>
<td>6 (3.4%)</td>
<td>178</td>
</tr>
<tr>
<td>Total Migraine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>80 (43.5%)</td>
<td>97 (52.7%)</td>
<td>7 (3.8%)</td>
<td>184</td>
</tr>
<tr>
<td>MO</td>
<td>26 (53.1%)</td>
<td>21 (42.8%)</td>
<td>2 (4.1%)</td>
<td>49</td>
</tr>
<tr>
<td>Total MO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For the GLUD1 rs4934292 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 0.17$, $p = 0.92$) or the allelic frequencies of cases and controls ($\chi^2 = 0.09$, $p = 0.76$). The GG genotype frequency was (3.9%) in the cases and (3.9%) in the controls and similarly the G allele was more frequent in cases (29.2%) compared to controls (28.3%). The observed minor allele frequency in the control group (G allele, 28.3%) was similar to expected allele frequencies as determined in the Hap-Map CEU population (G allele, 28%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 0.03$, $p = 0.9842$, Allelic $\chi^2 = 0.00$, $p = 0.9733$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 0.47$, $p = 0.7925$, Allelic $\chi^2 = 0.34$, $p = 0.5589$).
Table 7.3 shows the Hardy-Weinberg Analysis of GLUD1 and GLUD2 SNPs genotyped by Sequenom. Marker rs493326 failed to amplify and to be genotyped in the Sequenom plex and hence was not analysed. Marker rs191769566 the cases (10/17/71) are out of HWE due to the small number of samples genotyped. Marker rs9697983 had a small MAF (G allele, 0.023) and consequently only 2 genotypes in both cases (1/13/183) and controls (0/7/205) were obtained. Three markers rs17343630, rs5956265 and rs9697983 were in HWE and were analysed, genotype and allelic frequencies are shown in Table 7.4.

Table 7.3  Hardy-Weinberg Analysis of GLUD1 and GLUD2 SNPs genotyped by Sequenom.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>SNP</th>
<th>Alleles</th>
<th>HWE (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUD1</td>
<td>10</td>
<td>rs493326</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>GLUD2</td>
<td>10</td>
<td>rs17343630</td>
<td>T, C</td>
<td>0.7108</td>
</tr>
<tr>
<td>GLUD2</td>
<td>23</td>
<td>rs191769566</td>
<td>G, A</td>
<td>0.01825</td>
</tr>
<tr>
<td>GLUD2</td>
<td>23</td>
<td>rs5956265</td>
<td>G, T</td>
<td>0.2518</td>
</tr>
<tr>
<td>GLUD2</td>
<td>23</td>
<td>rs9697983</td>
<td>G, T</td>
<td>0.2418</td>
</tr>
</tbody>
</table>

Table 7.4  Genotyping results for the GLUD1 and GLUD2 SNPs genotyped by Sequenom.

<table>
<thead>
<tr>
<th>GLUD1 rs17343630</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Controls</th>
<th>Male</th>
<th>Female</th>
<th>Cases</th>
<th>Male</th>
<th>Female</th>
<th>Total Migraine</th>
<th>MA</th>
<th>MO</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>CT</td>
<td>CC</td>
<td>Total</td>
<td>T</td>
<td>C</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>41(14.8%)</td>
<td>124 (44.8%)</td>
<td>112 (40.4%)</td>
<td>277</td>
<td>206 (37.2%)</td>
<td>348 (62.8%)</td>
<td>554</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12 (14.6%)</td>
<td>38 (46.3%)</td>
<td>32 (39.0%)</td>
<td>82</td>
<td>62 (37.8%)</td>
<td>102 (62.3%)</td>
<td>164</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>29 (14.8%)</td>
<td>86 (44.1%)</td>
<td>80 (41.0%)</td>
<td>194</td>
<td>144 (37.0%)</td>
<td>246 (63.0%)</td>
<td>390</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>33 (12.6%)</td>
<td>122 (46.6%)</td>
<td>107 (40.8%)</td>
<td>262</td>
<td>188 (35.9%)</td>
<td>336 (64.1%)</td>
<td>524</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8 (12.5%)</td>
<td>25 (39.1%)</td>
<td>31 (48.4%)</td>
<td>64</td>
<td>41 (32.0%)</td>
<td>87 (68.0%)</td>
<td>128</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>25 (12.6%)</td>
<td>97 (49.0%)</td>
<td>76 (38.4%)</td>
<td>198</td>
<td>147 (37.1%)</td>
<td>249 (62.9%)</td>
<td>396</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Migraine</td>
<td>(\chi^2 = 0.6, p = 0.7)</td>
<td>(\chi^2 = 0.2, p = 0.7)</td>
<td>(\chi^2 = 0.1, p = 1.0)</td>
<td>(\chi^2 = 0.0, p = 0.8)</td>
<td>(\chi^2 = 2.3, p = 0.3)</td>
<td>(\chi^2 = 0.6, p = 0.4)</td>
<td>416</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>29 (14.0%)</td>
<td>94 (45.2%)</td>
<td>85 (40.8%)</td>
<td>208</td>
<td>152 (36.5%)</td>
<td>264 (63.5%)</td>
<td>416</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>4 (7.4%)</td>
<td>28 (51.9%)</td>
<td>22 (40.7%)</td>
<td>54</td>
<td>36 (33.3%)</td>
<td>72 (66.6%)</td>
<td>108</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p-values were calculated by \(\chi^2\)-analysis, significance was taken at p\leq 0.05.

For the GLUD1 rs17343630 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies \((\chi^2 = 0.58, p = 0.74)\) or the allelic frequencies of cases and controls \((\chi^2 = 0.20, p = 0.65)\). The TT genotype frequency
was (12.6%) in the cases and (14.8%) in the controls and similarly the T allele was less frequent in cases (35.9%) compared to controls (37.2%). The observed minor allele frequency in the control group (T allele, 37.2%) was slightly higher compared to expected allele frequencies as determined in the Hap-Map CEU population (T allele, 24%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 0.96, p = 0.61$, Allelic $\chi^2 = 0.00, p = 0.98$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 1.37, p = 0.50$, Allelic $\chi^2 = 1.20, p = 0.27$).

<table>
<thead>
<tr>
<th>GLUD2 rs5956265</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>GT</td>
<td>GG</td>
</tr>
<tr>
<td>Controls</td>
<td>52 (26.3%)</td>
<td>88 (44.4%)</td>
<td>58 (29.3%)</td>
</tr>
<tr>
<td>Male</td>
<td>14 (23.7%)</td>
<td>23 (39.0%)</td>
<td>22 (37.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>38 (27.3%)</td>
<td>65 (46.7%)</td>
<td>36 (25.9%)</td>
</tr>
<tr>
<td>Cases</td>
<td>52 (29%)</td>
<td>80 (44.7%)</td>
<td>47 (26.3%)</td>
</tr>
<tr>
<td>Male</td>
<td>16 (37.2%)</td>
<td>7 (16.3%)</td>
<td>20 (46.5%)</td>
</tr>
<tr>
<td>Female</td>
<td>36 (26.5%)</td>
<td>73 (53.7%)</td>
<td>27 (19.9%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td>36 (26.3%)</td>
<td>63 (46.0%)</td>
<td>38 (27.7%)</td>
</tr>
<tr>
<td>MA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total MA</td>
<td>$\chi^2 = 0.1, p = 0.9$</td>
<td>$\chi^2 = 0.0, p = 0.8$</td>
<td></td>
</tr>
<tr>
<td>Total MO</td>
<td>16 (38.0%)</td>
<td>17 (40.5%)</td>
<td>9 (21.4%)</td>
</tr>
<tr>
<td>Total MO</td>
<td>$\chi^2 = 2.6, p = 0.3$</td>
<td>$\chi^2 = 2.7, p = 0.1$</td>
<td></td>
</tr>
</tbody>
</table>

MA - migraine with aura, MO - migraine without aura.

p-values were calculated by $\chi^2$-analysis, significance was taken at p≤0.05.

For the GLUD2 rs5956265 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 1.77, p = 0.41$) or the allelic frequencies of cases and controls ($\chi^2 = 0.37, p = 0.54$). The TT genotype frequency was (29%) in the cases and (26.3%) in the controls and similarly the T allele was more frequent in cases (51.4%) compared to controls (48.5%). The observed minor allele frequency in the control group (T allele, 48.5%) was similar to expected allele frequencies as determined in the Hap-Map CEU population (T allele, 48%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 1.77, p = 0.41$, Female Allelic $\chi^2 = 0.37, p = 0.54$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 6.41, p = 0.04$, Male Allelic $\chi^2 = 0.09, p = 0.76$).
MA - migraine with aura, MO - migraine without aura.
p-values were calculated by $\chi^2$-analysis, significance was taken at $p \leq 0.05$.

For the GLUD2 rs9697983 polymorphism there was a statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 1.37$, $p = 0.50$) or the allelic frequencies of cases and controls ($\chi^2 = 0.06$, $p = 0.80$). The GG genotype frequency was (0.7%) in the cases and (1.3%) in the controls and similarly the G allele was more frequent in cases (3.4%) compared to controls (3.1%). The observed minor allele frequency in the control group (G allele, 3.1%) was slightly higher to expected allele frequencies as determined in the Hap-Map CEU population (G allele, 2.3%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 4.37$, $p = 0.11$, Female Allelic $\chi^2 = 3.63$, $p = 0.05$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 4.26$, $p = 0.11$, Male Allelic $\chi^2 = 3.87$, $p = 0.04$).
7.5 Discussion

Both migraine with aura and migraine without aura represent a spectrum and are complex diseases caused by a combination of genetic and environmental factors. Glutamate is the primary excitatory neurotransmitter in the central nervous system (CNS) synthesized from glutamine by glutaminase (GLS) and can be metabolized to GABA by glutamate decarboxylase 1 (GAD1). Glutamate and the receptors and transporters upon which it acts are intimately involved in migraine processes and have been implicated in both the hyperexcitability required for cortical spreading depression as well as activation of the trigeminovascular system required for the allodynia associated with migraine (Ramadan 2003). Overstimulation of glutamate is regarded as a contributing factor, through various mechanisms, to the pathology in migraine. We saw the GLUD1 and GLUD2 genes as potentially good candidate genes to investigate due to a role in glutamate metabolism.

The aim of this study was to clarify the contribution of the GLUD1 and GLUD2 genes in migraine susceptibility in an Australian case-control population. In the present association study we utilized an age (+/- 5yrs), gender and ethnicity matched Australian Caucasian population composed of 291 migraine cases diagnosed into one of two migraine subtypes, MA or MO matched to 314 controls. Study participants were recruited from the South East Queensland Region of Australia and our study population consisted of a slightly higher proportion of MA cases. In this study we genotyped one polymorphism in the GLUD1 gene by HRM and five polymorphisms in the GLUD2 gene by Sequenom to explore their potential role in susceptibility to migraine.

As seen in previous studies and in this current study, there are real difficulties in identifying genes involved in migraine due to the contribution of multiple loci as well as the confounding influence of environmental factors on this disease. Association analysis is more robust than linkage given a wider range of locus heterogeneity (though not allelic heterogeneity) and is a powerful method for mapping common alleles with small effect sizes. The results of this study have been inconclusive due to several reasons. Although the genotype and allele frequencies obtained in the tested population for the rs4934292 were similar to those obtained from the HapMap data on NCBI website, the percentage of the mutant genotype GG was present in lower numbers in the tested population compared to HapMap frequency. This may be a reason why the genotype frequencies of the tested population were not in Hardy
Weinberg Equilibrium. Another factor contributing to a deviation from Hardy Weinberg could also be the proportion of samples lost due to lack of amplification.

In addition, the HRM melting curves representing the homozygote CC and the homozygote GG were difficult to distinguish due to the very small difference in the specific temperatures that samples with these genotypes melted at. Although samples with ambiguous results were remelted and sequenced for accuracy, unfortunately there is a possibility that some samples may have been given inaccurate genotypes. Samples that did not amplify, sequence or whose genotype could not be accurately confirmed were removed from analysis. Some samples repeatedly could not amplify possibly due to poor DNA quality or degradation in those specific samples and in the specific polymorphic area we were trying to amplify. The presence of a SNP, which was supposedly rare in the forward primer designed for this study, could potentially have also interfered with the melt of the PCR product. Hardy-Weinberg Analysis of the 5 SNPs in GLUD1 and GLUD2 genotyped by Sequenom showed that 3 were in HWE rs17343630, rs5956265 and rs9697983 and were analysed for association with migraine. Chi-square ($\chi^2$) analysis indicated there was no statistically significant association between our case and control groups for either genotype or allele frequencies for any of the markers studied in the GLUD1 and GLUD2 genes and also for migraine subtype and gender.

Previously SNPs in an Italian population have also observed a significant association between SNPs in GRIA1 and GRIA3 genes and migraine (Formicola, Aloia et al. 2010). A recent study by Maher et al 2013 reported the significant association between SNPs in the GRIA3 and migraine in an Australian Caucasian population (Maher, Lea et al. 2013). Several studies have targeted glutamate and the Glutamatergic pathway when investigating migraine treatment options. Marin and Goadsby focused on the therapeutic potential of ADX10059, which is negative allosteric modulator (mGluR5) of metabotropic glutamate receptors 5, in migraine. Although ADX10059 has not continued further due to abnormalities of liver function tests it is an example of glutamatergic modulator that has shown through clinical trials to have the ability to reduce symptoms of acute migraine (Marin and Goadsby 2010).

Although the results of the current study did not reveal a significant association between the SNPs tested and migraine in our Australian population, further investigations need to be done to understand the potential role genes belonging to the glutamatergic system may play in
migraine. This is the first study to have investigated the association between the GLUD1 and GLUD2 genes and migraine. In the future additional SNPs in genes involved in glutamate homeostasis could be studied in the migraine population to fully understand if this neurotransmitter system may potentially play a role in migraine pathophysiology.

7.6 Conclusion
This study examined the possible association of polymorphisms in the GLUD1 and GLUD2 genes between migraine with and without aura. The results from this study suggest the GLUD1 and GLUD2 genes are not significantly associated with an increase in migraine risk and therefore do not play a significant role in disease susceptibility in our Australian-Caucasian population. Nevertheless the future of migraine genetics has a lot of potential. Investigating the genetic basis of migraine should improve our understanding of the pathogenesis of this disorder and may suggest pathways to which novel therapies can be targeted such as the glutamate pathway. There are already drugs for treatment of migraine attacks, such as Triptans which stimulate serotonin receptors and have the effect of reducing the dilation of blood vessels and block pre-synaptic receptors and inhibit the release of vasodilating peptides. Current migraine therapies work at differing efficacies and often have several side effects therefore more safe and effective treatment options for migraine are a high priority for future research. This is because migraine is a multifactorial disorder caused by several factors and genetic polymorphisms that together contribute to the variety of symptoms and triggers reported by migraineurs. Consequently gaining an in depth understanding of the genotypic profile of migraineurs is an active area of research that is necessary to improve the effectiveness of migraine treatment.
CHAPTER 8  Association Analysis of *ADARBI* and *ADARB2* gene variants in migraine

8.1 Abstract

Migraine causes crippling attacks of severe head pain along with associated nausea, vomiting, photophobia and/or phonophobia. A genetic aetiology is believed to play a significant role however only a small number of genes, the sum of which comprises only a portion of trait heritability, have at this point in time been conclusively linked to the migraine subtypes Migraine with Aura (MA) and Migraine without Aura (MO) and therefore current research is actively pursuing the identification of unknown migraine susceptibility genes. The genetic contributors identified include genes of small effect from neurological, vascular and hormonal pathways and most recently a functional variant in a potassium channel TRESK involved in neuronal excitability was identified by linkage analysis. The aim of this study was to investigate the association between migraine and SNPs in the ADARB1 and ADARB2 genes in an Australian case-control Caucasian population matched for age (+/- 5yrs), gender and ethnicity. Both genes are highly expressed in the CNS and fit criteria for migraine neuropathology. This is the first study to examine RNA editing genes in relation to migraine. ADARB2 was implicated in a pedigree-based GWAS using the genetic isolate of Norfolk Island, Australia, whereas the ADARB1 gene was concurrently chosen for investigation in this study due to its important function in editing neurotransmitter receptor transcripts. Disturbances in the Adenosine-to-Inosine RNA editing reaction catalysed by the ADARB1 enzyme have been linked to human cancer, viral infections and neurological diseases. Seven SNPs in ADARB1 and 14 in ADARB2 were chosen by inspecting blocks of LD in Haploview and genotyped using TaqMan and Sequenom genotyping platforms. Chi-square (χ²) analysis revealed most of the investigated SNPs in ADARB1 and ADARB2 are not associated with increased risk of migraine in our Australian outbred Caucasian population. Nominal association was detected in two SNPs in the ADARB2 gene, marker rs5015512 was positive for combined migraine, allelic χ² = 4.58, p = 0.0323 and MA χ² = 5.55, p = 0.01 and females χ² = 5.15, p = 0.02 and marker rs884861 was nominally positive for combined migraine at the allelic χ² = 3.93, p = 0.04 level with significance based on p-value. The ADARB2 gene warrants further investigation in a larger population.
8.2 Introduction

The International Headache Society (IHS) defines migraine as a recurrent headache disorder typified by painful headache attacks lasting 4-72 hours (IHS 2013). Classification criteria recognize two types of migraine, migraine with aura (MA) and migraine without aura (MO) (IHS 2013). Migraine generally affects 12% of the Caucasian population showing up more often in females a statistic correlated with instability of ovarian hormones (Lipton, Bigal et al. 2007). Interactions between the environment and the genotype are important in shaping the migraine phenotype. Although migraine has been described by many renowned physicians in the Roman era dating far back to BC time, its modern day aetiology and treatment are still unconquered.

Numerous theories and models regarding migraine mechanisms have emerged however it is generally accepted that the pain during migraine is not the result of vasodilation. The most accepted opinion is that a combination of vascular and neural events is involved in the initiation and perpetuation of a migraine attack (Edvinsson, Villalon et al. 2012; Amin, Asghar et al. 2013). Primarily the pain is thought to result from complex processes within the nervous system that activate the trigeminovascular system and cause dilation of blood vessels with an accompanying release of inflammatory markers. The aura on the other hand, occurring in only a minority of migraineurs, has been attributed to an electrophysiological phenomenon, first reported in epilepsy, termed Cortical Spreading Depression (CSD) (Dalkara, Zervas et al. 2006).

Migraine undeniably has a major impact on the wellbeing and quality of life of sufferers and their families due to absenteeism from the workplace and higher direct health-care costs (Leonardi, Steiner et al. 2005). Population based twin studies have confirmed a genetic influence ranging from 0.34 to 0.57 (Mulder, Van Baal et al. 2003; Svensson, Larsson et al. 2003). Genetic studies of a rare and more severe migraine subtype, familial hemiplegic migraine (FHM), have identified mutations in 3 causal genes that code for ion channels involved in neuronal signalling and have provided hypotheses applicable to common migraine (de Vries, Frants et al. 2009). Functional studies in cellular and animal models of mutant alleles provide direct evidence for neuronal hyperexcitability as one cellular mechanism underlying headache or aura in FHM (van den Maagdenberg, Pietrobon et al. 2004; Tottene, Conti et al. 2009; Garza-Lopez, Sandoval et al. 2012).
For the most part, however, genes causing more common types of migraine have been identified from neurological, vascular and hormonal pathways and recently the first functional variant to show linkage to familial MA was identified, TRESK a potassium channel involved in neuronal excitability (Lafreniere, Cader et al. 2010). The number and types of genes involved is still being arduously investigated, but until now, the hunt for migraine susceptibility genes has focussed on neurotransmitter-related pathways, as these pathways are considered to play a significant role in the migraine process. Therefore genes affecting synthesis and transport of neurotransmitters including RNA editing genes are potential candidates for involvement in disease susceptibility.

A pedigree-based GWAS using the genetic isolate of Norfolk Island, an island off the coast of Australia, identified four SNPs forming a 22kb haplotype block in the ADARB2 gene summarised in Table 8.1 and Figure 8.1 with strong association with migraine susceptibility (Cox, Lea et al. 2012a). The SNP rs2271275 confers a (Thr-Ala) amino acid change in the protein structure and has previously been associated with early-onset obsessive-compulsive disorder in some American families in a genome-wide linkage scan (Hanna, Veenstra-Vanderweele et al. 2007). The same pGWAS simultaneously implicated 2 SNPS in a glutamate receptor, metabotropic 7 (GRM7) and 1 SNP in the 5-hydroxytryptamine serotonin receptor 7 adenylate cyclase-coupled (HTR7) gene (Cox, Lea et al. 2012a). Collectively, association of variants in these neurotransmitter-related genes ADARB2; GRM7; HTR7 connected by a common neurological pathway supports current theories of a perturbed serotonin and glutamate mechanism in migraine in the Norfolk pedigree.

The ADARB2 gene was chosen as a candidate gene for further investigation in a large case-control Australian Caucasian population. ADARB2 is a member of the double-stranded RNA adenosine deaminase family of RNA-editing enzymes and has not previously been studied in relation to migraine susceptibility. The ADARB2 gene spans a genomic region of 9.5kb, is located on chromosome 10p15.3 and codes for a catalytically inactive protein, expressed in brain, amygdala and thalamus (Hogg, Paro et al. 2011). A second gene ADARBI encodes an enzyme involved in RNA editing and downstream regulation of neurotransmitters and is mainly expressed in the CNS (Seeburg and Hartner 2003; Maas, Kawahara et al. 2006). This gene is potentially more interesting as the editing reaction catalyzed by this enzyme changes the sequence of its mRNA substrates. Neurotransmitters are the predominant targets of
ADAR1 editing making this gene a good candidate. The ADAR1 gene spans a genomic region of 25 kb and comprises 10 exons of coding sequence and is located on chromosome 21q22.3 (Maas, Kawahara et al. 2006). The editing enzyme ADAR1 regulates its own expression through self-editing (Feng, Sansam et al. 2006).

The enzymatic activity of ADARs leads to the chemical modification of Adenosine-to-Inosine (A-to-I) in specific coding regions which are then translated as guanosines by the cells translational machinery (Figure 8.2) (Brennicke, Marchfelder et al. 1999). Adenosine-to-Inosine (A-to-I) RNA editing is a post-transcriptional process that permanently alters the nucleotide sequence of an RNA molecule resulting in the synthesis of proteins not encoded by the original gene sequence (Barbon and Barlati 2011). This is a form of chemical recoding that changes specific amino acid residues and alters the biological function of translated molecules, which is most clearly demonstrated in an alteration in channel properties including the Ca^{2+} permeability of GluRs (Maas 2010; Nishikura 2010).

Faulty A-to-I RNA editing has been implicated in human cancer (Akbarian, Smith et al. 1995; Maas, Kawahara et al. 2006; Paz, Levanon et al. 2007; Cenci, Barzotti et al. 2008; Shah, Morin et al. 2009; Galeano, Leroy et al. 2010) and viral infections and neurodegenerative/neurological diseases such as dyschromatosis symmetrica hereditaria (DSH), amyotrophic lateral sclerosis (ALS), Alzheimer’s disease, and Huntington’s disease epilepsy, depression, schizophrenia and is associated with a greater risk of suicide. In the disease amyotrophic lateral sclerosis (ALS) (MIM 105400) inefficient RNA editing fails to substitute an arginine for a glutamine residue in the GluR2 Q/R site of glutamate AMPA receptors in the spinal motor neurons and is proposed as a mechanism responsible for motor neuron death (Foran and Trotti 2009). Considering the positive association of ADAR2 in the Norfolk Island pGWAS and the function of ADAR1 in editing genes involved in neurotransmission we genotyped 7 SNPs in ADAR1 and 14 SNPs in ADAR2 using a combination of TaqMan and Sequenom methods to ascertain their role in migraine susceptibility in an Australian case-control cohort.
Table 8.1  SNPs in *ADARB2* associated with migraine in the Norfolk Island pGWAS sourced from (Cox, Lea et al. 2012a).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene</th>
<th>No. SNPs in Gene</th>
<th>NCBI dbSNP Ref No.</th>
<th>NCBI build 37.1 position (BP)</th>
<th>Function</th>
<th>Minor/Major allele</th>
<th>MAF</th>
<th>Beta</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10p15</td>
<td>ADARB2</td>
<td>4</td>
<td>rs10903399</td>
<td>1227868</td>
<td>Downstream</td>
<td>C/T</td>
<td>0.330</td>
<td>0.64</td>
<td>7.68E−05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rs1046914</td>
<td>1228206</td>
<td>3Prime utr</td>
<td>G/A</td>
<td>0.328</td>
<td>0.67</td>
<td>3.43E−05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rs2271275</td>
<td>1230968</td>
<td>Non-synon</td>
<td>G/A</td>
<td>0.368</td>
<td>0.65</td>
<td>2.67E−05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rs883248</td>
<td>1250184</td>
<td>Intrinsic</td>
<td>G/A</td>
<td>0.439</td>
<td>0.67</td>
<td>3.83E−06</td>
</tr>
</tbody>
</table>

Figure 8.1 Haplotype block of 4 SNPs implicated in the *ADARB2* gene in the Norfolk Island pGWAS sourced from (Cox, Lea et al. 2012a).
Figure 8.2  RNA editing genes and gene interactions sourced from (Cox, Lea et al. 2012a).
8.3 Materials and Methods

8.3.1 Study Population

The association study protocol was approved by the Griffith University Ethics Committee for experimentation in humans. The study population was composed of 291 migraine cases (70 Males, 221 Females) and 314 controls (89 Males, 225 Females). Affected individuals were diagnosed as having Migraine with Aura (MA) or without (MO) by an experienced clinical neurologist based on responses provided in a validated medical questionnaire in accordance with criteria determined by the International Headache Society (IHS) (IHS 2013). The migraine population consisted of individuals of Caucasian origin, recruited from the South East Queensland Region of Australia. This migraine population had a higher proportion of MA cases compared to MO which typically accounts for a third of total migraineurs (227 MA, 64 MO). The matched control (age ± 5 years, gender, and ethnicity) population samples were obtained via the Genomics Research Centre Clinic, Southport certified as having no history of personal or familial migraine. Signed informed consent was obtained from all patients before participation in the study.

8.3.2 Genotyping Methods

Genomic DNA was extracted from peripheral blood samples using a salting out method as described by Miller et al., 1988 (Miller S.A, Dykes D.D et al. 1988). DNA was quantified and normalised to a concentration of 20ng/µL for genotyping experiments. The genotypes for seven SNPs in \textit{ADARB1} and 14 SNPs in \textit{ADARB2} were determined by TaqMan and Sequenom genotyping platforms for all cases and controls.

8.3.3 Polymorphism Selection

HapMap CEU SNP genotype data was downloaded for each candidate gene and analysed for LD in Haploview v4.2 (Barrett 2009). Haploview was used to determine linkage disequilibrium blocks across the \textit{ADARBI} and \textit{ADARB2} genes to locate SNPs in different LD blocks so as to cover different regions of each gene. SNPs chosen were sampled in a European-Caucasian population and were mostly located in intronic regions of each gene.
8.3.4 Genotyping by TaqMan

The following SNPs rs2838771, rs1051367 in *ADARB1* and the following SNPs rs2271275, rs10903467, rs11250642 in *ADARB2* were genotyped using TaqMan® SNP Genotyping Assays from Applied Biosystems (Life Technologies, Carlsband, CA, USA). Detailed information regarding polymorphisms genotyped in the *ADARB1* and *ADARB2* genes by the TaqMan method and a summary of assay conditions and primer sequences for each polymorphism are listed in Table 8.2. The genotyping protocol for each marker was exactly the same except for the use of the specific Primer-Probe Mix. The final optimized PCR reaction conditions consisted of 20ng of genomic DNA template, TaqMan Universal PCR Master Mix (1X), SNP genotyping assay probe-primer mix (20X), DNase-free water in a 5 µL reaction volume. The PCR thermocycling conditions consisted of one cycle at 95°C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60°C for 1 min. The data were acquired during the annealing step and analysed using the 7900 system Sequence Detection System software Applied Biosystems (Life Technologies Corporation) in a 384-well plate format. Nuclease-free water was used as a negative control and DNA for each genotype included as positive controls.
Table 8.2  TaqMan Assay and SNP information.

<table>
<thead>
<tr>
<th>TaqMan Assay ID</th>
<th>Gene</th>
<th>rs number</th>
<th>Location</th>
<th>SNP Type</th>
<th>Allele Change</th>
<th>CEU MAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C__15831699_10</td>
<td>ADARB1</td>
<td>rs2838771</td>
<td>Chr.21: 46501576</td>
<td>Transversion Substitution</td>
<td>C:G</td>
<td>G=0.24</td>
</tr>
<tr>
<td>C_1211569_1_</td>
<td>ADARB1</td>
<td>rs1051367</td>
<td>Chr.21: 46641968</td>
<td>Silent Mutation</td>
<td>A:G</td>
<td>G=0.47</td>
</tr>
<tr>
<td>C_15959830_10</td>
<td>ADARB2</td>
<td>rs2271275</td>
<td>Chr.10: 1230968</td>
<td>Missense Mutation</td>
<td>C:T</td>
<td>C=0.44</td>
</tr>
<tr>
<td>C_32118695_20</td>
<td>ADARB2</td>
<td>rs10903467</td>
<td>Chr.10: 1535739</td>
<td>Intron, Transition Substitution</td>
<td>C:T</td>
<td>T=0.34</td>
</tr>
<tr>
<td>C__30856132_20</td>
<td>ADARB2</td>
<td>rs11250642</td>
<td>Chr.10: 1622644</td>
<td>Intron, Transition Substitution</td>
<td>C:T</td>
<td>T=0.45</td>
</tr>
</tbody>
</table>

ADARB1: adenosine deaminase, RNA-specific, B1; ADARB2: adenosine deaminase, RNA specific, B2; SNPs: single nucleotide polymorphisms; CEU MAF = HapMap Caucasian Minor Allele Frequency.
8.3.5 Genotyping by Sequenom

Additional polymorphisms in the \textit{ADARB1} and \textit{ADARB2} genes were genotyped using the Sequenom MassARRAY system 4 instrument and accompanying Typer 4.0 software (Sequenom, San Diego, CA) was used to carry out all genotyping work (MALDI-TOF; MassARRAY system, Sequenom Inc., San Diego, CA, USA). The oligos for PCR and iPLEX reactions were designed using the online Assay design suite 1.0 Sequenom software (available at: \url{www.mysequenom.com/tools}) and obtained from IDT and can be found in Table 8.3 (Integrated DNA Technologies, Carolville, Iowa, USA).

PCR and extension reactions were performed according to the manufacturer’s instructions, using Sequenom reagents. A master mix was made by multiplying the reagents by the number of samples. Genomic DNA 10ng was amplified in a 5\(\mu\)L reaction mixture containing, 2.3\(\mu\)L water, 0.5 10X PCR Buffer with 20mM MgCl\(_2\), 0.4\(\mu\)L MgCl\(_2\), 0.1\(\mu\)L dNTP mix, 1\(\mu\)L Primer mix containing forward and reverse primers, 0.2U enzyme. The PCR step was incubated at 95°C for 15 min, followed by 45 cycles, consisting of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s, and a final extension of 3 min at 72°C. PCRs were run on Applied Biosystems Veriti® Thermal Cyclers. After PCR, the remaining unincorporated dNTPs were dephosphorylated by adding 2\(\mu\)L of the SAP cocktail, containing 1.53\(\mu\)L of water, 0.17\(\mu\)L of SAP buffer (Sequenom) and 0.3\(\mu\)L of SAP enzyme (Sequenom). The 96-well plate was then sealed and placed in a thermal cycler with the following conditions: 37°C for 40 min, 85°C for 5 min and 4°C indefinitely.

After the SAP treatment, a primer extension reaction cocktail of 2\(\mu\)L, consisting of 1.728\(\mu\)L water; 0.2\(\mu\)L hME EXTEND mixture, containing 10X buffer and appropriate d/ddNTPs (Sequenom); 0.54ml of 10 mM each extension primer mixture and 0.018ml of 32U/ml ThermoSequenase (Sequenom) was added. After the hME cocktail addition, the plate was again sealed and placed in a thermal cycler with the following program: 94°C for 2 min followed by 55 cycles of 94°C for 5s, 52°C for 5s and 72°C for 5s. The completed genotyping reaction products were desalted with SpectroCLEAN resin to remove salts such as Na\(^+\), K\(^+\), Mg\(^{2+}\). Following the MassExtend reaction, completed genotyping reactions were spotted in nanoliter volumes onto a matrix arrayed silicon chip with 96 elements (Sequenom SpectroCHIP) using the MassARRAY Nanodispenser using the capillary action of slotted
pins. Spectro CHIPS were analyzed using the Bruker Autoflex MALDI-TOF mass spectrometer and the spectra were processed using the SpectroTYPER software (Sequenom).

Table 8.3 SNPs genotyped in \textit{ADARB1} and \textit{ADARB2} by Sequenom.

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Forward, Reverse and Extension Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADARB1</td>
<td></td>
</tr>
</tbody>
</table>
| rs407133 | F: 5'ACGTTGGATGCTGCTCATGTTAATCAG 3'  
          | R: 5'ACGTTGGATGTCCCTCTCTCTCTTTAACTCAG 3'  
          | E: TCGGACCAATGCTGA |
| rs2838787 | F: 5'AGCTGGATGTTGCTGTCACTTATCAG 3'  
           | R: 5'ACGTTGGATGTCCCTCTCTCTCTTTAACTCAG 3'  
           | E: ggATGGGCTGTATATACCA |
| rs2838815 | F: 5'AGCTGGATGTTGCTGTCACTTATCAG 3'  
           | R: 5'ACGTTGGATGTCCCTCTCTCTCTTTAACTCAG 3'  
           | E: TTAGGAAATAGAAATGTGA |
| rs1108982 | F: 5'AGCTGGATGTTGCTGTCACTTATCAG 3'  
           | R: 5'ACGTTGGATGTCCCTCTCTCTCTTTAACTCAG 3'  
           | E: cggATGGGCTGTATATACCA |
| rs422720 | F: 5'AGCTGGATGTTGCTGTCACTTATCAG 3'  
         | R: 5'ACGTTGGATGTCCCTCTCTCTCTTTAACTCAG 3'  
         | E: TTTAGGGAAAATAGAAATGTGA |
| ADARB2 |                                                   |
| rs10903520 | F: 5'AGCTGGATGTTGCTGTCACTTATCAG 3'  
         | R: 5'ACGTTGGATGTCCCTCTCTCTCTTTAACTCAG 3'  
         | E: AAGGAAAGCAGGGCCT |
| rs5015512 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
         | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
         | E: cggATGGGCTGTATATACCA |
| rs10903479 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
           | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
           | E: CCTTCATTGTCAGAGAT |
| rs2999399 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
         | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
         | E: ACCTTTCTATGCTGATA |
| rs2805533 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
         | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
         | E: aggcGTCAGGAGTCCCTTT |
| rs11250705 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
          | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
          | E: aggcGTCAGGAGTCCCTTT |
| rs884861 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
        | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
        | E: ACAGATAGACAAAGCCAATATAT |
| rs3793733 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
         | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
         | E: caggAAGCATAATGTCAGAGCCT |
| rs1909428 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
        | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
        | E: gTAACTGAGTCCCTACATCT |
| rs7094094 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
        | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
        | E: CATTCTGTTGCTTAATATATAT |
| rs7070629 | F: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
        | R: 5'ACGTTGGATGTTGCTGTCACTTATCAG 3'  
        | E:acctTGATGCAGCTTTTCTCCTGAC |

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8.3.6 Statistical Analysis

Chi-square ($\chi^2$) analysis was performed to determine if significant differences existed in the genotype and allele frequencies in the migraine population versus controls as well as the subgroups of migraineurs with aura versus controls and migraineurs without aura versus controls examined with significance threshold set at $p \leq 0.05$ (Table 7.1). This analysis was completed in the software PLINK v1.07 which is a free whole genome association toolset (Purcell, Neale et al. 2007). Hardy-Weinberg Equilibrium (HWE) was calculated for each SNP in case and control groups to check for genotyping error.

8.4 TaqMan Results

The aim of this study was to determine if polymorphisms in the $ADARBI$ and $ADARB2$ genes contribute to migraine susceptibility in an Australian case-control cohort. The SNPs were chosen by inspecting blocks of LD in Haploview using HapMap genotype data. The SNPs chosen provided good coverage of each gene and were not in LD with each other. The following SNPs rs2838771, rs1051367 in $ADARBI$ and the following SNPs rs2271275, rs10903467, rs11250642 in $ADARB2$ were genotyped by the TaqMan method. Figures 8.3-8.7 show the allelic discrimination plot for each marker genotyped by the TaqMan method and Tables 8.4-8.8 show the genotype and allele frequencies obtained for each marker.
8.4.1 ADARB1 rs2838771 Genotyping results

Figure 8.3 shows an allelic discrimination plot for the ADARB1 rs2838771 marker genotyping. CC genotypes are represented by the blue dots, CG genotypes are represented by the green dots and GG genotypes are represented by the red dots. Black squares represent no template controls and black crosses represent samples with undetermined genotypes.

Of the 291 cases and 314 controls genotyped, 261 cases (89%) and 247 (78%) controls were successfully genotyped for the marker. The cases and controls were found to be in Hardy Weinberg Equilibrium (p=0.6 and p=0.9 respectively). Table 8.4 shows that genotype and allele frequencies for the ADARB1 rs2838771 marker.

Figure 8.3 ADARB1 rs2838771 Marker Allelic Discrimination Plot.
Table 8.4 
ADARB1 rs2838771 genotype and allele frequencies.

<table>
<thead>
<tr>
<th>ADARB1 rs2838771</th>
<th>Genotypes</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>GC</td>
</tr>
<tr>
<td>Controls</td>
<td>100 (40.5%)</td>
<td>114 (46.2%)</td>
</tr>
<tr>
<td>Male</td>
<td>36 (57.2%)</td>
<td>22 (34.9%)</td>
</tr>
<tr>
<td>Female</td>
<td>64 (35.4%)</td>
<td>92 (50.8%)</td>
</tr>
<tr>
<td>Cases</td>
<td>112 (42.9%)</td>
<td>115 (44.1%)</td>
</tr>
<tr>
<td>Male</td>
<td>29 (46.8%)</td>
<td>24 (38.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>33 (41.7%)</td>
<td>91 (45.7%)</td>
</tr>
<tr>
<td>Total MA</td>
<td>91 (43.9%)</td>
<td>91 (43.9%)</td>
</tr>
<tr>
<td>Total MO</td>
<td>21 (38.9%)</td>
<td>24 (44.4%)</td>
</tr>
</tbody>
</table>

Total Migraine: $\chi^2 = 3.5$, p = 0.2

Total MA: $\chi^2 = 0.7$, p = 0.7

Total MO: $\chi^2 = 0.4$, p = 0.8

MA — migraine with aura, MO — migraine without aura.

p-values were calculated by $\chi^2$-analysis, significance was taken at p≤0.05.

For the ADARB1 rs2838771 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 3.47$, p = 0.17) or the allelic frequencies of cases and controls ($\chi^2 = 0.94$, p = 0.33). The GG genotype frequency was (13%) in the cases and (13.4%) in the controls and similarly the G allele was less frequent in cases (35.1%) compared to controls (36.4%). The observed minor allele frequency in the control group (G allele, 36.4%) was higher to expected allele frequencies as determined in the Hap-Map CEU population (G allele, 24%). Analysis by gender showed a slight trend toward association for the genotype frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 6.48$, p = 0.03, Allelic $\chi^2 = 3.38$, p = 0.06). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 1.98$, p = 0.37, Allelic $\chi^2 = 2.15$, p = 0.14).
8.4.2 ADARB1 rs1051367 Genotyping results

Figure 8.4 shows an allelic discrimination plot for the ADARB1 rs1051367 marker genotyping. AA genotypes are represented by the blue dots, AG genotypes are represented by the green dots and GG genotypes are represented by the red dots. Black squares represent no template controls and black crosses represent samples with undetermined genotypes.

Of the 291 cases and 314 controls genotyped, 274 cases (94%) and 300 (95%) controls were successfully genotyped for the marker. The cases and controls were found to be in Hardy Weinberg Equilibrium (p=0.3 and p=0.9 respectively). Table 8.5 shows the genotype and allele frequencies for the ADARB2 rs1051367 marker.
### Table 8.5 ADARB1 rs1051367 genotype and allele frequencies.

<table>
<thead>
<tr>
<th>ADARB1 rs1051367</th>
<th>Genotypes</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AG</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>96 (32%)</td>
<td>148 (49.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>79 (37.4%)</td>
<td>95 (45.0%)</td>
</tr>
<tr>
<td>Cases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>76 (27.8%)</td>
<td>145 (53.1%)</td>
</tr>
<tr>
<td>Female</td>
<td>61 (29.6%)</td>
<td>105 (51.0%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td>$\chi^2 = 1.3$, p = 0.5</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>64 (27.7%)</td>
<td>123 (53.2%)</td>
</tr>
<tr>
<td>Total MA</td>
<td>$\chi^2 = 1.2$, p = 0.5</td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>12 (28.6%)</td>
<td>22 (52.4%)</td>
</tr>
<tr>
<td>Total MO</td>
<td>$\chi^2 = 0.2$, p = 0.9</td>
<td></td>
</tr>
</tbody>
</table>

MA — migraine with aura, MO — migraine without aura.

p-values were calculated by $\chi^2$-analysis, significance was taken at p≤0.05.

For the ADARB1 rs1051367 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 1.26$, p = 0.53) or the allelic frequencies of cases and controls ($\chi^2 = 0.71$, p =0.40). The GG genotype frequency was (19.4%) in the cases and (18.7%) in the controls and similarly the G allele was more frequent in cases (45.8%) compared to controls (43.3%). The observed minor allele frequency in the control group (G allele, 43.3%) was lower than expected allele frequencies as determined in the Hap-Map CEU population (G allele, 46.7%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 2.87$, p = 0.23, Allelic $\chi^2 = 2.01$, p = 0.15). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 0.43$, p = 0.80, Allelic $\chi^2 = 0.35$, p = 0.55).
8.4.3 ADARB2 rs2271275 Genotyping results

Figure 8.5 shows an allelic discrimination plot for the ADARB2 rs2271275 marker genotyping. TT genotypes are represented by the blue dots, TC genotypes are represented by the green dots and CC genotypes are represented by the red dots. Black squares represent no template controls and black crosses represent samples with undetermined genotypes.

Of the 291 cases and 314 controls genotyped, 268 cases (92%) and 296 controls (94%) were successfully genotyped for the marker. The cases and controls were found to be in Hardy Weinberg Equilibrium (p=0.86 and p=0.49 respectively). Table 8.6 shows the genotype and allele frequencies for the ADARB2 rs2271275 marker.
Table 8.6  ADARB2 rs2271275 genotype and allele frequencies.

<table>
<thead>
<tr>
<th>ADARB2 rs2271275</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>TC</td>
<td>CC</td>
</tr>
<tr>
<td>Controls</td>
<td>86 (29.0%)</td>
<td>152 (51.3%)</td>
<td>58 (19.6%)</td>
</tr>
<tr>
<td>Male</td>
<td>20 (23.2%)</td>
<td>41 (47.7%)</td>
<td>25 (29.0%)</td>
</tr>
<tr>
<td>Female</td>
<td>66 (31.4%)</td>
<td>111 (52.8%)</td>
<td>33 (15.7%)</td>
</tr>
<tr>
<td>Cases</td>
<td>123 (42.9%)</td>
<td>118 (44%)</td>
<td>27 (17.5%)</td>
</tr>
<tr>
<td>Male</td>
<td>34 (53.1%)</td>
<td>26 (40.6%)</td>
<td>4 (6.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>89 (43.7%)</td>
<td>92 (45%)</td>
<td>23 (11.3%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>101(47.9%)</td>
<td>88 (41.7%)</td>
<td>22 (10.4%)</td>
</tr>
<tr>
<td>Total MA</td>
<td>174(49.9%)</td>
<td>120 (41.7%)</td>
<td>31 (9.2%)</td>
</tr>
<tr>
<td>MO</td>
<td>22 (38.6%)</td>
<td>30 (52.6%)</td>
<td>5 (9%)</td>
</tr>
<tr>
<td>Total MO</td>
<td>226(46.6%)</td>
<td>150 (48.6%)</td>
<td>31 (6.1%)</td>
</tr>
</tbody>
</table>

MA — migraine with aura, MO — migraine without aura.
p-values were calculated by $\chi^2$-analysis, significance was taken at $p \leq 0.05$.

For the ADARB2 rs2271275 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 4.16$, $p = 0.12$) or the allelic frequencies of cases and controls ($\chi^2 = 0.09$, $p = 0.76$). The CC genotype frequency was (17.5%) in the cases and (15.8%) in the controls and similarly the C allele was less frequent in cases (32.1%) compared to controls (41%). The observed minor allele frequency in the control group (C allele, 41%) was similar to expected allele frequencies as determined in the Hap-Map CEU population (C allele, 44%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 4.52$, $p = 0.10$, Allelic $\chi^2 = 0.14$, $p = 0.71$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 0.19$, $p = 0.90$, Allelic $\chi^2 = 0.07$, $p = 0.79$).
8.4.4 ADARB2 rs10903467 Genotyping results

Figure 8.6 shows an allelic discrimination plot for the ADARB2 rs10903467 marker genotyping. TT genotypes are represented by the blue dots, CT genotypes are represented by the green dots and CC genotypes are represented by the red dots. Black squares represent no template controls and black crosses represent samples with undetermined genotypes.

![ADARB2 rs10903467 Marker Allelic Discrimination Plot](image)

Of the 291 cases and 314 controls genotyped, 251 cases (86%) and 289 controls (92%) were successfully genotyped for the marker. The cases and controls were found to be in Hardy Weinberg Equilibrium (p=0.59 and p=0.36 respectively). Table 8.7 shows that genotype and allele frequencies for the ADARB2 rs10903467 marker.
Table 8.7 ADARB2 rs10903467 genotype and allele frequencies.

<table>
<thead>
<tr>
<th>ADARB1 rs10903467</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>CT</td>
<td>CC</td>
</tr>
<tr>
<td>Controls</td>
<td>59 (20.4%)</td>
<td>151 (52.4%)</td>
<td>78 (27.0%)</td>
</tr>
<tr>
<td>Male</td>
<td>16 (19.0%)</td>
<td>41 (48.8%)</td>
<td>27 (32.1%)</td>
</tr>
<tr>
<td>Female</td>
<td>43 (21.0%)</td>
<td>110 (53.9%)</td>
<td>51 (25%)</td>
</tr>
<tr>
<td>Cases</td>
<td>46 (18.3%)</td>
<td>118 (47.0%)</td>
<td>87 (34.6%)</td>
</tr>
<tr>
<td>Male</td>
<td>11 (18.6%)</td>
<td>30 (50.8%)</td>
<td>18 (30.5%)</td>
</tr>
<tr>
<td>Female</td>
<td>35 (18.2%)</td>
<td>88 (45.8%)</td>
<td>69 (35.9%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td>$\chi^2 = 3.6$, p = 0.2</td>
<td>$\chi^2 = 2.6$, p = 0.1</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37 (18.6%)</td>
<td>97 (48.9%)</td>
<td>64 (32.3%)</td>
</tr>
<tr>
<td>Total MA</td>
<td>$\chi^2 = 1.6$, p = 0.5</td>
<td>$\chi^2 = 1.2$, p = 0.3</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9 (16.9%)</td>
<td>21 (39.6%)</td>
<td>23 (43.3%)</td>
</tr>
<tr>
<td>Total MO</td>
<td>$\chi^2 = 5.8$, p = 0.1</td>
<td>$\chi^2 = 3.5$, p = 0.1</td>
<td></td>
</tr>
</tbody>
</table>

MA — migraine with aura, MO — migraine without aura.

For the ADARB2 rs10903467 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 3.63$, p = 0.16) or the allelic frequencies of cases and controls ($\chi^2 = 2.57$, p = 0.10). The TT genotype frequency was (18.3%) in the cases and (20.4%) in the controls and similarly the T allele was less frequent in cases (41.8%) compared to controls (46.7%). The observed minor allele frequency in the control group (T allele, 46.7%) was higher to expected allele frequencies as determined in the Hap-Map CEU population (T allele, 34%). Analysis by gender showed a slight trend toward significant differences between genotype and allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 5.61$, p = 0.06, Allelic $\chi^2 = 3.80$, p = 0.05). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 0.06$, p = 0.96, Allelic $\chi^2 = 0.01$, p = 0.91).
8.4.5 ADARB2 rs11250642 Genotyping results

Figure 8.7 shows an allelic discrimination plot for the ADARB2 rs2271275 marker genotyping. TT genotypes are represented by the blue dots, CT genotypes are represented by the green dots and CC genotypes are represented by the red dots. Black squares represent no template controls and black crosses represent samples with undetermined genotypes.

Of the 291 cases and 314 controls genotyped, 255 cases (87%) and 270 controls (86%) were successfully genotyped for the marker. The cases and controls were found to be in Hardy Weinberg Equilibrium (p=0.55 and p=0.86 respectively). Table 8.8 shows that genotype and allele frequencies for the ADARB2 rs11250642 marker.
**Table 8.8**  ADARB2 rs11250642 genotype and allele frequencies.

<table>
<thead>
<tr>
<th>ADARB2 rs11250642</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>CT</td>
<td>CC</td>
<td>Total</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Controls</td>
<td>54 (20.0%)</td>
<td>135 (50.0%)</td>
<td>75 (30.0%)</td>
<td>270</td>
<td>243 (45.0%)</td>
<td>297 (55.0%)</td>
</tr>
<tr>
<td>Male</td>
<td>17 (21.0%)</td>
<td>45 (55.5%)</td>
<td>19 (23.5%)</td>
<td>81</td>
<td>79 (48.7%)</td>
<td>83 (51.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>43 (22.7%)</td>
<td>90 (47.6%)</td>
<td>56 (29.6%)</td>
<td>189</td>
<td>176 (46.5%)</td>
<td>202 (53.4%)</td>
</tr>
<tr>
<td>Cases</td>
<td>51 (20.0%)</td>
<td>117 (45.8%)</td>
<td>87 (34.1%)</td>
<td>255</td>
<td>222 (41.8%)</td>
<td>288 (58.1%)</td>
</tr>
<tr>
<td>Male</td>
<td>13 (20.3%)</td>
<td>30 (46.8%)</td>
<td>21 (32.9%)</td>
<td>64</td>
<td>56 (43.8%)</td>
<td>72 (56.2%)</td>
</tr>
<tr>
<td>Female</td>
<td>38 (20%)</td>
<td>87 (45.5%)</td>
<td>66 (34.5%)</td>
<td>191</td>
<td>163 (42.6%)</td>
<td>219 (57.3%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\chi^2 = 2.1$, p = 0.3</td>
<td>$\chi^2 = 1.0$, p = 0.3</td>
</tr>
<tr>
<td>MA</td>
<td>40 (20.0%)</td>
<td>95 (47.7%)</td>
<td>64 (32.2%)</td>
<td>199</td>
<td>175 (44.0%)</td>
<td>223 (56.0%)</td>
</tr>
<tr>
<td>Total MA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\chi^2 = 0.8$, p = 0.6</td>
<td>$\chi^2 = 0.4$, p = 0.5</td>
</tr>
<tr>
<td>MO</td>
<td>11 (19.6%)</td>
<td>22 (39.3%)</td>
<td>23 (41.0%)</td>
<td>56</td>
<td>44 (39.2%)</td>
<td>68 (60.7%)</td>
</tr>
<tr>
<td>Total MO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\chi^2 = 3.8$, p = 0.2</td>
<td>$\chi^2 = 1.7$, p = 0.2</td>
</tr>
</tbody>
</table>

MA — migraine with aura, MO — migraine without aura.

p-values were calculated by $\chi^2$-analysis, significance was taken at p≤0.05.

For the ADARB2 rs11250642 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 0.33$, p = 0.84) or the allelic frequencies of cases and controls ($\chi^2 = 0.23$, p = 0.63). The TT genotype frequency was (18%) in the cases and (20%) in the controls and similarly the T allele was less frequent in cases (41.8%) compared to controls (45%). The observed minor allele frequency in the control group (T allele, 45%) was similar to expected allele frequencies as determined in the Hap-Map CEU population (T allele, 45%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 1.17$, p = 0.55, Allelic $\chi^2 = 1.16$, p = 0.28). In the male subpopulation, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 1.66$, p = 0.43, Allelic $\chi^2 = 0.72$, p = 0.39).
8.5 Sequenom Results

Among the 21 markers genotyped in total in both the \textit{ADARB1} and the \textit{ADARB2} genes in this study, the following 4 SNPs in the \textit{ADARB1} gene rs2838771, rs407133, rs422720, rs1051367, and the following 9 SNPs in the \textit{ADARB2} gene rs2271275, rs5015512, rs7070629, rs10903467, rs10903479, rs11250642, rs10903520, rs7094094, rs884861 were in Hardy-Weinberg Equilibrium and analysed for association. Table 8.9 shows the Hardy-Weinberg Analysis of \textit{ADARB1} and \textit{ADARB2} SNPs genotyped by the Sequenom method. As seen in Table 8.9, 2 markers rs407133 and rs422720 were in HWE and the genotype and allele frequencies are displayed in Table 8.10 whereas 3 markers rs2838787, rs11088982 and rs2838815 in the \textit{ADARB1} gene were out of HWE. Although SNP rs2838787 was genotyped and in HWE the proportions for cases (8/14/14) and controls (6/9/11) were really small to carry out association testing. Chi-square ($\chi^2$) analysis was performed for combined migraine (cases versus controls) and the migraine subtypes MA, MO and gender.

Table 8.9 Hardy-Weinberg Analysis of \textit{ADARB1} and \textit{ADARB2} SNPs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>SNP</th>
<th>Alleles</th>
<th>ALL</th>
<th>AFF</th>
<th>UNAFF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADARB1</td>
<td>21</td>
<td>rs2838787</td>
<td>A G</td>
<td>0.06914</td>
<td>0.3015</td>
<td>0.2176</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>rs407133</td>
<td>G A</td>
<td>0.144</td>
<td>0.4528</td>
<td>0.237</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>rs422720</td>
<td>C A</td>
<td>0.5852</td>
<td>0.6968</td>
<td>0.7981</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>rs11088982</td>
<td>A G</td>
<td>1.982E-07</td>
<td>0.0001628</td>
<td>0.0004145</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>rs2838815</td>
<td>C T</td>
<td>9.326E-45</td>
<td>2.039E-20</td>
<td>7.714E-26</td>
</tr>
<tr>
<td>ADARB2</td>
<td>10</td>
<td>rs2805533</td>
<td>C T</td>
<td>0.008848</td>
<td>0.003467</td>
<td>0.3912</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs5015512</td>
<td>C T</td>
<td>1</td>
<td>0.7373</td>
<td>0.8336</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs3793733</td>
<td>A G</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs7070629</td>
<td>A G</td>
<td>0.7781</td>
<td>1</td>
<td>0.7917</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs10903479</td>
<td>T A</td>
<td>0.9309</td>
<td>0.9012</td>
<td>0.8076</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs2999399</td>
<td>C T</td>
<td>0.0006935</td>
<td>0.07034</td>
<td>0.00382</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs7094094</td>
<td>G T</td>
<td>0.9191</td>
<td>0.7659</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs10903520</td>
<td>A G</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs11250705</td>
<td>A G</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs1909428</td>
<td>G A</td>
<td>6.44E-18</td>
<td>1.825E-08</td>
<td>1.207E-10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>rs884861</td>
<td>G C</td>
<td>0.02986</td>
<td>0.2282</td>
<td>0.08511</td>
</tr>
</tbody>
</table>
In the $ADARB2$ gene 5 markers were out of HWE rs2805533, rs3793733, rs2999399, rs11250705, rs1909428. For marker rs2805533 the cases are in HW disequilibrium but not controls, for marker rs3793733 this was also out of HWE due to the fact that it had a very small MAF 0.008 and only 2 out of 3 genotypes were recorded in the experiment and for marker rs2999399 both the cases and controls are out of HWE and finally for rs11250705 only 2 genotypes were recorded and for rs1909428 cases and controls are out of HWE.

Therefore in summary a total of 2 markers in the $ADARB1$ gene passed HWE and were analysed for association with migraine these included rs407133 and rs422720 and the genotype and allele frequencies are displayed in Table 8.10. Six markers in the $ADARB2$ gene passed HWE and were analysed for association with migraine these included rs5015512, rs7070629, rs10903479, rs7094094, rs10903520, rs884861 and the genotype and allele frequencies are displayed in Table 8.11. Chi-square ($\chi^2$) analysis was performed for all SNPs analysed for combined migraine (cases versus controls) and the migraine subtypes MA, MO and gender.
Table 8.10  Genotyping results for the \textit{ADARB1} SNPs genotyped by Sequenom.

<table>
<thead>
<tr>
<th>ADARB1 rs407133</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
<th>G</th>
<th>A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
<td>Total</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>Male</td>
<td>52 (17.9%)</td>
<td>154 (52.9%)</td>
<td>85 (29.2%)</td>
<td>291</td>
<td>258 (44.3%)</td>
<td>324 (55.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>36 (17.4%)</td>
<td>104 (50.4%)</td>
<td>66 (32.0%)</td>
<td>206</td>
<td>176 (42.7%)</td>
<td>236 (57.3%)</td>
</tr>
<tr>
<td>Cases</td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
<td>Total</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>Male</td>
<td>47 (17.8%)</td>
<td>137 (51.9%)</td>
<td>80 (30.3%)</td>
<td>264</td>
<td>231 (43.8%)</td>
<td>297 (56.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>34 (16.9 %)</td>
<td>108 (40.0%)</td>
<td>59 (29.4%)</td>
<td>201</td>
<td>176 (43.8%)</td>
<td>226 (56.2%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td>( \chi^2 = 0.1, p = 1.0 )</td>
<td>( \chi^2 = 0.0, p = 0.8 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>39 (18.7%)</td>
<td>106 (50.7%)</td>
<td>64 (30.6%)</td>
<td>209</td>
<td>184 (44.0%)</td>
<td>234 (56.0%)</td>
</tr>
<tr>
<td>MO</td>
<td>8 (14.5%)</td>
<td>31 (56.4%)</td>
<td>16 (29.1%)</td>
<td>55</td>
<td>47 (42.7%)</td>
<td>63 (57.3%)</td>
</tr>
<tr>
<td>Total MO</td>
<td>( \chi^2 = 0.4, p = 0.8 )</td>
<td>( \chi^2 = 0.1, p = 0.7 )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p-values were calculated by \( \chi^2 \)-analysis, significance was taken at \( p \leq 0.05 \).

For the ADARB1 rs407133 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies \( (\chi^2 = 0.08, p = 0.95) \) or the allelic frequencies of cases and controls \( (\chi^2 = 0.04, p = 0.84) \). The GG genotype frequency was (17.8%) in the cases and comparable to that (17.9%) in the controls and similarly the G allele was (43.8%) in cases compared to controls (44.3%). The observed minor allele frequency in the control group (G allele, 44.3%) was slightly higher compared to expected allele frequencies as determined in the Hap-Map CEU population (G allele, 46.3%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic \( \chi^2 = 0.46, p = 0.79 \), Allelic \( \chi^2 = 0.09, p = 0.75 \)). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic \( \chi^2 = 2.78, p = 0.24 \), Allelic \( \chi^2 = 0.61, p = 0.43 \)).
<table>
<thead>
<tr>
<th>ADARB1 rs422720</th>
<th>Genotypes</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td>CC</td>
<td>CA</td>
</tr>
<tr>
<td>Controls</td>
<td>38 (13.8%)</td>
<td>132 (48.0%)</td>
</tr>
<tr>
<td>Male</td>
<td>14 (18.0%)</td>
<td>40 (51.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>24 (12.2%)</td>
<td>92 (46.7%)</td>
</tr>
<tr>
<td>Cases</td>
<td>40 (15.7%)</td>
<td>127 (49.8%)</td>
</tr>
<tr>
<td>Male</td>
<td>11 (17.7%)</td>
<td>30 (48.4%)</td>
</tr>
<tr>
<td>Female</td>
<td>29 (15.0%)</td>
<td>97 (50.3%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td>$\chi^2 = 1.0, \ p = 0.6$</td>
<td>$\chi^2 = 0.9, \ p = 0.4$</td>
</tr>
<tr>
<td>MA</td>
<td>33 (16.3%)</td>
<td>97 (47.8%)</td>
</tr>
<tr>
<td>Total MA</td>
<td>$\chi^2 = 0.6, \ p = 0.7$</td>
<td>$\chi^2 = 0.5, \ p = 0.5$</td>
</tr>
<tr>
<td>MO</td>
<td>7 (13.5%)</td>
<td>30 (57.7%)</td>
</tr>
<tr>
<td>Total MO</td>
<td>$\chi^2 = 1.9, \ p = 0.4$</td>
<td>$\chi^2 = 0.7, \ p = 0.4$</td>
</tr>
</tbody>
</table>

For the ADARB1 rs422720 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 0.89, \ p = 0.64$) or the allelic frequencies of cases and controls ($\chi^2 = 0.85, \ p = 0.35$). The CC genotype frequency was (15.7%) in the cases and (13.8%) in the controls and similarly the C allele was more frequent in cases (41.6%) compared to controls (37.8%). The observed minor allele frequency in the control group (C allele, 37.8%) was slightly higher to expected allele frequencies as determined in the Hap-Map CEU population (C allele, 34%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic, $\chi^2 = 1.89, \ p = 0.38$ Allelic $\chi^2 = 1.77, \ p = 0.18$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 0.16, \ p = 0.92$, Allelic $\chi^2 = 0.08, \ p = 0.78$).
Table 8.11  Genotyping results for the *ADARB2* SNPs genotyped by Sequenom.

<table>
<thead>
<tr>
<th>ADARB2 rs5015512</th>
<th>Genotypes</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>Controls</td>
<td>14 (13.9%)</td>
<td>50 (49.5%)</td>
</tr>
<tr>
<td>Male</td>
<td>4 (13.8%)</td>
<td>14 (48.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (13.9%)</td>
<td>36 (50.0%)</td>
</tr>
<tr>
<td>Cases</td>
<td>4 (13.8%)</td>
<td>20 (46.6%)</td>
</tr>
<tr>
<td>Male</td>
<td>1 (6.7%)</td>
<td>8 (53.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>3 (7.9%)</td>
<td>12 (31.6%)</td>
</tr>
</tbody>
</table>

For the *ADARB1* rs5015512 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 4.90, p = 0.08$) or the allelic frequencies of cases and controls ($\chi^2 = 4.58, p = 0.03$). The CC genotype frequency was (13.8%) in the cases and (13.9%) in the controls and similarly the C allele was less frequent in cases (37.2%) compared to controls (38.6%). The observed minor allele frequency in the control group (C allele, 38.6%) was almost twice as high as expected allele frequencies as determined in the Hap-Map CEU population (C allele, 19%). Analysis by gender showed a slight trend toward association in the genotype and allele frequencies for female migraineurs compared to controls however the number of gender-specific samples are small (Female Genotypic, $\chi^2 = 6.02, p = 0.04$, Allelic $\chi^2 = 5.15, p = 0.02$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 0.50, p = 0.77$, Allelic $\chi^2 = 0.18, p = 0.67$).

MA — migraine with aura, MO — migraine without aura.
p-values were calculated by $\chi^2$-analysis, significance was taken at $p \leq 0.05$. 

MA — migraine with aura, MO — migraine without aura.
p-values were calculated by $\chi^2$-analysis, significance was taken at $p \leq 0.05$. 

For the *ADARB1* rs5015512 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 4.90, p = 0.08$) or the allelic frequencies of cases and controls ($\chi^2 = 4.58, p = 0.03$). The CC genotype frequency was (13.8%) in the cases and (13.9%) in the controls and similarly the C allele was less frequent in cases (37.2%) compared to controls (38.6%). The observed minor allele frequency in the control group (C allele, 38.6%) was almost twice as high as expected allele frequencies as determined in the Hap-Map CEU population (C allele, 19%). Analysis by gender showed a slight trend toward association in the genotype and allele frequencies for female migraineurs compared to controls however the number of gender-specific samples are small (Female Genotypic, $\chi^2 = 6.02, p = 0.04$, Allelic $\chi^2 = 5.15, p = 0.02$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 0.50, p = 0.77$, Allelic $\chi^2 = 0.18, p = 0.67$).
<table>
<thead>
<tr>
<th>ADARB2 rs7070629</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>GA</td>
<td>GG</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>35 (12.5%)</td>
<td>124 (44.4%)</td>
<td>120 (43%)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (13.6%)</td>
<td>37 (45.7%)</td>
<td>33 (40.7%)</td>
</tr>
<tr>
<td>Cases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>35 (13.8%)</td>
<td>118 (46.6%)</td>
<td>100 (39.5%)</td>
</tr>
<tr>
<td>Female</td>
<td>9 (14.8%)</td>
<td>22 (36.0%)</td>
<td>30 (49.2%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>27 (13.5%)</td>
<td>99 (49.5%)</td>
<td>74 (37.0%)</td>
</tr>
<tr>
<td>MO</td>
<td>8 (18.6%)</td>
<td>19 (44.2%)</td>
<td>26 (60.5%)</td>
</tr>
</tbody>
</table>

For the ADARB2 rs7070629 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 0.70$, $p = 0.70$) or the allelic frequencies of cases and controls ($\chi^2 = 0.66$, $p = 0.41$). The AA genotype frequency was (13.8%) in the cases and (12.5%) in the controls and similarly the A allele was more frequent in cases (34.8%) compared to controls (37.2%). The observed minor allele frequency in the control group (A allele, 37.2%) was similar to expected allele frequencies as determined in the Hap-Map CEU population (A allele, 35.5%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic, $\chi^2 = 2.27$, $p = 0.32$, Allelic $\chi^2 = 1.67$, $p = 0.19$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 1.37$, $p = 0.50$, Allelic $\chi^2 = 0.40$, $p = 0.52$).
For the ADARB2 rs10903479 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 2.16, p = 0.33$) or the allelic frequencies of cases and controls ($\chi^2 = 2.01, p = 0.15$). The TT genotype frequency was (19.5%) in the cases and (15.3%) in the controls and similarly the T allele was more frequent in cases (44%) compared to controls (39.8%). The observed minor allele frequency in the control group (T allele, 39.8%) was similar to expected allele frequencies as determined in the Hap-Map CEU population (T allele, 37%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 1.23, p = 0.54$, Allelic $\chi^2 = 1.21, p = 0.27$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 1.31, p = 0.52$, Allelic $\chi^2 = 0.70, p = 0.40$).
For the ADARB2 rs7094094 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 0.36, p = 0.83$) or the allelic frequencies of cases and controls ($\chi^2 = 0.24, p = 0.62$). The GG genotype frequency was (10.4%) in the cases and (10%) in the controls and similarly the G allele was slightly more frequent in cases (37.2%) compared to controls (32.5%). The observed minor allele frequency in the control group (G allele, 37.2%) was higher to expected allele frequencies as determined in the Hap-Map CEU population (G allele, 32%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 1.88, p = 0.39$, Allelic $\chi^2 =0.04, p = 0.84$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 3.10, p = 0.21$, Allelic $\chi^2 =1.32, p = 0.25$).
<table>
<thead>
<tr>
<th>ADARB2 rs10903520</th>
<th>Genotypes</th>
<th>Alleles</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>GA</td>
<td>GG</td>
</tr>
<tr>
<td>Controls</td>
<td>42 (14.5%)</td>
<td>138 (47.6%)</td>
<td>110 (38.0%)</td>
</tr>
<tr>
<td>Male</td>
<td>13 (15.5%)</td>
<td>41 (48.8%)</td>
<td>30 (35.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>29 (14.1%)</td>
<td>97 (47.1%)</td>
<td>80 (38.8%)</td>
</tr>
<tr>
<td>Cases</td>
<td>32 (12.3%)</td>
<td>138 (53.1%)</td>
<td>110 (42.1%)</td>
</tr>
<tr>
<td>Male</td>
<td>5 (8.0%)</td>
<td>25 (40.0%)</td>
<td>33 (52.0%)</td>
</tr>
<tr>
<td>Female</td>
<td>27 (13.6%)</td>
<td>94 (47.5%)</td>
<td>77 (38.8%)</td>
</tr>
<tr>
<td>Total Migraine</td>
<td>$\chi^2 = 1.2$, $p = 0.5$</td>
<td>$\chi^2 = 0.6$, $p = 0.4$</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>24 (11.6%)</td>
<td>94 (45.4%)</td>
<td>89 (43.0%)</td>
</tr>
<tr>
<td>Total MA</td>
<td>$\chi^2 = 1.7$, $p = 0.4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>8 (14.8%)</td>
<td>25 (46.3%)</td>
<td>21 (38.9%)</td>
</tr>
<tr>
<td>Total MO</td>
<td>$\chi^2 = 0.0$, $p = 0.1$</td>
<td>$\chi^2 = 0.0$, $p = 0.9$</td>
<td></td>
</tr>
</tbody>
</table>

MA - migraine with aura, MO - migraine without aura.

p-values were calculated by $\chi^2$-analysis, significance was taken at $p \leq 0.05$.

For the ADARB2 rs10903520 polymorphism there was no statistically significant difference between cases and controls for either the genotype frequencies ($\chi^2 = 1.18$, $p = 0.55$) or the allelic frequencies of cases and controls ($\chi^2 = 0.59$, $p = 0.44$). The AA genotype frequency was (12.3%) in the cases and (14.5%) in the controls and similarly the A allele was less frequent in cases (36%) compared to controls (38.3%). The observed minor allele frequency in the control group (A allele, 38.3%) was higher to expected allele frequencies as determined in the Hap-Map CEU population (A allele, 31.6%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 0.02$, $p = 0.99$, Allelic $\chi^2 = 0.01$, $p = 0.94$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 4.67$, $p = 0.09$, Allelic $\chi^2 = 4.66$, $p = 0.03$).
For the ADARB2 rs884861 polymorphism there was a statistically significant difference between cases and controls for the allelic frequencies ($\chi^2 = 3.93, p = 0.04$). The CC genotype frequency was (40.1%) in the cases and (34%) in the controls and similarly the C allele was less frequent in cases (62%) compared to controls (56%). The observed minor allele frequency in the control group (C allele, 56%) was much higher to expected allele frequencies as determined in the Hap-Map CEU population (C allele, 38.9%). Analysis by gender showed no significant differences between genotype or allele frequencies for female migraineurs compared to controls (Female Genotypic $\chi^2 = 2.71, P = 0.25$, Allelic $\chi^2 = 2.63, P = 0.10$). Similarly for the male sub-population, there was no significant difference between migraineurs and controls for either genotypes or alleles (Male Genotypic $\chi^2 = 1.33, P = 0.51$, Allelic $\chi^2 = 1.35, P = 0.24$). Table 8.9 and 8.10 show a summary of Chi-square ($\chi^2$) analysis for all migraine groups for SNPs in the ADARB1 and ADARB2 genes respectively.
Table 8.12  Chi-square ($\chi^2$) analysis of all migraine groups for SNPs in the *ADARB1* gene.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency Comparison</th>
<th>Genotypic</th>
<th>Allelic</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2838771</td>
<td>Combined</td>
<td>$\chi^2 = 3.47$, p = 0.17</td>
<td>$\chi^2 = 0.94$, p = 0.33</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>$\chi^2 = 0.59$, p = 0.74</td>
<td>$\chi^2 = 0.56$, p = 0.45</td>
</tr>
<tr>
<td></td>
<td>MO</td>
<td>$\chi^2 = 0.40$, p = 0.81</td>
<td>$\chi^2 = 0.23$, p = 0.63</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>$\chi^2 = 1.98$, p = 0.37</td>
<td>$\chi^2 = 2.15$, p = 0.14</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>$\chi^2 = 6.48$, p = 0.03</td>
<td>$\chi^2 = 3.38$, p = 0.06</td>
</tr>
<tr>
<td>rs407133</td>
<td>Combined</td>
<td>$\chi^2 = 0.08$, p = 0.95</td>
<td>$\chi^2 = 0.85$, p = 0.35</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>$\chi^2 = 0.24$, p = 0.88</td>
<td>$\chi^2 = 0.01$, p = 0.92</td>
</tr>
<tr>
<td></td>
<td>MO</td>
<td>$\chi^2 = 0.40$, p = 0.81</td>
<td>$\chi^2 = 0.10$, p = 0.75</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>$\chi^2 = 2.78$, p = 0.24</td>
<td>$\chi^2 = 0.61$, p = 0.43</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>$\chi^2 = 0.46$, p = 0.79</td>
<td>$\chi^2 = 0.09$, p = 0.75</td>
</tr>
<tr>
<td>rs422720</td>
<td>Combined</td>
<td>$\chi^2 = 0.89$, p = 0.64</td>
<td>$\chi^2 = 0.85$, p = 0.35</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>$\chi^2 = 0.62$, p = 0.73</td>
<td>$\chi^2 = 0.01$, p = 0.92</td>
</tr>
<tr>
<td></td>
<td>MO</td>
<td>$\chi^2 = 0.40$, p = 0.81</td>
<td>$\chi^2 = 0.10$, p = 0.75</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>$\chi^2 = 0.16$, p = 0.92</td>
<td>$\chi^2 = 0.08$, p = 0.78</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>$\chi^2 = 1.89$, p = 0.38</td>
<td>$\chi^2 = 1.77$, p = 0.18</td>
</tr>
<tr>
<td>rs1051367</td>
<td>Combined</td>
<td>$\chi^2 = 1.26$, p = 0.53</td>
<td>$\chi^2 = 0.71$, p = 0.40</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>$\chi^2 = 1.20$, p = 0.54</td>
<td>$\chi^2 = 0.58$, p = 0.44</td>
</tr>
<tr>
<td></td>
<td>MO</td>
<td>$\chi^2 = 0.21$, p = 0.90</td>
<td>$\chi^2 = 0.11$, p = 0.74</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>$\chi^2 = 0.43$, p = 0.80</td>
<td>$\chi^2 = 0.35$, p = 0.55</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>$\chi^2 = 2.87$, p = 0.23</td>
<td>$\chi^2 = 2.01$, p = 0.15</td>
</tr>
</tbody>
</table>
Table 8.13  Chi-square ($\chi^2$) analysis of all migraine groups for SNPs in the ADARB2 gene.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency Comparison</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Genotypic</td>
<td>Allelic</td>
</tr>
<tr>
<td>rs2271275</td>
<td></td>
<td>$\chi^2 = 4.16$, p = 0.12</td>
<td>$\chi^2 = 0.09$, p = 0.76</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td>$\chi^2 = 0.86$, p = 0.65</td>
<td>$\chi^2 = 0.65$, p = 0.42</td>
</tr>
<tr>
<td>MA</td>
<td></td>
<td>$\chi^2 = 5.41$, p = 0.06</td>
<td>$\chi^2 = 0.00$, p = 0.99</td>
</tr>
<tr>
<td>MO</td>
<td></td>
<td>$\chi^2 = 0.19$, p = 0.90</td>
<td>$\chi^2 = 0.07$, p = 0.79</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td>$\chi^2 = 4.52$, p = 0.10</td>
<td>$\chi^2 = 0.14$, p = 0.71</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>$\chi^2 = 4.90$, p = 0.08</td>
<td>$\chi^2 = 4.58$, p = 0.03</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td>$\chi^2 = 5.75$, p = 0.05</td>
<td>$\chi^2 = 5.55$, p = 0.01</td>
</tr>
<tr>
<td>MO</td>
<td></td>
<td>$\chi^2 = 1.91$, p = 0.38</td>
<td>$\chi^2 = 0.01$, p = 0.92</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td>$\chi^2 = 0.50$, p = 0.77</td>
<td>$\chi^2 = 0.18$, p = 0.67</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>$\chi^2 = 6.02$, p = 0.04</td>
<td>$\chi^2 = 5.15$, p = 0.02</td>
</tr>
<tr>
<td>rs5015512</td>
<td></td>
<td>$\chi^2 = 3.63$, p = 0.16</td>
<td>$\chi^2 = 2.57$, p = 0.10</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td>$\chi^2 = 1.76$, p = 0.41</td>
<td>$\chi^2 = 1.22$, p = 0.26</td>
</tr>
<tr>
<td>MA</td>
<td></td>
<td>$\chi^2 = 1.36$, p = 0.50</td>
<td>$\chi^2 = 0.12$, p = 0.72</td>
</tr>
<tr>
<td>MO</td>
<td></td>
<td>$\chi^2 = 2.27$, p = 0.32</td>
<td>$\chi^2 = 1.67$, p = 0.19</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td>$\chi^2 = 1.37$, p = 0.50</td>
<td>$\chi^2 = 0.40$, p = 0.52</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>$\chi^2 = 2.16$, p = 0.33</td>
<td>$\chi^2 = 2.01$, p = 0.15</td>
</tr>
<tr>
<td>rs7070629</td>
<td></td>
<td>$\chi^2 = 0.68$, p = 0.71</td>
<td>$\chi^2 = 0.66$, p = 0.41</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td>$\chi^2 = 5.75$, p = 0.05</td>
<td>$\chi^2 = 3.54$, p = 0.05</td>
</tr>
<tr>
<td>MA</td>
<td></td>
<td>$\chi^2 = 0.66$, p = 0.96</td>
<td>$\chi^2 = 0.01$, p = 0.91</td>
</tr>
<tr>
<td>MO</td>
<td></td>
<td>$\chi^2 = 5.61$, p = 0.06</td>
<td>$\chi^2 = 3.80$, p = 0.05</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td>$\chi^2 = 1.31$, p = 0.52</td>
<td>$\chi^2 = 1.21$, p = 0.27</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>$\chi^2 = 1.23$, p = 0.54</td>
<td>$\chi^2 = 1.21$, p = 0.27</td>
</tr>
<tr>
<td>rs10903467</td>
<td></td>
<td>$\chi^2 = 2.10$, p = 0.34</td>
<td>$\chi^2 = 1.00$, p = 0.31</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td>$\chi^2 = 0.80$, p = 0.66</td>
<td>$\chi^2 = 0.39$, p = 0.53</td>
</tr>
<tr>
<td>MA</td>
<td></td>
<td>$\chi^2 = 3.76$, p = 0.15</td>
<td>$\chi^2 = 1.70$, p = 0.19</td>
</tr>
<tr>
<td>MO</td>
<td></td>
<td>$\chi^2 = 1.66$, p = 0.43</td>
<td>$\chi^2 = 0.72$, p = 0.39</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td>$\chi^2 = 1.17$, p = 0.55</td>
<td>$\chi^2 = 1.16$, p = 0.28</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>$\chi^2 = 1.18$, p = 0.55</td>
<td>$\chi^2 = 0.24$, p = 0.62</td>
</tr>
<tr>
<td>rs11250642</td>
<td></td>
<td>$\chi^2 = 1.65$, p = 0.43</td>
<td>$\chi^2 = 1.65$, p = 0.19</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td>$\chi^2 = 0.03$, p = 0.98</td>
<td>$\chi^2 = 0.00$, p = 0.95</td>
</tr>
<tr>
<td>MA</td>
<td></td>
<td>$\chi^2 = 4.67$, p = 0.09</td>
<td>$\chi^2 = 4.66$, p = 0.03</td>
</tr>
<tr>
<td>MO</td>
<td></td>
<td>$\chi^2 = 0.02$, p = 0.99</td>
<td>$\chi^2 = 0.01$, p = 0.94</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td>$\chi^2 = 0.36$, p = 0.83</td>
<td>$\chi^2 = 0.24$, p = 0.62</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>$\chi^2 = 0.15$, p = 0.92</td>
<td>$\chi^2 = 0.00$, p = 0.96</td>
</tr>
<tr>
<td>rs7094094</td>
<td></td>
<td>$\chi^2 = 5.93$, p = 0.05</td>
<td>$\chi^2 = 2.22$, p = 0.13</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td>$\chi^2 = 3.10$, p = 0.21</td>
<td>$\chi^2 = 1.32$, p = 0.25</td>
</tr>
<tr>
<td>MO</td>
<td></td>
<td>$\chi^2 = 1.88$, p = 0.39</td>
<td>$\chi^2 = 0.04$, p = 0.84</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td>$\chi^2 = 3.69$, p = 0.15</td>
<td>$\chi^2 = 3.93$, p = 0.04</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td>$\chi^2 = 3.03$, p = 0.22</td>
<td>$\chi^2 = 3.32$, p = 0.06</td>
</tr>
</tbody>
</table>
MA - migraine with aura, MO - migraine without aura.

\[ \chi^2 = 3.03, p = 0.22 \]
\[ \chi^2 = 1.49, p = 0.22 \]

\[ \chi^2 = 1.33, P = 0.51 \]
\[ \chi^2 = 1.35, P = 0.24 \]

\[ \chi^2 = 2.71, P = 0.25 \]
\[ \chi^2 = 2.63, P = 0.10 \]

MA - migraine with aura, MO - migraine without aura.

p-values were calculated by \( \chi^2 \)-analysis, significance was taken at \( p \leq 0.05 \).

Overall chi-square (\( \chi^2 \)) analysis found no significant association in the SNPs in the \( ADARB1 \) gene. In contrast, a nominal association was detected in two SNPs in the \( ADARB2 \) gene, marker rs5015512 located in LD block 10 was positive for combined migraine, allelic \( p = 0.03 \) and MA \( p = 0.01 \) and females \( p = 0.02 \). Marker rs884861 located in LD block 56 was also nominally positive for combined migraine at the allelic \( p = 0.04 \) level with significance based on p-value.

### 8.6 Discussion

Susceptibility to migraine is conferred by genetics and by exposure to intrinsic and environmental triggering factors, with the latter varying considerably between patients. Although numerous association studies have detected positively associated SNPs in various neurotransmitter-related genes including \( DBH \) (Fernandez, Colson et al. 2009) and \( SLC6A3 \) (Todt, Netzer et al. 2009) with migraine, as have other neurological, hormonal and vascular candidate genes, in certain migraine populations the associations have not been consistently replicated and this is probably related to factors of biological and genetic heterogeneity. The fact that migraine is co-morbid with a number of medical conditions including stroke, psychiatric disorders and epilepsy also complicates the identification of migraine unique genes in out-bred populations. To overcome some of these obstacles migraine has also been analysed in the genetic isolate of Norfolk Island, an ideal population for genetic studies due to well documented family histories and increased prevalence of disease.

Pinpointing causal SNPs associated with disease has been one of the goals in GWAS and one of many approaches used to dissect the genetic basis of migraine. A recent pGWAS conducted in the Norfolk Island pedigree has implicated 4 SNPs in the RNA editing gene \( ADARB2 \) based on statistical significance (Cox, Lea et al. 2012a). RNA editing genes \( ADARB1 \) and \( ADARB2 \) were chosen to investigate in this study because they fit criteria of migraine neuropathology i.e. genes that are known to a) be expressed in the brain or central
nervous system (CNS) b) regulate neurological pathways (e.g. neurotransmitters) c) be plausibly related to migraine neuropathology (e.g. cellular hyperexcitability, ion channel disruption). Retyping top-ranking SNPs from GWAS data in independent case-controls cohorts is important to determine the validity of GWAS findings and risk within different population groups.

To date only a few association studies have investigated SNPs in RNA editing genes \textit{ADARB1} and \textit{ADARB2} none have been done in migraine. A GWAS of US centenarians revealed that 18 single nucleotide polymorphisms SNPs in \textit{ADARB1} and \textit{ADARB2} are associated with extreme old age in three independent populations of different genetic backgrounds (Sebastiani, Montano et al. 2009). Amore et al., 2004 identified a common neutral polymorphism in three out of seven patients with BD in the \textit{ADARB1} gene but no major alteration (Amore, Strippoli et al. 2004). In a separate study, Kostyrko et al., 2006 screening of the coding sequence of \textit{ADARB1} and their association with bipolar affective disorder did not reveal any mutations except one already known transition (Kostyrko, Hauser et al. 2006). Oguro et al., 2012 identified a longevity-associated SNP in \textit{ADARB2} “rs2805533” which may modulate human longevity by regulating metabolic factors such as abdominal obesity and lipid profiles (Oguro, Kamide et al. 2012).

RNA editing is a physiologically important and conserved process necessary for proper development and functioning of neuronal cells. RNA editing of glutamate receptors is essential to survival (Higuchi, Maas et al. 2000). Mice deficient in the RNA-editing enzyme \textit{ADARB1} die at birth and show early onset epilepsy (Higuchi, Maas et al. 2000). This phenotype results from under editing of a critical position that determines calcium permeability in excitatory neurons. The codon for this arginine is created by site-selective adenosine deamination of an exonic glutamine Gln codon at the pre-mRNA level, by the extent of Q/R-site editing

\textit{ADARB1} plays an important role in ensuring neurotransmitter receptor transcripts are properly edited at respective points (Barbon and Barlati 2011). The particular editing function of \textit{ADARB1} upon the AMPA glutamate receptor subunit (\textit{GRIA2}) pre-mRNA makes the investigation of \textit{ADARB1} in migraine interesting, because glutamate is a major mediator in the central nervous system and its regulation has been studied as a possible mechanism
causing migraine. It is hypothesised that defective RNA-editing of glutamate receptors may lead to poor ionic conductance and consequently synaptic dysfunction. Defective A-to-I editing of mRNA encoding the GRIA2 subunit of glutamate AMPA receptors has been linked to the disease amyotrophic lateral sclerosis (ALS) (Rosenthal and Seeburg 2012).

Seven SNPs in ADARB1 and fourteen SNPs in the ADARB2 genes were genotyped using TaqMan and Sequenom genotyping platforms to investigate if they are associated with migraine susceptibility. The SNPs were chosen by inspecting blocks of LD in Haploview using HapMap genotype data and although the majority of SNPs were intronic, there was one SNP rs2271275 that changes the amino acid sequence of the ADARB2 protein (Thr-Ala) and was originally identified in the Norfolk Island pGWAS.

Although Sequenom is a powerful high-throughput multiplexing genotyping technique it has some limitations as we found in our study that a small percentage of SNPs did not yield balanced results and had to be excluded from the analysis. Of 21 markers genotyped in the ADARB1 and ADARB2 genes 4 in the ADARB1 and 9 in the ADARB2 gene were in HWE and were analysed by Chi-square ($\chi^2$) for association (see Table 8.10 and Table 8.11). No association was detected in any of the SNPs in the ADARB1 gene. Two SNPs in the ADARB2 gene, marker rs5015512 was positive for combined migraine, allelic p = 0.03 and MA p = 0.01 and females p = 0.02. The second marker rs884861 was nominally positive for combined migraine, allelic p = 0.04 with significance based on p-value.

This result is consistent with the original positive result obtained in the Norfolk Island population implicating 4 SNPs in a 22kb haplotype block in the ADARB2 gene. Both SNPs rs5015512 and rs884861 are located downstream, in LD block 10 and 56 respectively, from the original four SNPs forming a 22kb haplotype block in the ADARB2 gene and should be tested in an independent population with larger numbers to see if they can be replicated. The SNP identified in the Norfolk Island pGWAS in the ADARB2 gene rs2271275 which changes the amino acid sequence of the protein (Thr-Ala) was not found to be significant when genotyped in this out-bred population. Our study could be improved by using a larger population.
The next line of inquiry in future studies could be to investigate candidate genes of the adenosinergic system. The relationship between migraine and adenosine is suggested based on the vasodilator properties of adenosine and its capacity to affect cerebral and meningeal blood flow (Phillis 2004). In addition intravenous adenosine infusion is reported to cause intense headache (Sollevi 1986; Brown and Waterer 1995). The purine adenosine exists in all nervous system cells (neurones and glia) and plays an important role in energy production, in fine-tuning synaptic transmission, it can also influence potassium channels and affect the release and action of many neurotransmitters and is also an important modulator of cardiovascular physiology (Tabrizchi and Bedi 2001; Ribeiro and Sebastiao 2010). It may be worthwhile to investigate the enzymes that metabolize adenosine such as adenosine kinase (Adk) and, to a lesser extent, adenosine deaminase (ADA) and adenosine receptors particularly in view of the study by Hohoff which identified a six-marker haplotype in the adenosine A2 receptor that is more frequent in MA (p=0.01) patients than in the control group (Hohoff, Marziniak et al. 2007). Potential sequencing of the components of the adenosinergic system in migraine patients may reveal novel unidentified mutations in particular in the ADARB2 gene.

8.7 Conclusion

In this case-control study of migraine using an Australian Caucasian population matched for age (+/- 5yrs), gender and ethnicity, we found that the investigated SNPs in ADARB1 and ADARB2 are not associated with increased risk of migraine. Among the markers analysed, two SNPs in the ADARB2 gene, marker rs5015512 and marker rs884861 were nominally positive. These results suggest that RNA editing genes may not be primarily implicated in the pathogenesis of migraine in our population and that the ADARB2 gene should be further investigated. Given that the genotypic profile of migraineurs may influence the type of treatment, identification of mutated alleles specific to migraine will help to produce more specific pharmacotherapy for the patient. The heterogeneity of migraine and variable phenotypic presentation exalt the need for new DNA sequencing technologies that can thoroughly screen functional groups of genes in migraine patients. Especially when considering that migraine is caused by the interactions of multiple loci in various genes the identification of genetic polymorphisms that can forecast disease status should increase understanding of disease mechanisms and lead to improved strategies for prevention and treatment. Also because we now have the genetic knowledge and tools to enable high-
throughput approaches an evident example being the recent discovery via sequencing of the first functional variant TRESK a potassium channel involved in neuronal excitability. Future efforts should be directed at further gene characterization at the molecular level including functional characterization of implicated variants and gene expression studies in models and humans as well as studies in other populations.
CHAPTER 9  Exome Sequencing Variants in Migraine X-linked Families
9.1 Introduction

Migraine is a genetically based disease stemming within the central nervous system. Presentation can include symptoms of nausea, emesis, photophobia, phonophobia, and occasionally, visual sensory disturbances (IHS 2013). The headache is described as moderate to severe in intensity, has a pulsating quality and occurs unilaterally. Additional symptoms often present in the migraineur and these include nausea, vomiting, neurological disturbance and sensory hypersensitivity, photophobia and phonophobia. The majority (about 70%) of migraineurs have migraine without aura whilst the remaining 30% experience migraine with aura accompanied by neurological symptoms that manifest as fully reversible, visual, sensory and/or dysphasic speech disturbances in conjunction with their headache (Viana, Sprenger et al. 2013).

Migraine incurs a ponderous burden to society due to the inability of migraine sufferers to perform work related activities when affected and the requirement for bed rest. There is strong evidence in the literature that supports the idea that the headache is caused by activation of the trigeminovascular system which carries pain signals from the meninges and the blood vessels and the aura by spreading depression, a slow propagating wave of neuronal and glial depolarization that spreads across the cortex (Hadjikhani, Sanchez Del Rio et al. 2001; Goadsby, Charbit et al. 2009). Imaging studies with MRI also support the view that migraine is a disease of neurovascular origin (Hadjikhani, Sanchez Del Rio et al. 2001; Borsook and Hargreaves 2010; Sprenger and Borsook 2012).

Migraine is a phenotypically and genetically heterogeneous disorder with no single variant capable of explaining the entire underlying genetic component across different families and populations. Migraineurs are genetically vulnerable possessing a combination of aberrant genes that interact with environmental factors to increase their likelihood of developing migraine. Population based twin studies have confirmed a genetic influence ranging from 0.34 to 0.57 (Mulder, Van Baal et al. 2003; Svensson, Larsson et al. 2003). The genes thus far identified as causing Migraine in the monogenic FHM are molecular components of ion transport channels and involved in neuronal signalling. Three specific genes have been isolated and characterized for monogenic FHM in human families and outbred populations. The functional effects of the mutated alleles provide direct evidence for neuronal hyperexcitability as one cellular mechanism underlying headache or aura in FHM.
The inequality in the gender ratio 3:1 of female migraine sufferers observed in epidemiological data is a unique feature of this disorder that may be explained by hormones (Merikangas 2013). Indeed 7-8% of migraineurs are diagnosed into Pure Menstrual Migraine and genetic studies have demonstrated a reproducible association with polymorphisms in the estrogen receptor gene ESR1 in ethnically different populations (Russell 2010). Sex hormones are synthesized and metabolized in the brain and have potent effects on endogenous opioid, serotonergic, noradrenergic, β-adrenergic, dopaminergic, and GABAergic systems and mitochondrial energy production (Shuster, Faubion et al. 2011). However at present it is still unclear how ovarian hormones contribute to migraine, an alternative hypothesis is that that there may be hidden genes on the X chromosome contributing to the migraine phenotype due to the unusual majority of females suffering from migraine and the fact that women have two copies of the X chromosome while men only have one X and one Y chromosome. The uneven gender distribution of migraine and the high proportion of male probands with affected first-degree relatives have led to the investigation of genomic factors on the X-chromosome. Genetic studies investigating the X chromosome have identified three susceptibility loci at Xp22, Xq13 and Xq24-q28 as seen in Figure 9.1.

![Figure 9.1 X chromosome regions implicated in Migraine adapted from (Maher 2011).](image_url)
Wessman et al., 2002 identified nominal linkage for Xp in a genome scan study involving 50 Finnish MA families (Wessman, Kallela et al. 2002). Antilla’s study also used a Finnish population and the TCA, LCA and ICHD-II classification methods demonstrated linkage at the Xp22 locus (Anttila, Nyholt et al. 2008). Weiser et al, screened the entire X-chromosome and identified linkage on Xp22 in 61 families of European descent (Wieser, Pascual et al. 2010).

Oterino and colleagues studied a four-generation pedigree co-segregating MA and X-linked Charcot-Marie-Tooth disease and identified the C552T mutation in the connexion 32 gene at the Xq13 region (2001). Eleven markers from Xp12 – Xq28 were genotyped in 42 members of this pedigree and results demonstrated linkage of MA to the Xp13 region.

The X-chromosome has been the target of pedigree linkage studies which have identified linkage to the Xq24–28 region. Susceptibility loci in this region Xq24–28 were initially identified in two Australian migraine pedigrees by Nyholt et al. (Nyholt, Dawkins et al. 1998; Nyholt, Curtain et al. 2000). Further studies by Maher et al., examined six new migraine pedigrees and identified two families that independently showed evidence of excess allele sharing to opposite regions (MF879 at Xq27 - LOD 1.38 p=0.005; MF47 at Xq28 - LOD 1.5 p = 0.004) (Maher, Kerr et al. 2012). This same study also identified microsatellite markers from both regions which may affect migraine in the general population, particularly in the female cohort (Xq27 – DXS8043 P = 0.009, Xq28 – DXS8061 P = 0.07) (Maher, Kerr et al. 2012). The Xq27 region that has now been identified in three different cohorts, these include three migraine families, the Norfolk Island population and the migraine association population.

The X chromosome was also investigated in the Norfolk Island population using a new pedigree-based association approach. The population forms a large core-pedigree composed of 288 individuals, 76 were affected with migraine and were analysed. This population has characteristics that facilitate the mapping of susceptibility loci due to a high prevalence of migraine and its well characterised pedigree structure in which genetic relationships can be traced back to the island's original founders. Another advantage of using this population in
Candidate genes residing in the Xq24-Xq28 region have been investigated for association with migraine including 5-hydroxytryptamine (serotonin) receptor 2C (5HT2C), Glutamate Receptor ionotropic AMPA3 (GRIA3), gamma-aminobutyric acid A receptor epsilon (GABRE), gamma-aminobutyric acid receptor theta (GABRQ) and gamma-aminobutyric acid A receptor 3 (GABRA3). A common theme that links all these genes together is that they have a neurological role and belong to the neurotransmitter class of genes supporting current concepts of a neurological dysfunction in migraine. There are however additional genes in the Xq27-Xq28 region that have not been investigated and could potentially be involved and therefore considered in future studies.

In this study exome sequencing of the X chromosome was undertaken in 3 individuals from 3 different families (MF47, MF14, MF6 see Appendix for pedigree structure) and identified 12 mutations in 8 genes as listed in Table 9.1 and Table 9.2. The mutations identified range from single nucleotide polymorphisms (SNP) to deletions and insertions of a few nucleotides (Table 9.2). The results of the exome sequencing project were filtered for variants that do not pass GATK sequencing QC, variants that are synonymous and variants with reported MAF \( >0.1 \) in 1000 genomes. Remaining genes were filtered for those that are common in all three affected individuals and that contain identified mutations which are predicted to be deleterious. The top candidate genes identified are listed in Table 9.1. The variants are all located on the q arm of the X chromosome with the exception of FAM104B, which is located at Xp11.21. The aim of this study was to determine if the exome sequencing variants detected were real by undertaking Sanger sequencing of the 3 individuals from the 3 pedigrees MF14 (MU67), MF47 (MU480), MF6 (MU1088).
### Table 9.1  Genes identified in Exome sequencing project NCBI Build 37.2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Location</th>
<th>Start (bp)</th>
<th>Stop (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM104B</td>
<td>Family with sequence similarity 104, member B</td>
<td>Xp11.21</td>
<td>55169535</td>
<td>55187628</td>
</tr>
<tr>
<td>IL13RA2</td>
<td>Interleukin 13 receptor, alpha 2</td>
<td>Xq13.1-q28</td>
<td>114238538</td>
<td>114252207</td>
</tr>
<tr>
<td>HDX</td>
<td>Highly divergent homeobox</td>
<td>Xq21.1</td>
<td>83572882</td>
<td>83757487</td>
</tr>
<tr>
<td>RBMX</td>
<td>RNA binding motif protein, X-linked</td>
<td>Xq26.3</td>
<td>135951353</td>
<td>135962939</td>
</tr>
<tr>
<td>GPR112</td>
<td>G protein-coupled receptor 112</td>
<td>Xq26.3</td>
<td>135383122</td>
<td>135499047</td>
</tr>
<tr>
<td>SPANXN1</td>
<td>SPANX family, member N1</td>
<td>Xq27</td>
<td>144329107</td>
<td>144337728</td>
</tr>
<tr>
<td>TMEM185A</td>
<td>Transmembrane protein 185A</td>
<td>Xq28</td>
<td>148678216</td>
<td>148713487</td>
</tr>
<tr>
<td>MAML1D1</td>
<td>Mastermind-like domain containing 1</td>
<td>Xq28</td>
<td>149531551</td>
<td>149682448</td>
</tr>
</tbody>
</table>
Table 9.2 Mutations identified in Exome sequencing project NCBI Build 37.2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Ref sequence</th>
<th>Type of Mutation</th>
<th>Migraine Family in which mutation was identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDX</td>
<td>NM_001177478:c.249_250insCC:p.T83fs</td>
<td>Frameshift insertion CC</td>
<td>MF14, MF47, MF6</td>
</tr>
<tr>
<td>RBMX(1)</td>
<td>NM_001164803:c.2_3insA:p.M1fs</td>
<td>Frameshift insertion A</td>
<td>MF14, MF47</td>
</tr>
<tr>
<td>RBMX(2)</td>
<td>NM_002139:c.316_317insTT:p.P106fs</td>
<td>Frameshift insertion TT</td>
<td>MF47</td>
</tr>
<tr>
<td>GPR112(1)</td>
<td>NM_153834:c.4972_4973insC:p.T1658fs</td>
<td>Frameshift insertion C</td>
<td>MF14</td>
</tr>
<tr>
<td>GPR112(2)</td>
<td>NM_153834:c.7966_7968del:p.2656_2656del</td>
<td>Nonframeshift deletion, CTG</td>
<td>MF47, MF14</td>
</tr>
<tr>
<td>GPR112(3)</td>
<td>NM_153834:c.C9045A:p.S3015R</td>
<td>Nonsynonymous SNV, A</td>
<td>MF14</td>
</tr>
<tr>
<td>FAM104B</td>
<td>NM_001166700:c.C331T:p.R111X</td>
<td>Stopgain SNV, T rs1047054</td>
<td>MF14, MF47, MF6</td>
</tr>
<tr>
<td>MAMLD1</td>
<td>NM_001177466:c.C1000T:p.P334S</td>
<td>Nonsynonymous SNV, T rs41313406</td>
<td>MF47, MF14</td>
</tr>
<tr>
<td>SPANXN4</td>
<td>NM_001009613:c.T33A:p.N11K</td>
<td>no rs number missense mutation</td>
<td>MF14, MF47, MF6</td>
</tr>
<tr>
<td>IL13RA2</td>
<td>NC_000023.10</td>
<td>T&gt;C change in 5’UTR</td>
<td>MF14, MF47, MF6</td>
</tr>
<tr>
<td>TMEM185A</td>
<td>NM_032508.2</td>
<td>SNP, rs200479899, T372G c.T148G aa W50G missense mutation within predicted transmembrane region</td>
<td>MF14, MF47, MF6</td>
</tr>
</tbody>
</table>
9.2 Sample Collection and Phenotyping

This study examined 3 Australian Caucasian pedigrees, showing linkage to the X chromosome including MF6, MF47 and MF14. Pedigree structures are shown in Figures 9.3 to 9.5. Migraine Families were collected by the Clinic at the Genomics Research Centre, Griffith University, Gold Coast. This involved provision of blood or saliva for DNA and completion of a detailed questionnaire regarding the patient’s health and history for migraine diagnosis. Ethical clearance was granted by the Griffith University Human Research Ethics Committee prior to initiation of this project and all participants provided signed informed consent. Migraineurs were diagnosed as having either migraine with aura or migraine without aura in accordance with ICHD-II diagnostic criteria using questionnaires and interviews with clinical neurologists. Information was also obtained regarding age of onset, frequency, triggers, medication and associated symptoms (IHS 2004). Study participants were recruited from the east coast of Australia and were Caucasians of European descent with emigrating ancestors within the British Isles and other parts of Europe.

9.3 Materials and Methods

Blood samples were collected in EDTA tubes and DNA from the Migraine Families was extracted from white blood cells using a standard salting out method as described by Miller et al., 1988 (Miller S.A, Dykes D.D et al. 1988). DNA was then quantified to a concentration of 20ng/µL for genotyping experiments. The concentration and purity of the DNA yields were determined spectrophotometrically using the NanoDrop ND-1000 (NanoDrop Technologies, Inc.). Forward and reverse primers were designed by downloading the sequence surrounding the polymorphism and entering this into NCBI Primer-3 and NCBI primer-blast (available at: www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Primers were designed for twelve variants in eight genes and were obtained from IDT (Integrated DNA Technologies) in sequencing quality at a concentration of 100µM in liquid form. Primer sequences were checked for specificity and complementary sequences in primer pairs or within a single primer. Aliquots for each set of primers were made at a concentration of 5µM in H₂O for use in PCR optimization experiments. Initial test PCRs were run on Applied Biosystems Veriti® Thermal Cyclers. PCR buffers, MgCl₂, Go-Taq
polymerase (Promega Corp., Madison, WI, USA), dNTPs, restriction enzymes, and enzyme buffers were from (New England Biolabs, Ipswich, MA, USA), SYTO9 dye was from (Invitrogen, Melbourne, Australia). Detailed information regarding primer sequences and amplicon size for each of the 12 mutations tested is listed in Table 9.3.

### 9.3.1 Sanger Sequencing of 12 mutations

The PCR thermocycling conditions were standardized for all 12 mutations as follows: 95°C for 5mins, then 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for 45 seconds for 30 cycles, followed by a final extension step of 72°C for 5 minutes. The PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide for 30mins at 90V, and then visualised under ultraviolet light to verify product amplification which can be seen in Figure 9.2. Successfully amplified PCR products were then Sanger sequenced following GRC established protocol to confirm the exome sequencing results.

### Table 9.3 Primer Sequences used for Sequencing.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Product size</th>
</tr>
</thead>
</table>
| HDX        | F: 5’ AAGCCAGGAGTCTTCTTTGGA 3’  
               R: 5’ TTCCAGCATTTAGCACACTTG 3’ | 284bp        |
| RBMX(1)    | F: 5’ GCTTCCTACTGGGCTGGAGG 3’  
               R: 5’ CCATATAGCCACCACCAATG 3’ | 235bp        |
| RBMX(2)    | F: 5’ CCCATAACTGCAATTGGAAAGG 3’  
               R: 5’ CAAACCATCCTGACTTG 3’ | 347bp        |
| RBMX(3)    | F: 5’ CTTTTCTTGATTTCTTGAGTATAAT 3’  
               R: 5’ TCCACTGCTGACTGGAACGT 3’ | 192bp        |
| GPR112(1)  | F: 5’ CTATGGCAACGTCCACTCTCT 3’  
               R: 5’ CTGGGGGTAAACTGAAGC 3’ | 286bp        |
| GPR112(2)  | F: 5’ TGCAAGGAGGATGGGTTTTA 3’  
               R: 5’ GGCTGATTGGCTCTCAGTTC 3’ | 264bp        |
| GPR112(3)  | F: 5’ TTTTTCTGCTATCTTCATGTTAGG 3’  
               R: 5’ TCAGCCAAGAATGAGTCGAG 3’ | 333bp        |
| FAM104B    | F: 5’ CCCAGAGAGAGCAAGTGAGGAC 3’  
               R: 5’ CCAATTTAATAAACTGCCATT 3’ | 312bp        |
| MAMLD1     | F: 5’ AAGCAAGGGTCTGCTACAAA 3’  
               R: 5’ GGAGAGTGCTACCCCGAAAG 3’ | 202bp        |
| SPANXN4    | F: 5’ GCTGTGGAATCTGCACCCTA 3’  
               R: 5’ ACCCTACCTTCCCTTTCA 3’ | 173bp        |
| IL13RA2    | F: 5’ TCTCAGTCTCTAGACGTCTGCT 3’  
               R: 5’ GGATTCTAGTCTCACTTG 3’ | 150bp        |
| TMEM185A   | F: 5’ TGCTGACAAAATGTGGCTTCTT 3’  
               R: 5’ TGCTCCCTCATGAGAC 3’ | 313bp        |
9.4 Results

9.4.1 Sanger Sequencing Results of 12 mutations

In this study we investigated the possible involvement of 12 mutations in 8 genes reported in an exome sequencing project of the X chromosome. Each of the variants were firstly sequenced in each of the three individuals from the three migraine pedigrees (MF14, MF47, MF6) to ascertain the existence of the mutation reported in the exome sequencing project. The PCR amplicons amplified for each of the 9 variants were visualized by agarose gel electrophoresis under UV light. An example of the agarose gel is shown in Figure 9.2 below, the amplicon size ranges from smallest 150bp to largest 347bp as seen in Table 9.3.

![Figure 9.2 PCR amplicon for each of the 9 variants.](image)

PCR amplicon products were run on a 2% agarose gel at 90 V for 30min with 100bp ladder in the first well and negative control in every fifth well.

The samples were then Sanger sequenced and Table 9.3 shows the results obtained and a comparison of exome sequencing results with Sanger Sequencing. Sanger sequencing identified 6 out of 12 variants as matching the exome sequencing results these were: RBMX(1) - Insert A, MAMLD1 - C, FAM104B - Insert C, SPANXN4 - T, IL13RA2 - A, TMEM185A - A. The sequence Chromatograms for each of the 12 mutations sequenced are shown in Table 9.4 using the software program Chromas. In the sequence Chromatograms the sequence and location of the mutations is shown. The HDX - Insert CC, GPR112(1) - Insert C and GPR112(2) - Del CTG and the RBMX(2) - Insert TT are mutations that were not observed in the sequence chromatograms in any of the 3 individuals whose exome was
sequenced. The sequence Chromatogram of GPR112(3) and RBMX(1) mutations is not
displayed as it was ambiguous to interpret. These two mutations could not be sequenced
accurately due to the difficult nature of the sequence which contains repetitive sequences that
interfere with sequence reactions.
Table 9.4 Comparison of Exome sequencing results with Sanger Sequencing.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expected result</th>
<th>MF14 (MU67)</th>
<th>MF47 (MU480)</th>
<th>MF6 (MU1088)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDX</td>
<td>Insert CC</td>
<td>No insert CC</td>
<td>No insert CC</td>
<td>No insert CC</td>
</tr>
<tr>
<td>GPR112(1)</td>
<td>Insert C</td>
<td>No insert C</td>
<td>No insert C</td>
<td>No insert C</td>
</tr>
<tr>
<td>GPR112(2)</td>
<td>Del CTG</td>
<td>Ambiguous</td>
<td>No del CTG</td>
<td>No del CTG</td>
</tr>
<tr>
<td>GPR112(3)</td>
<td>C</td>
<td>Ambiguous</td>
<td>Ambiguous</td>
<td>Ambiguous</td>
</tr>
<tr>
<td>RBMX(1)</td>
<td>Insert A</td>
<td>Ambiguous</td>
<td>Ambiguous</td>
<td>Ambiguous</td>
</tr>
<tr>
<td>RBMX(2)</td>
<td>Insert TT</td>
<td>No insert TT</td>
<td>No insert TT</td>
<td>No insert TT</td>
</tr>
<tr>
<td>RBMX(3)</td>
<td>G</td>
<td>GG</td>
<td>GG</td>
<td>GG</td>
</tr>
<tr>
<td>FAM104B</td>
<td>Insert C</td>
<td>CT</td>
<td>CT</td>
<td>CT</td>
</tr>
<tr>
<td>MAML1D1</td>
<td>C</td>
<td>CT</td>
<td>CT</td>
<td>CC</td>
</tr>
<tr>
<td>SPANXN4</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>IL13RA2</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>TMEM185A</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
</tr>
</tbody>
</table>
Table 9.5  Sequence Chromatograms of 12 Mutations.

<table>
<thead>
<tr>
<th>Mutation and Expected Result</th>
<th>MF14 (MU67)</th>
<th>MF47 (MU480)</th>
<th>MF6 (MU1088)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDX - Insert CC</td>
<td><img src="image1" alt="Chromatogram" /></td>
<td><img src="image2" alt="Chromatogram" /></td>
<td><img src="image3" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Not found</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPR112(1) - Insert C</td>
<td><img src="image4" alt="Chromatogram" /></td>
<td><img src="image5" alt="Chromatogram" /></td>
<td><img src="image6" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Not found</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPR112(2) - Del CTG</td>
<td><img src="image7" alt="Chromatogram" /></td>
<td><img src="image8" alt="Chromatogram" /></td>
<td><img src="image9" alt="Chromatogram" /></td>
</tr>
<tr>
<td>Not found</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>RBMX(2) - Insert TT</strong></td>
<td>Not found</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RBMX(3) – G</strong></td>
<td>Found</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FAM104B - Insert C</strong></td>
<td>Found</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Found</td>
<td>Sequence</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>----------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>MAML1 - C</td>
<td>Found</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPANXN4 - T</td>
<td>Found</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL13RA2 – A</td>
<td>Found</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The sequence chromatograms show a short stretch of 20bp of sequence for each of the 3 individuals sequenced and the location of the expected mutation is highlighted with a black circle. The GPR112(3) – C mutation and RBMX(1) - Insert A mutations were sequenced however the result is not included in this table as it was ambiguous and needs to be repeated.
9.5 Discussion

Although there is evidence of involvement of the X chromosome through studies that have identified susceptibility loci in three distinct regions at Xp22, Xq13 and Xq24-q28 no specific causal gene has yet been identified. The Xq24-q28 region is more strongly implicated than the other two regions in a number of pedigrees. The X chromosome has been studied in relation to migraine because it was thought to harbour migraine susceptibility genes and to account for the increased preponderance of affected females versus males.

In this study, we have sought to verify the results of an exome sequencing project of the X chromosome. The exome sequencing project identified 12 mutations in 8 genes on the X chromosome in three individuals showing X-linked inheritance using a refinement strategy. The variants are all located on the q arm of the X chromosome with the exception of FAM104B, which is located at Xp11.21. Little is known about the group of genes refined on the X chromosome locus the only thing they share in common is that they are all located to the X chromosome. The genes code for proteins that do not have any obvious role in migraine aetiology.

The sequence Chromatograms for each of the 12 mutations sequenced verified 6 out of 12 mutations as matching the exome sequencing results. Although Exome sequencing is an emerging technology on the genomic horizon that is being used more and more frequently due to savings on cost and time it has some limitations. In our project the sequencing results performed by the Sanger method did not confirm all exome sequencing results. This was an early new technology and clearly was not as accurate as we had hoped or as now would be available. Most variants were not validated but 6 were including RBMX(3), MAMLD1, FAM104B, SPANXN4, IL13RA2 and TMEM185A. The Genomics Research Centre will in the future investigate the 6 validated novel X variants and will undertake NGS and exome sequencing in more individuals in these families and other families using the now more accepted and accurate technologies which may reveal if the X-chromosome is responsible for genetic predisposition to migraine.
9.6 Conclusion

The X chromosome has shown some interesting results however examination of the role genomic factors may play in migraine are yet to be understood. Although three susceptibility loci at Xp22, Xq13 and Xq24-q28 have been identified and a number of candidate genes investigated at the Xq24-q28 region, apart from GRIA3 at Xq24, nothing has to date been found that identifies the causative factor. Not all candidate genes have been investigated in these regions therefore further analysis of migraine pedigrees for linkage to these loci may shed some light on the contribution of the X chromosome to migraine predisposition. This study has confirmed the existence of 6 novel variants including RBMX(3), MAMLD1, FAM104B, SPANXN4, IL13RA2 and TMEM185A and occurring in all 3 migraine sequenced individuals but not in normal controls. There is a possibility that these variants may play a significant role in migraine and future studies in extended migraine pedigrees are warranted in order to define whether these variants are involved in migraine. Although this is beyond the scope of the present thesis it is certainly something of future potential.
Note: The red circle in the pedigree structure is highlighting the individual whose X chromosome was exome sequenced.
Figure 9.4 MF14
Figure 9.5  MF47
10.1 Thesis Summary

Migraine continues to be a great source of morbidity in the developed world with a prevalence of 12% in the Caucasian population (Lipton, Bigal et al. 2007). Migraine is a complex polygenic disorder that occurs much more commonly in females than in males and that is most likely a product of multiple genes interacting with environmental triggers. Causes of migraine are not very clearly understood and therefore research into the aetiology of migraine is necessary to advance treatments. Monogenic migraine syndromes such as FHM and overlapping disorders have helped to guide research and identify a few key genes. The degree of migraine predisposition varies among individuals and variants are likely to involve gene-environment and gene-gene interactions.

The aim of this research was to identify migraine susceptibility genes using a candidate gene study of an Australian Caucasian population of cases and controls. Candidate genes were selected based on previous studies supporting their involvement for example disruption of glutamate-mediated neurotransmission and RNA-editing genes which fit criteria for migraine neuropathology. At present the majority of genes identified regulate neurological, vascular, hormonal and mitochondrial functions and have been identified by taking into account prior knowledge of the pathophysiology of the disorder. The emerging picture of migraine pathogenesis is that it is a heterogeneous and complex disorder at both the population and molecular levels.

Given that genetic variations are involved in the patient's response to treatment, identification of mutated alleles specific to migraine will help to produce more specific pharmacotherapy for the patient. There is a constant need to find more effective treatments which are safe and effective for the patient. There is no doubt that as further experiments are performed additional genes perhaps unsuspected to play a role in migraine will be uncovered. Importantly as technology progresses and new ways of accessing neurons through the use of induced pluripotent stem cells and cellular surrogates of migraine, such as neuronal excitability, synaptic strength, CGRP release or response to PGE2 directly from patients with migraine are identified exciting prospects to understand the molecular pathogenesis of migraine become a reality.
10.1.1 GRIA2 and GRIA4

This thesis has investigated susceptibility genes in neurotransmitter related genes for association with migraine. The broad aim of this research was to identify genetic factors that may contribute to migraine susceptibility. The candidate genes GRIA2 and GRIA4 were chosen to investigate based on a previous study reporting a positive association with 2 SNPs in GRIA1 (rs548294 MO allelic P=0.008, rs2195450 MA allelic P=0.0005) and 1 SNP in the GRIA3 promoter (rs3761555 MA Females allelic p = 0.003) in an Italian population (Formicola, Aloia et al. 2010). There are four GRIA subunits that assemble to form ionotropic AMPA receptors that upon binding to glutamate the primary excitatory neurotransmitter in the central nervous system (CNS) mediate fast excitatory neurotransmission. AMPA receptors have been targeted in a variety of neurological diseases including schizophrenia (Carter 2006; Magri, Gardella et al. 2008; Chaki and Hikichi 2011). Ion channel genes are particularly favourable targets in the study of migraine as they may promote Cortical Spreading Depression (CSD) and contribute to neuronal excitability. Three polymorphisms in the GRIA2 and four polymorphisms in the GRIA4 genes were genotyped in an Australian case-control cohort by HRM and RFLP genotyping methods. Chi-square ($\chi^2$) analysis revealed a negative association between migraine and the variants tested.

10.1.2 GLUD1 and GLUD2

The GLUD1 gene codes for an important mitochondrial enzyme expressed in all tissues, glutamate dehydrogenase 1 that catalyzes reversible oxidative deamination of L-glutamate to 2-oxoglutarate. Although there is no direct evidence to implicate the GLUD1 gene in migraine aetiology activating mutations in the GLUD1 gene cause the hyperinsulinism–hyperammonaemia (HI/HA) syndrome and it is found at the interface of glutamate-glutamine cycling and is therefore an interesting candidate gene to pursue. The study of genes involved in glutamate-mediated neurotransmission is an important part of the ongoing research into migraine and its treatment. In this association study 1 SNP was genotyped by HRM, the MAF of this SNP was low but was demonstrated to be similar in the control group (G allele, 28.3%) to expected allele frequencies as determined in the Hap-Map CEU population (G allele, 28%). Five additional SNPs were genotyped in a migraine multiplex using Sequenom MassArray technology. One SNP rs493326 was excluded because it did not amplify. Three of the five SNPs...
genotyped in the *GLUD1* and *GLUD2* genes were analysed and were not significant after chi-square (χ²) analysis for migraine, migraine subtype and gender. The results obtained from this study although negative have built on the results of pre-existing studies. The lack of available data on the rest of genes involved in glutamate homeostasis means that this family of genes could by an alternative class of genes to investigate for involvement in migraine aetiology. Enzymes that cycle glutamate-glutamine between neurons and astrocytes are key targets to further investigate.

10.1.3 **ADARB1 and ADARB2**

The *ADARB2* gene was identified as a candidate gene to investigate based on results of a pedigree based GWAS of the isolated population of Norfolk Island (Cox, Lea et al. 2012a). The Norfolk Island pGWAS identified several novel migraine genes, 3 of which were related through their effects on neurotransmitter pathways regulating serotonin and glutamate and provided the initial basis for pursuing this gene further. Norfolk Island is a unique population to study from geneticists point of view which may increase the chance of identifying susceptibility genes due to its isolated nature and uniform lifestyle factors but more importantly because the heritability of migraine in this pedigree is (H² = 0.53) and the prevalence of migraine 25.5% is double that observed in outbred populations (Bellis, Cox et al. 2008). *ADARB2* was also identified in a genome-wide linkage scan in American families with early-onset obsessive-compulsive disorder (Hanna, Veenstra-Vanderweele et al. 2007). Polymorphisms in a second RNA-editing enzyme *ADARB1* were also genotyped for association with migraine. This gene performs an RNA editing reaction which alters RNA sequences encoded by DNA. Glutamate and serotonin receptor gene RNAs are two RNA-editing substrates modified by *ADARB1* adenosine deamination and are of interest to this study (Maas, Rich et al. 2003).

In this study a total of 21 markers in the RNA editing genes *ADARB1* and *ADARB2* were genotyped including 7 in the *ADARB1* gene and 14 in the *ADARB2* gene using a TaqMan and Sequenom platform approach. The program Haploview using HapMap genotype data was used to locate SNPs in different blocks of LD in the *ADARB1* and *ADARB2* genes. Thirteen of the 21 markers were in HWE and were analysed for association with migraine. Analysis included gender and migraine subtype with and without aura, overall SNPs in the *ADARB1* gene were not associated however two SNPs
in the *ADARB2* gene, marker rs5015512 was positive for combined migraine, allelic, $p = 0.03$ and MA, $p = 0.01$ and females, $p = 0.02$ and the second marker rs884861 was nominally positive for combined migraine, allelic, $p = 0.04$ with significance based on p-value. This study suggests the *ADARB2* is a candidate gene that should be further investigated. Typically the next line of inquiry would be to replicate the association in a larger cohort and to pursue other variants in RNA-editing pathways which in this case the adenosinergic system and its receptors could be worthwhile to investigate.

### 10.1.4 Exome sequencing of the X chromosome

There is evidence from studies done in pedigrees implicating three susceptibility loci at Xp22, Xq13 and Xq24-q28 on the X chromosome. Exome sequencing of three individuals from three different migraine pedigrees revealed 12 mutations in 8 genes on the X chromosome as listed in Table 9.2. Sanger sequencing of each of the 12 mutations was performed to validate the exome sequencing results. Sanger sequencing validated 6 out of 12 mutations as matching the results of the exome sequencing project. These 6 new X-linked mutations included RBMX(3), MAMLD1, FAM104B, SPANXN4, IL13RA2 and TMEM185A. This is a very interesting result that will be further investigated by the Genomics Research Centre which will be undertaking NGS exome sequencing in more individuals and in the three families in which the mutations were first identified. The X chromosome remains an interesting target to investigate for migraine susceptibility genes.
10.2 Thesis Conclusions

This research has investigated genetic variation in the candidate genes \textit{GRIA2} and \textit{GRIA4} and \textit{GLUD1} and \textit{GLUD2} which belong to the glutamatergic system due to evidence surrounding glutamate involvement in CSD and in the aetiology of migraine including genetic, biochemical and clinical in the form of modulators of glutamatergic signalling as potential treatments of migraine. Association in the variants genotyped was not detected for migraine, migraine subtype and gender however this result does not rule out the possibility of the involvement of variants in other components of the glutamatergic system in causing or predisposing to migraine. Secondly the RNA-editing genes \textit{ADARB1} and \textit{ADARB2} were investigated based on their neurological role and support for the \textit{ADARB2} gene from the pGWAS study in the genetic isolate on Norfolk Island. Out of 21 markers genotyped in both genes two markers rs5015512 and rs884861 in the \textit{ADARB2} gene were marginally associated with migraine a finding that warrants replication in a larger population. This is a very interesting result that should be further investigated. Finally 12 mutations in 8 genes on the X chromosome were Sanger sequenced to validate results of an exome sequencing project of the X chromosome, 6 out of 12 novel variants were implicated. It is possible that these variants may play a role in migraine and therefore the 6 mutations will be further investigated in more migraine families and using NGS and exome sequencing, the next major approach that could be used to identify migraine genes.
10.3 Future Directions

The most promising gene and therapeutic target yet identified in migraine research has been MTHFR, a gene that has been associated with MA in a number of different populations and supported by meta-analysis (Rubino, Ferrero et al. 2009). MTHFR is an enzyme of the folate-methionine metabolism pathway, which functions to regulate DNA synthesis and DNA methylation, and thus is involved in epigenetic regulation of gene expression (Sohn, Jang et al. 2009). In addition to contributing to the condition Hcy, MTHFR TT carriers have also been reported to have a decrease in global DNA methylation levels (Friso, Choi et al. 2002). Therefore DNA hypomethylation may also play a role in migraine aetiology and this is a direction that is being explored as well as the investigation of other genes involved in the folate metabolism pathway, i.e MTRR, MTR and enzymes that break down methionine such as MAT1A, GNMT, AHCP may also be potential candidate genes.

Nutrigenomic manipulation of the well-studied C667T polymorphism in MTHFR with simple administration of B vitamins has resulted in lower homocysteine levels and reduced migraine pain severity and disability (Lea, Colson et al. 2009; Menon, Lea et al. 2012). This is a clear example of the successful exploitation of pharmacogenetic relationships. Further work around the homocysteine metabolic pathway which involves the conversion of Hcy to methionine is necessary as there has been little investigation into how the treatment might actually be working and the mechanism by which the MTHFR C667T polymorphism and other variants in genes essential for folate metabolism modulate risk in various diseases has not been fully elucidated. The GRC in future studies will explore the potential for genotype/methylation/environmental factor interaction with the goal to model enviro-genomic risk profiles.

Another line of inquiry as previously mentioned in Chapter 8 could be to investigate candidate genes of the Adenosinergic system. The relationship between migraine and adenosine is suggested based on the vasodilator properties of adenosine and its capacity to affect cerebral and meningeal blood flow (Phillis 2004). In addition intravenous adenosine infusion is reported to cause intense headache (Sollevi 1986; Brown and Waterer 1995). The purine adenosine exists in all nervous system cells (neurones and glia) and plays an important role in energy production, in fine-tuning synaptic transmission, it can also influence potassium channels and affect the release and action of many neurotransmitters (Tabrizchi and Bedi 2001; Ribeiro and Sebastiao 2010).
The molecule adenosine is rapidly metabolized by adenosine kinase and, to a lesser extent, adenosine deaminase (ADA). The enzyme adenosine kinase (AdK) takes phosphate from Adenosine triphosphate (ATP) and adds it to adenosine to form Adenosine monophosphate (AMP) (Tabrizchi and Bedi 2001). In addition to regulating the cellular concentration of Ado, AdK plays a second role in the maintenance of methylation reactions. S-adenosylhomocysteine (SAH) is the product of S-adenosylmethionine-dependent transmethylation reactions in cells. Adenosylhomocysteinase is an enzyme responsible for cleavage of SAH into Ado and homocysteine. A buildup of SAH occurs due to poor activity of AdK. Missense mutations causing a deficiency in AdK have been shown to cause hypermethioninemia, encephalopathy and abnormal liver function (Bjursell, Blom et al. 2011). Adenosine deaminase is an enzyme that catalyzes the hydrolysis of adenosine to inosine. ADA is widely distributed in many mammalian cells and tissues particularly in the heart, vascular smooth muscle and endothelial cells (Schrader and West 1990). The biological and gene constituents of the adenosinergic System are displayed in Table 10.1.

Table 10.1  The biological and gene constituents of the Adenosinergic System.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>Chromosome (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine Kinase</td>
<td>ADK</td>
<td>10q22</td>
</tr>
<tr>
<td>Adenosine Deaminase</td>
<td>ADA</td>
<td>20q13.12</td>
</tr>
<tr>
<td>Adenosylhomocysteinase</td>
<td>AHCY</td>
<td>20q11.22</td>
</tr>
<tr>
<td>Receptor Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1, adenosine A1 receptor</td>
<td>ADORA1</td>
<td>1q32.1</td>
</tr>
<tr>
<td>A2A, adenosine A2a receptor</td>
<td>ADORA2A</td>
<td>22q11.23</td>
</tr>
<tr>
<td>A2B, adenosine A2b receptor</td>
<td>ADORA2B</td>
<td>17p12</td>
</tr>
<tr>
<td>A3, adenosine A3 receptor</td>
<td>ADORA3</td>
<td>1p13.2</td>
</tr>
</tbody>
</table>

Adenosine controls signaling pathways involved in regulating many body functions, especially in cardiovascular physiology by binding to four subtypes of receptors A1, A2A, A2B, and A3 (Burnstock, Fredholm et al. 2011). Adenosine receptors are coupled to G proteins and exist abundantly in nerve endings of cerebral and meningeal vessels and on platelet membranes (Guieu, Devaux et al. 1998; Burnstock, Fredholm et al. 2011). Adenosine mediates vasodilation by acting through A2A, A2B receptors. The Adenosine A1 receptor protein is localized in human trigeminal ganglia (Schindler, Harris et al. 2001) and have been shown to influence pain transmission and adenosine
A1 receptor agonists could be used to inhibit the trigeminal nerve (Sawynok 1998; Goadsby, Hoskin et al. 2002; Ribeiro and Sebastiao 2010).

A study by Hohoff identified a six-marker haplotype in the adenosine A2 receptor that is more frequent in MA (p=0.01) patients than in the control group (Hohoff, Marziniak et al. 2007). The adenosine A2 receptor is the molecular target of caffeine (Diener, Pfaffenrath et al. 2005), sometimes used in migraine treatment and is expressed in basal ganglia, vasculature, T lymphocytes and platelets. The encoded protein facilitates effects of calcitonin gene-related peptide and vasoactive intestinal peptide, two important neuropeptides in migraine pathophysiology (Hohoff, Marziniak et al. 2007). The six-marker haplotype could be investigated in future studies on the pharmacogenomics of caffeine and migraine.

In support of the adenosinergic pathway pharmacological studies investigating Adenosine A1 receptor agonists, GR79236 (Gurden, Coates et al. 1993) and GR190178 (Sheehan, Wilson et al. 2000) have demonstrated inhibition of the peripheral release of CGRP in the cranial circulation as well as at the central trigeminal synapse, thereby preventing activation of central trigeminal neurons. As there are no data on the role of adenosine and its receptors in migraine patients this could be an area to focus on in future studies leading to the potential use of adenosine antagonists in the treatment of migraine.

In the future, another research avenue to specifically focus upon is the glutamate pathway to systematically assess whether genetic variants influencing glutamate levels are associated with migraine. There is biochemical evidence to implicate glutamate in migraine, reviewed in Chapter 4. The molecular components of the glutamatergic system involved in glutamate transport, metabolism and homeostasis have not been thoroughly investigated at a genetic level. Perturbation of serotonin and/or glutamate homeostasis, and cerebral vasodilatation, are all observed during migraine attacks. In particular as there is little research on this topic and there is evidence linking excess glutamate with CSD development in animal models, coming up with a panel of genes regulating glutamate homeostasis should be easy with the advent of NGS.
The progression of the Norfolk Island Health Study through ongoing recruitment of pedigree members will be paramount to characterising functional variants and their effects on gene expression and to aid in clarifying the genetic basis of complex disease in this unique population to further support current migraine research. In addition the establishment of subgroups such as the menstrual migraine population will be invaluable to the analysis of genetic factors responsible for the variable expression of migraine subtypes. Implementation of strategies to minimise phenotypic and genetic heterogeneity (e.g. stratifying participants by comorbid disorders or migraine trait components) will also be essential to achieving consistency in future genetic studies.

10.4 Future Project Applications

The field of migraine genetic research is progressing thanks to the development of next generation sequencing (NGS) techniques and the field of bioinformatics. Already causal variants for about 3,000 different Mendelian disorders have been discovered and archived in the Online Mendelian Inheritance in Man (OMIM, http://www.ncbi.nlm.nih.gov/omim) (Ku, Naidoo et al. 2011). These techniques will soon enough help uncover the genetic basis of more complex heterogenous disorders such as migraine and it is therefore an exciting time to be working in this field and to think of what the future holds. Gene discovery approaches have also changed from focussing on whole-genomes to more targeted and exome sequencing approaches to better meet the demands of modern day projects. This section will discuss strategies and techniques to progress the future of molecular genetic research in migraine.

10.4.1 Phenotypic Heterogeneity of Migraine

Migraine is a multifactorial disease and has a significant genetic basis, with heritability estimates of 40-65% (Gervil, Ulrich et al. 1999b). Studies have shown the risk of migraine to be significantly higher in first degree relatives of migraineurs compared to relatives of controls. Migraine families are continuously being investigated to determine the mode of genetic transmission and most studies have concluded in favour of a multifactorial inheritance pattern. Migraine is a complex disorder involving multiple chromosomal loci and substantial genetic heterogeneity, factors which complicate the identification of the crucial genomic risk factors. Although the exact cause of migraine is not known, genetic studies have demonstrated that migraine is a
Genetic variation in families and populations is what explains differences in allele frequencies and severity of disease and is vital to answering a myriad of genetic research questions. Genetic heterogeneity is the term used to describe genetic disorders whereby disease aetiology is different in different individuals due to the possession of different genetic profiles. In migraine heterogeneity is evident at both the locus (different genes) and allelic level (different mutation in the same gene) with susceptibility variants identified in neurological, vascular, hormonal and more recently mitochondrial gene families (Strauch, Fimmers et al. 2003). Similar to many other complex diseases, migraine aetiology remains obscure but evidence from many genetic studies suggests that polymorphisms in different families of susceptibility genes act synergistically to increase the risk of migraine occurrence. To date, geneticists have interrogated the genome using a variety of methods including case-control association studies, GWAS and linkage studies in different population cohorts including out-bred populations, family pedigrees and genetic isolates. These techniques have yielded both consistent and conflicting results but are a necessary part of progressing the understanding of the genetic basis of migraine.

A clearly defined phenotype is the essential starting point along with formal diagnostic criteria that is consistently applied. Reliable classification criteria are particularly important in the study of complex genetic disorders involving various subtypes in order to achieve consistency and for results to be replicated in different populations. Problems encountered with replicating results of genetic studies are due to the heterogeneity of the cohorts utilized which can sometimes be composed of individuals with differing genetic profiles and can affect the overall results and interpretation therein. Classification of migraine samples into MA and MO as two separate
phenotypes has been used in this research. The occurrence of both subtypes (MA and MO) in the same family and in the same individual as well as symptomatic overlap has confused geneticists trying to identify a common mode of inheritance. It is unclear whether MA and MO are etiologically distinct entities and remains to be confirmed. Some studies report migraine sub-types as distinct disorders (Russell and Olesen 1995). While other findings suggest that different aetiology may exist for MO and MA, with MO caused by both genetic and environmental factors and MA determined largely by genetic factors (Ligthart, Boomsma et al. 2006).

The most common issues hampering replication in genetic association studies include phenotype definition, overestimation of the magnitude of initial association, testing of multiple hypotheses, publication bias, population-specific differences in underlying linkage disequilibrium, and gene-gene and gene-environment interactions (Knight 2009). Another factor to be considered is the power of the study; quite often the sample sets used in genetic studies are too small to detect risk loci of small effect. This is a well-recognised limitation that can only be overcome with the use of well-defined case material and continued collaborations utilising large datasets that we will move forward and overcome some major challenges such as clinical heterogeneity, compounded by multiple treatments and co-morbidities. Even within a phenotype there is variation related to aetiology or other factors, and minimizing such heterogeneity through consistent cohort recruitment is important to obtain significant results. Furthermore, limited sample size and consequent drop in statistical power in some subgroups is another important issue that precludes comprehensive analysis of genetic factors responsible for the variable expression of clinical end-points.

Not everyone carries the same combination of genes and that is why some people get migraine more severely than others and is also the reason medications are not effective in all individuals. In an attempt to better understand the underlying etiological processes of different migraine subtypes alternative phenotyping methods such as LCA latent-class analysis, and TCA trait component analysis have been applied to identify migraine symptoms to chromosomal regions within the entire migraine ‘end diagnosis’ phenotype (Ligthart, Boomsma et al. 2006). Such strategies try to identify genetic variants that may be influencing the risk of specific characteristics such as headache severity and duration, age of onset, triggers and certainly responses to specific types of
medication and symptoms rather than the disease as a whole and try to identify the relationship of such genotypic variation on phenol-subtypes.

Meta-analysis is a powerful technique that draws together the results of many individual studies to help reach a consensus about the involvement of a particular gene in a disease. This technique involves combining results from many different studies and obtaining a weighted average that relates back to the sample sizes within the individual studies (Greco, Zangrillo et al. 2013). Meta-analysis can help identify patterns among study results unseen in individual studies and bring to light interesting relationships in the context of multiple studies to identify the overall risk contribution (Greco, Zangrillo et al. 2013).

10.4.2 Next Generation Sequencing (NGS)

Sequencing is the most straightforward and sure approach to directly identify the disease causing variants in people affected by a disease. Traditionally the approach taken when investigating the genetic basis of disease is to sequence the suspected gene and to search for genetic variation that can explain the clinical phenotype. The Sanger method has been the most prevalent method for DNA sequencing and the gold standard during the past 30 years. More recently next generation sequencing technologies have taken the lead catapulting genomic medicine to the forefront in just the last five years from studying the sequence of one or two genes in a month to studying the sequence of tens of thousands of genes in a single afternoon. Next Generation Sequencing (NGS) refers to post-Sanger sequencing methods which involve massively parallel sequencing and other high throughput methods. Compared with Sanger sequencing, the powerful throughput and flexible environment of NGS methods have rendered this technology more economical to the budgets of westernized nations and reduced the time necessary to provide comprehensive genetic diagnosis and to help inform patient treatment. Decoding the structure of genomes is becoming a tangible prospect that will allow the linkages between genomics and biology to be revealed along with the function of the myriad encoded proteins. The ultimate aim is to develop more individualized treatments targeted to the specific genetic causes underlying the individual forms of disease.
Currently there are three NGS platforms on the market, first was the 454 sequencer (now owned by Roche), but this sequencer was low throughput compared to the Solexa (now Illumina owned) and SOLiD/5500 sequencers (Life Technologies) (Hui 2012). A comparison of the three NGS platforms available is shown in Table 10.2. These instruments are able to generate three to four orders of magnitude more sequence in less time and are considerably more economical than the traditional Sanger method (Harismendy, Ng et al. 2009). To put this into perspective scientists can get the sequence of at least 10 bacterial genomes in a week on an Illumina instrument. The potential capacity for sequencing is evident in the 100's of Gigabases of sequence data generated in a single run. Accuracy in NGS is achieved by sequencing a given region multiple times, enabled by the massively parallel process, with each sequence contributing to “coverage” depth.
Table 10.2  A summary and a comparison between three NGS platforms in current use adapted from (Hui 2012).

<table>
<thead>
<tr>
<th>Companies</th>
<th>Roche GS FLX</th>
<th>Illumina-Solexa</th>
<th>SOLiD/5500 sequencers/Life Technologies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platforms</td>
<td>GS FLX Titanium, GS 20</td>
<td>HiSeq 2000, Genome Analyzer II (GA II), Solexa platform</td>
<td>ABI SOLiD, SOLiD 4</td>
</tr>
<tr>
<td>Chemistry</td>
<td>Pyrosequencing (sequencing-by-synthesis with pyrophosphate)</td>
<td>Reversible dye terminators</td>
<td>Sequencing by ligation</td>
</tr>
<tr>
<td>Sample</td>
<td>1 µg for shotgun library, 5µg for paired end</td>
<td>&lt;1 µg for single or paired-end</td>
<td>&lt;2 µg for shotgun library, 5-20µg for paired end 2-4</td>
</tr>
<tr>
<td>Sequencing run time</td>
<td>10-24 hours</td>
<td>1 to 10 days (based on the sequencer)</td>
<td>6 days</td>
</tr>
<tr>
<td>Method of feature generation</td>
<td>Bead-based/emulsion PCR</td>
<td>Isothermal bridge PCR amplification on flow cell surface</td>
<td>Bead-based/emulsion PCR</td>
</tr>
</tbody>
</table>
The latest NGS sequencer is the Ion Torrent (Life Technologies, Inc), a personal genomic machine (PGM) available as a benchtop instrument in research and clinical laboratories. The Ion Torrent works by measuring a change in pH which is correlated with the release of a hydrogen ion during each nucleotide incorporation by DNA polymerase. By measuring the release of hydrogen ions (1 per base added per DNA strand) the DNA sequence is determined. In the developmental pipeline is Oxford’s nanopore technology which uses the scanning tunneling electron microscope (TEM) to measure variations of conductivity across a nanopore while a single DNA molecule is passing through (Hui 2012).

Next generation sequencing (NGS) is going to revolutionize how genetic research projects are approached and how the genome is investigated from whole-genome to whole-exome and targeted sequencing. Although whole-genome sequencing (WGS) is the best way to have all the information of one genome including the noncoding bases and structural and haplotype phasing information it is very time consuming due to the copious amounts of data produced. As a result researchers have turned to targeted sequencing to cater for specific clinical needs and as a means to focus in on specific genomic regions of interest or selected genes related to a specific disorder. Essentially targeted sequencing refers to sequencing a selected region of interest and is being applied in a clinical setting in the form of a targeted panel of disease genes followed by NGS. One important application of targeted sequencing is in the area of oncology for cancer diagnosis, prognosis, and precision therapy using a targeted gene mutation panel analysis (Hui 2012). This approach is cost-effective for some laboratories and can suffice for knowing about the disease causing variant rather than sequencing the whole genome.

From a business perspective target enrichment can be a highly effective way of saving on sequencing time and costs, and has the power to bring the field of genomics into smaller laboratories, as well as being an invaluable tool for the detection of disease-causing variants of large effect. Next generation sequencing (NGS) are also being used to confirm and extend linkage and GWAS findings harbouring causative variants which can be enriched and sequenced to identify additional rarer variants within the associated region via resequencing. Although many reference sequences exist in the bioinformatics databases, as the population grows and develops and new mutations
accumulate more and more genomes from humans to viruses and microbes will need to be sequenced time and time again to fully reflect the current spectrum of genetic diversity. This is of consequence in genetic studies that use human populations with diverse ancestry as different populations have different polymorphisms and may not respond equally well to medications.

The most common application of NGS is variant discovery by resequencing targeted regions of interest as well as whole genomes to provide new reference sequences in public databases, resequencing is in itself an ideal method for discovering genetic variation, validating sequences and performing diagnostics (Nowrousian 2010). As sequencing becomes more economical deciphering genes and genomes is becoming an attainable goal. Next Generation Sequencing (NGS) have diverse applications ranging from documenting human ancestry to the characterization of the genetics of different communities such as de novo assemblies of viral, bacterial and lower eukaryotic genomes, cataloguing the transcriptomes of cells, tissues and organisms (RNA-seq), genome-wide profiling of epigenetic marks and chromatic structure using other seq-based methods (ChiP-seq, methyl-seq and Dnase-seq), and species classification and or gene discovery by metagenomics studies (Metzker 2010). Additional examples demonstrating the utility of NGS in current clinical practice include the detection of mutations in genes for the diagnosis of cardiomyopathy, X-linked congenital diseases, comprehensive mutation detection in genes known to cause congenital disorders of glycosylation, and various other autosomal disorders (Hui 2012).

In addition to genetic variants increasing disease risk, it has been hypothesized that epigenetic variation, including DNA methylation at certain gene loci may also lead to increased disease susceptibility. Epigenetics is concerned with heritable and reversible changes such as DNA methylation, histone modification and miRNA regulation that in turn affect gene expression and protein translation without changing the actual DNA sequence itself (Jaenisch and Bird 2003). Epigenetic modifications can influence the activity of genes and this phenomenon has been well recognized in cancer cells which exhibit aberrant epigenetic signatures that contribute to the disease (De Carvalho, Sharma et al. 2012; You and Jones 2012). Epigenetic mechanisms have been noted in depression and epilepsy disorders that are co-morbid with migraine. In depression changes in epigenetic programming of stress related genes, for example BDNF
(Tsankova, Berton et al. 2006; Wilkinson, Xiao et al. 2009) have been noted and in epilepsy also, increased DNA methylation was found at the promoter of Reelin in the brain of temporal lobe epilepsy (Kobow, Jeske et al. 2009). DNA methylation occurs specifically at cytosine residues present in a symmetric CpG dinucleotide and has been associated with many genes including that encoding the estrogen receptors (Eising, Datson et al. 2013).

There is currently great interest in whether epigenetic modifications which control gene expression, such as DNA methylation, may play a role in causing or predisposing an individual to a disease, in a similar way as DNA mutations (Hesson, Hitchins et al. 2010). This is a promising avenue in migraine research that future projects conducted by the Genomics Research Centre are investigating if an epigenetic link with migraine exists and if so which gene loci are susceptible to epigenetic modification and could be implicated in the aetiology of migraine. Emerging technologies for high-throughput assaying of genome-wide methylation profiles of human DNA samples such as Next generation sequencing and DNA methylation arrays are being applied to analyze human exomes and epigenetic marks in the context of migraine research. These technologies may facilitate discovery of unknown/unidentified epigenetic marks that predispose to migraine and when combined with other existing genomic data, including microsatellite (STR), single nucleotide polymorphism (SNP) and copy number variant (CNV) information, as well as environmental factor information may help explain a greater component of the trait variance and enable identification of comprehensive ‘envirogenomic’ profiles for predicting migraine risk.

10.4.3 Exome sequencing

Exome sequencing (also known as targeted exome capture) is an emerging technology that enriches for only the exonic regions of the genome which are the protein-coding regions expected to be most relevant in disease. Although exons constitute just 1% of the human genome, these are the most important regions where 85% of the disease-causing mutations are located with large effects on disease-related traits (Majewski, Schwartzentruber et al. 2011). For this reason exome sequencing has become the main tool to investigate Mendelian disorders. In this approach 180,000 exons from more than 20,000 genes are interrogated in the exome therefore minimizing the overall variants to be filtered (Ng, Turner et al. 2009). Exome sequencing therefore represents an efficient
strategy for identifying variants with large effect sizes in a highly enriched subset of the genome. The identification of causative mutations and the responsible genes in Mendelian diseases will impact molecular diagnosis and carrier testing of the patient and his or her family which will be important for family counseling as well as contributing to understanding the pathophysiology of rare diseases (Singleton 2011).

Exome sequencing is a cost-effective strategy for variant identification as it eliminates the time-consuming and costly data analysis required in whole genome sequencing. The advantage of exome sequencing is that by concentrating on only the coding regions this method can be effectively applied to diagnose monogenic diseases such as Cystic Fibrosis (Gilissen, Hoischen et al. 2012). The disadvantage of exome sequencing is that disease causing mechanisms in regulatory regions may not be identified. The reason why researchers are turning to exome sequencing approaches is that it is more focussed and saves time and money by reducing the data to analyse.

Exome sequencing is performed using in-solution whole exome capture kits currently supplied by three vendors (Agilent, Illumina and Nimblegen). The kits supplied target the exome portion of the genome which consists of all the exons that are transcribed into mature RNA. The exonic segments targeted by the different commercial platforms are derived from combinations of different databases of mRNA coding sequences including RefSeq, UCSC KnownGenes and Ensembl (Clark, Chen et al. 2011). The platforms differ in performance and design characteristics such as targeted capture areas or how the exome (fraction of the genome targeted) is defined, number of genes, exons captured and capture probe sequence composition (Coonrod, Durtschi et al. 2013).

Agilent’s Sure-Select capture kit targets 50 Mb of the genome with biotinylated RNA probes incorporating both exons and non-coding RNAs. The recently released version 3.0 of Roche NimbleGen’s SeqCap EZ Human Exome Library in solution capture kit targets 64 Mb of the genome but with DNA probes (Clark, Chen et al. 2011). Illumina offers two solution-capture systems, the stand-alone TruSeq™ Exome Enrichment Kit, which captures 62 Mb of genomic sequence using more than 340,000 95-mer probes, and the Nextera® Exome Enrichment Kit (Clark, Chen et al. 2011). Illumina kits cat detect a greater total number of variants as they also capture untranslated regions, which
are not targeted by the Nimblegen and Agilent platforms. The three platforms are capable of capturing greater than 96% of RefSeq genes (Bras and Singleton 2011).

A powerful application of exome sequencing is to directly compare the exome of affected and unaffected individuals to directly identify disease causing variants in homogenous pedigrees or with Mendelian disorders with recessive, dominant, and de novo inheritance patterns (Robinson, Krawitz et al. 2011). Chromosomal regions harbouring susceptibility genes may be known and therefore sequencing only the exons from the genes in these regions can help to reduce the variations to be filtered and data to analyse. Exome sequencing can thus be advantageous in this case and may identify rare variants that are only present in the affected individuals composing the pedigree.

Exome sequencing has identified over 100 genes in several Mendelian disorders and is predicted to coexist with other NGS-based strategies, namely the targeted NGS and WGS in molecular diagnostics for some time to come (Gilissen, Hoischen et al. 2011; Rabbani, Mahdieh et al. 2012). Exome sequencing is a practical approach because it is focussed on only the most obvious disease-influencing variants which are the functional variations which can help explain disease in different populations and give an answer as to why certain drugs only work in a subset of the population. Future projects are going to be dramatically reliant on this technology and likely to be followed by functional work of identified proteins and pathways to elucidate disease mechanisms. Importantly future studies should aim to collect both DNA and RNA samples for large-scale association studies and should analyse both the genome and transcriptome together to derive more concrete knowledge of the interplay between possession of a gene and the expression of that information. Integration of RNA-Seq data or expression data (the “Systems Biology” approach) with GWAS results and genotyping studies and pathway analysis is a more robust and favourable strategy that can more comprehensively inform the underlying processes of a disease.

10.4.4 Bioinformatics

The development of bioinformatics tools and software solutions has been spurred by the need to breakdown the vast quantity of genomic information generated by NGS projects and has resulted in new insights into the complex interactions genes have with other genes, protein-protein interactions, metabolic pathways and regulatory networks
involved in health and disease. Bioinformatics is an established scientific field that scientists can specialize in by undertaking specific courses and that is rapidly expanding and that much genetic research of today and tomorrow will be dependent upon. Bioinformatics combines disciplines of computer science, statistics, mathematics and engineering to process biological data and derive greater understanding of the complex dynamics observed in nature (Romano, Giugno et al. 2011).

Bioinformatics not only entails the development of software tools for organizing and analyzing genetic data but also the creation and management of databases, algorithms, computational and statistical techniques, for solving problems and answering biological questions. The bioinformatics community aims to develop robust and flexible software solutions and programs accessible through the internet through collaborative research efforts and database architectures that can be coupled to web applications offering ongoing curation functions, intuitive upload interfaces, search, visualization and download tools, and direct integration into analysis functions (Luscombe, Greenbaum et al. 2001). In a collaborative research setting bioinformatics services aim to establish suitable models and technologies to properly support interfaces that facilitate human-machine interactions. In an interdisciplinary field like bioinformatics there must be several entry points and one of them is using the language that is familiar to computer scientists to reach a broader audience and enable "reproducible workflows" which can be understood, reproduced and experimentally validated (Luscombe, Greenbaum et al. 2001).

Unravelling the complexities of a biological system requires the integration of multi-layer information from high-throughput ‘omics’ technologies that consider the expression and abundance of biological molecules of a whole biological system. The complexities of cellular functions can be traced to the genome, transcriptome, proteome and metabolome dynamic ‘omics’ entities, whose presence, abundance and function of each transcript, protein and metabolite must be carefully analysed.

The establishment of internet has catapulted the development of the bioinformatics field making it more accessible to both the general public and the scientific community. Bioinformatics has provided researchers with the tools to manage the complexity of biological data thanks to improvements in computing power (CPU), computers are the
tools of the trade and have become irreplaceable to genetics research. Computation, as understood here, is not just about the ‘hardware’, but is fundamentally concerned with the algorithms that allow more sensitive discovery of new relationships, faster and more accurate retrieval of those already known, better and more scalable use of resources in parallel, and new ways of visualising large data sets. Commercial bioinformatics software solutions are able to perform computations for analysing gene pathways and making predictions regarding pathogenicity of genetic variants. The Galaxy framework provides both infrastructure and reusable workflows while the R/Shiny framework provides powerful foundations for reactive exploration of data.

In the field of genetics and genomics, bioinformatics plays an important role in extracting useful information from large amounts of raw data. Bioinformatics is helping to narrow the gap between the availability of new sequence data and its use in protein identification and having a scientific understanding of that information. With sequences for over 40 organisms completed and more constantly being sequenced, bioinformatics will be responsible for the modelling of genetic and metabolic networks. Another aspect of bioinformatics in sequence analysis is annotation. Annotation is the process of identifying the locations of functional sequences within a genome and making sense of the genomic information. Obtaining the sequence information of a genome is just the first step in solving the puzzle the next step is to annotate it to make sense of it. Bioinformatics projects ultimately aim to create databases for sequence variant annotation (e.g., locus specific database, HGMD/ Biobase, OMIM, SeattleSeq, and 1000 Genome program) (Hui 2012). Functional annotation consists of understanding how annotated regions interact with each other and attaching biological information to genomic elements. For this, functional prediction programs such as PolyPhen and SIFT have a role to play in the interpretation of new or uncommon sequence variants (Hui 2012). Bioinformatics is helping to convert the terabytes of data generated from simple genotypes into meaningful clinical outcomes by looking for new links between our genes and disease.
10.5 Conclusion

From mapping the first genetic marker in Huntington’s disease in the early 80s, genetic investigation of inherited disease has blossomed into a marvellous genomics era that promises to unveil the mystery of the human genome. The revolution in molecular biology has been possible because of the development of a series of powerful laboratory techniques and the availability of biobanks of DNA samples from large numbers of affected people. Continued advances in automation and development of vast genomic database resources have brought the molecular genetics field to the forefront of medical science. Recognition of the potential for SNPs to have interacting effects with endogenous and exogenous environmental triggers and investigation of the mechanisms behind this association is an important next step in advancing research. The future availability of next generation sequencing (NGS) technology, which allows high throughput sequencing of either desired regions of the genome, all exons of the genome (the so-called exome), or the entire genome, in conjunction with novel high-throughput genotyping techniques is driving the future of migraine research forward and will reveal the extent to which migraine is a genetically determined disease. Together NGS techniques and bioinformatics approaches will provide researchers with a rich research roadmap and a practical set of tools for the investigation of disease which will carry us well into the 21st Century. In conclusion, a true multidisciplinary approach involving academic institutions, hospitals, government agencies and industries is necessary to harvest this knowledge and translate it to health interventions in medical practice that will help migraine patients.
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