Migraine Molecular Genetic and Pharmacogenetic studies

By

Saraswathy Menon BSc (Hons)

School of Medical Science, Griffith Health
Griffith University

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy (PhD).

August 2011
ABSTRACT

Migraine is a painful neurological disease that affects at least 12% of the Australian population. It is generally characterized by recurrent head pain usually accompanied by nausea, vomiting, neurological disturbance, photo-and phonophobia. Migraine has been classified by the international headache society (IHS) into two most common types, migraine with aura (MA) and migraine without aura (MO). The underlying pathophysiology of this debilitating disease is still partially understood and there are no known diagnostic markers for these common types of migraine. Current diagnosis of migraine is based on patient reported symptoms. Studies have shown several health conditions such as epilepsy, depression and stroke to be co-morbid with migraine. Current migraine treatments work with varying efficacy and often have adverse side effects. A greater understanding of this debilitating and painful disease is thus pertinent for developing new and improved migraine treatment.

Both familial clustering and twin studies have shown evidence for significant genetic mechanisms to underlie migraine pathogenesis. Migraine is thus currently defined as a complex multifactorial disorder which involves an interaction between genetic and environmental factors. We have not yet identified all migraine genes but a number of genes, causative mutations and susceptibility variants have been identified and are already of significant clinical relevance. Currently the detection of 3 rare subtypes of migraine (Familial Hemiplegic Migraine 1, 2 and 3) and several related conditions with symptom overlap (Episodic Ataxia 2, Spinocerebellar Ataxia Type 6 and Cerebral Autosomal Dominant Arteriopathy with Sub cortical Infarcts and leucoencephalopathy (CADASIL) is undertaken by sequencing, with susceptibility variants for common types of migraine detected by sequencing or genotyping.
This study was directed towards identifying genes that play a role in migraine susceptibility, migraine endophenotypes and migraine treatment response. Specifically this research involved an investigation of a number of potential susceptibility genes, as well as investigating whether symptoms of migraine correlate with specific genotypes and whether genes involved in a specific pathway correlate with migraine treatment response. A significant focus of this thesis related to the methylenetetrahydrofolate reductase (MTHFR) gene and related pathway. The specific aims of this research were thus to investigate the potential association of migraine clinical variables with MTHFR genotype, to study the role of MTHFR and MTRR genotypes in relation to migraine treatment response and finally to explore the role of a number of potential candidate genes in migraine.

The MTHFR variant C677T has been implicated as a genetic risk factor in migraine susceptibility, particularly in MA. MA and MO have many diagnostic characteristics in common. It is postulated that migraine symptomatic characteristics might themselves be influenced by MTHFR. The C677T variant is associated with enzyme function whereby, carriers of the homozygous mutant allele T exhibit 50% in enzyme activity. MTHFR is involved in the remethylation of homocysteine, a highly reactive amino acid that has been previously shown to cause endothelial cell injury in both animal and cell culture studies when present in elevated levels. Apart from reducing the enzyme activity of MTHFR, the C677T polymorphism also leads to increased levels of circulating homocysteine. Homocysteine related endothelial dysfunction might be involved in the initiation and maintenance of a migraine attack.

This study first investigated the role that the MTHFRC677T variant may have on migraine symptomatic characteristics. Two hundred and sixty seven adult Caucasian
migraineurs previously collected at the Genomics Research Centre (GRC) and diagnosed as having either MA or MO by the IHS criteria, were genotyped for the C677T variant. Chi square test and regression analyses were performed to assess the association of the C677T variant with all migraine clinical variables after adjusting for gender. Results of this analysis found the MTHFR genotype to be associated with specific clinical variables of migraine, specifically the homozygous TT genotype was found to be significantly associated with MA (P< 0.0001) and unilateral head pain (P= 0.002). While the CT genotype was significantly associated with physical activity discomfort (P< 0.001) and stress as a migraine trigger (P= 0.002). Overall the results indicated that the presence of the T allele to be associated with the most number of migraine symptoms and triggers, suggesting that although the TT genotype appears to have a recessive effect on MTHFR enzyme levels, perhaps both heterozygous and homozygous states of the T allele may contribute to the phenotypic expression of migraine. It is also plausible that the resulting levels of homocysteine conferred by the T allele may contribute to selected migraine phenotypic expression.

Other than the MTHFRC677T variant, there are other genes and functional variants or possible nutritional deficiencies in cofactors of the homocysteine metabolism cascade that may be reasons for hyper-homocysteinemia. The MTRR A66G variant resulting in the replacement of methionine with isoleucine in the enzyme has also been associated with less efficient enzyme activity and increased plasma homocysteine levels. The second part of this study examined the genotypic effects of MTHFR C677T and MTRR A66G variants on vitamin supplementation treatment response in homocysteine lowering and migraine disability including frequency and severity. This was a 6-month randomised, double blind placebo controlled trial of daily B vitamin supplementation (B6, B9 and B12) on homocysteine lowering and
reduction of migraine disability in 206 female patients diagnosed with migraine with aura. The results of this study showed that vitamin supplementation reduced homocysteine levels (P<0.001), the prevalence of high migraine disability (P=0.022) and head pain severity (P=0.017), compared to the placebo group. When the vitamin treated group was stratified by genotype, the C allele carriers of the MTHFR C677T variant showed a higher reduction in homocysteine levels (P<0.001), migraine pain severity (P=0.01) and percentage of high migraine disability (P=0.009) compared to TT genotypes. Similarly the A allele carriers of the MTRR A66G variants showed a higher level of reduction in homocysteine levels (P<0.001), migraine pain severity (P=0.002) and percentage of high migraine disability (P=0.006) compared to GG genotypes. Genotypic analysis for both genes combined indicated that the treatment effect modification of the MTRR variant was independent of the MTHFR variant.

Overall this study provided evidence that vitamin supplementation is effective at reducing migraine disability and that the C allele carriers of the MTHFRC677T, and the A allele carriers of the MTRRA66G are genetically more efficient metabolisers of homocysteine and are better responders to vitamin supplementation treatment then the mutant homozygote allele carriers of both variants. This trial also observed both MTHFR and MTRR gene variants to be acting independently to influence treatment response in female migraineurs.

The final part of this study focussed on the OPRM-1, Notch 3 and CALCA genes as potential candidate genes in migraine head pain severity and migraine in general. The opioid system plays an important role in diverse biological functions including analgesia, stress responsivity, drug response and pain reduction. The A118G SNP in exon of the OPRM-1 gene has been extensively studied in a variety of populations and has been associated with elevated pain responses and decreased pain threshold.
Genetic factors have been identified to influence treatment options for some migraine subtypes. This study investigated the effect of the OPRM-A118G SNP on migraine pain severity in the view of optimising pain treatment for migraineurs. A total of 154 adult female Caucasian MA sufferers collected as part of the clinical trial detailed above were assessed for migraine pain using the Migraine Disability Assessment Score (MIDAS) instrument and were genotyped for the A118G SNP. Results of this study showed the A118G SNP of the OPRM-1 gene to be significantly associated with migraine pain severity in the test population of female MA sufferers ($P=0.0225$). Participants of this study who were G118 allele carriers had demonstrably higher pain scores than A118 allele carriers. These findings suggest that OPRM-1 gene mutations may influence migraine associated pain.

The next study focussed on the Notch 3 gene implicated in CADASIL, a rare inherited autosomal dominant disease caused by mutations in the Notch 3 gene that shares common symptoms with migraine. More than 65% of all CADASIL mutations occur within exons 3 and 4 of the Notch 3 gene. In addition, a number of synonymous polymorphisms have also been identified in these exons. This study investigated whether two Notch 3 polymorphisms, specifically the C381T and G684A variants in exons 3 and 4, respectively of the Notch 3 gene, contribute to the risk of migraine in an Australian Caucasian population. The two variants were tested in two independent populations previously collected in the GRC. Population 1 was comprised of 275 migraineurs and 275 age, gender and ethnicity matched controls while the second population comprised of 300 cases and 300 matched controls. All individuals were diagnosed as having either MA or MO according to IHS criteria. The results of this study found a significant association between the C381T variant and migraine ($\chi^2=6.64$, $P=0.005$) and when analysed by subtype of migraine, the C381T variant
was observed to be significantly associated with MO ($\chi^2=8.36$, $P=0.002$) in the first population tested. However when the C381T variant was tested in the second population, only a trend towards significance was observed in the MO group. A significant association of the G684A variant with the migraine group was also observed in the first population studied ($\chi^2=7.21$, $P=0.015$). Further analysis found the G684A variant to be significantly associated with MA ($\chi^2=10.43$, $P=0.001$). In the second population tested, a significant association was again demonstrated between the G684A variant and MA ($\chi^2=7.97$, $P=0.003$). Taken together, the positive results from both populations strongly suggest the G684A variant to be associated with migraine, specifically sufferers of MA as compared to MO sufferers. The results of this study indicate a correlation between the Notch 3 gene and migraine and suggest that variants in the Notch 3 gene may be playing a role in susceptibility that influences both the severity and subtype of migraine.

This research next focussed on the role of the CGRP gene in migraine susceptibility. Migraine is a neurological disorder that is associated with increased levels of calcitonin gene-related peptide (CGRP) in plasma. CGRP being one of the mediators of neurogenic inflammation, a phenomenon implicated in the pathogenesis of migraine headache, is thus suggested to have an important role in migraine pathophysiology. Polymorphisms of the CALCA gene have been linked to Parkinson’s disease, ovarian cancer and essential hypertension, suggesting a functional role for these polymorphisms. Given the strong evidence linking CGRP and migraine, it was hypothesised that polymorphisms in the CALCA gene may play a role in migraine pathogenesis. Seemingly non functional intronic polymorphisms are capable of disrupting normal RNA processing or introducing a splice site in the transcript. A 16 bp deletion in the first intron of the CALCA gene has been reported
to be a good match for the binding site for a transcription factor expressed strongly in neural crest derived cells, AP-2. This deletion also eliminates an intron splicing enhancer (ISE) that may potentially cause exon skipping. This study investigated the role of the 16bp intronic deletion in the CALCA gene in migraineurs and age, gender and ethnicity matched control individuals previously collected in the Genomics Research Centre and diagnosed as suffering from MA or MO as per IHS criteria. 600 individuals were genotyped for the deletion by polymerase chain reaction followed by fragment analysis on the 3130 Genetic analyser. The results of this study showed no significant association between the intronic 16bp deletion in the CALCA gene and migraine in the tested Australian Caucasian population. However, given the evidence linking CGRP and migraine, further investigation of variants with this gene are warranted.

In conclusion, the research described in this thesis has firstly provided evidence for the involvement of a genetic variant in the MTHFR gene to influence migraine clinical phenotypes. This study has also provided strong evidence for the homocysteine lowering effect of vitamin supplementation that could potentially decrease migraine disability and associated head pain severity in a subgroup of migraineurs. This effect has also been shown to be dependent on the MTHFRC677T and the MTRRA66G genotypes raising the possibility of a pharmacogenetic relationship leading to the opportunity of introducing personalised migraine treatment. In addition the study of migraine candidate genes described in this thesis has shown the first association of Notch 3 gene variants with migraine and opioid receptor variants with migraine associated head pain. This clearly proves that a plethora of genes may be involved in the pathophysiology underlying migraine. Although this study has implicated variants in Notch 3 gene and opioid receptor in
migraine, replication studies in larger independent populations are required to further understand their role in migraine pathogenesis. However, this research has provided some novel and compelling results in relation to migraine molecular genetics.
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude to everyone who had given me their support and contributed in one way or the other to this work. Firstly I would like to thank Prof. Lyn Griffiths for giving me the opportunity to undertake my PhD at the Genomics Research Centre. I truly appreciate all your contributions of time, valuable feedbacks and positive encouragements which added considerably to my PhD experience. The enthusiasm you have for your research has been both contagious and motivational for me, especially during the trying times of my PhD pursuit.

I would also like to thank Dr. Rod Lea for his expertise, time and guidance throughout the course of my PhD journey. I would have been well and truly lost in the abyss of statistical world if it was not for you and your patience. A very special thank you goes out to Dr. Larsia Haupt for her guidance, assistance, good advice and good company. I appreciate your great efforts in explaining things clearly and simply and making sure I stayed focused. I would also like to thank Dr. Robert Smith for always being there when I was in need of help. Your vast knowledge in almost anything I came to you about was both awe inspiring and greatly valuable.

I am indebted to many student colleagues at the GRC for providing a stimulating and an enjoyable environment to work in. A big thank you to Bishaka Roy, Carlos Aya Bonilla, Emily Camilleri, Jacob Goodwin, Javed Fowder, Pam Gabrovska and Rebecca Grealy. You have all played an important part in this journey. A special thank you goes out to Michelle Hanna and all the volunteers of the Genomics Research Clinic for working tirelessly together with me to meet my datelines and in getting this thesis completed.
I would like to thank my family for their love and encouragement. Their support and trust in me has not only made me realize my dreams but also made me work to make them a reality. Special thanks go to my parents in law for their love, encouragement and enduring support throughout this journey. Your genuine interest in my work and your recognition of my little successes along the way has made me strive for the best. I would also like to say thank you to Kartika Menon for babysitting and entertaining her nephew when I had to run off to the Lab. I don’t know how I could have managed without your help in those desperate times.

I would like to thank my amazing husband. I would not be writing this section if it was not for you. I couldn’t have done it without your love and unwavering support. You have been there every step of the way and words can’t express how grateful I am for all that you have been for me. I would also like to say a big thank you to Yuvi and Sahana for their smiles and cuddles which make my day everyday. I dedicate this thesis to my mother, who I continue to miss dearly.

Thank you.
Statement of Originality

This work has not been previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

-----------------------------------

Saraswathy Menon
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ix</td>
</tr>
<tr>
<td>Statement of Originality</td>
<td>xi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xxi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xxiii</td>
</tr>
<tr>
<td>Publications Arising From Work Described In This Thesis</td>
<td>xxviii</td>
</tr>
<tr>
<td>Conference Presentations</td>
<td>xxviii</td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1. overview</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Aims</td>
<td>2</td>
</tr>
<tr>
<td>1.3. Significance</td>
<td>6</td>
</tr>
<tr>
<td><strong>1.0 Migraine Background</strong></td>
<td>8</td>
</tr>
<tr>
<td>1.1. Migraine</td>
<td>9</td>
</tr>
<tr>
<td>1.1.1. Definition</td>
<td>9</td>
</tr>
<tr>
<td>1.1.2. Classification and diagnosis</td>
<td>9</td>
</tr>
<tr>
<td>1.1.3 Migraine without aura</td>
<td>11</td>
</tr>
<tr>
<td>1.1.4 Migraine with aura</td>
<td>11</td>
</tr>
<tr>
<td>1.2. Clinical symptoms</td>
<td>13</td>
</tr>
</tbody>
</table>
1.3. Epidemiology 14
1.4. Social and economic impact of migraine 14
1.5. Comorbidity 15
1.6. Migraine pathophysiology 17
1.7. Migraine treatment 21
   1.7.1 In ancient times 22
   1.7.2 In the 21st century 23

2.0 Migraine Genetics 27
2.1. Genetic studies 28
2.2. Mode of inheritance 29
2.3. Approaches in the search for migraine genes 30
2.4. Molecular genetics of migraine and related conditions 31
   2.4.1. Familial hemiplegic migraine 32
   2.4.2. Episodic and Spinocerebellar ataxias 35
   2.4.3. CADASIL 37
   2.4.4. Dopamine beta hydroxylase gene and migraine 38
   2.4.5. Serotonin related genes and migraine 40
   2.4.6. Hormone receptor genes and migraine 42
   2.4.7. Role of vascular genes in migraine susceptibility 45

3.0 Research Methodology 47
3.1. Study design 48
3.2. Ethical approval 49
3.3. Subject ascertainment and diagnosis 49
   3.3.1 Diagnosis 49
   3.3.2 Clinical trial participants 50
   3.3.3 Unrelated case-control samples 50
3.4. DNA extraction and quantitation 51
3.4.1. DNA extraction 51
3.4.2. DNA purification and quantitation 51

3.5. Genotyping overview 51
3.5.1. Primer design 51
3.5.2. Polymerase Chain Reaction 52
3.5.3. Agarose gel electrophoresis 52

3.6. Mutation Screening 53
3.6.1. Restriction enzyme digestion 53
3.6.2. Fragment Analysis- Genemapper 54
3.6.3. High resolution melt 55
3.6.4. DNA sequencing 57

3.7. Statistical Analysis 58
3.7.1. Intention to treat principle 58
3.7.2. Parametric and Non parametric tests 60
   3.7.2.1. ANOVA 62
   3.7.2.2. Pearson’s correlation test 63
3.7.3. Association analysis 63
   3.7.2.1. Power analysis 63
   3.7.2.2. Hardy Weinberg Equilibrium 64
   3.7.2.3. Chi- square 65
   3.7.2.4. Odds ratio 66
   3.7.2.5. Linkage Disequilibrium 67

3.8. Summary and results of research 68

4.0 Analysis of the MTHFR C677T variant and migraine phenotypes 70

4.1. Methylene tetrahydrofolate reductase gene and migraine 71
4.2. Analysis of MTHFR C677T variant and migraine phenotypes 72
4.3. Methods
4.3.1 Study subjects
4.3.2 Phenotype variables
4.3.3 Genotyping
4.3.4 Statistical analysis

4.4. Results
4.4.1 MTHFR genotypes associated with migraine clinical variables
4.4.2 Gender differences and the MTHFR genotype

4.5. Discussion

5.0 Genotypic effect on vitamin B treatment response in migraineurs

5.1. Introduction
5.2. Role of homocysteine metabolism
5.3 Homocysteine
5.4. Genes involved in homocysteine metabolism
5.4.1 MTHFR-Methylenetetrahydrofolate gene and migraine
5.4.2 MTR- Methionine synthase gene
5.4.3 MTRR- Methionine synthase reductase gene
5.4.4 CBS- Cystathionine β synthase gene
5.4.5 Folate receptor genes
5.4.6 MTHFD- Methylene tetrahydrofolate dehydrogenase gene

5.5. Vitamin supplementation studies
5.5.1 Folic acid supplementation and migraine
5.5.2 Riboflavin (B2) and cobalamin (B12) vitamin supplementation and migraine
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6 Effects of vitamin supplementation and the MTHFR C677T genotype</td>
<td>99</td>
</tr>
<tr>
<td>on migraine disability</td>
<td></td>
</tr>
<tr>
<td>5.6.1 Study design and participant group</td>
<td>100</td>
</tr>
<tr>
<td>5.6.2 Treatment</td>
<td>101</td>
</tr>
<tr>
<td>5.6.3 Baseline and follow up assessment</td>
<td>102</td>
</tr>
<tr>
<td>5.6.4 Dietary consumption</td>
<td>102</td>
</tr>
<tr>
<td>5.6.5 Migraine disability measurement</td>
<td>103</td>
</tr>
<tr>
<td>5.6.6 Genotyping</td>
<td>103</td>
</tr>
<tr>
<td>5.7 Statistical analysis</td>
<td>105</td>
</tr>
<tr>
<td>5.7.1 Modified intention-to-treat principle</td>
<td>105</td>
</tr>
<tr>
<td>5.7.2 Parametric and Non-parametric analysis</td>
<td>105</td>
</tr>
<tr>
<td>5.7.3 Linear regression analysis</td>
<td>105</td>
</tr>
<tr>
<td>5.8 Results</td>
<td>106</td>
</tr>
<tr>
<td>5.8.1 Baseline analysis</td>
<td>108</td>
</tr>
<tr>
<td>5.8.2 Six month follow-up analysis</td>
<td>109</td>
</tr>
<tr>
<td>5.8.3 Treatment response by MTHFR C677T and MTRR A66G genotype.</td>
<td>113</td>
</tr>
<tr>
<td>5.9 Summary of discussion</td>
<td>117</td>
</tr>
<tr>
<td>6.0 A candidate Gene analysis investigating migraine pain</td>
<td>123</td>
</tr>
<tr>
<td>6.1 Introduction</td>
<td>124</td>
</tr>
<tr>
<td>6.2 OPRM-1 gene</td>
<td>124</td>
</tr>
<tr>
<td>6.3 Methods</td>
<td>127</td>
</tr>
<tr>
<td>6.3.1 Subjects</td>
<td>127</td>
</tr>
<tr>
<td>6.3.2 Migraine disability assessment</td>
<td>127</td>
</tr>
</tbody>
</table>
6.3.3 OPRM-1 A118G genotyping
6.3.4 Statistical analysis
6.4 Results
6.5 Summary of discussion

7.0 Association study of Notch 3 gene and migraine

7.1. Migraine and chromosome 19
7.2. Notch 3 gene as a migraine candidate gene
7.3 Association analysis of the Notch 3 gene
   7.3.1 Materials and methods
      7.3.1.1 Subjects
      7.3.1.2 Notch 3 genotyping
      7.3.1.3 Statistical analysis
   7.3.2 Results
      7.3.2.1 SNP identification
      7.3.2.2 Results- C381T polymorphism
      7.3.2.3 Results- G684A polymorphism
   7.4 Linkage disequilibrium analysis of the Notch 3 C381T and G684A SNPs
   7.5 Summary of discussion of association analysis of Notch 3 gene

8.0 Association Study of CALCA polymorphism with migraine

8.1 Calcitonin gene (CGRP) and their receptors
8.2 CGRP as a migraine candidate gene
8.3 Association analysis of CGRP gene
8.3.1 Materials and methods

8.3.1.1 Subjects
8.3.1.2 CGRP genotyping
8.3.1.3 Statistic analysis

8.3.2 Results

8.3.3 Discussion of association study of CGRP gene and migraine

9.0 Research summary and future directions

9.1. Introduction
9.2 The MTHFR variant and migraine phenotypes
9.3 Interaction of vitamin supplementation, MTHFR genotype and homocysteine in migraine disability.
9.4 Migraine candidate gene studies
  9.4.1 OPRM-1 gene
  9.4.2 Notch 3 gene
  9.4.3 CALCA gene

9.5 Conclusion

References

Appendices
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Mechanism underlying a migraine episode</td>
<td>20</td>
</tr>
<tr>
<td>1.2</td>
<td>The Trigeminovascular system</td>
<td>21</td>
</tr>
<tr>
<td>1.3</td>
<td>Ancient Egyptian treatment for patient suffering from head pain</td>
<td>22</td>
</tr>
<tr>
<td>1.4</td>
<td>Possible sites of mechanism of anti-migraine therapy</td>
<td>25</td>
</tr>
<tr>
<td>2.1</td>
<td>Functional roles of the proteins coded by known FHM genes within a glutameric synapse</td>
<td>34</td>
</tr>
<tr>
<td>3.1</td>
<td>An example of an agarose gel stained with ethidium bromide after electrophoresis.</td>
<td>53</td>
</tr>
<tr>
<td>3.2</td>
<td>An example of a melt curve profile of DNA samples after HRM.</td>
<td>56</td>
</tr>
<tr>
<td>3.3</td>
<td>A sample chromatogram from an automated DNA sequencing Reaction.</td>
<td>58</td>
</tr>
<tr>
<td>5.1</td>
<td>Homocysteine metabolism and the genes involved in the pathway.</td>
<td>87</td>
</tr>
<tr>
<td>5.2</td>
<td>Participant flow chart for the trial</td>
<td>107</td>
</tr>
<tr>
<td>5.3a</td>
<td>Change in homocysteine levels after trial in the placebo and vitamin treated group.</td>
<td>110</td>
</tr>
<tr>
<td>5.3b</td>
<td>Change in folate levels after trial in the placebo and vitamin treated group.</td>
<td>110</td>
</tr>
<tr>
<td>5.3c</td>
<td>Change in B₆ levels after trial in the placebo and vitamin treated group.</td>
<td>111</td>
</tr>
<tr>
<td>5.3d</td>
<td>Change in B₁₂ levels after trial in the placebo and vitamin treated group.</td>
<td>111</td>
</tr>
<tr>
<td>5.4</td>
<td>Change in frequency of migraine disability over treatment period.</td>
<td>112</td>
</tr>
<tr>
<td>5.5</td>
<td>Change in head pain severity score over treatment period for placebo</td>
<td>112</td>
</tr>
</tbody>
</table>
and vitamin group.

Figure 5.6. Change in homocysteine levels over treatment period in the vitamin

  group stratified by the MTHFR variant.

Figure 5.7. Change in homocysteine levels over treatment period in vitamin

  group stratified by the MTRR variant.

Figure 5.8. Change in high migraine disability in MTHFR and MTRR

  genotype group.

Figure 7.1. Notch 3, C allele of the C381T variant

Figure 7.2. Notch 3, T allele of the C381T variant

Figure 7.3. Notch 3, G allele of the G684A variant

Figure 7.4. Notch 3, A allele of the G684A variant

Figure 8.1. Schematic diagram of the CGRP1 receptor displaying

  interactions of receptor activity modifying protein (RAMP) with

  Calcitonin receptor like (CLR) and receptor complement protein

  (RCP).

Figure 24a. Fragment analysis of a sample homozygous CGRP 16bp deletion

  displaying a single peak of 306bp.

Figure 24b. Fragment analysis of a sample heterozygous for CGRP 16bp

  deletion, displaying a peak at 306bp and another peak at 287bp.

Figure 24c. Fragment analysis of a sample homozygous for CGRP 16bp

  displaying a peak at 287bp.
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Migraine Diagnostic Types.</td>
<td>10</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>MO Diagnostic Criteria</td>
<td>11</td>
</tr>
<tr>
<td>Table 1.3</td>
<td>MA Diagnostic Criteria</td>
<td>12</td>
</tr>
<tr>
<td>Table 1.4</td>
<td>Migraine co–morbidity studies</td>
<td>17</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Ace genotype and allele distribution among controls and migraineurs in different studies.</td>
<td>45</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>OR and heterogeneity results of C677T MTHFR polymorphism in migraineurs, both MA and MO from all studies analyzed in the meta analysis.</td>
<td>73</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Chi-squared ($\chi^2$) analysis of MTHFR genotype for individuals experiencing clinical variables versus all migraineurs who do not.</td>
<td>78</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Genotypic frequency distribution of MTHFR and statistically significant variables in relation to MTHFR genotype.</td>
<td>79</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Frequency distribution of CC/CT vs TT genotype significant variables</td>
<td>79</td>
</tr>
<tr>
<td>Table 4.5</td>
<td>Genotypic frequency distribution of MTHFR for statistically significant clinical variable in relation with gender</td>
<td>80</td>
</tr>
<tr>
<td>Table 4.6</td>
<td>Statistically significant clinical variables by gender and MTHFR genotype</td>
<td>80</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Clinical characteristics of participant groups at baseline</td>
<td>108</td>
</tr>
<tr>
<td>Table 5.2</td>
<td>Regression analysis model predicting homocysteine reduction after trial.</td>
<td>112</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>Association of OPRM 118A&gt;G with different variables</td>
<td>130</td>
</tr>
</tbody>
</table>
Table 7.1 Distribution of the C381T polymorphism in Notch 3 gene in migraineurs and controls of original sample in two studied population.

Table 7.2 Chi-squared ($\chi^2$) analysis of the allelic and genotypic frequencies in all migraine groups against controls for the C381T polymorphism in the two studied populations.

Table 7.3 Distribution of the G684A polymorphism in the Notch 3 gene in migraineurs and controls of original samples with the two studied populations.

Table 7.4 Chi-squared ($\chi^2$) analysis of the allelic and genotypic frequencies in all Migraine groups against controls for the G684A polymorphism in the two studied populations.

Table 8.1 Genotype and allele frequency distribution for CALCA 16bp deletion in migraine and control groups.

Table 8.2 Genotype and allele frequency distribution for CALCA 16bp deletion for the different genders in migraine and control groups.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-formyl-THF</td>
<td>10-formyl tetrahydrofolate</td>
</tr>
<tr>
<td>5-MTHF</td>
<td>5- methyltetrahydrofolate</td>
</tr>
<tr>
<td>3’ UTR</td>
<td>3’ Untranslated Region</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>5 HT&lt;sup&gt;1B/1D&lt;/sup&gt;</td>
<td>Serotonin receptor agonists</td>
</tr>
<tr>
<td>ABI</td>
<td>Applied Bio Science Laboratories</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin I converting enzyme</td>
</tr>
<tr>
<td>AFLP</td>
<td>Amplified fragment length polymorphism</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>AM</td>
<td>Adrenomedullin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>ATPase</td>
</tr>
<tr>
<td>ATP1A2</td>
<td>ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BOLD</td>
<td>Blood oxygen level dependant</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>CACNA1A</td>
<td>Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy</td>
</tr>
<tr>
<td>CALCA</td>
<td>calcitonin gene-related polypeptide-alpha</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CALCBB</td>
<td>calcitonin gene-related polypeptide- beta</td>
</tr>
<tr>
<td>CBS</td>
<td>cystathionine b-synthase</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CH₂-THF</td>
<td>5, 10-methylenetetrahydrofolate</td>
</tr>
<tr>
<td>CH₃-THF</td>
<td>5-methyltetrahydrofolate</td>
</tr>
<tr>
<td>CLR</td>
<td>Calcitonin-like receptor</td>
</tr>
<tr>
<td>CNCbl</td>
<td>cyanocobalamin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSD</td>
<td>Cortical Spreading Depression</td>
</tr>
<tr>
<td>DBH</td>
<td>Dopamine beta-hydroxylase</td>
</tr>
<tr>
<td>DDC</td>
<td>1-dopadecarboxylase</td>
</tr>
<tr>
<td>DF</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>DHE</td>
<td>Dihydroergotamine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy-ribo Nucleic Acid</td>
</tr>
<tr>
<td>DRD2</td>
<td>Dopamine receptor D2</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygotic</td>
</tr>
<tr>
<td>EA2</td>
<td>Episodic Ataxia Type 2</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESR1</td>
<td>Estrogen receptor 1</td>
</tr>
<tr>
<td>ERPs</td>
<td>Event-related potentials</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FHM</td>
<td>Familial Hemiplegic Migraine</td>
</tr>
<tr>
<td>FR-α</td>
<td>Folate receptor alpha</td>
</tr>
<tr>
<td>GRC</td>
<td>Genomics research centre</td>
</tr>
<tr>
<td>GH-1</td>
<td>Growth Hormone</td>
</tr>
<tr>
<td>H₄B</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HRM</td>
<td>High resolution melt</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy Weinberg Equilibrium</td>
</tr>
<tr>
<td>IDT</td>
<td>Integrated DNA technologies</td>
</tr>
<tr>
<td>IGHC-II</td>
<td>isolated growth hormone deficiency</td>
</tr>
<tr>
<td>IHS</td>
<td>International Headache Society</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage Disequilibrium</td>
</tr>
<tr>
<td>MA</td>
<td>Migraine with Aura</td>
</tr>
<tr>
<td>MAOA</td>
<td>Monoamine oxidase A</td>
</tr>
<tr>
<td>MAOB</td>
<td>Monoamine oxidase B</td>
</tr>
<tr>
<td>MAP 1</td>
<td>Migraine population 1</td>
</tr>
<tr>
<td>MAP 2</td>
<td>Migraine population 2</td>
</tr>
<tr>
<td>MeCbl</td>
<td>Methylcobalamin</td>
</tr>
<tr>
<td>MELAS</td>
<td>Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MIDAS</td>
<td>Migraine Disability Assessment Score</td>
</tr>
<tr>
<td>MO</td>
<td>Migraine without Aura</td>
</tr>
<tr>
<td>MMA</td>
<td>Methylmalonic acid</td>
</tr>
<tr>
<td>MTHFR</td>
<td>5,10-methylene-tetrahydrofolate reductase</td>
</tr>
<tr>
<td>MTRR</td>
<td>Methionine synthase reductase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Methionine synthase</td>
</tr>
<tr>
<td>MSB</td>
<td>Mean square between</td>
</tr>
<tr>
<td>MSE</td>
<td>Mean square error</td>
</tr>
<tr>
<td>MZ</td>
<td>Monodizygotic</td>
</tr>
<tr>
<td>Naᵥ,1.1</td>
<td>Neuronal voltage-gated sodium channel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NR3C3</td>
<td>Nuclear receptor subfamily 3, group 3</td>
</tr>
<tr>
<td>NTD</td>
<td>Neural tube defect</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OHCb1</td>
<td>Hydroxocobalamin</td>
</tr>
<tr>
<td>OPRM-1</td>
<td>μ-opioid receptor 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PGR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>RAMP</td>
<td>Receptor activity-modifying protein</td>
</tr>
<tr>
<td>RER</td>
<td>Replication error</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>SCA6</td>
<td>Spinocerebellar Ataxia Type 6</td>
</tr>
<tr>
<td>SCN1A</td>
<td>Sodium channel, voltage-gated, type I, alpha subunit</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single Strand Conformation Polymorphism</td>
</tr>
<tr>
<td>STR</td>
<td>Short Tandem Repeat</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>TPH</td>
<td>tRNA</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>TVS</td>
<td>Trigeminal vascular System</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>VAL</td>
<td>Valine</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number tandem repeat</td>
</tr>
</tbody>
</table>
vWF  Willebrand factor
Publications arising from work described in this thesis


Conference presentations arising from work described in this thesis

Abstracts

2008


2009


2010

INTRODUCTION

1.1 Overview

Migraine is a painful and debilitating disorder that affects about 12% of the general population worldwide with higher prevalence in females than males (Maizels 2001; Lipton, Stewart et al. 2002; Tepper, Dahlof et al. 2004). The disease is generally characterized by recurrent head pain usually accompanied by nausea, vomiting, neurological disturbance, photo-and phonophobia (Kara, Sazci et al. 2003). The lack of clear symptom definitions and precise diagnostic criteria has led to variability in diagnosis. The International Headache Society has however prepared a classification for headache that has made diagnosis more precisely defined. This system uses the presence of specific attributes to establish diagnosis and has classified migraine into two most common types, migraine with aura (MA) and migraine without aura (MO) (HCCIHS 2004).

Migraine has been known as a medical abnormality for quite some time and a variety of methods have been applied throughout the centuries to cure the disabling pain from migraine. While they may have seemed appropriate given the time of the century, today they seem at best amusing and at worst torturous (Villalon, Centurion et al. 2003). There are 9,000 year old Neolithic skulls that exist with evidence of trepanation, one of the earliest and drastic methods taken in response to headache. Many years later in the 2nd century AD Galenus of Pergamon used the term “hemicrania” to describe the painful disorder affecting approximately half the head. “Hemicrania” slowly transmuted to become “migraine” some 600 years later (Villalon, Centurion et al. 2003). It was not until the late 17th century through to 20th
century that the vascular and neurological theories behind migraine emerged and almost simultaneously the first anti-migraine efficacy of ergotamine was established. Despite the substantial advances in understanding this disease the pathophysiology of migraine is still not fully understood and the search for an effective treatment has not ceased (Edmeads 1991; Rapoport and Edmeads 2000).

One of the most significant findings in recent times is the involvement of genetics in migraine aetiology. Genetic linkage and association studies have both implicated a number of susceptibility genes, causative mutations and susceptibility variants that are of significant clinical relevance in migraine. However not all migraine genes have been uncovered and therefore further research is warranted to determine the definitive molecular genetics of migraine.

1.2 Aims

This research focuses on the molecular genetics of migraine in an attempt to understand the possibility of a pharmacogenetic relationship and personalized treatment options for migraineurs based on their genetic predisposition to this disorder.

This research encompasses 3 broad aims investigating migraine endophenotypes, migraine treatment response and migraine potential candidate genes. The specific aims are:

1. To investigate the potential association of migraine clinical variables with MTHFR genotype.
Since the first discovery of the MTHFRC677T polymorphism association with migraine in a Japanese population, several studies have replicated the findings in different independent populations (Kowa, Yasui et al. 2000). Similarly the GRC found the C677T variant to be associated with migraine, with significant overrepresentation of the TT genotype in individuals with MA and not MO subtype (Lea, Ovcaric et al. 2004). With multiple genes reported to contribute to migraine susceptibility, it is plausible that different genotypes in these genes may cause varying disease manifestations. The genotypes could have an effect on migraine subtypes, triggers, severity, symptoms and response to medication. These genotype influenced clinical profiles may be highly crucial for developing effective and personalized treatment for migraine. Thus the first aim of this study investigated the genotype to phenotype correlation between the MTHFR C677T SNP and migraine clinical profiles. This study was conducted on migraineurs, who were drawn from two large age and sex matched case/ control populations that were previously recruited by the GRC from the east coast of Australia.

2. To investigate the role of MTHFR and MTRR genotypes in relation to migraine vitamin treatment response.

The TT genotype of the MTHFR C677T SNP has been shown to be associated with a 50% reduction in enzyme activity resulting in increased levels of circulating homocysteine (Lea, Ovcaric et al. 2004). Hyperhomocysteinemia is linked to an increase risk of atherosclerotic vascular disease. MA sufferers have an increased risk of vascular brain lesions and ischemic stroke. Cortical spreading depression (CSD), which is characteristic of MA, and the changes in cerebral blood flow, are both shared by migraine and stroke (Lea, Ovcaric et al. 2004). Based on this comorbidity between
the two disorders and the potential role that homocysteine might play in disturbing the cerebrovascular system; it is possible that increased levels of homocysteine may be involved in the pathophysiology underlying certain subtypes of migraine and stroke.

The MTRR gene reduces inactive cobalamin II to active cobalamin I and methylates it to methylcobalamin using S-adenosylmethionine as the methyl donor (Elmore, Wu et al. 2007). MTRR therefore plays a pertinent role in maintaining adequate supply of active cobalamin I and may also be a critical determinant of homocysteine concentrations (Gaughan, Kluijtmans et al. 2001). A common variant, A66G in the MTRR gene results in the replacement of methionine with isoleucine in the enzyme. The MTRR A66G polymorphism is also associated with an increase in plasma homocysteine, with the GG genotype having a greater effect than AG genotype (Gaughan, Kluijtmans et al. 2001). In addition the coexistence of the MTHFR 677TT genotype and either the AG or GG genotypes for the MTRR A66G polymorphism has been reported to magnify the effect of the MTHFR 677TT genotype alone (Vaughn, Bailey et al. 2004).

Homocysteine levels can be inexpensively lowered with vitamin supplementation including folic acid and vitamin B₆ and B₁₂ (Lea, Colson et al. 2009). A recent study by Lea et al examined vitamin B supplementation and its effect on plasma homocysteine and migraine disability, as well as the effect of MTHFRC677T genotype on the treatment response in fifty two MA sufferers (Lea, Colson et al. 2009). Results of this trial revealed that vitamin supplementation significantly reduced plasma homocysteine levels and migraine disability, in a small subgroup of migraineurs (Lea, Colson et al. 2009). These results were supported by an open-
labelled study by Di Rosa et al (Di Rosa, Attina et al. 2007) who also provided evidence that homocysteine lowering through folic acid coupled vitamins B₆ and B₁₂ may reduce migraine disability in a subgroup of patients (Di Rosa, Attina et al. 2007; Lea, Colson et al. 2009).

The second aim of this study thus investigated the genotypic effects of both the MTHFR and the MTRR gene on folate and vitamin B treatment response in homocysteine-lowering and migraine disability including frequency and severity. This was conducted as a randomised, double-blind, placebo-controlled trial of 6 months of daily vitamin supplementation of 2mg of folic acid, 25mg vitamin B6 and 400ug of vitamin B12 in 206 female patients diagnosed with migraine with aura.

3. To investigate the role of a number of potential candidate genes in migraine.

Candidate gene association analysis is a popular method of identifying disease genes by investigating potential gene changes that may be linked to the pathophysiological characteristics and biochemical pathways implicated in a disorder. Association studies investigate the candidate disease gene by comparing the frequency of alleles of a candidate gene SNP or mutation between gender, age and ethnicity matched case control (migraine vs non- migraine) populations. The third aim of this study investigated the role of candidate genes in migraine pathogenesis. Investigation of candidate genes was carried out by association analysis in large case/control cohorts. This study investigated a common variant in the µ-opioid receptor 1 gene (OPRM1) for association with migraine disability and variants in the Notch 3 gene and Calcitonin gene related peptide (CGRP) for association with migraine.
1.3 Significance

The World Health Organisation lists migraine in the top 10 health disabilities. Migraine undeniably has a major impact on the well being and quality of life. The economic burden of migraine on society at large is also extensive (Cerbo, Pesare et al. 2001). The Australian Brian Foundation in 1995 estimated a migraine economic cost of ~ $721 million per annum comprising of treatments as well as absenteeism and lost productivity at work. Diagnosing migraine and its specific sub types is severely hampered by the lack of a simple diagnostic test. Although IHS has greatly improved the validity of migraine diagnosis differentiation from other stroke-like, cerebrovascular and ataxia disorders that have symptomatic overlap with migraine, diagnosis is still difficult. Misdiagnosis can lead to incorrect therapy suggestions and failure to treat the disorder.

Current migraine treatments fall into two general categories, abortive therapy and preventive therapy. In abortive therapy, migraine is treated with nonsteriodal anti-inflammatory drugs (NSAIDs) such as ibuprofen or aspirin, or with specific anti migraine drugs such as triptans (MacGregor, Brandes et al. 2003). In preventive therapy β-adrenoceptor- blockers, propanolol and metaprolol, the antiepileptic drug sodium valporate and calcium channel blockers such as verapamil, have been demonstrated to be effective (Ferrari 1998) against migraine. Antidepressants, anti-seizure drugs and supplements such as magnesium, coenzyme Q¹⁰, and the herbs Feverfew have also been used for reducing pain, frequency and duration of migraine. The responses to these therapies differ among migraineurs, in some producing adverse side effects. These therapies are only effective in about 55% of migraineurs and that too with only about 50% reduction in migraine frequency.
Preventive therapy also includes vitamin and mineral supplementation for migraine. Although they are usually not first in line as a treatment option for migraineurs, some vitamin therapies have been proven to be effective in migraine clinical studies. The complexities underlying migraine pathophysiology and diagnosis have thus made the treatment options varied and continuously improved. However there is still much to be ventured into when it comes to treating this disabling disorder.

The underlying pathophysiology of migraine remains to be determined. Cortical spreading depression (CSD), a depolarization wave that progresses over the cortical surface is often suggested to be the underlying mechanism for the migraine aura (Mathew 2001). The cerebral blood flow change during the spreading has been suggested to activate the trigeminal vascular system (TVS). The activation and sensitisation of TVS are thought to be the cause of most migraine symptoms, including head pain (Mathew 2001; Buzzi and Moskowitz 2005). Therefore identifying factors that have the potential to disrupt this phenomenon leading to CSD and/ or affecting the TVS may contribute to the development of migraine treatment.

In recent years the molecular genetics underlying the pathophysiology of migraine has taken a pivotal role in research. With current migraine medication working with differing efficacy in migraineurs, it may be possible that the underlying genetic predisposition to the disease might be affecting treatment suitability in individuals. Thus rigorous research is warranted to expand our knowledge on the molecular genetics behind migraine in order to explore better treatment options for this painful disease.
Chapter 1

Migraine Background
1.1 Migraine

1.1.1 Definition

The word “Migraine” originated more than 2500 years ago from the Greek “hemikrania”, which means half skull. The present day term of migraine is French in origin. Migraine is clinically defined as an episodic headache lasting about 4-72 hours and accompanied by nausea, vomiting and light/sound sensitivity. This neurological disorder is common and can often be disabling. Migraines can be experienced from as little as once a year to as frequently as three times a week. Their severity, duration and symptoms vary within and among individuals (HCCIHS 1988).

1.1.2 Classification and Diagnosis

Current diagnosis of migraine relies on traits and symptoms reported by the patients, due to the lack of clear diagnostics markers and the episodic nature of the disorder. The IHS established new international diagnostic criterion in 1988 (HCCIHS 1988). This criterion was later updated in 2004 and it aides in the diagnosis and classification of a broad range of headache disorders including migraine (HCCIHS 2004). Although the criterion has greatly improved, the validity of migraine diagnosis still relies on clinical features, which may not be present in every migraine episode or in every patient (Ducros, Tournier-Lasserve et al. 2002).

Migraine has been classified as a primary headache disorder and divided into two major subtypes by the current international diagnostic criterion: migraine without aura (MO) and migraine with aura (MA) which was previously termed as ‘common migraine’ and ‘classical migraine’ respectively. The IHS classification of migraine subtypes based on the current diagnostic criterion is as follows:
### Table 1.1: Migraine Diagnostic Types (HCCIHS 2004)

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
   1.2.5 Sporadic hemiplegic migraine
   1.2.6 Basilar-type migraine

1.3 **Childhood periodic syndromes that are commonly 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 migrainosus
   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
1.1.3 Migraine without aura (MO)

The diagnostic criteria for MO, the most common type of migraine, by the International Headache Society Classification of Primary Migraine Disorders are outlined in Table 2.

<table>
<thead>
<tr>
<th>Table 1.2: MO diagnostic criteria (HCCIHS 2004)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. At least 5 attacks lasting 4-72 hours untreated or unsuccessfully treated, accompanied by at least two of the following characteristics and fulfils criteria B</td>
</tr>
<tr>
<td>1. Unilateral localization</td>
</tr>
<tr>
<td>2. Pulsating quality</td>
</tr>
<tr>
<td>3. Moderate to severe intensity</td>
</tr>
<tr>
<td>4. Aggravation by walking stairs or similar routine physical activity</td>
</tr>
<tr>
<td>B. During headache at least one the following occurs</td>
</tr>
<tr>
<td>1. Nausea and/ or vomiting</td>
</tr>
<tr>
<td>2. Photophobia and phonophobia</td>
</tr>
<tr>
<td>C. History, physical and neurological examinations do not suggest that headache is attributed to another disorder</td>
</tr>
<tr>
<td>D. Presence of another disorder found in history and/or during physical and/or neurological examination ruled out after appropriate investigations</td>
</tr>
<tr>
<td>E. First migraine attack did not occur in close temporal relation with the other Disorder.</td>
</tr>
</tbody>
</table>
1.1.4 Migraine with Aura (MA)

The diagnostic criteria for MA, the least common type of migraine, by the International Headache Society Classification of Primary Migraine Disorders are outlined in Table 3.

Table 1.3: MA diagnostic criteria (HCCIHS 2004)

A. At least two episodes fulfilling criteria B to D

B. Fully reversible visual, sensory and aphasic aura symptoms

C. Headache has at least two of the following characteristics

1. Homonymous positive features and/or unilateral sensory symptoms

2. At least one of them gradually develops over more than five minutes or two or more occur subsequently.

3. Each symptom lasts between five to sixty minutes

D. Headache follows aura with free interval of at least 60 minutes, which may also simultaneously start with aura

E. History, physical and neurological examinations do not suggest that headache is attributed to another disorder

F. Presence of another disorder found in history and/or during physical and/or neurological examination ruled out after appropriate investigations

G. First migraine attack did not occur in close temporal relation with the other disorder
1.2 Clinical symptoms

A typical migraine episode may consist of five phases, namely the prodrome, aura, headache, resolution and the postdrome. Not all phases are present in a migraine attack and not all migraineurs experience all five phases. Most migraineurs experience at least one phase and that may vary between the different episodes of migraine (Wessman, Terwindt et al. 2007).

The prodrome phase is approximately experienced by at least 60% of migraineurs. It can start hours to days before a migraine episode and includes symptoms such as food craving, mood changes, neck stiffness and fatigue. This acts as warning sign for many migraineurs for the impending migraine headache. The aura is the most familiar phase and it begins after the prodrome phase. In migraine with aura sufferers, the aura phase usually lasts for less than an hour and may include a wide range of syndromes such as visual distortion, muscular weakness, olfactory hallucinations, partial paralysis and reduced sensation (Blau 1987).

The headache phase may begin at any time of the day and can last from one to three days. The phase starts with a moderate headache and progresses to severity over time before declining to resolution. The often unilateral and pulsating pain in this phase may be worsened by physical activity. Age and triggering factors have been implicated to have an impact on the duration of the headache phase (Linde 2006). During the resolution phase, headache and other migraine symptoms slowly subside. Migraineurs may take a couple of hours to a couple of days to fully recover and may experience symptoms such as fatigue and depression (Schreiber 2006).
1.3 Epidemiology

To understand the impact of migraine on society, it is important to determine its prevalence. Migraine epidemiology has faced methodological difficulties over the years and this has been significantly improved with the implementation of the new IHS Classification (Ducros, Tournier-Lasserve et al. 2002). According to epidemiological studies done based on the new IHS criteria, migraine is estimated to affect about 12% of the population worldwide and prevalence varies by age, gender and race (Mathew 2001).

Migraine onset often occurs during childhood especially during puberty; however it can also begin during adulthood (Jensen, Oldfield et al. 2009). Age at migraine onset is earlier in males than in females and it peaks between 25-55 years in both genders. Although the pre-pubertal incidence of migraine is similar in both genders, migraine prevalence is twice as common in females as in males after puberty (Lipton and Stewart 1997). Studies have implicated the fluctuation of hormones, oestrogen and progesterone in increased risk for migraine and their severity in some females (Colson, Lea et al. 2005).

1.4 Social and economic impact of migraine

Migraine has a significant impact on both individuals and society at large (Mennini, Gitto et al. 2008). The cost of migraine can be divided into 3 categories, direct, indirect and intangible costs. Direct costs concern mainly expenses incurred by the medical care system in managing, diagnosing and treating migraine. With migraine affecting the majority of population during the most productive period of their life, the indirect costs arising due to loss of productivity at work, reduced working
proficiency and remuneration represent a large portion of the total costs of migraine (Cerbo, Pesare et al. 2001). Intangible costs refer to the pain, suffering and reduction in quality of life as a result of migraine. The impact on emotional and physical well being of the migraineurs and their family are difficult to interpret in monetary terms and are rarely included when studying the cost of illness, however they are still a consequence suffered by migraineurs and their families (Cerbo, Pesare et al. 2001; Dartigues, Michel et al. 2003).

1.5 Co-morbidity

Co-morbidity refers to the concomitant but unrelated existence of two or more disorders in the same individual (Scher, Bigal et al. 2005). Migraine has been linked to a variety of diseases, including well defined medical conditions, such as coronary heart disease, epilepsy, stroke, hypertension, diabetes, asthma and depression (Scher, Bigal et al. 2005; Tietjen, Herial et al. 2007) (Refer to Table 4). It has also been associated with a multitude of idiopathic syndromatic medical disorders such as fibromyalgia, irritable bowel syndrome and psychiatric disorders (Rose 1986; Stewart 1994; Peres 2001; Breslau 2003; Novi 2005). Epilepsy is closely linked with migraine with a prevalence of 6% in migraineurs compared to 0.5% in non migraineurs and recent research suggests a strong relation between epilepsy and MA (Simone, Ranieri et al. 2007). Although the underlying epidemiology linking migraine and epilepsy is still vague, a shared neuronal hyperexcitability appears to be the phenomena accounting for their comorbidity (Parisi, Piccioli et al. 2008).

The association between migraine and stroke have been well documented in recent years through several population studies. The association has been observed to be strongest in woman, especially MA sufferers, under the age of 55 years. The risk
increases significantly for women with MA who smoke or use oral contraceptives. This risk was however not seen in women above 55 years of age. Similarly an association between increased risk for stroke and migraine was only observed in men below the age of 55 years and not in the older population (Kurth, Gaziano et al. 2007). Migraine has also been closely associated with white matter abnormalities in several studies. A population-based study done by Kruit et al demonstrated that clinically silent cerebellar lesions, mostly in the white matter were more apparent in MA sufferers with frequent attacks (Kruit, van Buchem et al. 2004). Women with migraine were also found to be about twice as likely to have deep white matter lesions compared with non-migraineurs (Kruit, Launer et al. 2005).

Migraineurs particularly those with aura also pose several classic risk factors, such as high blood pressure and parental history of early myocardial infarction, for the onset of cardiovascular disease (Scher, Terwindt et al. 2005). Several inherited neurological disorders, CADASIL and mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) have also been associated with migraine. These autosomal dominant disorders suggest that a potential genetic component may contribute to the migraine-stroke association.
### Table 1.4: Migraine Co-morbidity studies

<table>
<thead>
<tr>
<th>Data Source</th>
<th>Country</th>
<th>Year</th>
<th>Comorbid condition</th>
<th>Source of participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cook [7]</td>
<td>USA</td>
<td>2002</td>
<td>CHD</td>
<td>Health professionals</td>
</tr>
<tr>
<td>Rose [8]</td>
<td>USA</td>
<td>2004</td>
<td>Rose angina</td>
<td>Population</td>
</tr>
<tr>
<td>Koth [4]</td>
<td>USA</td>
<td>2004</td>
<td>Stroke</td>
<td>Health professionals</td>
</tr>
<tr>
<td>Lecou [12]</td>
<td>Italy</td>
<td>2003</td>
<td>Vascular disorders in family</td>
<td>Patients (pediatric)</td>
</tr>
</tbody>
</table>

### Congenital heart defects

- Caster [16]   : Italy     2003, Atrial septal aneurysm, Patients
- Lorry [18]   : Multicenter 2003, PFO, stroke, Patients
- Schuermann [13]: Switzerland 2003, PFO, Patients
- Vakhashit [21]: UK        2004, PFO, ASD, Patients
- Mavardi [20]: Italy      2003, PFO closure, Patients (final)
- Pesti [21]: Belgium      2004, PFO closure, Patients (final)
- Requenas [18]: USA       2005, PFO closure, Patients (final)
- Schuermann [17]: Switzerland 2004, PFO closure, Patients (final)

### Psychiatric

- Breslau [23]: USA       2005, Depression, Population
- McWilliams [20]: USA     2004, Depression, anxiety, Population
- Wimmer [21]: USA        2004, Depression, MHO participants
- Smolarek [23]: USA      2005, Panic attacks, Population
- Szewczak [21]: Poland   2005, Depression, anxiety, Population

### Pain

- El Malek [22]: Finland   2004, Musculoskeletal pain, Third, Fifth grade Sufferers
- Hartvig [22]: Denmark    2004, Low back pain, Population
- Ver Korf [21]: USA      2004, Chronic pain, Population

ASD, atrial septal defect; CHD, coronary heart disease; HMO, health management organization; PFO, patent foramen ovale; WML, white matter lesions.

(Adapted from Scher et al 2005)

### 1.6 Migraine Pathophysiology

The pathogenesis of migraine is now better described than it has been in the last four decades. The aetiology of this disabling disorder is continuously being explored to facilitate the understanding of the mechanism underlying the pain and the accompanying symptoms. Migraine is currently termed as a neurological model due to its symptoms being a derivative of a biochemical, vascular or neurological event. Although the exact pathogenesis of migraine is yet to be determined, a number of theories have been proposed to explain and understand the mechanism behind this disorder (Bussone 2004).
1. **The Vascular theory.** Harold G. Wolff introduced the vascular theory in the late 1930s. Wolff’s research findings proposed that cerebral vasoconstriction and vasodilatation were the cause of the neurological symptoms and headache experienced during MA. The dilated blood vessels may stimulate stretch receptors, which increase the release of neuropeptides mainly calcitonin gene-related peptide (CGRP). Plasma extravasations and degranulation of mast cells accompanies the oedema response and ultimately cause throbbing head pain and other related symptoms (Mathew 2001). This theory was further supported when vasoconstrictors were observed to decrease arterial dilation and improve head pain in migraineurs, while vasodilators were observed to intensify head pain (Graham and Wolf 1938). However inconsistencies with the vascular theory were recognised when further research was undertaken. Blau JN et al described the Phase model in migraine in 1987. He found the Prodrome phase of the migraine, although clinically common, could not be completely explained by the vascular theory (Blau 1987). Olesen demonstrated that vasoconstriction occurred during migraine, but it did not coincide with the headache phase. Headache occurred while regional blood flow remained decreased, and in some patients headache disappeared even though blood flow remained increased (Olesen, Friberg et al. 1990).

2. **Cortical Spreading Depression.** Cortical spreading depression (CSD) is often implicated in the aura or prodrome phase experienced with initiation of a migraine episode and the associated blood flow changes (Lauritzen 2001). CSD begins at the occipital lobe at the onset of migraine attack. It involves a temporary but a severe disruption in ion homeostasis, causing a brief neuronal excitation. This propagates a wave of depolarisation followed by suppression that spreads over the cortical surface and down into the depths of the sulci (Arulmozhi et al 2005). The depolarising wave
is considered to be the mechanism underlying the scintillations during the Aura phase of migraine. Neuronal depolarisation and cerebral blood flow changes activate the trigeminal nerve leading to an inflammatory response which results in the painful period of the migraine episode (Lauritzen 2001) (refer to Figure 1.1).

This electrical phenomenon can also be triggered by trauma and chemicals such as potassium and glutamate (Lauritzen 2001; James et al 2001; Arulmozhi et al 2005). CSD phenomenon was first described by Aristides Leao in the 1940s and it has being well characterized in many animal models except man possibly due to technical difficulties (Bolay, Reuter et al. 2002). Potassium injection into the caudate nucleus or hippocampus initiated CSD and elicited waves of cortical depolarization at a rate of 3.2mm/min, consistent with the propagation rate of CSD in rodents.

Bures et al obtained the first indication that human grey matter in vivo supports CSD. Studies examining magnetic resonance imaging (MRI) and Blood oxygen level dependant (BOLD) changes during migraine in humans have reported that the BOLD signal change propagated at 3.5mm/min over the occipital cortex, as the visual aura started concurrently. Although CSD is able to explain certain aspects of migraine such as the prodrome phase, which is difficult to explain based on the vascular theory, the specific role CSD plays in the generation of headache is still unclear.
3. The trigeminovascular theory. The trigeminovascular theory also known as the neurogenic dural inflammation theory, proposes to address the inconsistencies of the vascular and the CSD theories. Moskowitz and colleagues described in 1992 that neuropeptides released from primary sensory nerve terminals that innervate the dural blood vessels, result in neurogenic dural inflammation (Moskowitz 1992). Migraine pain is thus proposed to be the result of the inflammation and dilation of meningeal arteries, particularly those present within the dura, a membrane surrounding the brain (Arulmozhi et al 2005).

The trigeminovascular system is the innervation of cerebral vessels and the dura mater by pseudo-unipolar neurons whose cell bodies are located in the trigeminal ganglion (Arne May and Goadsby 1999). The peripherally projecting fibers of the trigeminovascular system terminate at the cranial vessels and the centrally projecting fibres, carrying nociceptive information, synapse in the caudal brain stem. The
peripherally and centrally projecting fibres constitute the pathway in the trigeminovascular system through which sensory information such as pain signals are transmitted to the thalamus and cortical pain areas (Borsook, Burstein et al. 2006) (refer to Figure 1.2).

Figure 1.2: The Trigeminovascular system (Adapted from Goadsby PJ et al., 2002)
The stimulation of the trigeminal ganglion, releases vasoactive peptides such as substance P and CGRP, which promote neurogenic inflammatory response in the dura mater. The release of these vasoactive peptides during migraine causes vasodilation, endothelial changes and plasma protein extravasation which results in further inflammation and increases the consequent transmission of pain signal to the brain (D.K. Arulmozhi 2005).

1.7 Migraine Treatment
1.7.1 In ancient times

Migraine has been described in detail in Neolithic period dating back to 8500-7000 BC. Archaeological findings have reports that show the use of trepanation which involved the drilling of a hole through a part of the skull and removing circular chunks of the skull, which was believed to release the evil spirits that were causing the pain in the individual’s head (Edmeads 1991). In ancient Egypt itself, migraine treatment evolved from simply rubbing a fried fish on the side of the headache to tying a clay crocodile with a herb bundle in its mouth to the head of the patient using a linen that had the names of those gods they believed would heal the pain (refer to Figure 1.3).

![Figure 1.3: Ancient Egyptian treatment for patient suffering from head pain.](Adapted from Villalon et al., 2003)

Migraine treatment in ancient Rome consisted of tying a black torpedo fish or electric ray to the head in the hope that the electric shock would cure them of the pain. The father of medicine himself, Hippocrates was one of the earliest Greek physicians to describe the aura that preceded a migraine attack in about 400 BC. Hippocrates released migraine from the realms of the supernatural by attributing the migraine associated head pain to the rising vapours from the stomach to the head as a reaction
to the imbalance of natural elements, by the body (Edmeads 1991; Rapoport, Edmeads 2000).

The early 17th century would have been the earliest indication of doctors understanding migraine pain as a result of cranial vasodilation. However the emerging theories behind migraine pathophysiology did not change treatment options immediately. In 1660, William Harvey, an English physician was still recommending trepanation as a migraine treatment. Migraine patients continued to endure a bizarre array of alleged remedies and medication before the early 1900 when the first drugs specifically designed to target migraine pain, known as ergots was discovered.

1.7.2 In the 21st century

Ergotamine a drug derived from rye fungus was first introduced in 1938 for the acute treatment of migraine and they led to the development of the drug dihydroergotamine (DHE)(Dahlof and Goadsby 2000). This potent vasoconstrictor is not widely prescribed any longer due to drawbacks associated with its use, including lack of receptor specificity, nausea, vomiting and vasoconstriction of systemic and coronary arteries (Group, 3a Divisione di Neurologia et al. 1999; Adelman and Belsey 2003; Mett and Tfelt-Hansen 2008)

Migraine treatment today falls into two broad categories: Abortive or acute treatment and prophylactic treatment. Migraine is treated symptomatically in abortive therapy with nonsteriodal anti-inflammatory drugs (NSAIDs) such as ibuprofen or aspirin, or with specific anti migraine drugs such as triptans (MacGregor 2003) (refer to Figure 1.4). Simple analgesics are frequently used as first line therapy for mild to moderate
attacks in most patients. However, they are often not sufficiently effective and their overuse may lead to dependence, ulcers and rebound headaches (Mett and Tfelt-Hansen 2008).

The latest class of drugs to be introduced for the treatment of moderate to severe migraine are the triptans (Adelman and Belsey 2003). Triptan was created in an effort to make an equally effective drug as the ergots with fewer side effects. The first line of Triptan drugs that was approved by the “Food And Drug Administration (FDA) in 1993 and between 1996 and 2003 several new Triptan drugs that improve migraine pain within five minutes of consumption and complete relief after one or two hours have been discovered. Since 2003 several other triptan drugs have been tested out in clinical trials around the world (Mett and Tfelt-Hansen 2008) (Refer to Table 2). These selective serotonin receptor agonists (5 HT_{1B/1D}) have 3 distinctive pharmacological actions at sites implicated in the pathophysiology of migraine. These 5 HT_{1B/1D} agonists act on the 5-HT receptors to cause cranial vasoconstriction, inhibition of pro-inflammatory neuropeptide release by trigeminal nerves and inhibition of nociceptive neurotransmission (Ahn and Basbaum 2005). Although Triptans are well characterized and have high receptor specificity, in some patients they have resulted in recurring migraine pain after initial high efficacy (Smith, Sunshine et al. 2005; Brandes, Kudrow et al. 2007).
Preventive medications such as β-adrenoceptor- blockers, propanolol and metaprolol, the antiepileptic drug sodium valporate and calcium channel blockers such as verapamil, have been demonstrated to be effective (Ferrari 1998). Antidepressants, anti seizure drugs and supplements such as magnesium, coenzyme Q10, and the herbs Feverfew have also been used for reducing pain, frequency and duration of migraine. High doses of riboflavin showed promising results as a novel prophylactic anti-
migraine therapy in a recent trial as well (Schoenen, Jacquy et al. 1998). However, migraine prophylactics are only effective in about 55% of migraineurs with only 50% reduction in migraine frequency. The responses to these medications differ among migraineurs, in some producing adverse side effects (Welch 1993; Tfelt-Hansen and Brosen 2008).
Chapter 2

Migraine Genetics
2.1 Genetic Studies

Genetic epidemiological studies undertaken over the last few decades have convincingly demonstrated that both genetic and environmental factors underlie migraine (Ducros, Tournier-Lasserve et al. 2002). Studies have shown the risk of migraine to be significantly increased in first degree relatives of migraineurs compared to relatives of controls. Twin studies comparing concordance rates between monozygotic and dizygotic twins have been used to assess the role of environmental and genetic factors contributing to migraine (Ducros, Tournier-Lasserve et al. 2002). Many of these studies have produced inconsistent results, as they were performed before the introduction of the HCCIH criteria for diagnosing migraine and involved lack of separation between MA and MO. Two studies of a Danish twin cohort which adapted the HCCIH in diagnosing their participants found higher concordance among MZ than DZ twins for both MO and MA, indicating that genetic factors do contribute to both MO and MA (Wessman, Terwindt et al. 2007).

Russell et al studied the relative risk of MO and MA in relatives and spouses of migraine probands and found a different pattern of familial risk for both MO and MA. The first degree relatives of probands with MO had 1.86 times the risk of this disease compared to the general population and the first degree relatives of probands with MA had 3.79 times the risk of MA compared to the general population (Russell, Hilden et al. 1993; Russell, Iselius et al. 1996). Although these findings reaffirm that migraine indeed has familial aggregation, it does not ascertain that this familial aggregation could be due to genetic factors and thus inheritance. Specific environmental factors acting within a family could have influenced the observed familial aggregation (Montagna 2001). However when the authors further studied the comparative risk of migraine in spouses of migraine probands versus relatives of migraine probands who
share similar environment but have distinct genotypic backgrounds, no increased risk of migraine was found in spouses, implying familial aggregation to be hereditary rather than environmentally determined (Russell, Hilden et al. 1993; Russell, Iselius et al. 1996; Montagna 2001).

Further studies have also shown this genetic influence on migraine to be stronger in the case of MA, with a disease risk for first degree relatives ranging from 2 to 6 times that found in the general population, compared to MO. This findings suggest that a different aetiology may exist for MO and MA, with MO caused by both genetic and environmental factors and MA determined largely by genetic factors (Russell, Iselius et al. 1996). However there is increasing evidence for a high co-occurrence and symptomatic overlap of MO and MA in twins and their relatives as well (Ligthart, Boomsma et al. 2006). Thus overwhelming evidence from various genetic studies infers migraine to have a significant hereditary component. Whether MA and MO are aetiologically different cannot be determined with certainty.

2.2 Mode of Inheritance

Migraine pedigrees are constantly analysed to determine the genetic transmission pattern involved. Many such studies have found no consistent patterns or have concluded in favour of a multifactorial inheritance pattern, even in those families that have displayed an apparently autosomal dominant inheritance (Ulrich, Russell et al. 1997; Montagna 2008). A segregation analysis study undertaken on a large Danish population, which included 126 families with MO and 127 families with MA found both MA and MO to have a non-mendelian multifactorial mode of inheritance (Russell, Iselius et al. 1995). A Danish twin study proposed a combination of both additive genetic and environmental factors (Gervil, Ulrich et al. 1999; Gervil, Ulrich
Various other modes of suggested inheritance include autosomal dominant with reduced penetrance, autosomal recessive with 70% penetrance, polygenic and X-linked (Montagna 2004). These diverse modes of suggested inheritance reflect the complexity of migraine as a disorder. Nevertheless it is largely agreed upon that migraine has a multifactorial mode of inheritance influenced by both genetic and environmental factors (Ducros, Tournier-Lasserve et al. 2002).

### 2.3 Approaches in the search for Migraine genes

In non-mendelian diseases, the search for causative mutations can be approached by different strategies. In family studies, linkage analysis of localised markers is utilised to identify the genetic location of a disease gene. Discovery of the susceptibility locus is then followed by fine-mapping and positioned cloning approaches to enable the detection of the disease causing mutation (Ducros, Tournier-Lasserve et al. 2002).

Linkage analysis is based on the fact that genes that reside physically close on a chromosome are linked and remain linked during meiosis, and thus are often inherited jointly. Linkage analysis has been successfully used to identify pathogenetic mutations in diseases such as Cystic fibrosis, Huntington disease, Alzheimer and Parkinsons diseases (Pulst 1999). However it is much more difficult to estimate penetrance values and allele frequencies of the more complex non mendelian diseases using linkage analyses due to the genetic complexity and heterogeneity inherent in them. There have been several studies that have failed to find credible candidate genes or mutations despite finding evidence of linkage (Botstein and Risch 2003).
Association studies are another more commonly used approach for fine mapping complex diseases. Unlike linkage analysis, these studies do not have difficult methodological considerations and can be performed in both family and homogeneous population studies. The principle underlying association studies is the comparison of the allele frequency of a polymorphic genetic marker between a group of randomly selected diseased individuals (cases) and healthy individuals (controls). A significant difference in the frequency of the polymorphic genetic marker between the groups suggests that the polymorphism is located either within the susceptibility gene or is in linkage disequilibrium with the susceptibility gene (Ducros, Tournier-Lasserve et al. 2002). However association studies only reveal the link between polymorphisms and complex diseases and they do not provide any information on the normal function of the gene or its abnormal function leading to the disease being studied.

2.4 Molecular Genetics of Migraine and Related Conditions

Genetic approaches provide powerful tools for studying and identifying genes responsible for several mendelian disorders. However, it is still a challenge to identify susceptible genes for complex diseases such as migraine, due to its polygenic determination and the compounding influence of environmental factors (Wessman, Terwindt et al. 2007). At present, the type and number of genes involved in migraine is still unclear, although a number of genes have already being identified. Several researchers in their quest to find genes involved in migraine have focused on studying genes involved in familial hemiplegic migraine (FHM), a rare, severe autosomal dominant form of MA, as these genes may also be involved in other more common sub types of migraine, MA and MO (Ducros, Tournier-Lasserve et al. 2002).
A number of genes that cause related neurogenetic disorders with migraine symptoms have also been identified. These include mutations within the calcium channel gene CACNA1A that causes FHM1, episodic ataxia type 2 (EA2) and spinocerebellar ataxia type 6 (SCA6) and mutations in the Notch 3 gene resulting in Cerebral autosomal dominant arteriopathy and subcortical infarcts and leukoencephalopathy (CADASIL). Other genes implicated in migraine susceptibility include genetic variants that relate to neurotransmitter, hormonal and vascular function, including Dopamine Beta-Hydroxylase (DBH), Methylenetetrahydrofolate reductase (MTHFR), Angiotensin converting enzyme (ACE) and Estrogen (Supornsilpchai, Sanguanrangsirikul et al.) and Progesterone (PGR) hormone receptor genes.

2.4.1 Familial Hemiplegic Migraine

FHM is a severe subtype of MA and is the only variety of migraine to conform to a mendelian type of genetic transmission (Montagna 2001). FHM attacks are characterised by the presence of some degree of hemiparesis and motor weakness of variable intensity. Certain clinical features, headache characteristics and triggers are shared by both FHM and typical migraine (Joutel, Bousser et al. 1993). FHM is genetically heterogeneous and is the result of heterozygous mutations in exonic regions of three genes, CACNA1A on chromosome 19p13, ATP1A2 on chromosome 1q23 and SCN1A on chromosome 2q24 (Wessman, Terwindt et al. 2007) (refer to Figure 2.1).

Joutel et al mapped the first FHM locus on chromosome 19p13 in 1993. In 1996 Ophoff et al identified that a missense mutation in the gene CACNA1A, which encodes the α1 subunit of the neuronal P/Q type voltage-gated calcium channel CaV2.1, resulted in FHM (Ophoff, Terwindt et al. 1996). Different mutations in
CACNA1A have also been implicated in at least two other autosomal dominant neurological disorders: episodic ataxia type 2 (EA2) and spinocerebellar ataxia type 6 (SCA6) (Wessman, Terwindt et al. 2007). FHM1 accounts for about 50% of all FHM patients, and is implicated in all families with FHM and permanent cerebellar symptoms (Ducros, Tournier-Lasserve et al. 2002).

The CaV2.1 is responsible for presynaptic Ca\(^{2+}\) entry and the release of neurotransmitters into the synaptic cleft. FHM mutations within the CACNA1A gene produce gain-of function of the CaV2.1 channel (Tottene, Fellin et al. 2002). This enhanced channel activity at negative potential leads to an increased Ca\(^{2+}\) influx and facilitates \textit{in vivo} stimulation and propagation of cortical spreading depression (Peitroban et al. 2007). Although several studies have been conducted to understand the mechanisms leading from the different CACNA1A mutations, the complexity caused by these mutations in the human brain is still not entirely known. However, studies in CACNA1A knockout mice have demonstrated that the absence of the gene causes severe ataxia at birth and death within few days (Jun, Piedras-Renteria et al. 1999). Mice carrying different mutations within CACNA1A display tottering, leaner and rocker phenotypes (Zwingman, Neumann et al. 2001). At least 17 different mutations in CACNA1A causing FHM1 have been identified in 40 families and six patients worldwide.

The second locus, FHM2, on chromosome 1q21-31 was discovered in 1997 in three French families and in one family of German-Native American descent (Ophoff, van Eijk et al. 1994). Marconi and colleagues further narrowed the FHM2 locus to a 0.9Mb region on chromosome 1q23, which led to the identification of mutations in the ATP1A2 gene in two Italian families (Marconi, De Fusco et al. 2003). ATP1A2 gene encodes a transmembrane transporter, Na\(^+\)/K\(^+\)-ATPase. FHM2 mutations produce a
loss-of-function of a single allele of Na\(^+/\)K\(^+\)-ATPase, decreasing the removal of K\(^+\) and glutamate from the synaptic cleft (De Fusco, Marconi et al. 2003). So far, there are 29 different known mutations in ATP1A2 associated with FHM2, most of which are found in the membrane spanning regions 4 and 5.

The third FHM locus was recently identified on chromosome 2q24 (Dichgans, Freilinger et al. 2005). A heterozygous missense mutation (Gln1489Lys) in SCN1A, the gene encoding the \(\alpha1\) subunit of the neuronal voltage-gated sodium channel Na\(_{\text{V}1.1}\), was found in 3 German families with FHM (Wessman, Terwindt et al. 2007). This mutation which is located in the highly conserved hinged-lid domain of the protein, greatly hastens recovery from inactivation. The Na\(_{\text{V}1.1}\) channel plays an important role in generating action potential in neurons and it is thus hypothesised that the mutation Gln1489Lys results in hyper excitable neurons (Dichgans, Freilinger et al. 2005).

Figure 2.1: Functional roles of the proteins coded by known FHM genes within a glutamatergic synapse (Adapted from Wessman, Terwindt et al. 2007).
When an action potential reaches the presynaptic terminal, the voltage-gated calcium channels open and let Ca\(^{2+}\) ions enter the neuron and this releases glutamate into the synaptic cleft. The \textit{CACNA1A} (FHM1) gene encodes the \(\alpha_1\) subunit of Ca\(_{2.1}\) channels, and FHM mutations within this gene increase release of glutamate. \textit{ATP1A2} encodes an \(\alpha_2\) subunit of a Na\(^+/K^+\)-ATPase expressed on astrocytes, which clears extracellular potassium and produces a Na\(^+\) gradient that is used in the uptake of glutamate from the synaptic cleft. Mutations slow the clearance of glutamate and K\(^+\) ions. Mutations in \textit{SCN1A}, encoding the \(\alpha_1\) subunit of the neuronal voltage-gated sodium channel Na\(_{1.1}\), are essential in the generation and propagation of action potentials, and the FHM mutation seems to cause accelerated recovery of the channel from fast inactivation.

### 2.4.2 Episodic and Spinocerebellar Ataxias

Episodic ataxia also known as acetazolamide-responsive/hereditary paroxysmal cerebellar ataxia (APCA/HPCA) is characterized by recurrent episodes of cerebellar ataxia often followed by other symptoms of cerebellar dysfunctions (Alonso, Barros et al. 2003). The disease is heterogeneous and displays an autosomal dominant inheritance (Terwindt, Ophoff et al. 1998). There are six known loci for EA, with 4 of the genes are distinguished. Episodic ataxia type 1 (EA1) is caused by missense mutations in a potassium voltage gated channel gene on chromosome 12p14. Symptoms of EA1 include interictal myokymia and brief episodes of ataxia and dysarthria (Terwindt, Ophoff et al. 1998; Alonso, Barros et al. 2003).

Episodic ataxia type 2 (EA2) shows bouts of ataxia, nausea, vertigo, fatigue, dysarthria, and nystagmus. Symptoms can last from anywhere between a few hours to a few days and can be precipitated by emotional stress, physical stress, coffee or
alcohol (Gancher and Nutt 1986). EA2 was mapped to the same location on chromosome 19 as FHM. A total of 19 mutations have now been identified to cause EA2. About 50% of EA2 sufferers also have MA or MO and some present with hemiplegia (Balah, Yue et al. 1997; Jen, Wan et al. 2001; Jen, Kim et al. 2004). Albeit the clinical differences between FHM and EA2, there are also some similarities. Both disorders display migraine like symptoms, dysarthria and progressive ataxia. Due to this symptomatic overlap, patients are often tested for mutation analysis of the CACNA1A gene for both FHM and EA2 (Terwindt, Ophoff et al. 1998).

Spinocerebellar ataxias (SCA) are a dominantly inherited group of progressive degenerative disorders that are both clinically and genetically heterogeneous. In addition to late onset gait ataxia, SCA also display symptoms such as ophthalmoplegia, pyramidal and extrapyramidal signs, peripheral neuropathy, pigmentary retinopathy and dysarthria (Alonso, Barros et al. 2003). Thirty distinct genetic types of SCA exist and the underlying mutations in 14 of them have been discovered. The most prevalent SCA types worldwide would include SCA type 3 also known has Machado-Joseph disease, SCA1, SCA2, SCA6, SCA7 and SCA8 (Terwindt, Ophoff et al. 1998).

SCA6 is caused by an unstable polymorphic triplet expansion of the CAG repeat in exon 47 of the CACNA1A gene, linked to the same location on the chromosome 19p as FHM and EA2 (Alonso, Barros et al. 2003). These CAG repeat expansion lead to an elongated stretch of glutamine greater than 19 in the CaV2.1 channel. An age-dependant process coupled with accumulations of mutant CaV2.1 channels which cause ovular intracellular aggregations and early cell death, have been implicated to be the underlying pathogenesis of SCA6 (Ishikawa, Tanaka et al. 1997; Matsuyama, Kawakami et al. 1997).
2.4.3 CADASIL

CADASIL a common hereditary form of stroke is caused by mutations in the Notch 3 gene on chromosome 19p13 resulting in neuronal white matter abnormalities. Migraine is a clinical hallmark of CADASIL. CADASIL is a rare inherited disorder characterised by recurrent cerebral ischaemic strokes, dementia, and mood disorders with depression and in 30% of the patients by MA (Wang, Sharma et al. 2000). The Notch 3 gene encodes a large single-pass transmembrane protein expressed in the arterial vascular smooth muscle cells (Joutel, Vahedi et al. 1997; Gridley 2003). Notch 3 is 34 exons long with ninety three mutations and 5 small deletions leading to CADASIL identified to date. Most of these mutations are missense mutations leading to the loss or gain of a cysteine residue, suggesting the occurrence of abnormal disulphide bridging and protein misfolding (Federico, Bianchi et al. 2005).

Mutations clustering in exons 3 and 4 of the Notch 3 gene account for approximately 65% of the all CADASIL patients (Joutel, Vahedi et al. 1997). Exons 3 and 4 are routinely screened as first stage and if no mutations are found the rest of the gene is sequenced in most diagnostic labs. Whether mutations in such a rare monogenic condition account for more common phenotypes is still yet to be discovered partly due to the lack of fast, reliable and readily accessible screening tests (Wang, Sharma et al. 2000).

Apart from the known CADASIL causative mutations identified in the Notch 3 gene, some functional and non-functional polymorphisms in the sequence have also been identified. The role these polymorphisms play is still largely unclear. The most common polymorphism, T6746C, resulting in an amino acid substitution Val/Ala at residue 2223 is found in the intracellular domain of the Notch 3 gene. Although this
polymorphism has been suggested to directly affect the function of the Notch 3 gene, a recent study by Borroni and colleagues revealed no relation between this polymorphism and migraine (Borroni, Brambilla et al. 2006).

Schwaag et al investigated the role of two non amino acid changing polymorphisms C381T and G684A occurring in exons 3 and 4 respectively of the Notch 3 gene in 97 migraineurs. Results of this study revealed a significant association of genotype, as well as alleles, of the G684A polymorphism with migraine and particularly with MO. It is of interest that the association stems from patients suffering from MO while most CADASIL patients have MA (Schwaag, Evers et al. 2006). These studies were conducted in small groups of migraineurs of a particular ancestry; therefore further studies are necessary to evaluate these results before drawing a conclusion if the G684A polymorphism of Notch 3 gene is associated with migraine.

2.4.4 Dopamine Beta Hydroxylase (DBH) Gene and Migraine

In addition to the migraine related genes such as CACNA1A, ATP1A2 and Notch 3, a number of susceptibility gene variants for the more typical forms of migraine have also been identified. Based on pharmacological evidence, several genetic association studies have investigated a possible pathogenic role of the dopaminergic system in migraine (Blin, Azulay et al. 1991; Barbanti, Fabbrini et al. 2000). Studies of several dopamine receptor genes have been performed in different populations with some contradictory results. A positive link between the D2 dopamine receptor genes and MA and MO has been reported in association studies (Peroutka et al 1997; Del-Zompo, et al 1998). These findings further increased the interest to investigate the involvement of dopaminergic system in migraine.
Several polymorphisms in the dopamine-beta-hydroxylase (DBH) gene have also been implicated in typical migraine. The DBH gene has been localised to chromosome 9q34 and is made up of 12 exons. DBH is a key enzyme that catalyses the conversion of dopamine to norepinephrine. It therefore plays a vital role in both dopaminergic and noradrenergic system (Lea, Dohy et al. 2000). Elevated serum levels of DBH enzyme have been observed in migraineurs during a migraine attack (Anthony 1981). A significantly increased in DBH enzyme activity has also been noted in migraineurs during the headache-free interval (Gotoh, Kanda et al. 1976). These findings suggested that DBH is a good candidate gene to be investigated for its involvement in migraine pathophysiology.

Studies in an unrelated British population reported the first association between DBH alleles of a short tandem repeat (STR), and DBH plasma concentration in 1997 (Wei, Ramchand et al. 1997). Cubells et al confirmed this DBH (STR) and have also showed that a DBH promoter insertion/deletion polymorphism was associated with phenotypic variation in DBH activity in plasma (Cubells, van Kammen et al. 1998). Lea et al investigated the prevalence of different alleles of markers, the DBH STR and DBH insertion/ deletion in an unrelated case-control association study and a transmission/disequilibrium analysis of migraineurs from 82 migraine families. Both studies revealed a distortion of allele transmission of the DBH STR marker in individuals suffering from both MA and MO (Cubells, van Kammen et al. 1998; Lea, Dohy et al. 2000).

More recently an association analysis performed in a larger population of case-controls examining DBH insertion/deletion found a significant association between the polymorphism and migraine, particularly in MA (Fernandez, Lea et al. 2006). Zabetian et al reported a single 10-kb block beginning from the insertion deletion
marker which houses the single nucleotide polymorphism (SNP) -1021 C to T, in the DBH gene to be linked to the phenotype of the enzyme (Zabetian, Buxbaum et al. 2003). An intragenic non-synonymous SNP polymorphism (+1603 C to T) indentified in exon 11 of the DBH gene has also been associated with the phenotype of the enzyme (Tang, Anderson et al. 2005). Taken together these findings clearly emphasise the importance of DBH and its significant association with migraine.

2.4.5 Serotonin related genes and Migraine

Several studies have implicated the serotonin system to play an important role in migraine pathophysiology. First, drugs known to facilitate 5-HT release are able to induce migraine attacks (Panconesi and Sicuteri 1997). In addition, 5-HT releasing agents and certain 5-HT receptor agonists have the ability to abort and treat migraine headaches (Silberstein 1994; Kim et al. 2005). 5-HT receptor antagonists such as ergotamine, methysergide and amntriptyline are also prescribed as antimigraine drugs (Schmuck, Ullmer et al. 1996; Porter, Benwell et al. 1999; Schaerlingler, Hickel et al. 2003). Furthermore, low 5-HT blood levels predispose to CSD in rats, the suggested event during a migraine episode (Supornsilpchai, Sanguanrangsirikul et al. 2006). Albeit the controversy, most studies suggest altered 5-HT levels in migraineurs both during attacks and interictally (Ferrari and Saxena 1993; Hamel 2007; Corominas, Sobrido et al.). Thus several studies in different populations have focused on investigating potentially dysfunctional genes in the serotoninergic pathway as potential candidates contributing to migraine susceptibility.

Polymorphisms within genes encoding the 5-HT transporter (SCL6A4), several 5-HT receptors (HTR1A, HTR1B, HTR1D, HTR2A, HTR2C) and the enzymes involved in 5-HT synthesis or degradation tryptophan hydroxylase (TPH), monoamine oxidase A
and B (MAOA, MAOB) and l-dopadecarboxylase (DDC), have been investigated in several populations and have provided conflicting results (Corominas, Sobrido et al.). Buchwalder et al investigated the role of 5HT2A and HT2C in migraine by linkage analysis in 18 pedigrees and reported no association between the 5-HT receptors and migraine (Buchwalder, Welch et al. 1996). Similarly Johnson et al reported no association between 5-HT2C and migraine using both linkage and association study approach (Johnson, Lea et al. 2003). Racchi et al investigated 5-HT1B/1D and 5-HT2C polymorphisms in MA and failed to find an evidence for a role of these polymorphisms in migraine (Racchi, Leone et al. 2004). Erdal et al reported a positive association between 102T/C polymorphism of the 5-HT2A and MA in a small population of 105 participants. However the results were not replicated in another study that investigated the same locus in a larger population (Erdal, Herken et al. 2001).

A recent association study of the serotonergic system and migraine in a Spanish population revealed risk haplotypes in three genes significantly associated with the migraine phenotype or with MO or MA subtype. Two marker risk haplotypes were identified in the HTR2B and MAOA genes conferring susceptibility to MO (Corominas, Sobrido et al. 2010). This receptor has been previously suggested to be implicated in migraine, although no case-control study has been performed (Panconesi and Sicuteri 1997). The 5-HT2B receptors located on endothelial cells of meningeal blood vessel were hypothesised to trigger migraine headache through nitric oxide dependent mechanism (Schmuck, Ullmer et al. 1996; Corominas, Sobrido et al. 2010). Three independent association studies have previously investigated the involvement of a functional variable number tandem repeat (VNTR) polymorphism in the promoter region of MAOA in migraine and only one identified a trend towards significance in a small subset of males with MO (Marziniak, Mossner et al. 2004;
Filic, Vladic et al. 2005; Johnson and Griffiths 2005). MAO inhibitors though not frequently, are still used to treat migraineurs and have been reported to be effective in some, supporting a role for MAOA in migraine susceptibility (Rapoport 2008; Corominas, Sobrido et al. 2010).

Additionally a four marker haplotype in DDC specific for MA was also reported in the study by Corominas et al (Corominas, Sobrido et al. 2010). This result was contrary to an earlier study that used a two - stage DNA pooling design to screen an insertion/deletion in the 5’UTR region of DDC and found no association with migraine (Johnson and Griffiths 2005). However this study did not analyse MA and MO patients separately. The results reported by Corominas et al suggest differential involvement of the serotonin related genes in the pathogenesis of MO and MA. However the study has to be repeated in another case - control data sets and family data set from another population to confirm the findings (Corominas, Sobrido et al. 2010).

**2.4.6 Hormone Receptor Genes and Migraine**

Prior to puberty migraine occurrence in both genders does not vary significantly. However after puberty, migraine prevalence in woman is more than three folds compared to that of men (Lipton and Stewart 1997). Migraine frequency and intensity have been shown to be influenced by hormonal events such as menstruation, pregnancy and menopause. Steroid hormones, oestrogen and progesterone, modulators of the menstrual cycle have thus been considered a triggering factor involved in the onset of many migraine attacks (Colson, Lea et al. 2006).
Both hormones play an important role in the central nervous system through their cognate receptors. Steroid hormone receptors initiate signal transduction for steroid hormones that lead to changes in gene expression by interacting with hormone response elements in the promoter region of genes. Steroid hormone receptors also interact with neurotransmitters and are involved in regulating ion channels (Cenni and Picard 1999; Kelly and Levin 2001). Ion channels are important factors involved in the mechanism of neurotransmitter release and cortical spreading depression phenomenon. As the hormonal milieu in humans is influenced by hormone receptors, it is thus reasonable to postulate that genetic variation in hormone receptor genes may also be involved in migraine susceptibility (Colson, Lea et al. 2006).

**Oestrogen receptor 1**

The Oestrogen receptor gene 1 (ESR1) which is mapped to chromosome 6q25.1 has been identified in different regions of the brain including the hypothalamus, hippocampus and the brain stem, as well as in other tissues (Osterlund, Grandien et al. 2000). There is a common synonymous polymorphism which consists of a guanine to adenine change at nucleotide 2014 in codon 594 of exon 8. Interestingly this polymorphism has also been associated with breast cancer, another disease affected by hormonal levels (Roodi, Bailey et al. 1995). Colson et al performed an association analyses of the ESR1, investigating the common synonymous polymorphism in a migraine case - control cohort of European descent in Australia. The results of this study revealed a significant association between ESR1 and migraine with statistical difference seen between case and control groups, in sub-categories of the test population including MA and MO and in a second independent case-control population (Colson, Lea et al. 2004).
**Progesterone receptor**

The progesterone receptor also known as nuclear receptor subfamily 3, group C, member 3 (NR3C3) is a steroid receptor encoded by the progesterone receptor gene (PGR). Human PGR resides on chromosome 11q22 and is expressed in many parts of the human brain including serotonergic neurons (Bethea, Lu et al. 2002). The progesterone ligand binds to PGR and dimerizes to form a complex that works as a transcription factor and controls the expression of genes necessary for mammary cell growth and differentiation (Cenni and Picard 1999). A 306bp Alu insertion occurs within intron 7 of PGR in some individuals (Rowe, Coughlan et al. 1995). A valine to leucine substitution in exon 4 and a synonymous C to T substitution in exon 5 of the receptor are linked to the Alu insertion (Rowe, Coughlan et al. 1995; Wang-Gohrke, Chang-Claude et al. 2000). These PGR polymorphisms termed the PROGINS are considered to have a deleterious effect on PGR expression, through recombination or mis-splicing (Donaldson, Crapanzano et al. 2002).

A positive association between the PGR PROGINS insert and migraine in two independent test populations was reported in 2005 by Colson et al. Results of this study revealed that individuals carrying the PROGINS insert were twice as likely to suffer from migraine than those who did not. Additional analyses of the PGR PROGINS polymorphism in combination with ESR revealed that carriers of at least one copy each of both the ESR 1 594 allele and the PGR PROGINS allele were approximately three times more likely to suffer from migraine than those who carried no copies from both alleles, suggesting that that both these variants may work synergistically to increase the risk of migraine (Colson, Lea et al. 2005). It is thus plausible that the presence of these hormone receptor gene variants may have implications to the use of hormonal treatments and factors that affect hormonal levels in some migraineurs.
2.4.7 Role of vascular genes in migraine susceptibility

While the role of vascular dysfunction in migraine is debatable, alterations in vascular function and cerebral blood flow has been identified in migraineurs (Silvestrini, Cupini et al. 1995). This has led to the exploration of genes involved in vascular functioning in search for migraine susceptibility genes. The study of vascular related genes in migraine identified a role for the Angiotensin I-converting enzyme (ACE) gene. ACE is an exopeptidase that is expressed in a wide variety of tissues including vascular endothelial cells and it catalyses the conversion of the decapeptide angiotensin I to the vasoconstricting octapeptide angiotensin II (Colson, Lea et al. 2006). ACE has long been known to play a vital role in the rennin angiotensin system that regulates blood pressure, and ACE inhibitors are used in treatment of hypertensive individuals. An insertion (I) or deletion (D) of a 278bp Alu sequence occurring with intron 16 of the ACE gene forms three possible genotypes: II, ID or DD. Genetically determined serum ACE levels have been reported to be influenced by ACE I/D polymorphism (Rigat 1990).

Table 2.1: ACE genotype and allele distributions among controls and migraineurs in different studies

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>N</th>
<th>DD(%)</th>
<th>ID(%)</th>
<th>II(%)</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paterna</td>
<td>201</td>
<td>75 (37.3)</td>
<td>101 (50.3)</td>
<td>25 (12.4)</td>
<td>251 (62.4)</td>
</tr>
<tr>
<td>Kowa</td>
<td>248</td>
<td>31 (12.5)</td>
<td>114 (46.0)</td>
<td>103 (41.5)</td>
<td>176 (35.5)</td>
</tr>
<tr>
<td>Lea</td>
<td>244</td>
<td>76 (31.1)</td>
<td>122 (50.0)</td>
<td>46 (18.9)</td>
<td>274 (56.1)</td>
</tr>
<tr>
<td>Migraine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paterna</td>
<td>302</td>
<td>146 (48.3)</td>
<td>129 (42.7)</td>
<td>27 (9.0)</td>
<td>421 (69.7)</td>
</tr>
<tr>
<td>Kowa</td>
<td>176</td>
<td>33 (18.7)</td>
<td>86 (48.9)</td>
<td>57 (32.4)</td>
<td>152 (43.2)</td>
</tr>
<tr>
<td>Lea</td>
<td>250</td>
<td>77 (30.8)</td>
<td>142 (56.8)</td>
<td>31 (12.4)</td>
<td>296 (59.2)</td>
</tr>
<tr>
<td>MwA subgroup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paterna</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Kowa</td>
<td>54</td>
<td>14 (25.9)*</td>
<td>26 (48.2)</td>
<td>14 (25.9)</td>
<td>54 (50.0)*</td>
</tr>
<tr>
<td>Lea</td>
<td>151</td>
<td>48 (31.8)</td>
<td>85 (56.3)</td>
<td>18 (11.9)</td>
<td>181 (59.9)</td>
</tr>
<tr>
<td>MoA subgroup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paterna</td>
<td>302</td>
<td>146 (48.3)*</td>
<td>129 (42.7)</td>
<td>27 (9.0)</td>
<td>421 (69.7)</td>
</tr>
<tr>
<td>Kowa</td>
<td>122</td>
<td>19 (15.6)</td>
<td>60 (49.2)</td>
<td>43 (35.2)</td>
<td>98 (35.2)</td>
</tr>
<tr>
<td>Lea</td>
<td>99</td>
<td>29 (29.3)</td>
<td>57 (57.6)</td>
<td>13 (13.1)</td>
<td>115 (58.1)</td>
</tr>
</tbody>
</table>

* Reported significant finding for genotype or allele frequencies
Clinical studies have provided evidence for several ACE inhibitors and angiotensin II receptor blockers to have an important prophylactic effect on migraine. Two independent studies by Paterna et al have both suggested a link between ACE-D genotype and MO (Paterna 1997; Paterna 2000; Paterna, Di Pasquale et al. 2000). Kowa et al investigated the role of ACE polymorphism in headache and found the ACE-D genotype to be a genetic risk factor of MA in the Japanese population (Kowa, Fusayasu et al. 2005). Lea et al has also shown a significant over representation of the ACE-D genotype in migraineurs compared to controls (refer to Table 2.1). Interestingly the same study also provided good evidence that the MTHFR and ACE genotypes act synergistically to further increase migraine susceptibility (Lea 2005).
Chapter 3

Research Methodologies
3.1 Study Design

The main objective of this study was to focus on the molecular genetics of migraine in an Australian cohort. This research was specifically aimed at studying possible pharmacogenetic relationships with a view to develop personalised treatments for migraineurs based on migraine candidate gene information. The study also aimed to identify novel candidate genes involved in migraine. The first objective of this study aimed to investigate genotype-phenotype correlations between the MTHFR C677T SNP and migraine clinical profiles. This study was conducted on migraineurs, who were drawn from two large age and sex, matched unrelated case/control populations that were previously recruited by the GRC from the east coast of Australia. Secondly, the genotypic effects of the MTHFRC677T and MTRRA66G on vitamin supplementation response in migraineurs was investigated by conducting a randomised double blind clinical trial of daily vitamin supplementation over a six month period in an Australian cohort. In addition to the clinical trial, this study also investigated markers within potential migraine candidate genes- CACNA1A, Notch 3 and OPRM-1 for involvement in migraine susceptibility and disability.

All participants recruited for this study were clinically diagnosed with MA or MO based on the criteria specified by the International Headache Society. The participants’ DNA was extracted using standard techniques. All marker alleles were amplified by PCR and discrimination of fragments was determined using either RFLP or HRM or fragment analysis techniques. The chi-square test was used to analyse all potential relationships between the MTHFR C677T genotype and migraine clinical variables. Regression analyses were performed to assess the association of C677T with all migraine clinical variables after adjusting for gender.
The resulting MTHFR C677T and MTRR A66G genotype data and the clinical outcomes from the clinical trial were analysed using parametric and non parametric statistical methods such as sample T-tests and Pearson’s correlation to identify the genotypic effects of the two variants on treatment response in homocysteine lowering and migraine disability. Statistical methods such as chi-square and CLUMP were used to determine if the investigated SNPs in the candidate genes, Notch 3 CACNA1A and OPIOID contributed to migraine susceptibility and migraine disability.

3.2 Ethical approval

Approval by the Griffith University Ethics Committee for experimentation on human subjects was obtained before the commencement of this research.

3.3 Subject ascertainment and diagnosis

3.3.1 Diagnosis

All participants in this study were of Caucasian origin recruited from the East Coast of Australia. Participants were interviewed and asked to complete a detailed questionnaire that was administered through Griffith University’s Genomic Research Centre (GRC), providing information including personal and family medical history, migraine symptoms, age of onset, frequency, severity and migraine treatment as previously described (Colson, Lea et al. 2004; Lea, Ovcarić et al. 2005). Those suffering from migraine were diagnosed as having MA or MO through interview by an experienced neurologist and/or in combination with their answers to the questionnaire that was prepared using the International Headache Society (IHS) criteria (HCCIHS 2004).
3.3.2 Clinical trial participants

Females suffering from MA were recruited for the clinical trial through a combination of local and national media coverage. Participants were included if they had suffered migraine for over 20 years and had a current diagnosis of MA (>90% of their migraine attacks were associated with aura), and a 1-year history of severe, long lasting attacks (at least 4 attacks lasting more than 48h), had a family history of migraine. Patients were excluded if they were currently taking vitamin supplementation, were pregnant or had been diagnosed with a clinically recognized cardiovascular or neuropsychiatric condition. The patient group was not selected on the basis of pre-existing folic acid, B12 or B6 deficiency or the MTHFR C677T genotype.

3.3.3 Unrelated case control samples

In total ~600 cases and an equivalent number of controls were collected over a period of several years by the Genomics Research Centre. The first study population collected comprised of 275 migraineurs and 275 matched controls and was called Migraine Population 1 (MAP 1). The follow-up second study population consisting of 300 migraineurs and 300 controls were collected later. The DNA was prepared as a second independent population and was called Migraine Population 2 (MAP 2) (Fernandez, Lea et al. 2006). The migraine case population had a median age of onset of ~19 years and average headache duration of ~20 hours with frequency of approximately ~30 per year. 90% of the migraine case population had a known family history of migraine or at least one - first degree relative suffering from the disorder. The control population was recruited from the same geographical location as the case population and was carefully matched to the case samples by age (+/- 5 years), sex and ethnicity.
3.4 DNA extraction and quantification

3.4.1 DNA extraction

DNA was extracted from 2ml blood samples using the either QIAGEN DNeasy Blood & Tissue Kit according to the protocol as detailed in Appendix A or by a modification of the salting out method salting used by Miller et al (1988) as detailed in Appendix B (Miller, Dykes et al. 1988)

3.4.2 DNA purification and quantification

200ul of the extracted DNA was added to 20ul of 3M sodium acetate and 400ul of absolute ethanol. To allow precipitation to occur the samples were stored in -80C for 2 hours. Centrifugation was then performed at 10 000rpm for 15mins at 4C and the supernatants were carefully removed from each sample. The pellet was resuspended in 70% ethanol and centrifuged again with the same specifications as described above. The supernatant was removed and the pellet was resuspended in 1ml of sterile water. The ug of DNA was quantified using a Nanodrop $^\text{TM}$ spectrophotometer to determine the optical density wavelength of 260nm, with an optical density (OD) of 1.0 representing 50ug/ml of double stranded DNA and diluted to a standard concentration of 20ng/ul. The diluted DNA was stored in 4 C until use.

3.5 Genotyping Overview

3.5.1 Primer Design

Primers obtained from published assays and designed using Primer 3 software, were checked for homology and specificity to the genomic regions of interest by using the Basic Local Alignment Search Tool (BLAST) (available at http://www.ncbi.nlm.nih.gov/BLAST/). All primers were obtained from Integrated
DNA technologies Inc, Australia and were provided either as a lyophilised pellet or in a concentrated liquid form. Primers were diluted to a working concentration of 5µM with Dnase free sterile water.

3.5.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to amplify specific genomics regions of interest. PCR amplifies a piece of DNA by *in vitro* enzymatic replication by using specific oligonucleotide primers flanking the region of interest, PCR buffers, magnesium chloride (MgCl₂), deoxynucleotide triphosphates and thermostable DNA polymerase. The PCR reaction was carried out in a final volume of approximately 20 to 25µl containing approximately 20ng of DNA, 5µm primer mix, 2.5mM dNTPs, 1.75mM MgCl₂, 10x PCR amplification buffer, 1 unit/µl of TAQ polymerase and sterile water. The PCR was then put through under the assumed following thermal cycle conditions. The PCR mix went through an initial heating stage at 94°C and held at 3mins, following which 35 cycles of denaturation, annealing and extension at 94°C for 15sec, 55°C for 15sec and 72°C for 30sec respectively was implemented. At the final stage each reaction was held at 72°C for 10mins and allowed to cool to 4°C. PCR conditions specific for each analysis fragment are detailed in the respective results chapters.

3.5.3 Agarose Gel electrophoresis

Gel electrophoresis allows the separation and identification of DNA fragments according to size, by moving negatively charged nucleic acid molecules through the pores of an agarose matrix to the positive node. Shorter fragments migrate faster and farther down the matrix than longer fragments. The location of the distinctive bands the DNA fragments form on the agarose gel is visualized by staining the gel with a
fluorescently intercalating dye such as ethidium bromide and viewed under a UV light source (refer to Figure 3.1). In the current study all PCR products were separated using agarose gel electrophoresis to identify if the right sized amplicon dictated by the primer sequences has been amplified by the PCR reaction. Furthermore, all products resulting from the RFLP technique were also subjected to this method to indentify the cleavage or lack of by the different restriction enzymes used for the different SNPs investigated in the current study.

**Figure 3.1: An example of an agarose gel stained with Ethidium bromide** after electrophoresis and visualised under the UV light, showing different sized bands fractionated on the gel.

### 3.6 Mutation screening

#### 3.6.1 Restriction Enzyme Digestion

Restriction enzyme digestion is the method of cleaving double stranded or single stranded DNA at specific sites called recognition sites using an enzyme. Restriction enzymes are largely isolated from bacteria in which they appear to play a host defence role by cleaving and inactivating foreign DNA within the bacterium. Polymorphisms, mutations, deletions or insertions in a DNA sequence which may result in a change in an enzyme restriction site can thus be identified by the success or failure of cleavage by the restriction enzyme. The resulting fragments of varying sizes may then be detected by either gel or capillary electrophoresis. This technique is termed
Restriction Fragment Length Polymorphism (RFLP). RFLP technique was used to identify variants alleles in the MTHFR, MTRR, Notch 3 and Opioid gene. All restriction enzymes and the appropriate buffers used were provided by Integrated DNA Technologies Inc (Linnebank, Schmidt et al.). Specific RFLP reaction conditions used for each SNP investigated in this study are detailed in the methods section of the different chapters. In general the reaction conditions were optimised using sufficient amount of the appropriate enzyme and buffer at 1x concentration to obtain complete exonuclease activity in the recommended time.

3.6.2 Fragment analysis – Genemapper

One of the major limitations with the use of gel electrophoresis is the difficulty in sizing PCR products that vary by only a few base pairs. This limitation could for example make the calling of a heterozygote genotype with alleles 3 bps apart from a homozygote genotype which has alleles of the same size, a very difficult task.

Ziegle et al first introduced in the 1992, the fluorescent based DNA sizing technology for genotyping microsatellite markers (Ziegle, Su et al. 1992). In this method target sequences are PCR amplified using fluorescently-labelled primers and products are detected by a laser after capillary electrophoresis. DNA sequences of defined length are used as internal size standards for a size calibration curve, which is then used to determine the length of labelled PCR product (Ziegle, Su et al. 1992). Internal size standards consist of fragments of known sizes, which are added and electrophoresed with every DNA sample to be investigated using capillary electrophoresis. The current study incorporated this technology by using the Applied Biosystems 3130 Genetic Analyser and the GeneMapper® Analysis Software.
GeneMapper® software is an analytical tool that creates a size calibration curve based on the mobility of all the known fragments incorporated in the internal size standard. The internal size standard used also minimise capillary-to-capillary and run-to-run variability. The length of the labelled PCR products is then automatically determined using this calibration curve and each peak is assigned a defined size. The GeneMapper® analysis tool has been routinely utilised for amplified fragment length polymorphisms (AFLP), detecting microsatellite instability of replication error (RER), determining loss of heterozygosity (LOH), and screening for single nucleotide polymorphisms (SNP) using single strand conformation polymorphisms (SSCP) or fluorescent allele specific PCR.

3.6.3 High Resolution Melt

High resolution melt (HRM) technique is a homogenous, mutation or polymorphism detecting post-PCR technique. The inclusion of an intercalating dye to the PCR mix and adding a HRM step after the PCR allows samples to be discriminated according to their sequence length, GC content and most importantly even single base changes can be detected. HRM is able to detect sequence variation by monitoring the progressive change in fluorescence caused by the release of the intercalating dye from a DNA duplex as it is being denatured by marginal increase in temperature (Krypuy, Ahmed et al. 2007). The melting behaviour of the PCR amplicon changes in the presence of even a single base change. The difference in the melt curves of the PCR amplicon is used to determine the possible genotype of the specific sample (Figure 8).

HRM analysis was performed using a Rotorgene 6000 Real-Time PCR machine. In HRM analysis, the temperature of the PCR products was raised from 80-85°C in 0.1°C increments, with a two-second hold at each increment. The high-resolution melt
curves were analysed using Rotor-gene series 6000 software version 1.7. In each analysis, the melt curve of a representative of each identified genotype was used as a control. A melt-curve profile for each unknown sample was then generated by graphing the normalised fluorescence emitted by each sample against temperature (refer to Figure 3.2). Following this, a confidence parameter

\[ C = 1.05^{e^{-0.3T_s}} \]

where \( S = \sum_{i=1}^{n} (\text{Unknown}_i - \text{Control}_i)^2 \), with \( T_s \) – start temperature, \( T_e \) – end temperature, and \( \text{Unknown}_i \) and \( \text{Control}_i \) being the height of the respective curves at temperature \( i \).

If the highest \( C \) for an unknown sample with respect to a control was \( \geq \) a given threshold, this unknown sample was allocated to the genotype corresponding to that control. If the highest \( C \) for an unknown sample was \( < \) the given threshold in relation to any control, the unknown was not assigned to a genotype.

Figure 3.2: An example of a melt curve profile of DNA samples after HRM. A melt curve for each sample was generated by plotting the normalised fluorescence
emitted by each sample against temperature. The three distinctive melt curves represent the 3 different genotypes detected in the set of samples tested.

### 3.6.4 DNA Sequencing

DNA sequencing determines the exact order of bases, A, T, C, G in a piece of DNA by using the dideoxy or chain termination method developed by Fred Sanger in 1977. New strands of DNA are synthesised on a single stranded DNA template of interest by primers. The primers used will determine the region of the sequence being read and the direction of the sequencing reaction. Sequencing is achieved by including dideoxy nucleotides in each reaction that cannot be extended and thus acts as a chain terminator. Since only low concentrations of dideoxy nucleotides are included, incorporation into the new DNA strand is a random event.

In automated sequencing each of the dideoxy nucleotides is labeled with a different coloured fluorescent dye. Each reaction therefore produces a set of differentially labeled DNA fragments of differing length by one nucleotide. The fluorescent signal of the electrophoretically resolved DNA fragments are then detected and the base is identified. The output results come in the form of a chromatogram as seen in Figure 3.3 and they are analyzed by specific software that are designed to interpret the resultant chromatogram files and generate reports. The base sequence of the DNA region of interest determined by sequencing is then compared to known wild-type sequences available in public database’s such as Genbank for the presence of mutations.
Automated sequencing and high-powered computer facilities has enabled the production of large amounts of high quality sequence data that can be analysed efficiently and the discovery of universal sequence primers has made it possible to sequence most taxa without prior knowledge of their sequence. Although DNA sequencing is accurate it is also a time consuming and expensive method of diagnosis. Furthermore, not all DNA samples can be sequenced with ease. Some samples do not sequence well due to the occurrence of secondary structures. While single or low copy number genes may need cloning prior to sequence analysis.

### 3.7 Statistic Analysis

#### 3.7.1 Intention- to- treat principle

Sir Austin Bradford first suggested in 1961 that the exclusion of participants after they have been randomly allocated to the different treatment groups, could affect the validity that randomisation aims to provide (Gravel, Opatrny et al. 2007). Over the past century randomised clinical trials and statistical analysis methods such as Intention-to-treat (ITT) have transformed scientific investigations. The ITT principle is defined as “The analysis of all randomised patients in the groups to which they were randomly assigned, regardless of whether they actually received treatment, and
regardless of subsequent withdrawal from treatment or deviation from the protocol” (Fisher, Dixon et al. 1990).

The ITT principle has two main objectives. Firstly the principle assures the similarity of the treatment groups apart from random variation (Hollis and Campbell 1999). The reason for random allocation is to ensure that trial participants’ risk factors that may potentially affect outcome of the trial are balanced between the studied treatments. This thus ensures that any outcome difference observed between the studied treatment groups could be attributed to the trial intervention (Heritier, Gebski et al. 2003). Secondly, ITT allows for non-compliance and deviations from protocol. Some deviations from randomised allocation may only occur within trial setting and would not be expected in clinical practice. However most types of deviations can be expected to occur in clinical practice and should be included when studying the effect of any treatment or intervention in a clinical trial setting (Hollis and Campbell 1999; Heritier, Gebski et al. 2003).

The complete ITT approach is only possible when complete outcome data are available for all randomised participants of the trial (Hollis and Campbell 1999). However the reality of clinical trials is that there will usually be deviations from protocol and non-compliers resulting in missing outcome. There are a number of strategies that have been adopted if the assumptions underpinning ITT are not satisfied (Heritier, Gebski et al. 2003). The Per-protocol (PP) or On-treatment analysis only includes participants who have sufficiently complied with the trials protocol. Compliance usually covers exposure to treatment, availability of outcome data and absence of major protocol deviation. This approach however would introduce bias related to exclusion of participants from analysis (Heritier, Gebski et al. 2003; Blumberg, Zhao et al. 2007). The other alternative is the Treatment-received (TR)
analysis, which analyses all the participants according to the treatment they received, regardless of what treatment group they were originally randomly assigned to. However, again this approach compromises the effect of randomisation and affects the assumptions underlying the statistical analysis, resulting in the difficulty of result interpretation (Heritier, Gebski et al. 2003).

The use of a modified or quasi ITT population, allowing for certain exclusions has also been introduced (Heritier, Gebski et al. 2003). This approach suggests that criteria from exclusion from analysis should be pre specified in the protocol and that researchers should be blinded to (i) treatment allocation and (ii) on the basis of information not related to allocated treatment or events or outcomes that occur after random allocation (Heritier, Gebski et al. 2003). The modified or quasi ITT population could potentially be beneficial when outcomes are not available for all participants (Heritier, Gebski et al. 2003). There is no consensus about how missing data should be treated and different studies have adopted different approaches when analysing such a data outcome (Hollis and Campbell 1999). There exists several definitions of the ITT approach and no single or excessively rigid approach is going to give the absolute credible clinical results. Considering these limitations, clinical research results should be treated as an imperfect approximation of what any treatment or intervention outcome will be in clinical practice (Blumberg, Zhao et al. 2007).

3.7.2 Parametric and Non parametric tests

Parametric and non parametric tests are two statistical tests available to analyse continuous outcome variables. Both of these tests were applied to assess the baseline
group means of the variable in the migraine clinical trial. The parametric tests require the observation within each group to be approximately normally distributed. If the observations are independent and the variables under study have underlying continuity the non-parametric tests is used. There is at least one non-parametric test equivalent to each parametric test. The 1-sample t-test is a parametric test that analyses whether the mean of a single variable differs from a specified constant (Chan 2003). If the normality distributions are not satisfied then the equivalent non-parametric sign test or the Wilcoxon Signed Rank test would be applied (Chan 2003). The Wilcoxon signed rank test is applied when testing the median difference in paired data. Paired data refers to the values in the two groups being compared as naturally linked and usually arise from groups being measured more than once. The test measures the magnitude of difference between the pairs of observations and thus the actual data values are measured on an interval scale similarly to the t-test (Conover 1980; Bland 1995).

The 2 sample T-test is an example of a parametric test used when comparing means between groups. The three assumptions of the 2-sample T-tear are firstly that the data is normally distributed, secondly, that the population variance are equal and thirdly that the 2 groups are independent random samples. When normality assumptions are violated for any or both the groups, the equivalent non-parametric Mann Whitney U test is applied. This test requires two independent groups of observations and is based on ordering and ranking of the data. The Mann Whitney test analyses the degree of separation or the amount of overlap between the experimental and control groups (Altman 1991; Conover 1980).

The Mann Whitney U statistic is defined as:

\[ U = n_1n_2 + \frac{n_1(n_1 + 1)}{2} - \sum_{i=n_1+1}^{n_2} R_i \]
Where samples of size $n_1$ and $n_2$ are pooled and $R_i$ are the ranks. $U$ can be resolved as the number of times observations in one sample precede observations in the other sample in the ranking (Altman 1991; Conover 1980).

3.7.2.1 Analysis of Variance (ANOVA)

Analysis of variance (ANOVA) is an expansion of the 2-sample T test and is applied when more than 2 groups are to be compared. ANOVA was first developed by Sir Ronald A. Fisher in the 1920. It is thus also known as the Fisher’s analysis of variance of Fisher’s ANOVA (Hinkelmann and Kempthorne 2008). A one-way ANOVA is applied when the experiment only involves a single factor. A two-way or multiple ANOVA is relevant when two or more factors are involved. A Factorial ANOVA is considered when there is replication at each combination of levels in a two way ANOVA. A Mixed-design ANOVA is applied when comparing a between-subjects variable and a within subjects variable. A Multivariate analysis of variance (MANOVA) is used when more than one independent variable is involved in the analysis. The three basic assumptions for the 2-sample T test also apply for the ANOVA (i) Cases are independent (ii) data are normally distributed in each of the groups (aimie D. Vaughn) homogeneity of variance. The tests in ANOVA are based on the F ratio: MSB or mean square between which is based on the variance of the sample means divided by MSE or mean square error which is based on the variance within samples (Freedman 2007; Hinkelmann and Kempthorne 2008).

$$F = \frac{MS_{(Between)}}{MS_{(error)}}$$

The null hypothesis of identical means, the value of F-ratio is ideally 1. Since variances are always positive if the null hypothesis is rejected than MSB is expected
to be larger than MSE and the F ratio will be more than 1 (Freedman 2007; Hinkelmann and Kempthorne 2008).

### 3.7.2.2 Pearson’s Correlation test

Pearson’s correlation is the method of measuring the correlation between at least two continuous variables. The relationship among biochemical variables in the migraine clinical trial was assessed using the Pearson’s correlation test. The Pearson’s correlation test is calculated by dividing the covariance of the two variables by the product of their standard deviation (Bland 1995; Freedman 2007).

\[
= \frac{\text{SUM}((x_i - \text{xbar})(y - \text{ybar}))}{((n - 1) * s_x * s_y)}
\]

Where x and y are the variables, \(x_i\) is a single value of x, \(x\text{bar}\) is the mean of all x's, n is the number of variables, and \(s_x\) is the standard deviation of all x's (Freedman 2007).

A coefficient value between 0.75 and 1 is considered high degree of correlation and a coefficient value between 0.25 and 0.75 is termed as moderate degree and a coefficient value below 1 is considered as no correlation.

Pearson’s correlation is a parametric test that assumes that the data is normally distributed, both variables are present at least at interval level or ratio and that a linear relationship is present between the two variables. This method not only provides information about the degree but it also provides information on the direction of correlation (Freedman 2007).

### 3.7.3 Association Analysis

#### 3.7.3.1 Power analysis
Power analysis is an important aspect of all studies involving a null hypothesis. It is defined as the probability of rejecting the null hypothesis when it is false. Power analysis is detrimental in association studies as it allows us to determine the sample size required in a study to maximise accurately detecting an effect (Schork 2002). In association studies, the extent of the effect that a risk allele is suspected to have on the disease, determines the power. This is often calculated as relative risk. The scale of the difference in the frequency of the risk allele between the cases and controls is then correlated to the relative risk measured and allows the sample size required to detect a genetic effect to be determined.

### 3.7.3.2 Hardy Weinberg

The law of Hardy Weinberg Equilibrium (HWE) was defined in 1908 by Godfrey H. Hardy and Wilhelm Weinberg (Hardy 1908). The HWE law basically states that the allele and genotype frequencies will reach equilibrium, defined by a binomial distribution and remain the same from generation to generation in large randomly mating populations (Hardy 1908). This law holds true under the assumption that the populations experience no migration, selection, mutation, or non-random mating. For biallelic markers the law utilises the binominal equation $p^2 + 2pq + q^2 = 1$, where $p$ and $q$ denotes allele 1 and 2 respectively. The HWE assumption allows genotype frequencies to be directly determined from allele frequencies and thus is used as a statistical control to indentify genotyping error (Yonan, Palmer et al. 2006). HWE expected and observed allele frequencies are calculated in a chi-squared analysis to determine how well the observed genotype distribution fits the expected. A P-value of < 0.05 signifies Hardy Weinberg disequilibrium and indicates a significant variation of the observed distribution from the expected (Hardy 1908).
3.7.3.3 Chi-square

Genotype and allele frequencies in the migraine and control populations were tested for association using standard Chi-square analysis. Chi-square is a non parametric test used to determine if a distribution of observed frequencies differs from the theoretical expected frequencies. Chi-square statistics use nominal (categorical) or ordinal level data thus uses frequencies instead of using means and variances. There are two basic types of chi-square analysis, tests for goodness of fit, used with a single nominal variable, and tests of Independence, used with two nominal variables. Both types of chi-square tests use the computational formula as below:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where, $\chi^2$ = symbol for chi-square,  
\[ \sum \] = the sum of,  
\[ O \] = observed frequency,  
\[ E \] = expected frequency.

All migraine association studies in this research used the chi-square statistic of independence to analyse the relationship between two nominal variables (in a 2 x 3 contingency table for genotype frequencies and in a 2 x 2 contingency table for allele frequencies). All Chi –square analysis was conducted using the Statistical Package for Social Sciences (SPSS version 17.0) program. The level of significance was set to \( P < 0.05 \).

For contingency tables greater than 2 x 3, the CLUMP program was used (Sham and Curtis 1995). CLUMP is a program designed to assess the significance of the departure of observed values in a contingency table from the expected values conditional on the marginal totals. This program produces a novel chi-squared value.
by “CLUMPing” columns together into a new two-by-two table, which maximises the chi-squared value obtained. The significance of the value is assessed using the Monte Carlo method, by performing repeated stimulations to generate tables having the marginal results as the one under consideration, and totalling the number of times that a chi-squared value associated with the real table is achieved by the randomly simulated data. This is undertaken to make sure that the assigned significant levels are unbiased and there is no need to take into account continuity corrections or small expected values (Sham and Curtis 1995).

### 3.7.3.4 Odds ratio

The odds ratio (OR) for disease is a measure of the escalated risk of disease conferred to an individual who carries the disease risk allele. An OR of 1.5 would indicate that a disease risk allele carrier is 50% more likely to develop the disease than a non-risk allele carrier. Whereas an OR of 1 indicates that the both allele carriers stand an equal chance of developing the disease. OR is thus a method of comparing whether the probability of a certain event is the same for two groups. An OR greater than 1 implies that the event is more likely in the first group and an OR less than 1 implies otherwise. The degrees of freedom for an allelic analysis is one (because there are two possible alleles) while the degrees of freedom for a genotypic analysis is two (as there are three possible genotypes). The OR can be calculated using the following formulas.

**OR for treatment group (migraine cases) is described below:**

\[
O_T = \frac{p_T}{1 - p_T} \quad \text{P}_T = \text{probability of migraine group}
\]

**Odds ratio for control group:**

\[
O_C = \frac{p_C}{1 - p_C} \quad \text{P}_C = \text{probability of control group}
\]
The Odds ratio between treatment and control:

\[
OR = \frac{\hat{O}_T}{\hat{O}_C} = \frac{p_T(1 - p_C)}{p_C(1 - p_T)}
\]

For this thesis OR was calculated with 95% confidence intervals, with a P value of less than 0.05 giving significance for the test. In cases of multiple testing the Bonferroni correction for multiple testing was applied by adjusting the alpha level by the number of comparisons run in the analysis.

### 3.7.3.5 Linkage Disequilibrium

The non random association of alleles at adjacent loci is called Linkage disequilibrium (LD). LD occurs when genotypes at the two loci are not independent of another and segregate together more often than expected (Reich, Cargill et al. 2001; Chen, Lin et al. 2006). One of the earliest measures of LD proposed in the measure of D (Lewontin 1964). D quantifies disequilibrium as the difference between the observed frequency of a two –locus haplotype and the expected frequency of the alleles if they are segregating at random. The measure of D can be represented by the following equation:

\[
D = P_{AB} - (P_A \times P_B)
\]

Where the two adjacent loci are A and B with two alleles (Aa and Bb) and at each locus the observed frequency of the haplotype consists of alleles A and B represented by \(P_{AB}\). \(P_A\) is then the frequency of allele A at the first locus and \(P_B\) is the frequency of allele B at the second locus. Under the assumption of the independent assortment of alleles at the two loci, the expected haplotype frequency is calculated as the product of the allele frequency of each of the two alleles \(P_A \times P_B\). The absolute value of \(D'\) is obtained by dividing D by its maximum possible value and a value of 1 is known as
complete LD. Values of $D' < 1$ suggests independent assortment (Lewontin 1964; Teare, Dunning et al. 2002; Tenesa, Wright et al. 2004; Pittman, Myers et al. 2005).
3.8 Introduction to research

In summary, this research investigated the genotype - phenotype correlation between the MTHFRC677T variant and migraine clinical profiles on migraineurs drawn from two large case sex and age matched case control populations. To determine the genotypic effect of MTHFR and MTRR variants on vitamin supplementation response in migraineurs, a randomised double blind clinical trial was conducted on an Australian cohort. Finally this thesis investigated variants in potential migraine candidate genes, Notch 3, CACNA1A and Opioid-1 receptor in migraineurs to understand their involvement in migraine susceptibility and disability. The following results chapters 4 to 8 represent a number of published papers and publications under review arising from the research described in this thesis.
Chapter 4

Analysis of the MTHFR C677T variant with migraine phenotypes
4.1 Methylene tetrahydrofolate reductase gene and migraine

The human MTHFR gene mapped to chromosome 1p36.3, consists of 11 exons and catalyses the nicotinamide adenine dinucleotide phosphate (NADPH) dependent conversion of 5, 10-methylenetetrahydrofolate (CH2-THF) to 5-methyltetrahydrofolate (CH3-THF), the principal circulatory form of folate and a cofactor for methylation of homocysteine to methionine (Goyette, Sumner et al. 1994; Goyette, Pai et al. 1998). An increase in circulatory homocysteine levels have been reported in patients with MA (Evers 1997). It is proposed that homocysteine acts as an excitatory amino acid in migraine pathophysiology, either by causing vasodilation of cerebral blood vessels or temporary thrombosis of cerebral blood vessels, reducing oxygen into the brain (Kara, Sazci et al. 2003; Oterino, Valle et al. 2004).

The C677T allele (rs1801133), a common variant of the MTHFR gene has a frequency of approximately 23-41% in the Caucasian population (Kara, Sazci et al. 2003). The MTHFR C667T allele results in an amino acid change at position 222, substituting alanine (Ala) for valine (Val). Individuals homozygous for this variant express approximately 30% of the mean activity of MTHFR enzyme levels, whereby, compared to baseline levels, the mean activity is 65% in individuals who are Ala/Val (Frosst, Blom et al. 1995; Geisel, Zimbelmann et al. 2001; Lea, Ovcaric et al. 2004). Individuals homozygous for the Val residue may exhibit mild elevation in plasma homocysteine levels particularly in combination with low dietary folate levels (Frosst, Blom et al. 1995; Geisel, Zimbelmann et al. 2001).

The TT genotype has been reported to be a modest, yet significant risk factor for stroke and hypertension (Kelly, Rosand et al. 2002; Ilhan, Kucuksu et al. 2008). The association of the C677T variant with MA was first reported in a Japanese population and subsequently replicated in both Turkish and Dutch population (Kowa, Yasui et al. 2008).
The atherothrombotic effects of hyperhomocysteinemia have been postulated to increase the risk of stroke and the decrease in MTHFR activity due to C677T mutation, affecting DNA repair and cell division, may result in hypertension (Marinho, Alho et al. 2007).

We investigated the MTHFR C677T variant in migraine in an Australian Caucasian population and have similarly shown significant over-representation of the TT genotype in individuals with MA in comparison to the control group (Lea, Ovcaric et al. 2004). This association was however not seen in a Finnish study that investigated the contribution of the C677T variant in MA and MO patients. Interestingly, Schurks et al. (Schurks, Zee et al. 2008) investigated the interrelationships of the MTHFR C677T variant, migraine and cardiovascular disease, with data suggesting a protective effect for the TT genotype against MA in their population (Schurks, Zee et al. 2008). This inconsistency may be a result of allelic heterogeneity, diagnostic variation or differences between the populations examined (Kaunisto, Kallela et al. 2006). However a recent meta analysis by Rubino et al that analysed all existing studies that evaluated allelic and genotypic frequencies of the C677T SNP in migraine concluded that the MTHFR gene is a genetic risk factor for MA only (Rubino, Ferrero et al. 2009) (refer to Table 4.1)

### 4.2 Analysis of MTHFR C677T variant and migraine phenotypes

As multiple genes have now been associated with migraine susceptibility, it is plausible to assume that different genotypes and susceptibility genes may cause varying disease manifestation (Lea, Ovcaric et al. 2005; Nyholt, Morley et al. 2005). Nyholt et al, through genome wide latent-class analysis (LCA) of migraineurs, identified significant linkage on chromosome 5q21 and suggestive linkage on
chromosomes 8, 10 and 13 in relation to migraine phenotypes (Nyholt, Morley et al. 2005).

Table 4.1: OR and heterogeneity results for C677T MTHFR polymorphism in migraineurs, both MA and MO from all studies analysed in the meta analysis. (Adapted from Rubino et al., 2009)

<table>
<thead>
<tr>
<th>Population</th>
<th>OR (95% CI)</th>
<th>P (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alleles T vs. C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>1.12 (1.01, 1.25)</td>
<td>1.16 (0.96, 1.40)</td>
<td>65</td>
</tr>
<tr>
<td>MA</td>
<td>0.94 (0.80, 1.10)</td>
<td>1.00 (0.79, 1.27)</td>
<td>69</td>
</tr>
<tr>
<td>MA</td>
<td>1.08 (0.89, 1.31)</td>
<td>1.21 (1.00, 1.46)</td>
<td>75</td>
</tr>
<tr>
<td>Homozygotes TT vs. CC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>1.34 (1.06, 1.69)</td>
<td>1.22 (0.94, 1.58)</td>
<td>69</td>
</tr>
<tr>
<td>MA</td>
<td>0.80 (0.71, 1.01)</td>
<td>0.93 (0.78, 1.12)</td>
<td>60</td>
</tr>
<tr>
<td>MA</td>
<td>0.86 (0.65, 1.14)</td>
<td>0.94 (0.72, 1.22)</td>
<td>75</td>
</tr>
<tr>
<td>Recessive model TT vs. CT + CC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>1.26 (1.04, 1.52)</td>
<td>1.40 (0.95, 2.03)</td>
<td>57</td>
</tr>
<tr>
<td>MA</td>
<td>0.90 (0.79, 1.03)</td>
<td>0.95 (0.79, 1.16)</td>
<td>50</td>
</tr>
<tr>
<td>MA</td>
<td>1.07 (1.19, 1.28)</td>
<td>1.23 (1.12, 1.35)</td>
<td>75</td>
</tr>
<tr>
<td>Dominant model TT + CT vs. CC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>1.26 (0.97, 1.65)</td>
<td>1.05 (0.75, 1.46)</td>
<td>60</td>
</tr>
<tr>
<td>MA</td>
<td>1.00 (0.82, 1.23)</td>
<td>1.15 (0.87, 1.53)</td>
<td>55</td>
</tr>
</tbody>
</table>

The study investigated the broad contribution of chromosomal loci to migraine clinical symptoms through linkage analysis, but did not consider the effects of specific genes or polymorphisms within genes (Nyholt, Morley et al. 2005). A recent study by Tietjen et al. (Tietjen, Herial et al. 2009), that investigated if angiotensin converting enzyme (ACE) and MTHFR gene variants are associated with von Willebrand factor (vWF) activity, an endothelial dysfunction marker, and with a distinct headache phenotype in premenopausal women with migraine, observed elevated vWF activity to be associated with the ACE DD genotype, which was highest when combined with the MTHFR TT genotype (Tietjen, Herial et al. 2009).

The aim of the current study was to investigate migraine phenotypes in relation to the MTHFR gene. This study examined the genotype-phenotype correlations between the...
C677T variant and the clinical phenotypes of migraine to determine if the MTHFR genotype was associated with migraine in general or more specifically with particular migraine sub-types, symptoms, severity, gender and/or response to medication.

4.3 Methods

4.3.1 Study subjects

Study participants (n = 267) were drawn from two large age (+/- 5 years) and sex matched case-control panels previously genotyped for the MTHFR C677T mutation (Lea, Ovcaric et al. 2004). All description of the study population is described in Chapter 3. There were 165 MA and 102 MO participants in this study. Participants who experienced both subtypes of migraine were classed as being affected with MA. Individuals reported affected with, or had a family history of known migraine or comorbid conditions such as mental illness (including depression and schizophrenia) cerebral vascular disease and alcohol, were excluded from the study. Written informed consent was obtained from all participants and the study was approved by the Griffith University Ethics Committee for Experimentation on Human Subjects.

4.3.2 Phenotype variables

The dependent (outcome) variables for the study were migraine clinical data related information of which there were four main categories. These were: i) migraine subtype diagnosis, ii) migraine triggers, iii) migraine treatment and iv) treatment for other conditions/pains. In total, there were around 50 outcome variables under these main categories, a summary of which are outlined in Table 1. Each of these dependent variables underwent statistical analysis against genotype. The independent MTHFR variable has three possible genotypes, CC, CT and TT.
4.3.3 Genotyping

Genomic DNA was isolated from whole blood by standard salting out method as previously described by Miller et al (Miller, Dykes et al. 1988). DNA fragments with the MTHFR C677T variant were PCR amplified and the resulting product was digested by the H\textit{inf}\textsubscript{I} enzyme and fractionated using a 5% ultra–high-resolution agarose gel. The genotype results were confirmed using an ABI-3130 genetic analyser. The detailed genotyping method for the samples used in this study has been previously published in Lea et al 2004 (Lea, Ovcaric et al. 2004).

4.3.4 Statistical Analysis

Statistical analyses of the variables were performed using SPSS for windows version 13. The chi-square statistical test was used to test for potential relationships between genotype and all migraine qualitative dependent variables as well as the relationship between migraine diagnosis and migraine dependent variables. For quantitative outcome variables, nonparametric tests of variance, the Kruskal-Wallis, Median, Mann-Whitney and Kolmogorov-Smirnov tests were used. Logistic and ordinal regression analyses were used to devise models where more than one independent variable (ie. Genotype, age and gender) was used to predict the outcome of dependent variables. Regression analyses were performed to identify whether the addition of age and gender improved the model compared to simply predicting with genotypes alone. The equivalent number of measured independent traits was measured using the matSpD interface (http://genepi.qimr.edu.au/general/daleN/matSpD) (Nyholt 2004; Li and Ji 2005). matSpD analysis determined that the original 50 variables correspond to approximately 14 independent traits. Bonferroni correction was applied to correct for multiple testing and to determine the significance of the results by dividing the
significance level by the number of independent traits. The level of significance was taken at P value of \(0.05/14 = 0.004\) (Distel, Ligthart et al. 2007).

### 4.4 Results

#### 4.4.1 MTHFR genotypes associated with migraine clinical variables

Table 4.2 shows results for MTHFR genotype analysis in relation to migraine clinical variables. There were 165 MA and 102 MO participants in this study group. Sixteen participants who experienced both MA and MO were classified as having MA as they may share inherited and acquired factors predisposing them to aura. The MTHFR group consisted of 27% males and 73% females. 94% of individuals with the MTHFR TT genotype, suffered from MA as compared to 61% and 55% of individuals carrying the CC and CT genotypes respectively. These results confirm our previous observations (Lipton, Stewart et al. 2001; Lea, Ovcaric et al. 2004).

Of the 50 variables tested and after Bonferroni correction for multiple testing, migraine diagnosis \((P<0.0001, \text{degree of freedom (df)}=2)\), unilateral head pain \((P=0.002, \text{df}=2)\), physical activity discomforts \((P<0.001, \text{df}=2)\) and stress as a migraine trigger \((P=0.002, \text{df}=2)\) were all factors that were significantly associated with MTHFR genotype (refer to Table 4.3). Further analyses demonstrated the TT genotype to be significantly associated with MA \((P<0.0001)\) and unilateral head pain \((P=0.002)\). Frequency data found 87% of migraineurs with the TT genotype experienced unilateral head pain compared to 62% in the CC group. The CT group showed an intermediate percentage of migraineurs who experienced unilateral head pain (77%). A smaller percentage of migraineurs carrying the CC genotype experienced discomfort associated with physical activity during or just prior to migraine (69%), while the TT group had an intermediate response (83%) and the CT (88%) group had the highest response \((P<0.001)\). The CT genotype group also had
the highest percentage of participants acknowledging stress as a migraine trigger (76%) (P= 0.002) (Table 4.3). These findings suggest that individuals carrying one and/or more copies of the T allele are more prone to unilateral head pain, are more likely to experience discomfort associated with physical activity during or prior to migraine and have stress as a migraine trigger compared to those carrying the CC genotype.

To further analyse the contribution of the recessive TT genotype with migraine clinical variables, the CC and the CT groups were reclassified into one group, and compared with the TT genotype group. The chi-square, Kruskal-Wallis and Kolmogorov-Smirnov Z tests between the two reclassified genotype groups and migraine clinical data revealed statistical significance of genotype with migraine diagnosis (P<0.000, df= 1) and visual disturbances (P= 0.001, df=1). Frequency data demonstrated the recessive TT genotype group had a significantly higher percentage of individuals with MA; while the CC/CT genotype group appeared to have an equal distribution of the two different migraine subtypes (refer to Table 4.4).

When age was included as an independent variable and tested with both the original (CC vs CT vs TT) and the reclassified (CC vs CT/TT) genotype group, statistical significance was obtained for none of the 50 migraine clinical variables tested.
Table 4.2: Chi square analysis of MTHFR genotype for individuals experiencing clinical variables versus all migraineurs who do not.

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>Pearson's chi-square</th>
<th>N</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Migraine Subtype Diagnosis</td>
<td>16.65</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Visual disturbances</td>
<td>5.57</td>
<td>96</td>
<td>0.062</td>
</tr>
<tr>
<td>Numbness &amp; Tingling</td>
<td>1.49</td>
<td>45</td>
<td>0.475</td>
</tr>
<tr>
<td>Speech problems</td>
<td>1.41</td>
<td>26</td>
<td>0.493</td>
</tr>
<tr>
<td>Nausea</td>
<td>0.15</td>
<td>165</td>
<td>0.927</td>
</tr>
<tr>
<td>Emesis</td>
<td>0.69</td>
<td>121</td>
<td>0.708</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>2.3</td>
<td>18</td>
<td>0.316</td>
</tr>
<tr>
<td>Phonophobia</td>
<td>2.16</td>
<td>153</td>
<td>0.34</td>
</tr>
<tr>
<td>Photophobia</td>
<td>0.85</td>
<td>177</td>
<td>0.654</td>
</tr>
<tr>
<td>Osmophobia</td>
<td>3.78</td>
<td>42</td>
<td>0.151</td>
</tr>
<tr>
<td>Altered Vision</td>
<td>0.52</td>
<td>81</td>
<td>0.77</td>
</tr>
<tr>
<td>Dizziness/ Double Vision</td>
<td>4.33</td>
<td>49</td>
<td>0.115</td>
</tr>
<tr>
<td>Speech problems</td>
<td>1.78</td>
<td>31</td>
<td>0.411</td>
</tr>
<tr>
<td>Numbness &amp; Tingling</td>
<td>0.1</td>
<td>49</td>
<td>0.952</td>
</tr>
<tr>
<td>Weakness</td>
<td>2.7</td>
<td>54</td>
<td>0.259</td>
</tr>
<tr>
<td>Pulsating &amp; throbbing head pain</td>
<td>1.23</td>
<td>158</td>
<td>0.542</td>
</tr>
<tr>
<td>Unilateral head pain</td>
<td>7.07</td>
<td>123</td>
<td>0.029</td>
</tr>
<tr>
<td>Bilateral head pain</td>
<td>2.76</td>
<td>66</td>
<td>0.251</td>
</tr>
<tr>
<td>Head movement discomfort</td>
<td>2.72</td>
<td>123</td>
<td>0.257</td>
</tr>
<tr>
<td>Eye Movement discomfort</td>
<td>3.59</td>
<td>97</td>
<td>0.166</td>
</tr>
<tr>
<td>Physical Activity discomfort</td>
<td>8.97</td>
<td>149</td>
<td>0.011</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Migraine triggers</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Menses</td>
<td>1.224</td>
<td>58</td>
<td>0.542</td>
</tr>
<tr>
<td>Weather Changes</td>
<td>0.98</td>
<td>36</td>
<td>0.613</td>
</tr>
<tr>
<td>Stress</td>
<td>7.3</td>
<td>123</td>
<td>0.026</td>
</tr>
<tr>
<td>Holiday &amp; Relaxation</td>
<td>3.29</td>
<td>17</td>
<td>0.193</td>
</tr>
<tr>
<td>Red Wine</td>
<td>2.5</td>
<td>33</td>
<td>0.286</td>
</tr>
<tr>
<td>Other Alcohol</td>
<td>1.48</td>
<td>35</td>
<td>0.478</td>
</tr>
<tr>
<td>Chocolate</td>
<td>0.5</td>
<td>46</td>
<td>0.799</td>
</tr>
<tr>
<td>Oranges</td>
<td>0.21</td>
<td>23</td>
<td>0.902</td>
</tr>
<tr>
<td>Ripe Cheese</td>
<td>4.32</td>
<td>23</td>
<td>0.115</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Migraine treatment</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes/No</td>
<td>0.04</td>
<td>58</td>
<td>0.981</td>
</tr>
<tr>
<td>5-HT1 Drugs</td>
<td>0.03</td>
<td>30</td>
<td>0.985</td>
</tr>
<tr>
<td>5-HT1 Drug treatment effectiveness</td>
<td>1.97</td>
<td>21</td>
<td>0.373</td>
</tr>
<tr>
<td>Pain killers Natural</td>
<td>5.02</td>
<td>137</td>
<td>0.081</td>
</tr>
<tr>
<td>remedy Medication</td>
<td>1.77</td>
<td>27</td>
<td>0.412</td>
</tr>
<tr>
<td>for nausea</td>
<td>2.75</td>
<td>31</td>
<td>0.253</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment for other conditions/pains</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes/No</td>
<td>0.67</td>
<td>17</td>
<td>0.716</td>
</tr>
<tr>
<td>Chronic neck pain</td>
<td>1.98</td>
<td>33</td>
<td>0.372</td>
</tr>
<tr>
<td>High blood pressure</td>
<td>0.08</td>
<td>16</td>
<td>0.916</td>
</tr>
<tr>
<td>Stroke</td>
<td>0.93</td>
<td>1</td>
<td>0.629</td>
</tr>
<tr>
<td>Heart Disease</td>
<td>1.5</td>
<td>7</td>
<td>0.473</td>
</tr>
<tr>
<td>Depression</td>
<td>1.68</td>
<td>23</td>
<td>0.431</td>
</tr>
<tr>
<td>Anxiety disorder</td>
<td>0.32</td>
<td>2</td>
<td>0.853</td>
</tr>
<tr>
<td>Panic disorder</td>
<td>0.21</td>
<td>6</td>
<td>0.899</td>
</tr>
<tr>
<td>Chronic fatigue</td>
<td>1.71</td>
<td>5</td>
<td>0.425</td>
</tr>
<tr>
<td>Irritable bowel</td>
<td>3.22</td>
<td>15</td>
<td>0.2</td>
</tr>
<tr>
<td>Menstrual problems</td>
<td>1.19</td>
<td>14</td>
<td>0.551</td>
</tr>
<tr>
<td>Chronic back pain</td>
<td>0.95</td>
<td>1</td>
<td>0.623</td>
</tr>
<tr>
<td>Contraceptive Pill</td>
<td>0.177</td>
<td>67</td>
<td>0.915</td>
</tr>
<tr>
<td>Passive Smoking</td>
<td>0.1</td>
<td>81</td>
<td>0.952</td>
</tr>
</tbody>
</table>

*P values are values of $\chi^2$ tests (2tailed) for the 2x3 tables (df=2).
Table 4.3: Genotypic frequency distribution of MTHFR and statistically significant clinical variables in relation to MTHFR genotype

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>Subtype (Total)</th>
<th>MTHFR Genotype %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CC</td>
</tr>
<tr>
<td>Migraine Diagnosis</td>
<td>MO (102)</td>
<td>39 (39%)</td>
</tr>
<tr>
<td>P&lt; 0.0001</td>
<td>MA (165)</td>
<td>61 (61%)</td>
</tr>
<tr>
<td>Unilateral head pain</td>
<td>Y (123)</td>
<td>40 (62%)</td>
</tr>
<tr>
<td>P&lt; 0.0001</td>
<td>N (47)</td>
<td>25 (38%)</td>
</tr>
<tr>
<td>Physical activity</td>
<td>Y (149)</td>
<td>51 (69%)</td>
</tr>
<tr>
<td>P&lt; 0.001</td>
<td>N (38)</td>
<td>23 (31%)</td>
</tr>
<tr>
<td>Stress</td>
<td>Y (123)</td>
<td>42 (61%)</td>
</tr>
<tr>
<td>P= 0.002</td>
<td>N (60)</td>
<td>27 (39%)</td>
</tr>
</tbody>
</table>

Significant P values after correction for multiple testing (Bonferroni correction).
MO=Migraine without aura, MA=Migraine with aura, Y= Yes and N= No.

Table 4.4: Frequency distribution of CC/CT vs TT genotype significant variables

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>Subtype (Total)</th>
<th>MTHFR Genotype %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CC/ CT</td>
</tr>
<tr>
<td>Migraine Diagnosis</td>
<td>MO (102)</td>
<td>100 (43%)</td>
</tr>
<tr>
<td>P&lt; 0.0001</td>
<td>MA (165)</td>
<td>135 (57%)</td>
</tr>
<tr>
<td>Visual Disturbance</td>
<td>Y (96)</td>
<td>78 (52%)</td>
</tr>
<tr>
<td>P&lt; 0.0001</td>
<td>N (74)</td>
<td>72 (48%)</td>
</tr>
</tbody>
</table>

Significant P values after correction for multiple testing (Bonferroni correction).
MO=Migraine without aura, MA=Migraine with aura, Y= Yes and N= No.

4.4.2 Gender differences and the MTHFR genotype

The inclusion of gender as an independent variable revealed gender specific statistical significance for a number of migraine clinical variables. In female migraineurs the TT genotype was significantly associated with unilateral head pain (OR= 0.35, CI-95=0.15-0.79, P<0.001). Conversely, male migraineurs experienced higher incidences of bilateral head pain, especially in those with the TT genotype, compared to female migraineurs (67% vs 19%)(refer to Table 4.5). In addition, female migraineurs with the CT genotype were more likely to suffer from nausea (OR= 3.41, CI-95=1.44-8.08,
P<0.001), osmophobia (OR= 3.39, CI-95=1.01-10.48, P=0.002) and use natural remedy as a migraine treatment (OR= 0.2, CI-95=0.05-0.92, P=0.003) compared to male migraineurs (refer to Tables 4.6).

Table 4.5: Genotypic frequency distribution of MTHFR for statistically significant clinical variable in relation with gender

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>Gender</th>
<th>MTHFR Genotype %</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CC(%)</td>
<td>CT(%)</td>
<td>TT(%)</td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>M</td>
<td>10(100%)</td>
<td>15(60%)</td>
<td>5(71%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>55(85%)</td>
<td>64(94%)</td>
<td>16(89%)</td>
<td></td>
</tr>
<tr>
<td>Osmophobia</td>
<td>M</td>
<td>1(13%)</td>
<td>3(14%)</td>
<td>0(0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>15(26%)</td>
<td>21(35%)</td>
<td>2(13%)</td>
<td></td>
</tr>
<tr>
<td>Unilateral head pain</td>
<td>M</td>
<td>4(50%)</td>
<td>15(65%)</td>
<td>3(50%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>36(63%)</td>
<td>48(81%)</td>
<td>17(100%)</td>
<td></td>
</tr>
<tr>
<td>Bilateral head pain</td>
<td>M</td>
<td>5(62%)</td>
<td>13(62%)</td>
<td>4(67%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>26(46%)</td>
<td>15(26%)</td>
<td>3(19%)</td>
<td></td>
</tr>
<tr>
<td>Natural Remedy</td>
<td>M</td>
<td>0(0%)</td>
<td>2(9%)</td>
<td>0(0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8(15%)</td>
<td>14(25%)</td>
<td>3(19%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.6: Statistically significant clinical variables by gender and MTHFR genotype

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>P-value</th>
<th>OR</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea</td>
<td>&lt;0.001</td>
<td>3.41</td>
<td>(1.44-8.08)</td>
</tr>
<tr>
<td>Osmophobia</td>
<td>=0.002</td>
<td>3.39</td>
<td>(1.01-10.48)</td>
</tr>
<tr>
<td>Unilateral head pain</td>
<td>&lt;0.001</td>
<td>0.35</td>
<td>(0.15-0.79)</td>
</tr>
<tr>
<td>Bilateral head pain</td>
<td>&lt;0.000</td>
<td>0.25</td>
<td>(0.11-0.55)</td>
</tr>
<tr>
<td>Natural remedy</td>
<td>=0.003</td>
<td>0.2</td>
<td>(0.05-0.92)</td>
</tr>
</tbody>
</table>

Significant P values after correction for multiple testing (Bonferroni correction)

4.5 Discussion

Migraine is most likely produced as a result of the interaction between multiple genes with environmental factors and triggers. As a consequence, variability and overlap is expected in the manifestations of the associated genetic defect/s. In a study of the clinical manifestations associated with mutations in the calcium- channel, voltage dependent, P/Q type, alpha A subunit gene (CACNA1A) in 28 families with Familial
Hemiplegic Migraine type 1, Ducros et al (2002), revealed significant genotype-phenotype correlations. In addition to clinical variability being partly due to the different CACNA1A mutations, the study also suggested that variability in phenotypic expression among patients with the same mutation could be influenced by other genetic or environmental factors (Ducros, Tournier-Lasserve et al. 2002).

The study of vascular genes in migraine identified a role for MTHFR gene. MTHFR synthesizes 5-methylenetetrahydrofolate, the major carbon donor required for efficient remethylation of homocysteine to methionine (Das 2003). The MTHFR C677T allele results in an amino acid change and reduces MTHFR enzyme activity leading to mild hyperhomocysteinemia (Frosst, Blom et al. 1995). Hyperhomocysteinemia have been suggested to produce endothelial cell injury in animal and cell culture studies; this homocysteine related dysfunction of the vascular endothelium may potentially influence migraine susceptibility, especially MA, through the activation of trigeminal fibres (Storer and Goadsby 1997; Chen, Stampfer et al. 2001; Parsons and Strijbos 2003). It is possible that the vascular disturbance connected to hyperhomocysteinemia trigger downstream neurological manifestations that are observed in MA sufferers.

The current study investigated genotype-phenotype correlations of the migraine susceptibility gene, MTHFR with 50 migraine clinical variables. Even after correction for multiple testing, analyses indicated the MTHFR genotype to be significantly associated with migraine diagnosis, unilateral head pain, physical activity discomforts, and stress as a migraine trigger. The homozygous MTHFR TT genotype was linked with MA and unilateral head pain and the heterozygous CT genotype was linked to physical activity discomforts during or prior to migraine and stress as a migraine trigger. While the TT and CC genotypes showed the highest and the lowest
percentage of participants suffering from unilateral head pain during or prior to a migraine respectively, the CT genotype clearly demonstrated an intermediate response. Frequency data of participants suffering from physical activity discomfort during or prior to migraine showed the CT and the CC genotypes to have the highest and the lowest percentage of participants respectively and the TT genotype showed an intermediate response. Interestingly, the CT and the TT genotypes showed the highest and the lowest number respectively, of migraineurs reporting stress as a migraine trigger. When the MTHFR genotype groups were examined by comparing CC and CT genotypes to the homozygous TT genotype, the resulting regression models did not alter drastically. The TT genotype remained significantly linked to MA and was also observed to be significantly associated with visual disturbance.

When age was investigated in relation to genotype and phenotype none of the migraine clinical variables showed statistical significance. Age, being an important factor in the onset, prevalence and type of migraine experienced needs to be further investigated in a larger cohort of migraineurs in the different age groups to accurately determine the distinct migraine phenotypes that vary with age. When gender differences were investigated in relation to genotype and phenotype it was found that bilateral head pain was observed more commonly in male migraineurs. In contrast, in females, nausea, unilateral head pain, osmophobia and the use of natural remedy as a migraine treatment were significantly associated with one or more copies of the T allele. The gender distribution in this study is not equal with 27% of the participants being males and 73% of the participants being females; some caution has to be exercised when interpreting the significant results. Gender differences in relation to genotype and phenotype have to be examined in a bigger cohort to look at the effect of MTHFRC677T genotype on migraine clinical symptoms diligently. The TT genotype of the MTHFR C677T variant has been shown in several studies including a
recent meta-analysis by Rubino et al (Rubino, Ferrero et al. 2009) to confer a modest risk for MA (Rubino, Ferrero et al. 2009). This study expanded on this association to examine genotype-phenotype correlations between the C677T variant and the clinical phenotypes of migraine. The presence of the T allele was associated with the largest number of migraine symptoms and triggers, suggesting that although the TT genotype appears to have a recessive effect on MTHFR enzyme levels, perhaps both heterozygous and homozygous states of the T allele may contribute to the phenotypic expression of migraine. As well as intrinsic enzyme levels, individuals with the CT genotype may be more susceptible to the environmental triggers associated with migraine attacks.

The effects of elevated levels of homocysteine on neurons have been reported to include DNA damage, altered DNA repair, disturbance in DNA methylation and oxidative stress (Buemi, Marino et al. 2001; Kruman, Kumaravel et al. 2002; Parsons and Strijbos 2003; Zieminska and Lazarewicz 2006). Animal studies have reported cytotoxic effects of high levels of homocysteine to included apoptosis in sensitive brain areas such as the striatum and cerebellum involved in motor function and altered neurobehavioural capacity in rat models (Blaise, Nedelec et al. 2007). It is thus plausible that the resulting levels of homocysteine conferred by the T allele may contribute to selected phenotypic expressions described in migraineurs.

The major limitation of the present study was the number of available subjects in comparison to the number of outcome variables examined. As such, further investigations utilising larger populations to clarify the genotype-phenotype interactions of the migraine susceptibility gene MTHFR are warranted.
Chapter 5
Genotypic effect on vitamin B treatment response in migraineurs
5.1 Introduction

Migraine affects about 303 million people in the world according to the World Health Organisation (WHO) and is estimated to affect about 12% of the Caucasian population (Lea, Colson et al. 2009; Dhillon, Singh et al.). The prevalence of migraine is higher in females but the sex ratio varies with age (Martin and Behbehani 2006; Dhillon, Singh et al. 2011). With the prevalence of migraine being highest during the peak productive years in both genders, the impact of migraine for both individuals and the society is enormous (Lipton and Stewart 1997). There have been a myriad of studies in the last decade investigating pathophysiology of migraine, triggers, genetics and environmental factors that impact migraine (Goadsby, Lipton et al. 2002; Goadsby 2005; Wessman, Terwindt et al. 2007)). Biochemical factors that could potentially disrupt the vascular endothelial function leading to cortical spreading depression that can activate and affect the trigeminovascular system (TVS) are primary candidates for involvement in migraine pathophysiology (Mathew 2001; Buzzi and Moskowitz 2005). The dilation of the cerebral blood vessels following the activation of the TVS may be the cause of the characteristic head pain experienced in both MA and MO sufferers (Tzourio, El Amrani et al. 2001).

5.2 Role of homocysteine in migraine

Homocysteine related dysfunction of the vascular endothelium may potentially influence migraine susceptibility (Chen, Stampfer et al 2001; Parsons and Strijbos 2003; Storer and Goadsby 1997). It is possible that the vascular disturbance connected to hyperhomocysteinemia trigger downstream neurological manifestations that are observed in migraine sufferers. The atherogenic effects of hyperhomocysteinemia have been shown to involve vascular endothelial dysfunction/ injury in both experimental and cell culture studies (Harker 1976; Wall 1980). Animal studies have
reported cytotoxic effects of high levels of homocysteine to include apoptosis in sensitive brain areas such as the striatum and cerebellum and altered neurobehavioural capacity in rat models (Blaise, Nedelec et al. 2007) Cell culture studies have demonstrated high levels of homocysteine to produce endothelial cell injury and alter blood coagulant properties (Hering-Hanit 2001). Storer et al (Storer and Goadsby 1997) found the rate of spontaneous trigeminal cell firing to be accelerated with the application of oxidised homocysteine derivative called D,L-homocysteic acid, that mimics the effect of homocysteine on arteries (Bouhassira 1987; Storer and Goadsby 1997).

Homocysteine induced endothelial cell injury includes impaired release of nitric oxide, which may switch the usual thrombotic nature of the vascular endothelium to a more thrombotic phenotype, thus significantly changing the vascular function and the coagulant properties of blood (Stamler 1993; Lea, Ovcaric et al. 2004). Elevated levels of plasma homocysteine are also understood to exert its effects through oxidative stress and activation of nuclear factor-κB resulting in the recruitment of leukocytes and monocytes (Au-Yeung 2003).

Trigeminal nerves heavily innervate the meninges and cerebral blood vessels. Homocysteine related dysfunction of the vascular endothelium may potentially activate trigeminal fibres leading to an inflammatory reaction occurring in the meninges, along with dilation of the large cerebral vessels. It is this reaction that is thought to participate in the characteristic head pain common to both MA and MO (Lea, Colson et al. 2009; Lea, Ovcaric et al. 2004; Parsons and Strijbos 2003). Therefore biochemical factors such as homocysteine, that have the potential to lead to and/or affect the trigeminovascular system are important targets to be investigated for involvement in migraine pathology.
5.3 Homocysteine

Homocysteine is an amino acid that is formed during methionine metabolism and is itself metabolised by two pathways: remethylation pathway to form methionine via a folic acid, B\textsubscript{12} and B\textsubscript{2} dependant pathway and the trans-sulfuration pathway which converts homocysteine to cysteine and then taurine via a B\textsubscript{6} dependant pathway. MTHFR, methionine synthase (MS) and methionine synthase reductase (MSR) are the enzymes involved in the remethylation pathway (Lea, Colson et al. 2009).

Various factors determine the levels of circulating plasma homocysteine, in particular dietary deficiencies in the co-factors such as folic acid, vitamin B\textsubscript{12} and B\textsubscript{6} essential for metabolising homocysteine and mutations in the genes of key enzymes participating in homocysteine metabolism such as cystathionine b-synthase (CBS), methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MS) and methionine synthase reductase (MTRR) (Silaste 2001) (refer to Figure 5.1).

![Homocysteine metabolism and the genes involved in the pathway.](image)

*Figure 5.1: Homocysteine metabolism and the genes involved in the pathway.*

(Adapted from Janosikova et al., 2005)
5.4 Genes involved in Homocysteine metabolism

5.4.1 MTHFR – Methylenetetrahydrofolate gene and migraine

The human methylene tetrahydrofolate reductase (MTHFR) gene consists of 11 exons spanning ~17kb and has been localised to chromosome 1p36. MTHFR synthesizes 5-methylenetetrahydrofolate, the major carbon donor required for efficient remethylation of homocysteine to methionine (Das 2003). Another polymorphism occurring in MTHFR apart from the C677T that has been discussed extensively in Chapter 4 section 4.1, is the A1298C, a glutamine to alanine substitution in the C-terminal regulatory domain of that has been reported to lead to decreased enzymatic activity as well (Lea, Ovcaric et al. 2004).

MTHFR activity is reduced by 30-40% in homozygotes of the A1298C variant (Weisberg, Tran et al. 1998). Unlike the C677T SNP, the A1298C SNP does not render MTHFR thermolabile. Studies that have investigated the effect of the A1298C SNP on MTHFR activity and homocysteine levels have reported that the A1298C SNP does not disrupt homocysteine metabolism as severely as the C677T SNP (Weisberg, Tran et al. 1998). Compound heterozygotes for both C677T and A1298C SNPs have been reported to have higher fasting homocysteine levels, suggesting that the A1298C SNP may be implicated in migraine aetiology, cardiovascular disease, neural tube defect and stroke (Weisberg, Jacques et al. 2001; Kara, Sazci et al. 2003; Ogino and Wilson 2003). Results of an independent study by Kara et al (2003) found individuals with T677T, C1298C and combined C677C/C’1298C genotypes to have even greater susceptibility to MA and MO than those with any other genotypes (Kara, Sazci et al. 2003). Individuals with the T677T/A1298A genotypes were found to be approximately more that 4 times likely to develop migraine compared to individuals
with the C677C/A1298A genotype (Kara, Sazci et al. 2003). It has been postulated that the homozygous combination of T677T/C1298C may lead to total inactivity of MTHFR enzyme leading to increased levels of homocysteine (Kara, Sazci et al. 2003).

5.4.2 MTR- Methionine synthase gene

Elevated homocysteine levels can also be contributed to inefficient methionine synthase function. Methionine synthase (MTR) is a vitamin B12-dependent enzyme. 5 methyltetrahydrofolate, produced in reactions catalysed by MTHFR is a substrate of MTR (Gaughan, Kluijtmans et al. 2001; Gos and Szpecht-Potocka 2002). MTR with the aid of CoBalam in as a cofactor, catalyses the remethylation of homocysteine to methionine and tetrahydrofolate using a methyl group donated by 5-methyltetrahydrofolate (Gos and Szpecht-Potocka 2002). The MTR gene is localised in the telomeric region of chromosome 1 (1q34) in humans. The gene is composed of 12 exons and has three domains. The N-terminal domain binds substrates for reaction, the central domain binds to cobalamin and the C-terminal domain is a regulatory domain that interacts with SAM (Chen, Liu et al. 1997; Mathew 2001).

The most commonly reported polymorphism in MTR gene is an A to G transition at nucleotide position 2756 resulting in substitution of glycine for aspartic acid at amino acid position 919, a potentially functional site of the protein (Chen 2001; Mathew 2001). This polymorphism has been suggested to decrease methionine synthase activity and increase levels of cellular homocysteine (Chen, Liu et al. 1997).

5.4.3 MTRR- Methionine synthase reductase gene
MTRR gene is essential for the NADPH-dependent reductive regeneration of methionine synthase. MTRR reduces inactive cobalamin II to active cobalamin I and methylates it to methylcobalamin using S-adenosylmethionine as the methyl donor (Elmore, Wu et al. 2007). MTRR therefore plays a pertinent role in maintaining adequate supply of active cobalamin I and thus may also be a critical determinant of homocysteine concentrations (Gaughan, Kluijtmans et al. 2001).

The human MTRR gene was identified with the aid of orthologous E.coli reducing system and was confirmed by detecting mutations in methionine synthase reductase in patients with defective reactivation of methionine synthase (Leclerc, Wilson et al. 1998). MTRR is a housekeeping gene that has 15 exons and is localised in chromosome 5 (5p15.2-p15.3). Its promoter region lacks a TATA box and its RNA can be alternatively spliced (Leclerc, Wilson et al. 1998). Defective methionine synthase reductase causing impaired methionine synthase activity leads to increased plasma homocysteine levels and decreased plasma methionine, megaloblastic anaemia as well as other neurological disorders such as neuropathy, nystagmus, seizures and dementia (Rosenblatt, Cooper et al. 1984; Watkins and Rosenblatt 1988; Fowler, Schutgens et al. 1997; Steen, Rosenblatt et al. 1997; Zavadakova, Fowler et al. 2005; Elmore, Wu et al. 2007).

Leclerc et al identified the most common polymorphism MTRR A66G SNP, leading to the substitution of an isoleucine to methionine in amino acid 22 (I22M) (Leclerc, Wilson et al. 1998). Although A66G SNP does not cause severe loss of enzyme function or change the catalytic activity of the protein, it has been implicated in increased levels of plasma homocysteine alone and in conjunction with MTHFR C677T patients (Wilson, Platt et al. 1999; Gaughan, Kluijtmans et al. 2001; Vaughn, Bailey et al. 2004). A66G SNP has also been reported to increase the risk of Neural...
tube defect (NTD) pregnancy outcome in women and Down syndrome, with hyperhomocysteinemia being the primary cause for both disorders (Wilson, Platt et al. 1999; Hobbs, Sherman et al. 2000).

5.4.4 CBS- Cystathionine β synthase gene

Cystathionine β synthase (CBS) is encoded by the CBS gene. The human CBS locus is localised to chromosome 21q22.3. CBS is a cytosolic homotetramer built of four identical monomers and it requires the cofactor pyridoxal 5’- phosphate (PLP) to convert homocysteine to cystathionine in the first step of the transsulfuration pathway (Kraus, Janosik et al. 1999). Mutations in the CBS gene are major cause of homocystinuria. Decrease or deficiency in CBS enzyme activity leads to increased levels of homocysteine and methionine in plasma and urine and decreased levels of cystathionine and cysteine (Kraus, Janosik et al. 1999). Clinical symptoms presented by patients deficient in CBS enzyme activity, include ectopia lentis, osteoporosis, skeletal deformities and mental retardation.

There have been ninety two mutations identified in the CBS gene. The majority of these mutations are missense mutations that are correlated to homocystinuria and 30% of the mutations lead to decreased protein catalytic activity (Kraus, Janosik et al. 1999). Pyridoxine-responsive I278T and the pyridoxine-nonresponsive G307S are two of the most common SNPs occurring in exon 8 of the CBS gene. The I278T SNP is pan-ethnic and accounts for approximately one –fourth of all homocystinuric alleles (Kraus, Janosik et al. 1999). The G307S SNP has been the reported to be the leading cause of homocystinuria in Ireland (Gallagher, Ward et al. 1995). It has also been frequently detected in people of Irish, Scottish, English, French, and Portuguese ancestry (Kraus, Janosik et al. 1999).
5.4.5 Folate receptor genes

A family of genes with three functional loci (α, β and γ) and a pseudogene localised in chromosome 11q13 encode folate receptors in humans (De Marco, Moroni et al. 2000). The human folate receptor alpha (FR-α) is a glycoprotein that has high binding affinity for 5-methyltetrahydrofolate (5-MTHF), the circulating form of folate in humans. It binds, sequesters and regulates the transport of the 5-MTHF to the membrane (Barber, Shaw et al. 1998). The 5-MTHF and FR-α, ligand-receptor complex is first transported into an internal membrane bound compartment known as the caveolae. The folate dissociates from the receptor in response to an acidic caveolar lumen. The high luminal concentration of 5-MTHF enables an anion carrier to transport the folate across the membrane into the cytoplasm (Kamen, Smith et al. 1991).

Abnormal receptors would be less efficient at binding and therefore result in lower 5-MTHF transport to the cytoplasm, and decrease in circulating forms of active folate. Folate receptors play a critical role during embryogenesis. Their decreased affinity or incorrect functioning increases the risk of fetal folate deprivation leading to embryonic lethality (Barber, Shaw et al. 1998). This explains the rarity of genetic variants of folate receptor encoding genes. However it must be highlighted that de novo mutations in the folate receptor gene may correlate with neural tube defects (NTD) (Barber, Shaw et al. 1998; Heil, van der Put et al. 1999; De Marco, Moroni et al. 2000).

5.4.6 MTHFD- Methylenetetrahydrofolate dehydrogenase gene
MTHFD is localised in chromosome 14q24 and has coding domains with catalytic activities that has an indirect role in folate and homocysteine metabolism (Hol, 1998). MTHFD is a trifunctional enzyme: NADP-dependant methylenetetrahydrofolate dehydrogenase (N-terminal), methylenetetrahydrofolate cyclohydrolase and ATP-dependent formyltetrahydrofolate synthase (C-terminal). It catalyses three sequential reactions which provides 5,10-Methlene-THF and its derivates, such as 10-formyl tetrahydrofolate (10-formyl-THF), which are essential cofactors for thymidylate and de novo purine synthesis (Hum, Bell et al. 1988; Schwahn and Rozen 2001; Krajinovic, Lemieux-Blanchard et al. 2004).

Christensen et al studied the essential role of MTHFD gene by inactivating it in embryonic stem cells and using them to generate spontaneously immortalised fibroblast cell lines. The study reported that this monofunctional synthase played an essential role in forming formate from formyltetrahydrofolate in the mitochondria of their studied model of mammalian one-carbon folate metabolism in embryonic and transformed cells (Christensen, Patel et al. 2005). Christensen further reported that further investigations of the primary structure of MTHFD protein exhibited mutations in key regions, which are required for dehydrogenase and cyclohydrolase activites (Christensen, Patel et al. 2005). A defective MTHFD gene may lead to deficiency of enzyme activity leading to disrupted folate and homocysteine metabolism, resulting in hyperhomocysteinemia (Cheng, Zhu et al. 2005).

Mutational analysis in patient with Neural tube defect (NTD) revealed a G878A substitution in one allele of MTHFD gene in a patient with familial NTD. The mutation resulted in an arginine to histidine substitution (R193H) in the MTHFD
protein (Hol, van der Put et al. 1998). Apart from mutations, several SNPs in the MTHFD gene have also been extensively studied in disease associated with serum folic acid and homocysteine levels. G1958A and T401C are two of the most widely studied polymorphisms in humans in association with birth defects (Brody, Conley et al. 2002; Shi, Caprau et al. 2003; Cheng, Zhu et al. 2005). These two polymorphisms and high plasma homocysteine levels have been highly associated with risk of gastric cancer in a high risk Chinese population (Wang, Ke et al. 2007). Have been significantly associated MTHFD polymorphisms have also been investigated in association with the risk of breast cancer, colorectal cancer and methotrexate sensitivity on acute lymphoblastic leukemia (Chen, Kyte et al. 2004; Krajinovic, Lemieux-Blanchard et al. 2004; de Jonge, Hooijberg et al. 2005; Li, Rong et al. 2006).

5.5 Vitamin Supplementation Studies

A variety of factors such as age, disease, drugs, enzyme, vitamin deficiencies and mutations in the genes involved in the homocysteine pathway, are associated with mild and moderate hyperhomocystinemia: of particular interest are the nutritional deficiencies in the vitamin cofactors that are involved in the homocysteine remethylation pathway: folate and vitamins B_{12} and B_{6}. Folate, vitamin B_{6} and B_{12} are important determinants of plasma homocysteine and their dietary intake has been shown to be inversely related to risks of mortality and morbidity from myocardial infraction (Selhub, Jacques et al. 1993; Rimm, Willett et al. 1998) (Figure 11). A meta analysis of clinical trials done by Wang et al (Wang, Qin et al. 2007) found evidence of folic acid and vitamin B_{6} and B_{12} supplementation to significantly decrease the risk of stroke (Wang, Qin et al. 2007).
Supplementation with folate and vitamins B₆ and B₁₂ have been demonstrated to lower plasma homocysteine levels and reduce the risk of coronary heart disease (Merle L. Diamond and Steven J. Greenberg) (Hyndman, Manns et al. 2003; Lee, Lin et al. 2003). In contrary, Lee et al found serum folate and not vitamins B₆ and B₁₂ to be significantly inversely correlated with plasma homocysteine concentration in individuals with risk factors for CAD (Lee, Lin et al. 2003). The importance and effects of the B vitamins in relation to plasma homocysteine levels is still unclear but folic acid seems to play a key role in homocysteine metabolism.

5.5.1 Folic acid supplementation and migraine

Folic acid is a member of the water-soluble B vitamin group that was isolated in 1946 from spinach leaves and its name comes from folium, the Latin word for leaf. Dietary folic acid is a combination of folates in the form of polyglutamates, which are lost during cooking. Folate coenzymes either donate or accept one carbon unit in key metabolic pathways (Miller and kelly 1996). Folates requiring reaction include synthesis of serine from glycine, synthesis of purine and pyrimidine bases, synthesis of transfer RNA and as a methyl donor to form methylcobalamin which is essential for the remethylation of homocysteine to methionine (Bailey and Gregory 1999). Other than the involvement in homocysteine metabolism, recent studies have suggested folic acid to play a role in improving endothelia dysfunction by increasing the availability of tetrahydrobiopterin (H₄B) and reducing superoxide anion generation (Das 2003).

Kopjas et al almost 40 years ago examined the use of folic acid for the treatment of acute vascular migraine (Kopjas 1969). In Kopjas study, 31 patients experiencing a migraine episode were injected with 15mg of folic acid. 60% of the patients found
their headache to stop an hour after having the injection and the remainder of the patients were reported to have had experienced a significant reduction in migraine headache intensity. The effects of folic acid on migraine headache led Kopjas to conclude his paper by stating that ‘folic acid therapy may not cure migraine, but certainly deserves a high place among the therapeutic agents for treatment, because of it’s safe, rapid and lasting pain – relieving properties’ (Kopjas 1969).

More recently a clinical trial by Di Rosa et al (Di Rosa, Attina et al. 2007) investigated the effects of folic acid supplementation on 16 migraineurs children aged between 8 and 18 with MO, who were found to have hyperhomocysteinemia and MTHFR polymorphisms. The results of this study quite interestingly found 100% resolution of migraine attacks in 10 patients, 75% reduction in 5 patients and a 50% reduction in 1 patient. The plasma levels of homocysteine were found to have decreased to the normal range in all 16 patients. However there was no statistically significant difference found when the reduction in migraine attacks was compared to the patient’s genotype possibly due to the small study group consisting of only MO patients were being tested (Di Rosa, Attina et al. 2007).

5.5.2 Riboflavin (B2) and Cobalamins (B12) vitamin supplementation and migraine

Studies in migraineurs between attacks by evoked potential, report abnormal cortical information processing with decreased habituation and strong intensity dependence (Wang, Timsit-Berthier et al. 1996). The lack of habituation and decrease in brain mitochondrial energy reserve favouring excessive cortical activation during repetitive stimulation may lead to the activation of the trigeminovascular system and thus to a
migraine attack (Welch, Levine et al. 1989; Barbiroli, Montagna et al. 1992; Montagna, Cortelli et al. 1994).

Riboflavin is a water soluble precursor to flavin mononucleotides necessary for electron transport in oxidation and reduction reactions in the mitochondria (MacLennan, Wade et al. 2008). It is important for ATP production and thus necessary for maintaining membrane stability and all cellular functions requiring energy (Evans and Taylor 2006). Riboflavin may be an effective prophylactic agent to treat migraine with no or minimal side effects caused in the CNS due to their capacity to improve mitochondrial function without altering neuronal excitability (Sandor, Afra et al. 2000). A randomized controlled trial involving riboflavin alone by Schoenen et al studied 55 patients and reported a marked reduction of migraine attacks in 59% of the study participants receiving a high-dose of riboflavin (400mg/d) (Schoenen, Jacquy et al. 1998). Three other open label studies investigating the efficacy of riboflavin in treating migraine also reported significant reduction in migraine frequency with response rates ranging from 53% to 80% to riboflavin (Schoenen, Lenaerts et al. 1994; Sandor, Afra et al. 2000; Boehnke, Reuter et al. 2004).

Contrary to previous findings, a recent study that studied the efficacy of high-dose riboflavin for migraine prophylaxis in 48 children found riboflavin to be not more effective than placebo in the study. Maizel et al investigated the efficacy of riboflavin comparison to a combination formulation of riboflavin, magnesium and feverfew in migraine prophylaxis in 49 migraineurs and reported no significant difference in migraine attacks in both groups as compared to baseline. Interestingly, 42% of the group that was on 25mg of riboflavin experienced a more than 50% reduction in the number of migraine attacks, while in the combination formulation the reduction was
44%. All of the trials discussed were small and there is the possibility that a larger dose or longer follow-up would have altered the outcome. A larger randomised controlled trial is required to understand the efficacy of riboflavin as an effective migraine treatment.

Cobalamins are vitamin B₁₂ compounds and the four most commonly known cobalamins are cyanocobalamin (CNCbl), methylcobalamin, 5-deoxyadenosylcobalamin, and hydroxocobalamin (OHCbl). In the serum, OHCbl and CNCbl are believed to function as storage or transport forms of the molecule. Cobalamins are present in active forms as methylcobalamin (MeCbl) and deoxyadenosylcobalamin in the plasma and are required for cell growth and replication (Katzung, 1989). Cobalamin serves as a coenzyme in the homocysteine methylation pathway. Methionine synthase utilises methylcobalamin as a cofactor for the remethylation of homocysteine to form methionine by using 5-methyltetrahydrofolate as a methyl donor (Das 2003). Adenosylcobalamin is required for the conversion of L-methylmalonyl-coenzyme A (CoA) to succinyl-CoA. A deficiency of vitamin B₁₂ results in an increase in total serum homocysteine concentrations as well as hypermethylmalonic academia. While elevated levels of methylmalonic acid (MMA) (Vogel, Dali-Youcef et al. 2009) may indicate a deficiency in Vitamin B₁₂, hyperhomocysteinemia may reflect either vitamin B₁₂ or folate deficiency. Adequate supply of B₁₂ vitamins is essential for growth, normal blood formation and neurological function. Hydroxocobalamin has also been proposed to possess scavenging properties against nitric oxide (Rajanayagam, Li et al. 1993).

One of the earliest studies by Urdahl-Aasen et al 1951 discovered vitamin B₁₂ treatment to resolve migraine attacks in a patient with anaemia perniciosa and
continued on to show that intramuscular administration of cyanocobalamin either diminished or in some patients abolished migraine attacks (Urdahl-Aasen 1951; Dalsgaard-Nielsen 1952; van der Kuy, Merkus et al. 2002). More than 18 years after the first observation, Dalsgaard-Nielsen et al 1970 performed a double blind placebo-controlled study on the effects of intramuscular administration of 2mg of cyanocobalamin every 2 weeks for 2 months on 29 patients and found cyanocobalamin to have no therapeutic effect.

5.6 Effects of vitamin supplementation and the MTHFR

C677T genotype on migraine disability

Lea et al in 2009 investigated the effects of folic acid and vitamins B₆ and B₁₂ supplementation on homocysteine lowering and migraine disability and whether it is dependent on MTHFR C677T genotype by conducting a randomised, double blinded and placebo controlled clinical trial for 6 months on fifty two MA sufferers. The results of this trial firstly showed a significant reduction in plasma homocysteine concentrations in the treatment group compared to the placebo group. The major finding of this trial was that the migraine group under vitamin treatment showed a 2 fold reduction in migraine disability according to the Migraine Disability Assessment Score (MIDAS) instrument. This decrease was greater overall compared with the group under placebo treatment. Furthermore, vitamin treated migraineurs also reported a significant decrease in migraine headache frequency and pain severity. When the treatment effect was stratified by the MTHFR C677T genotype, it was observed that the C allele carriers responded better to vitamin treatment compared with the TT genotypes.
The results of this trial were supported by the open-labelled study by Di Rosa et al (Di Rosa, Attina et al. 2007) and provided initial evidence that homocysteine lowering through folic acid coupled vitamins B₆ and B₁₂ may reduce migraine disability in a subgroup of patients. One of the limitations of this study was the limited number of participants in the trial. While fifty two participants were initially recruited, only forty seven participants completed the trial and were reassessed after 6 months of intervention. The second limitation of this trial was the unavailability of the participant’s diet information during the course of the trial. Variation in the participant’s diet is a potential confounding factor for this study. Although a significant reduction in homocysteine levels were reported in the vitamin treated group compared to the placebo group, it is not known if this effect is independent of the dietary variations between the two treatment groups.

Migraine is a polygenic multifactoral disorder. With multiple genes reported to contribute to migraine susceptibility, it is plausible that different genotypes in these genes may cause varying disease manifestations and also varying response to medication. The current study thus investigated the genotypic effects of both the MTHFR and the MTRR gene on folate and vitamin B treatment response of lowering homocysteine and migraine disability.

### 5.6.1 Study design and participant group

This study analysed the genotypic effect of MTHFRC677T and MTRRA66G polymorphisms on daily folic acid and B₆ and B₁₂ vitamin treatment for lowering homocysteine, migraine disability, frequency and pain severity by conducting a randomized, double blindered placebo controlled clinical trial over a 6 month period. The trial guidelines were designed using the guidelines for controlled trials of drugs in
migraine (Tfelt-Hansen, Block et al. 2000). The study recruited female Caucasian adult between the ages of 18 and 60, of European ancestry from all over Australia. All participants were interviewed and completed a detailed questionnaire that was administered through Griffith University’s Genomics Research Centre (GRC). Migraine is more prevalent in females and there may be a difference in migraine susceptibility and response in relation to treatment thus the current study only focussed on one gender. Females between the ages of 18 and 60 were recruited and participants were included if they had suffered migraine for over 20 years and had a current diagnosis of MA (>90% of their migraine attacks were associated with aura), and a 1-year history of severe, long lasting attacks (at least 4 attacks lasting more than 48h), had a family history of migraine. Confirmation of migraine diagnosis was carried out using IHS criteria. Participants who were currently taking vitamin supplementation, pregnant, or had been diagnosed with a clinically recognised co-morbid disease such as vascular disease, depression or epilepsy were excluded from the trial to reduce clinical and pathological heterogeneity. Participants that had taken part in another clinical trial or had received any experimental therapy within the last one month were also excluded from the trial. The patient group was not selected on the basis of pre-existing folic acid, B$_6$ or B$_{12}$ deficiency or their MTHFR C677T and MTRR A66G variant genotypes.

### 5.6.2 Treatment

245 female participants meeting the inclusion criteria were randomly assigned into either the placebo or the treatment group. A blocked random allocation sequence was generated using Microsoft Excel (Microsoft, USA). Participants and everyone involved in this trial were blinded to randomisation and group allocation. Participants received either vitamin tablets containing 2mg of folic acid, 25mg of vitamin B$_6$ and
400 µg of vitamin B₁₂ or the placebo tablet. Participants were instructed to take one tablet daily for 6 months. Both the vitamin and placebo tablets were produced by Blackmores and were identical in appearance.

5.6.3 Baseline and follow up Assessment

Before the treatment all participants were assessed for migraine disability using the Migraine Disability Assessment Score (MIDAS) instrument, which provides a measure of productive days lost to migraine headache in previous 3 months (i.e. migraine disability), as well as headache frequency and pain severity (Lea, Colson et al. 2009; Stewart 1999). Patients were asked to complete a daily diary during the trial period to record the details of their migraine symptoms as well as treatment compliance. Patients were also instructed to take their usual migraine treatment for acute attacks. A blood sample was collected for baseline measurement of plasma homocysteine (µmol/l), folate (nmol/l), vitamin B₁₂ and B₆ (pmol/l) concentration. 2ml of venous blood was collected for Genomic DNA extraction and genotyping purposes.

Patients were contacted after 3 months for headache diary and compliance checking. At the end of the 6 month trial the patients were reassessed at the GRC clinic. They were questioned about their migraine history in the last 6 months since the start of the trial. A second collection of blood samples was done for measurement of homocysteine, folate, B₁₂ and B₆ concentrations.

5.6.4 Dietary consumption
Variation in the participant’s diet is a potential confounding factor for this study. In order to control these effects, participants were required to keep a daily diary of food type, amount and frequency. Each participant was given 2 diary packs, each pack consisting of 7 days of daily diet intake to be recorded. The diet dairy was designed to estimate the usual dietary intake of nutrients such as B₆, B₁₂ and folate over a typical week. Participants were asked to complete their diet dairy once a fortnight, on only one day until each day of the week has been recorded. The nutrient intake of participants was analysed using the NUTTAB version 2010 database, which is based on the Australian New Zealand food standard code.

5.6.5 Migraine disability measurement

Migraine disability measured by the MIDAS instrument was the primary clinical outcome in this trial. Studies have shown that the MIDAS instrument is a valid and clinically useful instrument for assessing health-related quality of life in migraineurs (Appendix C). Based on the 5-question MIDAS rating, participants were arbitrarily categorized into a ‘low’ disability group if they had a MIDAS rating of 0-10 and into a ‘high’ disability group if they had a MIDAS rating greater than 11 (Lea, Colson et al. 2009; Stewart 1999). Secondary outcome variables, which are partly captured within the primary outcome, were migraine frequency and head pain severity. These were measured as number of days with headache (over a 3 month period) and a pain score (based on a scale of 1-10), respectively (Lea, Colson et al. 2009; Stewart 1999).

5.6.6 Genotyping

The 2ml of blood, which was collected from the participants at the beginning of the trial was analysed for the presence of MTHFRC677T and MTRR A66G
polymorphisms in the GRC laboratory using the following technique. Genomic DNA was extracted from 2ml of venous blood using the QIAGEN DNeasy Blood & Tissue Kit according to the protocol as detailed in Appendix A. DNA was checked for quality and quantity using the Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Genotyping of the C677T variant was conducted by polymerase chain- reaction fragment length polymorphism (PCR-RFLP) (Refer to chapter _, for detailed explanation of the method). Briefly C677T SNP was amplified using the forward primer 5’ TGA AGG AGA AGG TGT CTG CGG GA 3’ and reverse primer 5’ AGG ACG GTG CGG TGA GAG TG 3’ (name of Primer Company). The PCR amplification was performed as per the following conditions.

A total PCR reaction volume of 20ul was prepared containing 20ng of DNA, 1 unit of Taq polymerase, 1.75mM of MgCl2, 5mM dNTPs, 10x PCR buffer mix and 5um of each primer. Amplification conditions involved an initial denaturation for 3mins at 95°C, followed by 30 cycles of 1min at 94°C, 1min at 60°C and 1min at 72°C, with a 10min final extension at 72°C. After PCR amplification, 10ul of PCR product of 198bp was digested for 16hr at 37°C with Hinf I (NEB). The digested products were analysed by electrophoresis on a 3% agarose gel in the presence of ethidium bromide. The bands of the DNA fragments were visualized under ultraviolet light. Hinf I digestion of the C allele gave a band of 198bp. The T allele DNA created one restriction site and Hinf I digestion gave 175 and 23bps. The results of each genotype were confirmed in randomly selected individuals by direct sequence analysis. Figure _ shows an agarose gel electrophoretogram of all possible genotypes.

High resolution melt analysis was used to genotype the A66G polymorphisms. Primers used for genotyping the MTRR A66G polymorphism are “Forward 5’: GCA AAG GCC ATC GCA GAA GAC AT 3’ AND “Reverse 5’: AAA CGG TAA AAT
CCA CTG TAA CGG C 3’. The reaction mixture used HotStarTaq (Qiagen, Hilden Germany) and consisted of 40 ng of genomic DNA, 10× PCR buffer, 25 mM MgCl$_2$, 5uM of each primer, 2.5mM of dNTPs, 50 µM of SYTO 9 (Invitrogen, Carlsbad, USA), 0.5 U of HotStarTaq polymerase and PCR grade water in a volume of 25 µL. All PCR reactions were performed in duplicate. PCR cycling and HRM analysis was performed on the Rotor-Gene™ 6000 (Corbett Research, Mortlake, New South Wales, Australia). The PCR cycling conditions for the MTRR A66G were as follows; one cycle of 95°C for 5 minutes; 45 cycles of 95°C for 5 seconds, 45 cycles of 60°C for 10 seconds, 72°C for 20 seconds; one cycle of 95°C for 1 second, 72°C for 90 seconds and a HRM step from 75 to 85°C rising at 0.1°C per second.

Plasma homocysteine, folate, B$_6$ and B$_{12}$ levels were measured in an accredited pathology laboratory.

5.7 Statistical Analysis

All analyses were performed using the Statistical Package for Social Sciences (SPSS version 17.0).

5.7.1 Modified Intention-to-treat

The analysis for the current trial was conducted on a modified intention-to-treat (ITT) principle. The modified ITT population was composed of all randomised participants who started the trial and consumed study supplements on at least one occasion, excluding those who withdrew from the trial after the randomisation process had taken place but before the commencement of study supplement consumption.

5.7.2 Parametric and Non-parametric analysis
At baseline, group means were tested using unpaired samples t-tests. The median were compared using the Mann Whitney U tests and proportions were compared using the $\chi^2$ test of independence. The primary hypothesis of this trial, that vitamin supplementation reduced migraine disability was tested by comparing proportions of high disability migraineurs before and after the 6 month trial in both the vitamin and placebo groups. The $\chi^2$ test of independence was used to compare the proportion changes. Mean changes were compared before and after treatment using paired samples t-tests and median changes were compared using nonparametric Wilcoxin signed rank tests for related samples. The relationships among the baseline biochemical variables were assessed using the Pearson’s correlation tests. Post treatment unpaired samples t-tests were conducted where means for treatment and placebo groups were compared at 6 months. The significance threshold was set at $\alpha$ level of 0.05.

5.7.3 Linear regression analysis

Linear regression analysis was performed using the “successive steps” method, to determine the independent predictors of the difference in homocysteine levels before and after the trial, allowing the introduction of a new variable if the P value of the new model was less than 0.05, and excluding those yielding a P value higher than 0.10 in each step. The independent variables were age, genotype, treatment group (placebo vs vitamin), and dietary intake of B$_6$, B$_{12}$ and folate.

5.8 Results

Figure 5.2 illustrates the patient flow through the trial from January 2009 to January 2010. Six hundred and twenty nine migraine patients were assessed for eligibility prior to enrolment into trial. 384 migraine patients were excluded from enrolment due
to reasons such as not meeting inclusion criteria, refusal to participate in placebo controlled trial and other reasons. 245 participants were initially enrolled in the trial and were randomly assigned to either the placebo group or the vitamin treated group but 3 participants dropped out before the commencement of the trial and the remaining 242 participants received baseline assessment and commenced the trial. One hundred and nineteen participants were on the vitamin treated group and the remaining 123 participants were in the placebo treated group. Forty four participants were lost to follow up due to lack of compliance and 162 participants completed the trial (76 Vitamin: 86 placebo).

### Patient flow chart

<table>
<thead>
<tr>
<th>Enrollment</th>
<th>Assessed for eligibility (n= 629)</th>
<th>Excluded (n=384)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Randomised (n=245)</td>
<td></td>
</tr>
<tr>
<td>Allocation</td>
<td>Allocated to intervention (n=119)</td>
<td>Allocated to intervention (n=123)</td>
</tr>
<tr>
<td></td>
<td>Received intervention (n= 103)</td>
<td>Received intervention (n=103)</td>
</tr>
<tr>
<td></td>
<td>Did not receive intervention (n= 16)</td>
<td>Did not receive intervention (n=20)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>Discontinued intervention (n=27)</td>
<td>Discontinued intervention (n=17)</td>
</tr>
<tr>
<td>Analysis</td>
<td>Analysed (n= 103)</td>
<td>Analysed (n= 103)</td>
</tr>
</tbody>
</table>

**Figure 5.2: Participant flow chart for the trial**
5.8.1 Baseline Analysis

Table 5.1 shows the baseline clinical characteristics of the participant group. For the total migraine group (n= 206), mean folate concentration was 30.1 nmol/l, which is above the average for a general Caucasian population replete for folate (13.7nmol/l). The mean plasma homocysteine concentration for the migraine group was 11.5 µmol/l, which is also above the average for a general Caucasian population (8.9 µmol/l). The mean levels of B6 and B12 at baseline fell within the normal range for this patient group. For the total group, plasma homocysteine concentration was negatively correlated with plasma folate (Pearson’s \( r = -0.057 \), \( P = 0.438 \)). Vitamin B6 (Pearson’s \( r = -0.212 \), \( P = 0.05 \)), vitamin B12 (Pearson’s \( r = -0.279 \), \( P= 0.000 \)). The percentage of participants with high migraine disability did not differ significantly between the placebo and the vitamin treated group (P=0.18). Similarly the migraine attack frequency (P=0.41) and migraine pain severity (P= 0.38) did not differ significantly between the placebo and the vitamin treated group. There were no statistically significant differences between the vitamin and placebo groups for the test variables at baseline.

Table 5.1: Clinical characteristic of participant groups at baseline.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total</th>
<th>Vitamin</th>
<th>Placebo</th>
<th>( P )-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.patients</td>
<td>206</td>
<td>103</td>
<td>103</td>
<td>0.65</td>
</tr>
<tr>
<td>Age in years, mean(SD)(^c)</td>
<td>44(13)</td>
<td>42(13)</td>
<td>45(13)</td>
<td>0.65</td>
</tr>
<tr>
<td>Female</td>
<td>206</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate, mean (SD)(^b)</td>
<td>30.1(12.5)</td>
<td>31.4(12.8)</td>
<td>28.7(12.2)</td>
<td>0.56</td>
</tr>
<tr>
<td>Vitamin B12, mean (SD)(^b)</td>
<td>322.7(148.5)</td>
<td>315.7(114.9)</td>
<td>328.6(175.7)</td>
<td>0.15</td>
</tr>
<tr>
<td>Vitamin B6, mean (SD)(^b)</td>
<td>78.8(12.6)</td>
<td>77(11.9)</td>
<td>80.8(13.5)</td>
<td>0.82</td>
</tr>
<tr>
<td>Homocysteine, mean (SD)(^b)</td>
<td>11.5(3.4)</td>
<td>11.6(3.6)</td>
<td>11.5(3.3)</td>
<td>0.7</td>
</tr>
<tr>
<td>B12ug consumption, mean (SD)(^b)</td>
<td>1.01(0.5)</td>
<td>1.1(0.5)</td>
<td>0.9(0.5)</td>
<td>0.9</td>
</tr>
<tr>
<td>B6mg consumption, mean (SD)(^b)</td>
<td>1.7(0.6)</td>
<td>1.7(0.7)</td>
<td>1.7(0.6)</td>
<td>0.13</td>
</tr>
<tr>
<td>Folate ug consumption, median (range)(^a)</td>
<td>428.7(225.6)</td>
<td>433(239)</td>
<td>424(212)</td>
<td>0.74</td>
</tr>
<tr>
<td>High migraine disability (%)(^b)</td>
<td>69.7</td>
<td>74%</td>
<td>65.30%</td>
<td>0.18</td>
</tr>
<tr>
<td>Attack frequency median (range)(^a)</td>
<td>2 (1-6)</td>
<td>2(1-6)</td>
<td>2(1-4)</td>
<td>0.41</td>
</tr>
<tr>
<td>Head pain score median (range)(^a)</td>
<td>7(3.5-10)</td>
<td>7(1-10)</td>
<td>7(3.5-10)</td>
<td>0.38</td>
</tr>
<tr>
<td>MTHFR C677T (TT) genotype %</td>
<td>15.3</td>
<td>18.3</td>
<td>12.4</td>
<td>0.46</td>
</tr>
<tr>
<td>MTRR A66G (GG) genotype %</td>
<td>25.4</td>
<td>19.6</td>
<td>30.9</td>
<td>0.15</td>
</tr>
</tbody>
</table>

\( MTHFR \ C677T, \ methylenetetrahydrofolate \)
5.8.2 Six month follow-up analysis

A total of 162 participants completed the trial. 86 of these participants were on placebo and 76 were on vitamin supplementation. Figure 5.3a to 5.3d shows the post treatment change in plasma folate, B₆, B₁₂ and homocysteine concentration in vitamin and placebo group. After 6 months of treatment, the vitamin treated group had marked increases in folate, B₆, B₁₂ concentration compared with baseline and the placebo group (P< 0.001). In the placebo group, the mean folate levels increased by 11.8% after 6 months (28.7 – 32.1 nmol/I, P= 0.852). The B₁₂ levels decreased by 6.5% (328.6-307.1 pmol/I, P= 0.059) and the B₆ levels decreased by 7.8% (80.8- 74.5 pmol/I, P= 0.292).

In the vitamin treated group, the median homocysteine levels reduced by 20% after 6 months (11.5-9.2 μmol/I, P<0.001) compared with 4.8% reduction observed for the placebo group (P=0.121) (refer to Figure 5.3a). The effect of treatment on the reduction of homocysteine levels remained significant after correction for confounding factors such as age, genotype and dietary consumption of vitamin B₆, B₁₂ and dietary folate (r²=0.042; P = 0.019) (refer to Table 5.2). The dietary consumption of B₆, B₁₂ and dietary folate between the vitamin and placebo groups were not significantly different at the 6 month follow-up analysis (B₆, P=0.721; B₁₂, P= 0.891; Dietary folate, P= 0.373).
(a) Change in homocysteine levels after trial in placebo and vitamin group

(b) Change in folate levels after trial in placebo and vitamin group

Figure 5.3 (a) and (b): Change in (a) homocysteine and (b) folate levels over the treatment period in placebo and vitamin treated groups. Values are represented as mean ± SEM.
Figure 5.3 (c) and (d): Change in (c) B₆ and (d) B₁₂ levels over the treatment period in placebo and vitamin treated groups. Values are represented as mean ± SEM.
Table 5.2: Regression analysis model predicting homocysteine reduction after trial

<table>
<thead>
<tr>
<th>Model</th>
<th>Variable</th>
<th>R²</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Treatment</td>
<td>0.042</td>
<td>5.67</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Model 1: The correlation for difference in homocysteine levels after trial was corrected for age, MTHFRC677T genotype, and dietary consumption of B6, B12 and dietary folate

In the vitamin treated group the frequency of high migraine disability decreased after 6 months of supplementation from 74% to 56.9% (P=0.022). The reduction in the placebo group was not statistically significant (65.3% to 53.2% (P=0.098) (refer to Figure 5.4). Headache frequency did not decrease from a median of 2 for both the vitamin (P=0.46) and placebo (P=0.147) groups after 6 months. The vitamin treated group reported a decrease in pain severity from a median score of 7 to 6 (P= 0.017), whereas the placebo group reported no change (P>0.1) (refer to Figure 5.5).
**Figure 5.4**: Change in frequency of high-level of migraine disability as measured by the Migraine Disability Score (MIDAS) instrument (MIDAS > 11) over the treatment period in vitamin and placebo groups.

![Change in head pain severity score after trial in placebo and vitamin group](image)

**Figure 5.5**: Change in average pain severity score over treatment period for placebo and vitamin groups. Values are medians. Quartiles are not shown but reductions are statistically significant (P<0.05).

### 5.8.3 Treatment response by MTHFRC677T and MTRRA66G genotype

When the vitamin-treated group was stratified by MTHFRC677T genotype, the mean homocysteine reduction was 20% for both the CC and the CT group (P<0.001) compared to 13.3% for the TT carriers (P=0.095) (refer to Figure 5.6). For further analysis the genotypes (CC and CT) that showed the largest decrease in homocysteine
levels after vitamin supplementation were grouped and their effect on migraine frequency, pain severity and disability were analysed. There was no significant reduction in migraine frequency in both CC/CT and the TT genotype groups after the trial. The CC/CT genotype group showed a significant reduction in migraine pain severity (P= 0.01) with the mean pain scores decreasing from 7 to 6. This significant reduction was not observed in the TT genotype group (P=0.278) with the mean pain scores remaining at 6 before and after the trial. When migraine disability was analysed in the CC/CT and TT genotype groups, the percentage of highly disabled migraineurs decreased from 77% to 57% after the trial in CC/CT group (P= 0.009) and by 6.3% in the TT genotype group (P=0.568).

Change in homocysteine levels over trial period in the vitamin group for the MTHFR variant

![Graph showing change in homocysteine levels over treatment period in vitamin treated group stratified by MTHFRC677T variant.](image)

**Figure 5.6:** Change in homocysteine levels over the treatment period in vitamin treated group stratified by the MTHFRC677T variant. Values are represented as mean ± SEM.
When the vitamin-treated group was stratified by MTRR A66G genotype, the mean homocysteine reduction was 27% for the AA carriers (P<0.001), 16% for the AG carriers (P=0.001) compared to 13% for the GG carriers (P=0.03) (refer to Figure 5.7).

**Figure 5.7: Change in homocysteine levels over the treatment period in vitamin treated group stratified by the MTRR A66G variant.** Values are represented as mean ± SEM.

For further analysis the AA and the AG genotypes of the A66G variant, which showed the largest decrease in homocysteine levels after vitamin supplementation were grouped and their effect on migraine frequency, pain severity and disability were analysed. Again there was no significant change in migraine frequency between the two groups. The A allele carriers of the MTRR A66G variant showed a significant reduction in migraine pain severity with the pain scores
decreasing from 7.1 to 6.2 after the trial (P=0.002). In the GG genotype group however, the pain scores increased from 6.3 to 7 (P=0.05). When the percentage of high migraine disability was analysed in the two genotype groups, the percentage of highly disabled migraineurs decreased from 75.3% to 53.8% in the A allele carriers (P= 0.006), while it only decreased from 68.8% to 66.7% in the GG genotype group (P=0.45).

The C allele carriers of the MTHFRC677T polymorphism and the A allele carriers of the MTRRA66G polymorphism were compared with the homozygote mutant allele carriers for both genotypes (CC/CT/AA/AG vs TT/GG). There was no significant decrease in migraine frequency in the two groups. Migraine head pain severity was however significantly decreased after the 6 months of vitamin supplementation in the CC/CT/AA/AG group from a pain score of 7 to 6.2 (P= 0.002) compared to the TT/GG group, which remained at a constant score of 6 (P= 0.5). Similarly migraine disability was significantly reduced after the trial in the CC/CT/AA/AG group with the percentage of highly disabled migraineurs decreasing from 74.6% to 52.2% (P=0.015) compared to the TT/GG group, where the percentage of highly disabled migraineurs decreased from 50% to 33.3% (P= 0.64) (refer to Figure 5.8).
Figure 5.8: Change in high migraine disability in MTHFR and MTRR genotype group. Change in frequency of high-level of migraine disability as measured by the Migraine Disability Score (MIDAS) instrument (MIDAS > 11) over the treatment period in the MTHFR677, CC and CT genotypes combined with the MTRR66 AA and AG genotypes group and the MTHFR667 TT genotype combined with the MTRR 66 GG genotype group.

5.9 Discussion of Results

Migraine is a chronic and often disabling condition has an enormous impact on both the individual sufferer and on society at large (Leonardi, Steiner et al. 2005). There are several acute care therapies and medications currently available that have been successful in either treating migraine symptoms or decreasing migraine attacks and several other medications and therapies in various stages of development (Rapoport
However current medications and therapies work with differing efficacy in migraineurs and are often associated with adverse effects (Lea, Colson et al. 2009). Thus the search for effective, safe and inexpensive migraine therapies to combine with or replace current therapies still continues (Lea, Colson et al. 2009). One of the most significant findings in recent times is the involvement of genetics in migraine aetiology, which has added further complexity to understanding the pathophysiology underlying migraine. This has made migraine diagnosis and treatment options varied and continuously improved.

Mild hyper-homocysteinemia has been reported to increase the risk of artherosclerotic vascular disease (Wald, Law et al. 2002). MA sufferers have been linked to increased risk of vascular brain lesions and ischaemic stroke (Kurth, 2007; Merikangas, 1997; Tzourio, 2001). It is still not clear if homocysteine levels are raised in migraineurs; however there is evidence to suggest that the CSD phenomenon observed in MA can also occur during a stroke episode (Scher, Terwindt et al. 2006). Based on the potential role homocysteine may play on the cerebrovascular system and the co morbidity of migraine and stroke, it is plausible that homocysteine levels may be involved in the underlying pathophysiology of both MA and stroke (Silberstein 2001). The reasons for hyper homocysteinemia may be varied: mutations in the genes for MTHFR, MTRR and CBS or possible nutritional deficiencies in cofactors in the homocysteine metabolism (Selhub, 1993).

The current study examined the genotypic effects of the MTHFR and MTRR gene on folate and vitamin B treatment response in migraineurs. Vitamin supplementation in the current trial was well tolerated by the study group with no reports of adverse reactions. At baseline, the homocysteine levels were mildly elevated in the migraineurs.
group compared to the general Caucasian population with some but not all studies of homocysteine in migraine (Scher, Terwindt et al. 2006; Di Rosa, Attina et al. 2007; Lea, Colson et al. 2009). Results of this trial showed the vitamin treatment compared with the placebo significantly decreases the homocysteine levels at six months by an average of 2.2 µmol/l, an effect size in Australian Caucasian female MA sufferers. This decrease in homocysteine levels remained significant after correcting for dietary consumption of folate, B6 and B12. There was no significant difference in the consumption of folate and B vitamins in the vitamin treated group and placebo group at the end of the trial period.

The 2.2 µmol/l reduction in the homocysteine levels at 6 months in the current trial approximates the 2.0 µmol/l reduction observed in the “Vitamin Intervention for Stroke Prevention (VISP) trial, which randomised 3680 stroke survivors of primarily white origin from North America to receive high-dose (folic acid 2.5mg; B6 25mg; B12 0.4 mg) or low dose folic acid 20 µg; B6 200ug; B12 0.6 µg) (Toole, Malinow et al. 2004). However the 2.2 µmol/l reduction in homocysteine levels observed in the current trial is lower compared to the 4.0 µmol/l reduction observed in a study by Lea et al in 2009 that investigated the effect of vitamin supplementation and MTHFR (C677T) genotype on homocysteine- lowering and migraine disability in 52 Australian Caucasians (Lea, Colson et al. 2009). The smaller than expected treatment effect of the prescribed dosage of vitamins seen in the current trial is most likely attributable to the implementation on September 2009, of fortification of wheat flour for bread making with folic acid in Australia, which coincided with the conduct of the trial. This may also be the reason for the slight increase in folate levels observed in the placebo group at 6 months follow up assessment.
There was significant reduction in migraine pain severity and disability according to the MIDAS instrument scores in the vitamin treated group. This decrease was greater overall compared with the placebo effect, which was not statistically significant (Figure 3). However the frequency of migraine headache did not decrease from a median of 2 in the vitamin treated group, which is inconsistent to that observed in the pilot study by Lea et al that reported a decrease from a median of 4 to 1 in migraine headache frequency in the vitamin treated group. The absence of significant reduction in migraine headache frequency observed in this trial may be attributable to the fact that the trial population only had a median headache frequency of 2 at baseline. A population of median headache frequency of 4 and more at baseline may have yielded a significant reduction in headache frequency after the prescribed vitamin treatment.

When the effects of the MTHFRC677T variant on treatment effect in migraineurs was assessed in the current study, a significant reduction in homocysteine levels in the C allele carriers compared to the mutant homozygote TT genotype carriers was observed. Further analysis found the C allele carriers to respond better to vitamin supplementation in lowering migraine pain severity and high migraine disability compared to the TT genotype carriers. These results are supported by similar findings reported in the pilot study by Lea et al (Lea, Colson et al. 2009) and adds to the idea that individuals with TT genotypes, having a 50% reduction in their enzymatic rate, are genetically slower in homocysteine metabolism and thus would require an increased dosage of vitamins compared with the C allele carriers to experience the same reduction in homocysteine levels and consequent migraine symptoms.

In addition to the MTHFR C677T variant, the current trial also investigated the A66G variant in the MTRR gene. The MTHFR product, 5-methyl-THF, donates a methyl
group for the remethylation of homocysteine to methionine, which is catalysed by MTR in a vitamin B₁₂ dependent reaction. MTR may become inactive due to oxidation of its vitamin B₁₂ cofactor and restoration of MTR activity is dependent on reductive remethylation of vitamin B₁₂ by MTRR (Leclerc, Campeau et al. 1996). The functional effects of the MTRR A66G variant have not been fully understood, however in vivo experiments suggest that the A66G variant MTRR enzyme restores MTR activity less efficiently than wild-type (Olteanu, Munson et al. 2002), and has also been shown to increase plasma homocysteine levels in humans (Leclerc, Wilson et al. 1998; Wilson, Platt et al. 1999). When the MTHFR 677TT genotype occurred with either the homozygous or heterozygous genotype for the MTRR A66G variant, it may exacerbate the effect of the MTHFR variant alone (Vaughn, Bailey et al. 2004).

When the analysis was stratified by the MTRRA66G variant, the A allele carriers showed the largest reduction in homocysteine levels and migraine pain severity and high migraine disability under vitamin supplementation compared to the mutant homozygote GG genotype carriers. The results of MTRRA66G similar to the MTHFRC677T, showed the wildtype allele carriers to respond better to vitamin treatment in reducing homocysteine levels, migraine pain severity and disability, than the homozygote mutant allele carriers. We continued to investigate if the combined effect of both these variants was greater in reducing migraine related head pain and disability. The combined effects of the C and A allele carriers of the C677T and A66G variants respectively, showed significant reduction in migraine pain severity and migraine disability. However the combined effect of the two variants was not significantly different from the independent effect of the two variants on migraine pain severity and disability. This suggests that both the MTHFRC677T and MTRR
A66G appear to be acting independently in affecting vitamin treatment response in migraineurs.

Genes involved in the homocysteine pathway play a crucial role in the amount of homocysteine in the extracellular media such as plasma (Woodside, Yarnell et al. 1998). The allele groups of MTHFR and MTRR that showed the largest reduction in homocysteine levels showed the most significant reduction in migraine pain severity and disability under vitamin treatment. There is an undeniable relationship between homocysteine levels and migraine disability. The homozygote mutant allele carriers of the MTHFR and MTRR variant may need a higher dose of vitamin supplementation to experience the same effect as the wild type allele carriers of the variants, in migraine pain severity and disability reduction. Further clinical trials of higher doses of vitamin supplementation are required to make an evidence based argument of this idea. The effects of hyperhomocysteinemia may be a partial determinant for the neuro and/or vascular pathologies underlying MA and stroke (Lea, Colson et al. 2009). Other genes and functional variants associated with the homocysteine metabolism cascade other than the MTHFR and MTRR have to be investigated in relation to vitamin treatment response in migraineurs.
Chapter 6

A Candidate Gene Analysis Investigating Migraine Pain
6.1 Introduction

Migraine is a common and debilitating neurologic disorder that is classified as either Migraine with aura (MA) or without aura (MO) based on the criteria specified by the International Headache Society (IHS) (HCCIHS 2004). A typical migraine attack is characterised by throbbing headache aggravated by movement accompanied by nausea, vomiting, phonophobia and photophobia (Monteith and Goadsby 2011). A dysfunction in the neuromodulatory structures of the brainstem is the current theory behind this disabling condition (Afridi 2005). Acute migraine treatment consists of non specific agents such as NSAIDs, acetaminophen and aspirin. Migraine specific medications include ergotamine, DHE and the introduction of triptans has been highly effective for many migraineurs. However not all migraineurs have found the current medication options effective and hence the search for novel treatment options continues.

An individual’s pain experience is shaped by psychological, behavioural and biological factors (Vossen 2010). Apart from these factors, an individuals age, sex, ethnicity, anxiety, type of pain have also been implicated to impact on pain sensitivity. Furthermore, linkage disequilibrium, genome-wide association, case-controlled and family controlled studies have shown the heritability of complex traits and response to drug treatments, suggesting that genetic factors may play a role in the inter-individual differences in pain responses (Mague 2010; Lacroix-Fralish 2007; Vossen 2010).

6.2 OPRM-1 gene
The human µ-opioid receptor, coded by the µ-opioid receptor 1 gene (OPRM1), is the site of action for many endogenous opioid peptides and a significant target for opioid analgesics (Ikeda 2005; Zhang, Chen et al. 2006). Opioid analgesics, such as morphine, codeine and pethidine have been used by clinicians to treat severe cases of migraine headache (Williamson 2001). Opioids are reported to have inhibitory actions at the presynaptic nerve terminal to inhibit neurotransmitter release from the trigeminal sensory neurons innervating peripheral structures (Williamson 2001).

The human OPRM1 gene spans over 200k and is at chromosome location 6q 24-25 (Mague 2010; Zhang 2010). A commonly studied OPRM-1 SNP is the A118G in exon 1 that causes an Asn40Asp substitution at a putative glycosylation site in the extracellular domain (Bond 1998). This SNP has been widely investigated in human disease and drug response and has been implicated in disorders such as drug addiction, stress responsivity, drug responses, including dependence and pain reduction. However the functional consequences of this SNP are yet to be completely understood (Mague 2010; Vossen 2010; Zhang 2010).

A myriad of studies have investigated the relationship between pain thresholds and analgesic responses to opioid administration for the A118G SNP (Mague 2010). In most instances the G allele has been associated with decreased pain threshold and reduced response to opioids for patients receiving post operative care or chronic pain (Campa 2008; Chou 2006; Mague 2010; Tan 2009). Oertel et al in 2006 demonstrated that the G118 allele required higher concentrations of opioid analgesic for pain relief following electrical pain stimulation in healthy volunteers (Oertel 2006).
Contradictory to Oertels et al findings, Fillingim et al in 2005 reported that healthy volunteers with the G118 allele receiving different experimental pain procedures had reduced pain responses to pressure (Fillingim 2005). Additionally, the males in the experimental group with the G allele rated thermal pain lower than those with the A allele. However, the females carrying the G allele in the experimental group reported elevated pain response following thermal pain administration, consistent with previous studies (Fillingim 2005). Lötsch et al in 2006 investigated the role of G118 allele for nociceptive sensory processing using event-related potentials (ERPs) and found that the ERP amplitudes of the G118 carriers were higher than the non-carriers, suggesting decreased pain perception in G118 allele carriers (Lotsch, Hummel et al. 2006). The studies described above suggest that the role of the A118G SNP in influencing pain perception and analgesic requirement is debatable and not fully explicit.

To the best of our knowledge, there has been no study that has investigated the propensity of the A118G SNP in affecting pain perception in migraine sufferers. In this study we investigated the effect of the OPRM-1 A118G SNP on migraine disability, frequency, and pain severity. As with the 5-HT_{1B/1D} agonists, the opioids’ inhibitory actions within the trigeminal caudalis and neuropeptide release has made it a significant target for opioid analgesics, which are used in migraine pain treatment. Current migraine treatments and medications exhibit differing efficacy in sufferers and studies have shown that certain gene mutations influence treatment suitability and options for some migraine subtypes (Lea, Colson et al. 2009; Lea, Ovcacic et al. 2004; Wessman, Terwindt et al. 2007). Understanding the effect of the A118G SNP on pain sensitivity could aid in optimising pain treatment for migraineurs who receive opioid analgesics.
6.3 Methods

6.3.1 Subjects

The study involved a total of 159 Caucasian adult participants. European desents living in Australia, having emigrating ancestors within the last 160 years from various locations within the British Isles and other parts of Europe were recruited from East Coast of Australia and were interviewed and completed a detailed questionnaire that was administered through Griffith University’s Genomics Research Centre (GRC). Participants were included if they had suffered migraine for over 20 years and had a current diagnosis of MA (>90% of their migraine attacks were associated with aura), and a 1-year history of severe, long lasting attacks (at least 4 attacks lasting more than 48h), had a family history of migraine. Whole blood sample was collected from each participant, for genomic DNA extraction from white blood cells, according to approved protocol for experimentation on human subjects. All participants gave their informed and written consent for genetic analysis of their blood samples. Patients were excluded if they are currently taking pain medication or had been diagnosed with a clinically recognized neuropsychiatric condition. The study was approved by the Griffith University Ethics Committee for Experimentation on Human Subjects.

6.3.2 Migraine disability assessment

All participants were assessed for migraine disability using the Migraine Disability Assessment Score (MIDAS) instrument, which provides a measure of productive days lost to migraine headache in previous 3 months (i.e. migraine disability), as well as headache frequency and pain severity (Lea, Colson et al. 2009; Stewart 1999). Studies have shown that this is a valid and clinically useful instrument for assessing health-related quality of life in migraineurs. Based on the 5-question MIDAS rating,
participants were arbitrarily categorized into a ‘low’ disability group if they had a MIDAS rating of 0-10 and into a ‘high’ disability group if they had a MIDAS rating greater than 11 (Lea, Colson et al. 2009; Stewart 1999). Secondary outcome variables, which are partly captured within the primary outcome, were migraine frequency and head pain severity. These were measured as number of days with headache (over a 3 month period) and a pain score (based on a scale of 1-10), respectively (Lea, Colson et al. 2009; Stewart 1999).

6.3.3 OPRM-1 A118G genotyping

Genomic DNA was obtained from leucocytes following a salting out method as previously described by Miller et al (Miller, Dykes et al 1988). Genotyping of the A118G SNP was conducted by polymerase chain reaction (PCR). The A118G SNP was amplified using the forward primer (5’-GGTCAACTTGTCACCACCTAGATCGC-3’) and reverse primer (5’-AATCACATACATGACCAGGAAGTTT-3’). The PCR conditions used were an initial denaturation at 94°C for 3 minutes, followed by 38 cycles of 94°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute, with a final extension of 72°C for 10 minutes (Zhang 2010). Genotyping for the A118G polymorphism was performed by digesting PCR products with the Bst UI restriction enzyme. The digested PCR products were resolved using a 3% agarose gel, stained with ethidium bromide and visualised under ultra-violet light. The Bst UI digestion of the A allele gave a band of 193bp. The G allele gave 164bp and 24bp fragments. To confirm results of the restriction enzyme digest, the DNA samples were also genotyped using an ABI-3130 Genetic Analyser. Sequence electrophoretograms were examined visually using the ABI Sequencing Analysis software 5.0 to determine the alleles of the OPRM-1 A118G SNP.
6.3.4 Statistical analysis

Summary statistics were calculated for all variables (MIDAS, migraine duration, migraine pain severity) in each of the three genotypic groups. Hardy-Weinberg equilibrium was verified for observed genotype frequencies of the OPRM A118G SNP to detect deviation from the normal genotype distribution in the population. Statistics for all variables in the AG and GG genotypes were added together and presented as one independent variable. Data was first evaluated for normality of distribution using the Shapiro-Wilk test and non normal data was log transformed for further analysis. Bivariate correlation analyses were conducted to explore the association between genotype, age, MIDAS, migraine duration and migraine pain severity. To test the effects of genotype on MIDAS, migraine duration and migraine pain severity, comparisons by one-way ANOVA was performed. The p values derived from ANOVA were divided into 2 to accurately represent the one-tailed hypothesis of this study – G allele carriers have a higher average migraine pain severity scores compared to non carriers. The threshold of statistical significance was defined as 0.05, and all analyses were performed using the Statistical Package for Social Sciences (SPSS version 17.0).

6.4 Results

A total of 154 female MA sufferers were analysed in this study. All 154 migraineurs completed the MIDAS questionnaire, which was used for the analysis of the results of this study. Among the 154 participants, there were 119 wild–type homozygotes (A/A), 33 heterozygotes (A/G) and 2 mutant homozygotes (G/G). The genotype frequency was in Hardy-Weinberg equilibrium (p = 0.87). Bivariate correlations showed that age was not associated with genotype, MIDAS, migraine duration or migraine pain severity. One-way analysis of variance (ANOVA) revealed no
statistically significant association between genotypes and the MIDAS (p=0.158), with the AA group having the higher mean of 2.81(0.9) while the AG/GG group with the lower mean at 2.61 (1.2) (refer to Table 6.1). There was also no statistically significant association between migraine duration and the OPRM A118G genotype (p= 0.409), again with the AA group having the higher mean of 1.1(0.8) and the AG/GG group having the lower mean of 1.07 (0.7). Although not statistically significant the G allele carriers recorded lower MIDAS and migraine duration scores compared to A allele carriers. The comparisons of mean between the genotypes and migraine pain severity scores revealed a statistically significant association between A118G and migraine pain severity \[ F (1, 152) = 4.096, \; p= 0.0225 \].With the AA group having the lower mean of 6.86(1.7) and the AG/GG group with the highest mean of 7.54(1.8), the G allele carriers recorded higher migraine pain severity scores compared to the A allele carriers.

Table 6.1: Association of OPRM 118A> G with different variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>OPRM118 Genotypes</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA= 199</td>
<td>AG/GG= 35</td>
</tr>
<tr>
<td>MIDAS</td>
<td>2.8(0.9)</td>
<td>2.6(1.2)</td>
</tr>
<tr>
<td>Mig Duration</td>
<td>1.1(0.8)</td>
<td>1.1(0.7)</td>
</tr>
<tr>
<td>Pain severity</td>
<td>6.9(1.7)</td>
<td>7.5(1.8)</td>
</tr>
</tbody>
</table>

Data are expressed as mean (SD).
*Statistically significant difference found between the two groups.
AA= Wild-type homozygous; AG = variant heterozygous; GG = variant homozygous

6.5 Summary of discussion

Migraine is a debilitating and disabling neurovascular disorder affecting a significant percentage of the world’s population. The activation of the trigeminovascular system followed by the dilation of the cerebral blood vessels is thought to be the cause of most migraine symptoms, including the associated head pain. The human OPRM 1
gene is one of the primary candidates for pharmacogenetic study and is the site of
target for opioid analgesics such as morphine, codeine and pethidine, which are used
to treat severe cases of migraine headpain (Williamson 2001).

A number of SNPs have been indentified in the OPRM1 gene and one of the most
widely studied SNP is the A118G. This SNP has been reported to be associated with
functional alterations of all physiological and pathological functions related to the
opioid system (Lotsch, Hummel et al. 2006). The OPRM1 A118G SNP has been linked
to a vast number of disorders including treatment responses and pain reduction. In a
recent study that investigated the association of A118G with self-rated postoperative
pain and the amount of self-administered morphine in females, reported that the
A118G variant was associated with higher pain scores (Tan 2009). Other studies
investigating pain sensitivity have also found the G118 carriers are more sensitive to
electrical stimuli and chemically induce pain, as well as pressure pain. In terms of
response to analgesic treatment, studies have demonstrated the G118 carriers to
require higher amount of morphine for cancer pain and major abdominal surgery
(Fillingim 2005; Hayashida 2008; Klepstad 2004; Oertel 2006).

The current study investigated if the OPRM1 A118G SNP was associated with
migraine disability, frequency and pain severity. The aim of this study was to
elucidate the prosperity of the A118G SNP in affecting pain perception in
migraineurs, specifically in MA sufferers. A total of 154 females who have reported
as having migraine for over 20 years and are currently diagnosed as MA sufferers
were genotyped for the OPRM1 A118G SNP. 77.3% of the participants had the AA
genotype, 21.4% had the AG genotype and 1.3% had the GG genotype. The
distribution of the A and G allele frequencies were in accordance with that reported in
previous studies and were in Hardy Weinberg equilibrium. ANOVA analyses revealed no significant association between the A118G genotype and MIDAS or migraine duration. However participants with at least one G allele seemed to have reduced disability caused by migraine and a shorter duration of migraine episode compared to participants with the A allele.

Interestingly, the A118G genotype was associated with migraine pain severity and participants with at least one G allele reported higher pain scores compared to participants with the A allele. An inverse correlation between pain severity and migraine disability and duration was not expected. Participants with higher pain scores were expected to have increased disability due to migraine. It may be hypothesised that these participants with higher pain scores may be taking increased doses of pain medication resulting in quicker pain relief and reduced disability due to a migraine episode.

The major finding of this study is that the A118G SNP of OPRM1 was associated with migraine pain severity in female MA sufferers. We demonstrated that female migraineurs who are G118 allele carriers had demonstrably higher pain scores than the A118 carriers. The relationships between altered pain thresholds and responses to analgesic opioid administration and A118G SNP have been well investigated and characterised (Mague 2010). The G118 allele has been associated with increased pain responses and reduced response to opioid analgesics in patients receiving treatments for post operative or chronic pain, in a variety of populations over the last couple of years (Campa 2008; Chou 2006; Fillingim 2005; Hayashida 2008; Klepstad 2004; Oertel 2006).
The change of quantity and binding affinity of the U-opioid receptors brought about by the A118G SNP at the OPRM 1 gene has been implicated for the observed effects of this SNP (Zhang 2010). Zhang et al expressed both variants of the OPRM gene in Chinese hamster ovary cells and found lower mRNA and protein expression levels with the G118 variant (Zhang 2005). Using Mfold technology the authors have also shown that the G118 variant demonstrated altered folding compared to other permutations which could possibly affect mRNA stability (Zhang 2005). Pang et al proposed that the inactivation of three transcription factor binding sites and the creation of two new ones, including a p53 by the A118G SNP could explain the alterations in expression observed with the G118 variant (Pang 2009). Conversely Bond et al found the OPRM 1 G118 allele expressed in AV-12 cells had a threefold higher binding affinity than the A118 allele for β-endorphin (Bond 1998). Further studies are thus required to understand the exact molecular effect of the A118G SNP on the OPRM1 gene function that may elucidate the observed clinical effects of this SNP.

This is the first study to investigate the association between the OPRM1 A118G SNP and migraine pain severity. There are several limitations in this study. Firstly we have only studied one polymorphism in the OPRM1 gene in association with migraine pain severity, and numerous functional SNPs in the same gene and many other candidate genes have been proposed for study of pain and analgesic responses. Secondly this study has been confined to only female MA sufferers, we do not know if this finding will be replicated in female MO migraineurs and in male migraineurs. As such, this study has taken the first step towards investigating the association between the OPRM1 A118G SNP and migraine pain severity and has provided initial evidence that the presence of the G118 allele may increase the severity of migraine pain. In
conclusion the results from this study are suggestive that the OPRM1 A118G SNP may be associated with increased severity of migraine pain in female MA sufferers. Further investigations are needed to fully understand the effect of this SNP on migraine pain in both MA and MO sufferers of both gender groups. Identifying SNPs of migraineurs could aid in optimising treatment for migraine associated head pain.
Chapter 7

Association Study of the Notch 3 gene and Migraine
7.1 Migraine and Chromosome 19

The two main subtypes of migraine, MA and MO though genetically complex and the number of genes involved is still widely unknown, genes for a rare, monogenetic severe subtype of migraine, FHM have been identified on chromosome 19. More recently the gene for FHM type 2 has been identified on chromosome 1 (Ophoff, Terwindt et al. 1996; De Fusco, Marconi et al. 2003). FHM type 1 has been shown to be caused by mutations in the neuronal calcium channel gene (CACNA1A) on 19p13.1-13.2. The FHM locus has also been suggested to contribute to MA and MO. Nyholt et al in 1998 have reported linkage in one large typical migraine family to the CACNA1A region on chromosome 19 (Nyholt, Lea et al. 1998). Additionally Terwindt et al detected an FHM mutation in the CACNA1A gene in a typical migraine patient diagnosed with MA (Terwindt, Ophoff et al. 1998). Although various studies investigating migraine have shown positive linkage to markers in the C19p13 area, chromosome 19 and the mutations within have only been indentified in FHM (Ophoff, Terwindt et al. 1996; Smith, Curtain et al. 2008).

7.2 Notch 3 gene as a migraine candidate gene

Cerebral Autosomal Dominant Arteriopathy with Sub cortical Infarcts and Leuкоencephalopathy (CADASIL) is clinically typified by recurrent sub cortical ischemic strokes, migraine with aura (MA) and dementia in association with diffuse white matter abnormalities on neuroimaging (Joutel, Vahedi et al. 1997). CADASIL is a rare inherited autosomal dominant disease caused by mutations in the Notch 3 gene. The Notch 3 gene is located on chromosome 19p and encodes a large single-pass transmembrane protein expressed in the arterial vascular smooth muscle cells in adult human tissues (Joutel and Tournier-Lasserve 1998). Joutel et al 1997 reported
that this large gene, containing 33 exons, contained more than 64% of all CADASIL mutations occur within exons 3 and 4 of the Notch 3 gene (Joutel, Vahedi et al. 1997; Federico, Bianchi et al. 2005). Mutations reported in Notch 3 gene have all been missense mutations involving a loss or gain of a cysteine amino acid residue (Joutel, Vahedi et al. 1997).

As migraine shows symptomatic overlap with CADASIL, the Notch 3 gene has been suggested to be implicated in migraine as well. Hutchinson et al MRI studied 15 members of an Irish family using magnetic resonance imaging (MRI). 10 members of the family had evidence of CADASIL and the rest of the family had hemiplegic migraine. The study proposed that hemiplegic migraine may be an allelic disorder to CADASIL (Hutchinson, O'Riordan et al. 1995). Oberstein et al found individuals with Notch 3 gene mutations to display an increase in white matter hyperintensities on brain MRI compared to controls and MA was also found to be more common in these individuals compared to controls (Oberstein, van den Boom et al. 2003). However not all mutations in the Notch 3 may be involved in migraine pathogenesis, as an association study by Borroni et al that tested the notch 3 mutation T6746C in an Italian population found no significant association between the mutation and migraine. More recently, Smith et al analysed the Notch 3 gene by sequencing all exons with known CADASIL mutations through a family previously linked to C19p13 and found no evidence of any of the CADASIL mutations tested to be involved in migraine in the family (Smith, Curtain et al. 2008).

Apart from the already known CADASIL mutations in the Notch 3 gene, there are a few polymorphisms present in the Notch 3, whose role in the function of the gene are not clearly understood. The C381T and G684A occurring in exons 3 and 4,
respectively of the Notch 3 are among the few polymorphisms recognised. Studies of these polymorphisms in a cohort of ninety seven German migraineurs revealed an association between the G684A polymorphism and MO sufferers (Schwaag, Evers et al. 2006). Although synonymous polymorphisms have been commonly assumed to be functionally insignificant, recently, an increasing number of studies have reported non-functional polymorphisms in both coding and non-coding sequences that affect the splicing process as well as \textit{in vivo} protein folding and consequently, gene function (Kimchi-Sarfaty, Oh et al. 2007). Thus this research investigated the whether Notch 3 polymorphisms, specifically the C381T and G684A variants in exons 3 and 4, respectively of the Notch 3 gene contribute to the risk of migraine in an Australian Caucasian population.

7.3 Association analysis of Notch 3 gene

7.3.1 Materials and methods

7.3.1.1 Subjects

Written informed consent was obtained from all 1,150 participants of the study and the study was approved by the Griffith University Ethics Committee for Experimentation on Human Subjects. All participants were interviewed and completed a detailed questionnaire that was administered through Griffith University’s Genomics Research Centre (GRC). The questionnaire included demographic characteristics, family history for migraine, cerebrovascular, cardiovascular and neurological diseases, migraine symptoms, age of onset, frequency, severity and treatment as previously described (Lea, Dohy et al. 2000; Johnson, Lea et al. 2003). Migraine diagnosis was performed by an experienced clinical neurologist from responses provided on the questionnaire in accordance with
the International Headache Society (IHS) criteria, also as described previously (HCCIHS 2004; Fernandez, Lea et al. 2006). Questions used to define migraineurs included length and frequency of attack; pain location, type and intensity; associated symptoms such as nausea, vomiting, phonophobia, photophobia and other visual disturbances and neurological symptoms. Those participants who experienced both subtypes of migraine were classed as being affected with MA. The control group consisted of individuals with no family history of migraine and individuals who did not meet these criteria were excluded from the study (Lea, Ovcaric et al. 2005; Fernandez, Lea et al. 2006).

All participants were Caucasians of European descent living in Australia, having emigrating ancestors within the last 160 years from various locations within the British Isles and other parts of Europe and were matched for sex and age and were recruited in parallel at a similar time from the East Coast of Australia as previously described (Colson, Lea et al. 2005). In total ~600 cases and an equivalent number of controls were collected over a spread of several years. The first study population collected comprised of 275 migraineurs and 275 matched controls and called Migraine populations 1 (MAP 1). The follow-up second study population consisting of 300 migraineurs and 300 controls were collected later. The DNA was prepared as a second independent population and was called Migraine population 2 (MAP 2) (Fernandez, Lea et al. 2006). Samples used for the genotyping studies were all individuals, not families, with care taken not to include any related individuals in the case control population. All participants of both independent cohorts were recruited from in an around the South Eastern Australia region, with collections undertaken in the Genomics Research Centre Clinic at the Gold Coast, Queensland, Australia. To minimize potential bias the control group was matched for sex, age (+/- 5 years) and
ethnicity. Migraine patients were clinically defined and suitably matched with the control population (Colson, Lea et al. 2005; Fernandez, Lea et al. 2006).

7.3.1.2 Notch 3 genotyping

Genomic DNA was obtained from leucocytes following a salting out method as previously described by Miller et al. (Miller, Dykes 1988). Exons 3 and 4 of the Notch 3 gene were polymerase chain reaction (PCR) amplified using oligonucleotide primer pairs previously described by Wang et al. (Wang, Sharma et al. 2000), Not3X3F (5’TGT GCT GCC CAA CCA AGC CA 3’) and Not3-X3R (5’ ACT GAC CAC ACC CCC GAC 3’) specific for Exon 3 and Not3-X4F (5’ TAG TCG GGG GTG TGG TCA GT 3’) and Not3-X4R (5’CCT CTG ACT CTC CTG AGT AG 3’) specific for exon 4. The PCR conditions used were an initial denaturation at 95°C for 4 minutes, followed by 35 cycles of 94°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute, with a final extension of 72°C for 10 minutes (Lea, Ovcaric et al. 2005). Polymerase chain reaction (PCR) products of exon 3 and 4 were determined to be 224 base pairs (bp) and 420 bp respectively. Genotyping of the Notch exon 3 for the C381T polymorphism was performed by digesting PCR products with the Aci I restriction enzyme and genotyping of the exon-4 G684A polymorphism was performed by digesting PCR products with the Mwo I restriction enzyme. The Notch 3 exon 3*C allele resulted in digested fragments of 95bp and 7bp, while Notch 3 exon 3*T allele was characterized by an uncut fragment of 166bp. The G to A substitution at nucleotide 684 in exon 4 gene was distinguished by the uncut fragment of 168kb, while the wild type G allele was characterized by digested fragments of 107bp and 61bp (Wang, Sharma et al. 2000). Products were resolved using a 3% agarose gel, stained with ethidium bromide and visualised under ultra-violet light. To confirm results of the restriction enzyme digest, the DNA samples were also genotyped using
an ABI-3130 Genetic Analyser. Sequence electrophotograms were examined visually using the ABI Sequencing Analysis software 5.0 to determine the alleles of the two polymorphisms C381T and G684A. The human Notch 3 cDNA and protein sequences were obtained from GenBank (accession no. U97669). A sequence electropherogram of sample positive for C381T and the control appear in Figure 7.1 and 7.2 respectively. The sequence electropherogram of positive sample for G684A SNP and the control sample appears in Figure 7.3 and 7.4 respectively.

![Figure 7.1: Notch 3, C allele of the C381T variant](image1)

![Figure 7.2: Notch 3, T allele of the C381T variant](image2)

![Figure 7.3: Notch 3, G allele of the G684A variant](image3)

![Figure 7.4: Notch 3, A allele of the G684A variant](image4)
7.3.1.3 Statistical Analysis

Allele and genotype frequencies were first analysed using standard contingency tables, incorporating the chi-squared test. $\chi^2$ analysis for migraineurs MA, MO and combined migraine groups versus control subjects for both C381T and G684A polymorphisms were performed. CLUMP analysis was used if one allele or genotype occurred less than 5 times (Sham and Curtis 1995). Hardy-Weinberg equilibrium was verified for observed genotype frequencies of C381T and G684A to detect deviation from the normal genotype distribution in the population. Odds ratios (OR) with their associated 95% confidence intervals (95% CI) were calculated using logistic regression analysis. Due to multiple testing, Bonferroni correction was performed (Mantel and Haenszel 1959). This set the level of statistical significance at 0.025 (i.e., 0.05/2).

7.3.2 Results

7.3.2.1 SNP identification

The locations of the two known SNPs, with the dbSNP accession numbers rs3815188 (C381T) and rs1043994 (G684A), were annotated in bp using the NCBI Build 36.3 genomic assembly. The positional location of C381T and G684A on chromosome 19 based on build 36.3 are 14,871,514 and 14,871,333 respectively. Genotype and allele frequencies along with statistical measures of association for C381T and G684A in exons 3 and 4 of the Notch 3 gene in the two independent populations studied are summarised in Table 7.1 and 7.2. The distribution of genotypes for both the variants in both populations did not deviate significantly from Hardy Weinberg equilibrium ($P>0.05$). Internal controls consisting of repeat samples and negative controls were used in the confirmation of the genotypes and to account for potential genotyping
errors. A matched pair was only included in the analysis when the results for the repeat samples and the negative controls were obtained. The genotype and allele frequencies of both C381T and G684A from both populations did not differ significantly from those previously reported (Ungaro, Mazzei et al. 2009).

### 7.3.2.2 Results- C381T polymorphism

The distributions of the genotype and allele frequencies of C381T in the two independent populations studied are summarised in Table 7.1. 252 migraineurs and 247 controls were successfully genotyped for this variant in the first population. There were 192 females and 60 males in the migraine group. Results demonstrated a significant association of the C381T variant with migraine in the first population studied for both allelic ($\chi^2=6.64, \ P=0.005$) and genotypic frequencies ($\chi^2=6.59, \ P=0.02$). When analysed by subtype of migraine, the C381T variant was observed to be significantly associated with MO for both allelic ($\chi^2=8.36, \ P=0.002$) and genotypic ($\chi^2=8.16, \ P=0.007$) frequency distribution (refer to Table 7.2).

In order to replicate the findings of a significant link between the C381T variant and migraine, a second independent population of 260 migraineurs and 229 controls were successfully genotyped for C381T. There were 221 females and 39 males in the migraine group. The frequency distribution of the alleles and genotypes obtained for C381T in the second independent populations are summarised in Table 7.1. Although initial observations demonstrated significant association with MO for C381T, this was not replicated in the second population studied. There was no significant association between either allelic ($\chi^2=0.46, \ P=0.245$) or genotypic ($\chi^2=3.03, \ P=0.115$) frequencies for C381T variant and migraine in the second population (Table 7.2). However a trend towards significance was observed in the MO group for both allelic
(\chi^2=2.43, \ P= 0.06) and genotypic (\chi^2=4.42, \ P=0.051) frequencies for the C381T variant (Table 7.2)
Table 7.1: Distribution of the C381T polymorphism in Notch 3 gene in migraineurs and controls of original sample in two studied populations

<table>
<thead>
<tr>
<th>Group</th>
<th>N (alleles)</th>
<th>Alleles</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>First Population</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>504</td>
<td>423 (83.9%)</td>
<td>81 (16.1%)</td>
</tr>
<tr>
<td>MO</td>
<td>192</td>
<td>156 (81.2%)</td>
<td>36 (18.8%)</td>
</tr>
<tr>
<td>MA</td>
<td>312</td>
<td>267 (85.6%)</td>
<td>45 (14.4%)</td>
</tr>
<tr>
<td>Control</td>
<td>494</td>
<td>442 (89.5%)</td>
<td>52 (10.5%)</td>
</tr>
<tr>
<td>Total case vs control</td>
<td>$X^2=6.64$</td>
<td>$P=0.005$</td>
<td></td>
</tr>
<tr>
<td>Second Population</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>404</td>
<td>334 (82.7%)</td>
<td>70 (17.3%)</td>
</tr>
<tr>
<td>MO</td>
<td>82</td>
<td>67 (81.7%)</td>
<td>15 (18.3%)</td>
</tr>
<tr>
<td>MA</td>
<td>322</td>
<td>267 (82.9%)</td>
<td>55 (17.1%)</td>
</tr>
<tr>
<td>Control</td>
<td>458</td>
<td>403 (88%)</td>
<td>55 (12%)</td>
</tr>
<tr>
<td>Total case vs control</td>
<td>$X^2=0.46$</td>
<td>$P=0.245$</td>
<td></td>
</tr>
</tbody>
</table>

*MO migraine without aura, MA migraine with aura*
Table 7.2: Chi-squared ($\chi^2$) analysis of the allelic and genotypic frequencies in all migraine groups against controls for the C381T polymorphism in the two studied populations.

<table>
<thead>
<tr>
<th></th>
<th>Population 1</th>
<th>Population 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>$P$ value</td>
</tr>
<tr>
<td>Allelic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine vs. controls</td>
<td>6.64</td>
<td>0.005</td>
</tr>
<tr>
<td>MO vs. controls</td>
<td>8.36</td>
<td>0.002</td>
</tr>
<tr>
<td>MA vs controls</td>
<td>2.74</td>
<td>0.049</td>
</tr>
<tr>
<td>Genotypic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine vs. controls</td>
<td>6.59</td>
<td>0.02</td>
</tr>
<tr>
<td>MO vs. controls</td>
<td>8.16</td>
<td>0.007</td>
</tr>
<tr>
<td>MA vs controls</td>
<td>3.6</td>
<td>0.08</td>
</tr>
</tbody>
</table>

The allelic ($\chi^2 = 6.64, P = 0.005$) and genotypic frequencies ($\chi^2 = 6.59, P = 0.02$) in the first population show a positive association between this polymorphism and MO. However this was not seen in the second independent population.

7.3.2.3 Results- G684A polymorphism

258 migraineurs and 247 controls were successfully genotyped for G684A in the first population studied. There were 189 females and 69 males in the migraine group. A significant association of the G684A alleles with the migraine group was observed in the first population studied ($\chi^2 = 7.21, P = 0.015$) (refer to Table 7.3). Interestingly, when we divided the migraine population into the two subtypes, MA and MO, we found the G684A variant to be significantly associated with MA for both allelic ($\chi^2 = 10.43, P = 0.001$) and genotypic ($\chi^2 = 9.73, P = 0.004$) frequencies (refer to Table 7.4). The distribution and allele and genotype frequencies for the migraine and the control groups are summarised in Table 7.3.
G684A was then genotyped in a second independent population consisting of 254 migraineurs and 229 matched controls. There 214 females and 40 males in the migraine group. As observed in the first population studied the G684A alleles were observed to be significantly associated with migraine ($\chi^2=7.18$, $P= 0.004$) (refer to Table 7.3). Similarly, significant association was demonstrated between genotypic frequencies for the G684A variant and migraine ($\chi^2=7.21$, $P= 0.015$) (refer to Table 7.3). Further analyses on the migraine subtypes demonstrated the G684A variant to be significantly associated with the MA group again for both allelic ($\chi^2=7.97$, $P= 0.003$) and genotypic frequencies ($\chi^2=8.03$, $P= 0.005$) in the second population (refer to Table 7.4) Taken together, the positive results from both populations strongly suggest the G684A variant to be associated with migraine, specifically suffers of MA as compared to MO sufferers. The distribution and allele and genotype frequencies are summarised in Table 7.3.
Table 7.3: Distribution of the G684A polymorphism in Notch 3 gene in migraineurs and controls of original sample in the two studied population.

<table>
<thead>
<tr>
<th>Group</th>
<th>N (alleles)</th>
<th>Alleles</th>
<th>Genotypes</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>A</td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
<td></td>
</tr>
<tr>
<td>First Population</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>516</td>
<td>444 (86%)</td>
<td>72 (14%)</td>
<td>194 (75.2%)</td>
<td>56 (21.7%)</td>
<td>8 (3.1%)</td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>196</td>
<td>179 (91.3%)</td>
<td>17 (8.7%)</td>
<td>82 (83.7%)</td>
<td>15 (15.3%)</td>
<td>1 (1%)</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>320</td>
<td>265 (82.8%)</td>
<td>55 (17.2%)</td>
<td>112 (70%)</td>
<td>41 (25.6%)</td>
<td>7 (4.4%)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>494</td>
<td>447 (90.5%)</td>
<td>47 (9.5%)</td>
<td>203 (82.1%)</td>
<td>41 (16.6%)</td>
<td>3 (1.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X² = 4.785</td>
<td></td>
<td></td>
<td>P = 0.015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Population</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X² = 4.56</td>
<td>P = 0.056</td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>508</td>
<td>441 (86.8%)</td>
<td>67 (13.2%)</td>
<td>192 (75.6%)</td>
<td>57 (22.4%)</td>
<td>5 (1.96%)</td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>78</td>
<td>70 (89.7%)</td>
<td>8 (10.3%)</td>
<td>32 (82.1%)</td>
<td>6 (15.4%)</td>
<td>1 (2.56%)</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>430</td>
<td>371 (86.3%)</td>
<td>59 (13.7%)</td>
<td>160 (74.4%)</td>
<td>51 (23.7%)</td>
<td>4 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>458</td>
<td>422 (92.1%)</td>
<td>36 (7.9%)</td>
<td>194 (84.7%)</td>
<td>34 (14.8%)</td>
<td>1 (0.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X² = 7.18</td>
<td></td>
<td></td>
<td>P = 0.004</td>
<td></td>
<td>X² = 7.21</td>
<td>P = 0.015</td>
</tr>
</tbody>
</table>

MO migraine without aura, MA migraine with aura.
Table 7.4: Chi-squared ($\chi^2$) analysis of the allelic and genotypic frequencies in all migraine groups against controls for the G684A polymorphism in the two studied populations.

<table>
<thead>
<tr>
<th></th>
<th>Population 1</th>
<th>Population 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\chi^2$</td>
<td>$P$ value</td>
</tr>
<tr>
<td>Allelic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine vs. controls</td>
<td>4.80</td>
<td>0.015</td>
</tr>
<tr>
<td>MO vs. controls</td>
<td>0.12</td>
<td>0.366</td>
</tr>
<tr>
<td>MA vs controls</td>
<td>10.43</td>
<td>0.001</td>
</tr>
<tr>
<td>Genotypic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine vs. controls</td>
<td>4.56</td>
<td>0.056</td>
</tr>
<tr>
<td>MO vs. controls</td>
<td>0.11</td>
<td>0.474</td>
</tr>
<tr>
<td>MA vs controls</td>
<td>9.73</td>
<td>0.004</td>
</tr>
</tbody>
</table>

The allelic and genotypic frequencies in the first population show a positive association between this polymorphism and MA ($\chi^2 = 10.43, P = 0.001$, $\chi^2 = 9.73, P = 0.004$, respectively). This association was again observed in the second independent population ($\chi^2 = 7.21, P = 0.015$, $\chi^2 = 8.03, P = 0.005$).

### 7.4 Linkage disequilibrium analysis of the Notch 3 C381T and G684A

As the genotype analysis of the two Notch 3 SNPS C381T and G684A SNPs in exons 3 and 4 respectively revealed a significant association of the G684A SNP in migraineurs, especially in MA sufferers in the migraine populations tested, Linkage disequilibrium analysis was carried out to determine the extent of pair-wise linkage disequilibrium between the two SNPs. Linkage disequilibrium between Notch 3 exon 3 and exon 4 SNPs was analysed using the Haploview version 4.1 program (Barrett, Fry et al. 2005). Results of the analysis indicated no significant evidence of linkage disequilibrium between C381T and G684A in the first and the follow up second
independent population studied (D’=0.050, 95% CI -0.01, 0.12, R² = 0.00 and D’=0.041, 95% CI -0.01, 0.15, R² = 0.001, respectively).

7.5 Summary of discussion of association analysis of Notch 3 gene.

CADASIL, a common hereditary form of stroke, is caused by mis-sense mutations leading to the loss or gain of a cysteine residue (Federico, Bianchi et al. 2005). Migraine is one of the clinical hallmarks of CADASIL. The Notch 3 gene on C19p13 is implicated in CADASIL and also is also postulated to be linked to migraine. Studies undertaken by Hutchinson et al (1995) and Verin et al (1995) have suggested that hemiplegic migraine may be an allelic disorder of CADASIL (Hutchinson, O'Riordan et al. 1995; Verin, Rolland et al. 1995). Oberstein et al reported that an increase in white matter hyperintensities was observed in brain MRI among 6 individuals who carried mutations in the Notch 3 gene and experienced a higher frequency of MA when compared to controls (Lesnik Oberstein, van den Boom et al. 2003). Population based magnetic resonance imaging (MRI) CAMERA studies have reported that migraineurs had a significantly higher prevalence of cerebellar border zone infarct like lesions and white matter hyperintense lesions. Notably, these observations were more prevalent in MA sufferers than MO sufferers (Kruit, van Buchem et al. 2004; Kruit, Launer et al. 2005). Taken together these finding suggest a common genetic background underlying migraine, particularly MA and CADASIL (Smith, Curtain et al. 2008).

Exons 3 and 4 of the Notch 3 gene are hotspots for most of the CADASIL causative mutations (Joutel, Vahedi et al. 1997). Apart from the well known mutations
identified in the Notch 3 gene, some functional and non functional SNPs that do not cause CADASIL have also been identified. Although the role these SNPs play remains unclear, there are a growing number of studies investigating their possible association in different diseases. A recent study investigating the interaction of eight different variants in five candidate genes in unrelated stroke patients and non stroke controls found the interaction between one of the known Notch 3 SNPs, C381T with two other SNPs (MTHFR C677T and ALOX5AP T2354A) to be a significant contributor to thrombotic stroke (Liu, Sun; et al. 2009). The present study analysed the role of C381T and G684A in exons 3 and 4 respectively of the Notch 3 gene to determine their role in migraine pathogenesis. These results demonstrated significant association of the G684A SNP with migraine.

Previously, a genetic association study by Schwaag et al (Schwaag, Evers et al. 2006) that included 97 migraineurs, also reported significant association of genotypes, as well as alleles, of G684A with migraine. A weak association was also found between C381T and migraine. Interestingly, both SNPs were associated with MO rather than MA. Although it is unknown why an association was found between the SNPs examined and MO when MA is one of the clinical features that characterises CADASIL, these results support a correlation between the Notch 3 gene, CADASIL and migraine (Schwaag, Evers et al. 2006). However not all mutations in Notch 3 may be implicated in migraine. An association study on an Italian population by Borroni et al investigated whether the functional Notch 3 polymorphism T6746C, which is not causative for CADASIL, might be linked to migraine. They examined 156 migraineurs, and reported no significant association between the functional polymorphism and migraine (Borroni, Brambilla et al. 2006).
We have previously analysed the Notch 3 gene by sequencing all exons with known CADASIL mutations in a family previously linked to C19p13 and failed to find common migraine to be affected by any of the known CADASIL mutations analysed (Smith, Curtain et al. 2008). The present study tested two independent matched case-control populations for the C381T and G684A variants in exons 3 and 4 of the Notch 3 gene. Results of the study initially revealed an association between the allelic \( \chi^2=6.64, P=0.005 \) and genotypic \( \chi^2=6.59, P=0.02 \) frequency distribution of C381T variant and migraine, in particular the MO \( (P<0.05) \) subgroup in the first population. However, this association was not replicated in the follow-up independent study group, although a trend towards significance was observed in MO samples for both allelic \( \chi^2=2.43, P=0.06 \) and genotypic \( \chi^2=4.42, P=0.051 \) frequencies. The weak association between C381T and MO reported by Schwaag et al taken together with the inconsistent results observed in the current study, warrant further research into this variant and its role in migraine.

Interestingly, in the current study a significant association between the G684A variant and migraine in the first \( (P=0.015) \) and a second \( (P=0.004) \) independent population was shown. The percentage of participants with the A allele was significantly increased in both migraine (14% and 13.2%, respectively) and the MA subtype (17.2% and 13.7%, respectively) when compared to controls (9.5% and 7.9%, respectively) in both populations studied. Further, frequency data of both populations indicated a linear trend in the proportion of MA sufferers as the number of the A allele increased. This suggests a gene dosage effect, however with G684A being a synonymous polymorphism; it is not clear how, if at all, it may affect the activity of the Notch 3 gene.
G684A is a synonymous polymorphism, that does not alter coding sequences and therefore is expected to be non functional or silent. However recent studies have provided evidence that these seemingly non functional polymorphisms could still affect transcription, splicing, mRNA transport or translation, any of which could influence the resultant phenotype (Pagani and Baralle 2004; Kimchi-Sarfaty, Oh et al. 2007). Kimchi-Sarfaty et al. investigated the phenotypic effects of a synonymous polymorphism in a common haplotype in the multidrug resistance 1 (MDR1) gene and identified the variant responsible for the resultant altered effectiveness of inhibitors of the P-glycoprotein. The authors found the conformation-sensitive antibody binding and trypsin susceptibility of the variant to be different from that of the wild-type protein, suggesting that the need for the tRNAs to translate rare codons slows down translation and causes the protein to fold into a different conformation (Kimchi-Sarfaty, Oh et al. 2007). Whether this is the case for G684A is yet to be determined.

The results of this study indicate a correlation between the Notch 3 gene and migraine. Migraine is a multifactorial disorder and as such, the genetic aetiology is likely to encompass a variety of modest-effect susceptibility genes. It is possible that variants in the Notch 3 gene may be playing a role in susceptibility that influences both the severity and subtype of migraine. It is possible that the G684A variant is acting in combination with other functional variants in Notch 3 or an adjacent gene yet to be identified to influence MA. Given the association between MA and G684A in the current study, further investigation into this SNP and the Notch 3 gene is warranted to further elucidate the exact role of this gene in migraine.
Chapter 8
Association study of Calcitonin gene-related polypeptide-alpha gene polymorphism with migraine
8.1 Calcitonin gene related peptide (CGRP) and their receptors

The human calcitonin gene-related polypeptide-alpha gene (Kimchi-Sarfaty, Oh et al.) located on chromosome 11p15.2-p15.1, codes for both calcitonin and CGRP. CGRP is a 37 amino acid neuropeptide that was first indentified in 1983 in rats as being generated by alternative splicing of the calcitonin gene transcript (Rosenfeld 1983). CGRP was later named α-CGRP to differentiate itself from β-CGRP which is a product of the calcitonin gene-related polypeptide- beta (CALCB) gene (Amara 1985). Both isoforms of CGRP have similar biological activities; however α-CGRP has been reported to be predominantly expressed in trigeminal ganglia neurons and importantly is the main mediator of dilation in human cerebral arteries (Jansen-Olesen, Edvinsson et al. 1996).

The important physiological role CGRP plays can be determined by the wide distribution of CGRP-containing nerves and receptors in the peripheral and central nervous systems as well as in the cardiovascular, respiratory and gastrointestinal systems (van Rossum, Hanisch et al. 1997). CGRP containing neurons modulate the functioning of the immune, respiratory, endocrine, gastrointestinal, musculoskeletal and cardiovascular systems by innervating all major organs and joints in the body (Poyner, Sexton et al. 2002; Arulmani, Maassenvandenbrink et al. 2004). In the cardiovascular system, CGRP induces positive chronotropic and inotropic effects and increases coronary perfusion pressure and blood flow (Saetrum Opgaard, Hasbak et al. 2000; Kaygisiz, Erksap et al. 2003). It also mediates cardioprotective effects and produces vasodilation in capacitance blood vessels (Wimalawansa 1996; Brzozowski, Konturek et al. 2004). CGRP functions in the central nervous system by modulating
pain perception and enhancing the release of substance P as well as of excitatory amino acids from primary afferent fibres (Oku, Satoh et al. 1987; Powell, Ma et al. 2000). Peripherally CGRP co exists with acetylcholine-containing neurons and thus modulates the release of and the response to acetylcholine (Rossi, Dickerson et al. 2003). Most importantly, CGRP’s ability to cause cranial vasodilatation and to facilitate nociception has made it a prime candidate to be investigated in migraine pathophysiology (Arulmani, Maassenvandenbrink et al. 2004; Hargreaves 2007; Durham 2008).

The CGRP receptor consists mainly of a G-protein –coupled receptor namely the calcitonin-like receptor (CLR) and an accessory protein known as receptor activity-modifying proteins (RAMPs) (refer to Figure 8.1). CLR is a rhodopsin-like G-protein coupled receptor that was discovered in the 1990s. Although CLR forms the basic receptor protein for CGRP, RAMP determines the receptor specificity. RAMPs are cleavable signal peptides of about 148 to 175 amino acids in length. The variants of RAMP, namely RAMP 1, RAMP 2 and RAMP 3 have been identified in human tissues (Poyner, Sexton et al. 2002).
RAMPs are required for the intracellular transportation of the CLR-maturing protein to the plasma membrane and express CLR on the cell surface and hence determine the relative affinity of the receptor (McLatchie, Fraser et al. 1998; Foord and Marshall 1999). RAMP 1 presents the CLR as a matured glycoprotein and a CGRP receptor at the cell surface (Foord and Marshall 1999). Co expression of CLR with either RAMP 2 or RAMP 3 allows CLR to bind to another vasodilator called adrenomedullin (AM) (McLatchie, Fraser et al. 1998). By modulating the give pharmacology of the given CLR, RAMPs are able to modulate the sensitivity of the cell from one receptor to another receptor (Mallee, Salvatore et al. 2002).

8.2 CGRP as a migraine candidate gene

The activation of the trigeminovascular system followed by the dilation of the cerebral blood vessels is thought to be the cause of most migraine symptoms, including the associated head pain. The trigeminovascular system is the innervation of cranial vessels by trigeminal sensory nerves that store several vasoactive neuropeptides such as substance P (SP), CGRP and neurokinin A. CGRP is the most powerful vasodilating neuropeptide that is released when trigeminal sensory nerves are stimulated. CGRP has been shown to dilate cephalic arteries, facilitate nociception and stimulate sensory nerve transmission (Goadsby, 1988; Arulmani, Massenvandenbrink et al 2004 ; Goadsby, Edvinsson et al. 1990). CGRP mediates
cellular effects at several sites within the trigeminovascular system and is therefore implicated in migraine pathophysiology via activation of different CGRP receptors. For these reasons, selective CGRP receptor antagonists have been proposed to be investigated as effective anti-migraine therapies (Jansen-Olesen, Edvinsson et al 1996).

A notable number of previous studies show evidence of CGRP involvement in the pathophysiology of migraine. Goadsby et al in 1988 reported elevated levels of CGRP in external jugular blood of patients treated with trigeminal neuralgia treated with thermocoagulation. In a more recent set of studies Goadsby et al have demonstrated an increase in CGRP release in external jugular vein and not in cubital venous blood during migraine attacks and the successful cessation of migraine attacks and normalisation of CGRP levels after the administration of 5HT 1B/D receptor agonist, sumatriptan. A double blinded, placebo-controlled, cross over study, examining the effect of intravenous injection of CGRP in migraine patients, reported headache symptoms in 100% of patients 20 mins after CGRP injection, with noticeably no effect observed in the placebo group. The headache was reported to disappear or diminish after the CGRP infusion was stopped (Lassen, Haderslev et al 2002). This data adds to the growing evidence from animal and human studies indicating that CGRP may play a vital role in migraine pathogenesis.

Numerous studies have convincingly demonstrated that both genetic and environmental factors underlie migraine and a subset of migraine genes, causative mutations and susceptibility variants have been identified and are already of clinical relevance (Kara, Sazci et al. 2003). Given the strong evidence linking CGRP and migraine, polymorphisms in the Calcitonin/α-CGRP (Kimchi-Sarfaty, Oh et al.) gene
may provide evidence for a role of this gene in migraine pathogenesis (Buervenich 2001). It is hypothesised that polymorphisms in the CALCA gene may lead to elevations in CGRP levels reported to be evident in migraine pathogenesis. Four novel polymorphisms have been identified in the CALCA gene including a 16bp deletion in the first intron (Buervenich 2001). Intronic mutations have the capacity to disrupt normal RNA processing in different ways. Two intronic mutations in the CACNA1A gene, one introducing a cryptic splice donor site in exon 24 and the other causing the skipping of exon 41 have previously been identified in 2 episodic ataxia type 2 (EA2) families (Wan 2005). The aim of this study was to investigate whether the 16bp deletion in the first intron of the CALCA gene contribute to the risk of migraine in an Australian Caucasian population. The 16bp intronic deletion may also remove a triple G motive which may act as an Intron splice enhancer (ISE) for efficient splicing of transcript (Jensen, Oldfield et al. 2009). An average vertebrate gene usually consists of approximately 137 nucleotides which are generally separated by larger introns. The recognition of smaller exons amid longer introns is aided by ISE which modulate splice site selection (Berget 1995; McCullough and Berget 1997).

The splice site consensus sequences that drive this exon recognition are a repeat of three or more G nucleotides termed the ‘G- run (Jensen, Oldfield et al. 2009). This G-run motif when present downstream of an exon acts an ISE by promoting exon inclusion and when present in an exonic location acts as an exonic splicing silencer (ESS) by promoting exon skipping (McCullough and Berget 1997; Jensen, Oldfield et al. 2009). Multiple G-run motifs are present throughout the intron and each G triplet has been reported to additively maximise splicing efficiency (McCullough and Berget 1997). McCarthy et al in their study have demonstrated that mutations in an ISE
located in the intron of Human Growth Hormone -1 (GH-1) gene disrupts splicing and causes the skipping of 3 exons, resulting in an autosomal dominant form of isolated growth hormone deficiency, (IGHC-II) (McCarthy and Phillips 1998). It is thus possible that mutations or the complete deletion of ISEs may cause other genetic disorders.

The first intron of the CALCA gene is studded with triplet G-run motifs including one in the 16 bp deletion. Additionally the 94bp exon upstream of the deletion is smaller than that of an average vertebrate exon length. It is possible that the G-run motifs present in the first intron of the CALCA gene are minimal ISEs that additively enhance the splicing of the intron and thus promote exon inclusion. The 16bp deletion in the intron may disrupt this process resulting in altered mRNA transcripts and an increase in CGRP. The aim of this study was to investigate whether the 16bp deletion in the first intron of the CALCA gene contribute to the risk of migraine in an Australian Caucasian population.

8.3 Association analysis of CGRP gene

8.3.1 Materials and Methods

8.3.1.1 Subjects

A total of 320 migraineurs and 320 matched controls were utilised for the genotyping of the CGRP 16bp deletion. The samples used for the genotyping study were all unrelated individuals that were randomly selected from MAP 1 and MAP 2. The migraineurs and controls in MAP 1 and MAP 2 have been previously described in Chapter 8.3.1.1. The study was approved by the Griffith University Ethics Committee for Experimentation on Human Subjects.
8.3.1.2 CGRP genotyping

Genomic DNA was obtained from whole blood following a salting out method as previously described by Miller et al (Colson 2005). According to NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP/) the 16 bp deletion in [rs35815751; (11)] intron 1 of the CALCA gene (Accession No. X15943) results in one of the three possible genotypes: II (Homozygous, insertion/insertion); ID (Heterozygous, insertion/deletion); or DD (Homozygous, deletion/deletion) in an individual. The deletion was detected by polymerase chain reaction followed by fragment analysis on the 3130 Genetic Analyser (Applied Biosystems). The fluorescently 6-FAM-labeled forward primer and the appropriate reverse primer used to amplify the region of 16bp deletion in CALCA intron and subsequent fragment analyses were: 6FAM-5’TGGGGAGAAGGGTAGGACT3’, 3’GAACTTTTGGAAGCCCATGA 5’. The PCR conditions used were: an initial denaturation at 95°C for 10 minutes, followed by 30 cycles of 95°C for 45 seconds, 60°C for 45 seconds, 72°C for 45 seconds, with a final extension of 72°C for 4 minutes. The PCR products along with Hi-Di™ Formamide/GeneScan™ 500 LIZ® size standard were run on the 3130 Genetic Analyser (Applied Biosystems). The resulting fragments of 306bp (wildtype) or 287bp (deletion) (refer to Figures 8.2a to 8.2c) were visualised after fragment analysis run and called automatically by the GeneMapper software.
Figure 8.2a: Fragment analysis of a sample homozygous CGRP 16bp deletion, displaying a single peak of 306bp.

Figure 8.2b: Fragment analysis of a sample heterozygous for CGRP 16bp deletion, displaying a peak at 306bp and another peak at 287bp.

Figure 8.2c: Fragment analysis of a sample homozygous for CGRP 16bp, displaying a peak at 287bp.
8.3.1.3 Statistical Analysis

Hardy-Weinberg equilibrium was verified for observed genotype frequencies of the 16bp deletion to detect deviation from the normal genotype distribution in the population. Allele and genotype frequencies were analysed using standard contingency tables, incorporating the chi-squared test. $\chi^2$ analysis for migraineurs MA, MO and combined migraine groups versus control subjects for 16bp deletion was performed. CLUMP analysis was performed if one allele or genotype occurred less than 5 times. Odds ratios (OR) and their associated 95% confidence intervals (95% CI) were calculated using logistic regression analysis. All other computations were performed using the Statistical Package for Social Sciences (SPSS version 17.0). Power analysis indicated that if the CALCA 16 bp deletion were to confer a two-fold increase in relative risk of migraine, the total case and control groups used in this study were of sufficient size to have 90% power to detect an allelic association and 80% power to detect a genotypic association at the 0.05 significance level. Although migraine subgroups were analysed, it should be noted that the power to detect association in these subgroups was < 80%.

8.3.2 Results

A 16 bp deletion in intron 1 of the CALCA gene with dbSNP accession number rs 35815751 was tested for association in 600 individuals with 278 migraineurs and 322 control individuals successfully genotyped for the 16bp deletion in the CALCA gene. The migraine group consisted of 234 MA and 44 MO sufferers. Genotype and allele distribution and statistical measures are summarised in Table 8.1. A total of 504 females and 96 males were successfully genotyped for the 16bp deletion in this study. There were a total of 237 and 267 females in the migraine and control groups.
respectively and there were a total of 41 and 55 males in the migraine and control groups respectively. Genotype and allele distribution and statistical measure for the different genders are summarised in Table 8.2. The genotypes were demonstrated to be in Hardy-Weinberg equilibrium in migraine (migraineurs: HWpval= 0.735) and in the control group (controls: HWpval= 0.247). Both migraineurs and control individuals had a similar distribution of the ID genotype (migraineurs vs. controls: 13.3% vs. 12.2%), with the DD genotype only demonstrated in migraineurs (0.4%). Most of the migraine (86.3%) and control (87.9%) study populations had the II genotype. Genotypic frequencies of the 16bp deletion did not differ much by migraine subtype, however the DD genotype was only observed in the MA group. The frequency of the I allele was significantly greater in both the migraineurs and control individuals when compared to the frequency of the 2 allele. (Migraineurs: 93% vs. 7%, Controls: 93.9% vs. 6.1%). Chi square results did not suggest an association between the 16bp deletion in intron 1 of the CALCA gene with migraine for genotypes (P=0.575) nor alleles (P= 0.502). Further analyses on the migraine subtypes also did not show any association between the 16 bp deletion and MA (genotypes: P=0.666, alleles: P=0.7) or MO (genotypes: P=0.325, alleles: P=0.276), nor in sub-population analysis of females (genotypes: P= 0.792, alleles: P= 0.691) or males (genotypes: P= 0.515, alleles: P= 0.432).
Table 8.1: Genotype and allele frequency distributions for CALCA 16 bp deletion in migraine and control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotypes (No deletion)</th>
<th>Genotypes Deletion</th>
<th>Alleles</th>
<th>Alleles OR(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>ID</td>
<td>DD</td>
<td>P</td>
</tr>
<tr>
<td>Migraine</td>
<td>240(86.3%)</td>
<td>37(13.3%)</td>
<td>1(0.4%)</td>
<td>0.585</td>
</tr>
<tr>
<td>MA</td>
<td>204(87.2%)</td>
<td>29(12.4%)</td>
<td>1(0.4%)</td>
<td>0.666</td>
</tr>
<tr>
<td>MO</td>
<td>36 (81.8%)</td>
<td>8(18.2%)</td>
<td>0 (0%)</td>
<td>0.325</td>
</tr>
<tr>
<td>Control</td>
<td>283(87.9%)</td>
<td>39(12.1%)</td>
<td>0 (0%)</td>
<td>-</td>
</tr>
</tbody>
</table>

*MA* - Migraine with aura, *MO* - Migraine without aura

Chi-squared ($\chi^2$) analysis of all migraine groups against controls for the CALCA 16bp deletion did not show significance in both allelic and genotypic frequencies of this marker in migraine.

Values are total number of alleles or genotypes. 95% CI = 95% confidence interval. Significance was taken at $P \leq 0.05$. 
Table 8.2: Genotype and allele frequency distributions for CGRP 16 bp deletion for the different genders in migraine and control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>CGRP Genotypes (%)</th>
<th>CGRP Alleles (%)</th>
<th>N alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>ID</td>
<td>DD</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>203 (85.7%)</td>
<td>33 (13.9%)</td>
<td>1 (0.4%)</td>
</tr>
<tr>
<td>Control</td>
<td>231 (86.5%)</td>
<td>36 (13.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Total case vs. control</td>
<td>P= 0.792</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>37 (90.2%)</td>
<td>4 (9.8%)</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>52 (94.5%)</td>
<td>3 (5.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Total case vs. control</td>
<td>P= 0.515</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MA - Migraine with aura, MO - Migraine without aura

CLUMP and Chi-squared ($\chi^2$) analysis revealed no significant association between either genotypic or allelic frequencies for the CALCA 16bp deletion and migraine in both genders. Values are total number of alleles or genotypes. Significance was taken at $P \leq 0.05$.

8.3.3 Summary and discussion of association analysis of CGRP gene

CGRP, the most abundant neuropeptide expressed in the cerebral vasculature and trigeminal ganglia seems to be a neuropeptide potentially involved in the origin and development of migraine (Arulmani 2004). A number of studies have highlighted its role in migraine pathogenesis which has led to the development of the first effective CGRP antagonists for use as an anti-migraine therapeutic. Goadsby et al was one of the earliest to demonstrate elevated plasma concentrations of CGRP in the external jugular venous blood during migraine headache phase in 22 patients ranging in age from 22 to 58 years (Goadsby, Edvinsson et al. 1990). Lassen et al demonstrated the infusion of human $\alpha$ CGRP intravenously for 20 minutes caused migraine like headache in 12 episodic MO sufferers in a double blinded crossover study (Lassen, Haderslev et al 2002). Plasma concentrations of CGRP levels during migraine attacks...
were also found to be significantly correlated with the intensity of headache in 15 female migraineurs who were administered with Sublingual nitroglycerin to invoke migraine (Juhasz 2003). Additionally CGRP receptor antagonists have been proven to be as effective as triptans in terminating migraine (Tepper 2008).

The CALCA gene codes for both calcitonin and the $\alpha$-CGRP through alternative splicing of gene transcripts. Recently polymorphisms in the CALCA gene have been reported to have a possible association with Parkinson’s disease, ovarian cancer, bone mineral density and essential hypertension suggesting a functional role for these polymorphisms (Luo 2008; Magana 2006; Goodman 2005; Buervenich 2001). Four novel polymorphisms in the CALCA gene were indentified in 2001, one of which was a 16bp micro deletion in the first intron of the gene (Buervenich 2001). This deletion has been reported to be a good match for the binding site for a transcription factor expressed strongly in neural crest derived cells, AP-2 (Buervenich 2001). It also removes a triple G-run motif that may potentially be an ISE required for efficient exon inclusion (Jensen, Oldfield 2009). Multiple copies of this ISE present in introns of some gene modulate splice site selection and work additively for efficient inclusion of small exons in the transcript (McCullough and Berget 1997). Mutations of ISEs have also been demonstrated to disrupt splicing; resulting in exon skipping that produces a disease phenotype (McCarthy and Phillips 1998).

The present study examined the relationship between the 16bp deletion occurring in the first intron of the CALCA gene and migraine in an Australian population. This study genotyped a total of 600 individuals (278 migraineurs and 322 controls) for the presence of the deletion. More than 90% of the control individuals and migraineurs had the I-insertion allele, with only 7% of migraineurs and 6% of control individuals
possessing the D-deletion allele. The distribution of the D-deletion allele frequency in our control individuals is in accordance with that reported in a previous study and is in Hardy Weinberg equilibrium (Buervenich 2001). Chi square analyses did not reveal any significant association between the 16bp deletion and migraine. Additionally, stratified analyses of migraine subtypes did not show any trend towards significance specifically attributed to the MA or MO subtype group for both allelic and genotypic frequencies of the CALCA 16bp deletion.

This is the first study to investigate the association between the 16bp deletion in intron 1 of the CALCA gene with migraine pathogenesis. The results of this study suggest that the 16bp deletion is a frequently occurring polymorphism that may be an unlikely candidate for involvement in migraine. It is possible that this deletion does not result in any deleterious effects on the CALCA gene, thus leading to unaltered mRNAs encoding for CGRP. However given the important role that CGRP potentially plays in migraine pathogenesis, other polymorphisms in the CALCA gene should be investigated.
Chapter 9
Discussion and Future Directions
9.1 Introduction

Genetic epidemiological studies performed in the last few decades have convincingly reported that significant genetic mechanisms underlie migraine. When the recurrence rate among first degree relatives of a MO proband was investigated, the resulting risk was estimated to be about twice that incurred by a random member of the general population while the first degree relatives of probands of MA carry a recurrence risk of MA that is almost four times that of the general population (Russell, Iselius et al. 1996). Migraine is currently understood to be a polygenic multifactorial disorder, where interactions between various susceptibility genes, along with environmental factors, cause an increased risk of the disease (Ophoff, van den Maagdenberg et al. 2001).

The search for migraine susceptibility genes and the role they play in treating this disorder is of significant medical importance. The current study has attempted to understand the possibility of a pharmacogenetic relationship and personalized treatment options for migraineurs based on their genetic predisposition to migraine. The results of this research have provided evidence that the genetic predisposition of migraineurs may significantly affect treatment option and response. This study has also provided evidence that genetic variants of markers involved in the vascular and endorphin system may play a role in migraine susceptibility and migraine associated head pain.

9.2 MTHFR variant and migraine- A pharmacogenetic relationship
The human MTHFR gene catalyses the NADPH dependent conversion of the CH2-THF to CH3-THF, the principle circulatory form of folate and a cofactor for methylation of homocysteine to methionine. Research has provided evidence that an increase in circulatory homocysteine levels is observed in MA patients. It has been postulated that homocysteine plays the role of an excitatory amino acid in migraine pathophysiology, either by causing vasodilation of cerebral blood vessels or temporary thrombosis of cerebral blood vessels. The C677T allele, a common variant of the MTHFR gene, has been shown to decrease the MTHFR activity, and the TT genotype has been indirectly linked to mild hyper-homocysteinemia. The TT genotype has also been reported to be significantly over-represented in MA sufferers compared to controls in a number of populations, including the Australian Caucasian population. This thesis first investigated the genotype-phenotype correlations between the C677T variant and the clinical phenotypes of migraine to determine if the MTHFR genotype was associated with migraine in general or more specifically with particular migraine subtypes also termed endophenotypes. As multiple genes have now been associated with migraine susceptibility, it is plausible to assume varying disease manifestations may be attributable to different genotypes and susceptibility genes.

This study investigated the genotype-phenotype correlations of the MTHFRC677T variant with fifty migraine clinical variables and found the presence of the T allele to be associated with the most number of migraine symptoms and triggers (MA, unilateral head pain, visual disturbance, physical activity discomfort during or prior to migraine and stress as a migraine trigger). When gender differences were investigated in relation to genotype and phenotype, it was found that females with the T allele were associated with more clinical variables compared to the homozygous C allele carriers and males. However the gender distribution in the current study was not equal
with 27% of the participants being males and 73% being females and therefore caution has to be exercised when interpreting the significant results.

The TT genotype carriers express approximately 50% of the mean activity of the MTHFR enzyme levels, as compared to the CT and CC genotype carriers. Although the TT genotype appears to have a recessive effect when it comes to the MTHFR enzyme levels, from the results of this study it appears that the resulting levels of homocysteine conferred by both the heterozygous and homozygous states of the T allele may contribute to the phenotypic expressions described in migraineurs.

To conclude, the current study has examined the contribution of the MTHFRC677T variant to migraine subtypes, triggers, severity, symptoms and response to medication and has provided initial evidence that the MTHFR genotype can relate to selected migraine clinical symptoms. As such, the major limitation of this study was the number of available participants in comparison to the number of outcome variables examined. Further investigations utilising a larger cohort of equal gender distribution to increase statistical power to clarify the genotype-phenotype interactions of migraine susceptibility gene MTHFR are warranted.

9.3 Genotypic effect of MTHFR (C677T) and MTRR (A66G) on vitamin treatment response in migraineurs.

Current migraine treatments show variable efficacy and often have adverse side effects. The pharmaceuticals currently available to treat migraine symptoms mostly impart broad based effects and/or are ineffective for a large proportion of sufferers. Migraine sufferers are concerned about the cost involved in treatment as well as the toxicity of prescription medication (Evans and Taylor 2006). There is a real necessity
to develop safe, effective and inexpensive alternative to combine with or replace current treatments for this debilitating disorder. Relatively little research has been conducted on complementary and alternative medicines for the treatment of migraine so there is a paucity of evidence currently available. Several trials have suggested that magnesium, feverfew, riboflavin, coenzyme Q10 and B12 supplementation may have some effect on reducing migraine symptoms but these studies were quite small and inconclusive (Vandalia 1969; Welch 1993; Schoenen, Jacquy et al. 1998; Tfelt-Hansen, Block et al. 2000; Rozen, Oshinsky et al. 2002; van der Kuy, Merkus et al. 2002).

The MTHFRC677T genotype results in increased levels of circulating homocysteine and it has been previously shown that additional dietary folate and increase in vitamin B levels can have an important effect in reducing these levels (Ho, Eikelboom et al. 2006). The MTRR gene plays an important role in maintaining adequate supply of active Cobalamin I and thus may also be a critical determinant of homocysteine concentrations. The MTRR A66G variant is associated with increased plasma homocysteine, with the GG genotype having a greater effect than the AG genotype. Furthermore genetic studies have demonstrated the coexistence of the MTHFR 677TT genotype and either AG or GG genotypes of the MTRR A66G polymorphism may magnify the effect of the MTHFRC677T genotype alone. This study thus investigated the genotypic effects of both the MTHFR and MTRR gene on folate and vitamin B treatment response in lowering homocysteine and migraine disability.

Results of this trial demonstrated that vitamin treatment significantly decreased homocysteine levels compared to placebo. A significant reduction in percentage of high migraine disability according to the MIDAS instrument scores was also observed.
in the vitamin treated group. This reduction was statistically significant overall compared with the placebo group. The vitamin treated group also saw a significant reduction in migraine pain severity, which was not seen in the placebo effect.

When the results were stratified by MTHFRC677T genotype, it was observed that the C allele carriers displayed a statistically significant reduction in homocysteine levels, migraine pain severity and migraine disability compared to the T allele carriers, on the prescribed vitamin supplementation. The MTHFR gene is associated with enzyme function and the TT genotype of the C677T variant exhibit approximately 50% reduction in enzyme activity. It was thus expected that the TT genotype carriers would observe a significant reaction to the vitamin supplementation, a bigger reduction in homocysteine levels and migraine disability, compared to the C allele carriers. The results of the current trial however propose the idea that individuals with the TT genotype, having a reduced enzymatic activity are slower in homocysteine metabolism and may require an increased dose of vitamins compared with the C allele carriers to display the same reduction in homocysteine levels and migraine disability (Lea, Colson et al. 2009).

Similarly the A allele carriers of the MTRR A66G variant showed the highest level of reduction in homocysteine levels, migraine pain severity and percentage of high migraine disability under vitamin supplementation compared to the mutant GG genotype carriers. The functional effect of MTRR A66G variants are not fully elicit yet, however in vivo studies suggest that the A66G variant MTRR enzyme restores MTR activity less efficiently than wild type, and has also been shown to increase plasma homocysteine levels in humans (Olteanu, Munson et al. 2002). And in combination, the MTHFR677TT genotype with either the homozygous or
heterozygous genotype for the MTRRA66G variant may exacerbate the effect of the MTHFR variant alone (Vaughn, Bailey et al. 2004).

The C and A allele carriers of the C677T and A66G variants respectively in a combined analysis showed the most significant reduction in migraine pain severity and percentage of high migraine disability. However the level of reduction observed in the combined analysis was not greater than the independent effect of the two variants on treatment response in migraineurs. This suggests that both the MTHFRC677T and MTRRA66G appear to be acting independently from one another in affecting vitamin treatment response in migraineurs.

The results of the current trial have added evidence that homocysteine lowering through vitamin supplementation may reduce migraine disability in a subgroup of patients. In the current study the allele groups of MTHFR and MTRR that showed the largest reduction in homocysteine levels showed the most significant reduction in migraine pain severity and disability under vitamin treatment. The homozygote mutant allele carriers of the MTHFR and MTRR variant may need a higher dose of vitamin supplementation to experience the same effect as the wild type allele carriers of the variants, in migraine pain severity and disability reduction. Further clinical trials, testing varied but safer dosages of vitamin supplementation including folic acid, vitamins B₆ and B₁₂ are warranted to provide a clearer understanding of the role the mutant homozygote genotypes of the MTHFRC677T and MTRRA66G variants play as a genetic modifier of the treatment effect.

Hyper - homocysteinemia may be a partial determinant for the neuro and/or vascular theories underlying both MA and stroke (Lea, Colson et al. 2009). There is an inverse
relationship between homocysteine levels and migraine disability. Genes involved in the homocysteine pathway are important determinants of plasma homocysteine levels and thus may be involved in migraine pathophysiology. Other genes and functional variants in the homocysteine metabolism cascade would be good candidates for further studies investigating genotypic effects on treatment response in migraineurs.

9.4 Migraine candidate gene studies

9.4.1 OPRM-1 Gene

Several case-control population studies have explored various susceptibility genes and indicated their association with MA and MO. In these association studies, migraine has been associated with variants in oestrogen, progesterone (Colson, Lea et al. 2006) and insulin receptor genes (McCarthy, Hosford et al. 2001), dopamine receptor genes (Mochi, Cevoli et al. 2003) as well as promoter variants of the tumour necrosis factor α (Trabace, Brioli et al. 2002), angiotensin-converting enzyme (Kowa, Fusayasu et al. 2005), serotonin transporter gene (Heils, Teufel et al. 1996) and the MTHFR gene (Lea, Ovcaric et al. 2004). Most of the association studies are single reports on specific populations and await confirmatory replication in different populations. There is no denying that migraine is a multifactorial disease that is modified by both environmental and genetic factors and has a polygenic determination. However the proof of the involvement of definite genes in still lacking, hence typical migraine still represents a genetic challenge. This thesis investigated genetic variant of the opioid receptor gene in association with migraine pain severity and genetic variants of the Notch 3 gene and CALCA gene in association with migraine susceptibility.
The human OPRM-1 gene is an important candidate in pharmacogenetic study as it is the site of target for opioid analgesics such as morphine, codeine and pethidine, which are occasionally used to treat severe cases of migraine head pain (Ikeda 2005; Williamson 2001; Zhang 2010). Psychological, behavioural and genetic factors all play a part in influencing an individual’s pain experience (Vossen 2010). Although the extent to which genetic factors are involved in individual human pain perception is still unknown, animal studies propose large and heritable differences in both nociceptive and analgesic sensitivity (Mogil 1999; Vossen 2010). The A118G variant in exon 1 of the OPRM-1 gene has been extensively studies and associated with elevated pain responses and decreased pain threshold in a variety of populations (Mague 2010). This thesis investigated the effect of the A118G variant on migraine pain severity in the view of optimising pain treatment for migraineurs.

The results of this study have indicated that the A118G variant of the OPRM-1 gene is significantly associated with migraine pain severity, as assessed by the MIDAS instrument, in the tested population of MA sufferers. The comparison between the genotypes and severity scores revealed a statistically significant association between the A118G variant and migraine pain severity (P=0.021). With the G allele carriers reporting a higher migraine pain severity score compared to the AA genotype carriers. There are several limitations in this study that need to be addressed. The first one being, he study group utilised for this investigation only consisted of female MA sufferers. Another limitation of this study was the small sample size. Further studies are therefore warranted in an attempt to replicate the current findings in a larger sample cohort consisting of both genders. It is also essential to answer the query if the association observed between the OPRM-1 A118G variant and migraine pain severity is specific to MA sufferers or is present in both subtypes of migraine. The current
study has only investigated one variant of the OPRM-1 gene in association with migraine pain severity, further studies to investigate the other functional variants in the same gene and also other pain candidate genes implicated in pain and analgesic responses may add relevant data for the development of migraine drugs and therapeutics

9.4.2 Notch 3 Gene

MA is much less prevalent than MO with 33% of migraineurs affected by it. MA is defined as the more severe type of migraine and both subtypes can occur within the same family. Although the number of genes involved in migraine is still unknown, genes for the rare subtype of migraine FHM have been identified on chromosome 19 and FHM type 2 has been identified on chromosome 1 and FHM 3 has been identified on chromosome 2 (Ophoff, Terwindt et al. 1996; De Fusco, Marconi et al. 2003; Dichgans, Freilinger et al. 2005). Families with FHM type 1 have missense mutations in the neuronal calcium channel gene CACNA1A. As studies have indicated that the FHM locus may contribute to both subtypes of migraine, it is postulated that calcium channel genes may also be involved in both subtypes of migraine (May, Ophoff et al. 1995). Terwindt et al detected an FHM mutation in the CACNA1A gene in migraine patient with MA, suggesting that FHM may be a rare and severe form of MA (Terwindt, Ophoff et al. 1998). Although chromosome 19 has been extensively studied and positive linkage between causal markers in the C19p13 area and migraine have been shown, only in FHM has the chromosome 19 gene and mutations within, been identified.
Mutations in the Notch 3 gene on chromosome 19 have been shown to cause CADASIL, an inherited stroke syndrome leading to dementia (Joutel, Corpechot et al. 1997; Joutel, Vahedi et al. 1997). The Notch 3 gene may also be implicated in migraine as migraine is one of the clinical hallmarks of CADASIL. MRI Studies have found some correlations between the two disorders (Hutchinson, O'Riordan et al. 1995). Exons 3 and 4 of the Notch 3 gene harbour approximately 64% of the CADASIL causative mutations (Joutel, Vahedi et al. 1997). There are some functional and non-functional polymorphisms that do not cause CADASIL also present in the Notch 3 gene. Although the role these polymorphisms play is still unclear, they are investigated in association with different diseases (Liu, Sun et al. 2009). The current study investigated the role of the C381T and G684A in exons 3 and 4 respectively of the Notch 3 gene to determine their role in migraine pathogenesis.

Results of analysis of the G684A polymorphism indicated a positive association with migraine in 2 large independent cohorts. This positive association was seen in the migraine group and the MA subgroup in both the independent populations tested. Results of chi square analysis comparing the GG genotypes with the GA and AA genotype frequencies together in both populations strongly suggested that the 684A allele carriers are more likely to suffer from migraine, specifically MA (P<0.05) than the GG genotype carriers. The C381T polymorphism in exon 3 of the Notch 3 gene was also tested in population 1.

Results showed the C381T variant to be significantly associated with migraine (P<0.05) and when analysed by subtype of migraine, the variant was observed to be significantly associated with MO (P<0.05). When the C381T polymorphism was tested in the second independent population, there was no association between either
allelic (P=0.245) or genotypic (P=0.115) frequencies of the C381T polymorphism and migraine. However a trend towards significance was observed for the polymorphism in the MO subgroup. This weak association between C381T and MO observed in the current study has been previously noted in a study by Schwagg et al, who investigated the C381T and G684A polymorphisms in association with migraine in a cohort of 97 MA and MO patients (Schwaag, Evers et al. 2006). The inconsistent results obtained for the C381T observed in the current study warrant further research into this polymorphism and its role in migraine and the MO subtype.

The significantly associated G684A polymorphism does not lead to an amino acid change and therefore is expected to be non functional or silent. However recent studies have provided evidence that these seemingly non functional polymorphisms could still affect the splicing process, which could influence the resulting phenotype (Pagani and Baralle 2004; Kimchi-Sarfaty, Oh et al. 2007). Alternatively, the G684A polymorphism might be in linkage disequilibrium with another functional variant in the Notch 3 gene or another gene in close proximity, and this yet to be identified variant may play a role in migraine pathogenesis.

To conclude, the results of this study have indicated a correlation between the Notch 3 gene and migraine. Further studies are required to study the role of other functional polymorphisms in linkage with the tested variants in the Notch 3 gene, in migraine. Gene expression studies have to be undertaken to determine the mechanism by which alterations in the Notch 3 gene may be involved in the underlying migraine pathogenesis.
9.4.3 CALCA Gene

Immunoreactive fibres of CGRP originate in the trigeminal ganglion and innervate the cranial cerebral blood vessels (Uddman, Edvinsson et al. 1985). When stimulated these sensory nerve fibres have been shown to cause antidromic release of CGRP followed by vasodilation of the cerebral vasculature (Goadsby, Edvinsson et al. 1990). Subsequently after the discovery of CGRP, Goadsby et al showed that CGRP levels, but not of other neuropeptides, are increased in external jugular venous blood during migraine attacks (Goadsby, Edvinsson et al. 1990). The 5HT_1b/D receptor agonist sumatriptan aborts migraine attacks by normalising CGRP levels. CGRP plays an important role in migraine pathophysiology and CGRP receptor antagonists possess antimigraine properties with an efficacy similar to that of triptans.

The human CALCA gene codes for both CGRP and calcitonin (Rosenfeld 1983). Polymorphisms in the CALCA gene have been linked to Parkinson’s disease, ovarian cancer and essential hypertension, suggesting a functional role for these polymorphisms (Buervenich 2001; Goodman 2005; Luo 2008; Magana 2006). Given the strong evidence for the involvement of CGRP in migraine, it was hypothesised that polymorphisms in the CALCA gene may lead to elevations in CGRP levels reported to be observed in migraine pathogenesis. This thesis investigated the role of a 16bp intronic deletion in the CALCA gene in a migraine case control population. Although this is an intronic deletion found in the first intron of the CALCA gene, it has been reported to be a good match for the binding site for a transcription factor expressed strongly in neural crest derived cells, AP-2 (Buervenich 2001). Furthermore this deletion also eliminates an intron-splicing enhancer (ISE) that may potentially cause exon skipping (McCarthy and Phillips 1998).
Results of CALCA gene analysis did not show a significant association between the 16bp deletion and migraine. Stratified analyses of migraine subtypes also showed no trend towards significance specifically attributed to MA or MO subtype group for both allelic and genotypic frequencies of the CALCA 16bp deletion. The lack of association suggest that the 16bp intronic deletion in CALCA gene may not result in any deleterious effects on CALCA gene and leads to unaltered mRNAs encoding for CGRP. There is no denying the potentially important role that CGRP may play in migraine pathogenesis. Although the current study did not reveal a significant association between the investigated deletion in CALCA gene and migraine, further studies are warranted to study the effects of other functional polymorphisms in this gene and their role in migraine.

9.5 Conclusion

In conclusion, this research has provided further evidence that genetics not only plays an important role in migraine clinical phenotypes and endpoints, it also has the potential to influence treatment options for at least a subgroup of migraineurs. In particular this study has provided evidence that nutritional supplementation targeted to specific mutations associated with migraine may effectively reduce migraine associated disability and head pain. This study has also identified new variants in migraine candidate genes that show evidence of association with migraine pain severity and susceptibility in general. Further studies are required to understand the function of these genes in the disease process and investigate their potential role in migraine therapy. The global burden of migraine on individuals and society is immense. Current migraine therapies do successfully treat and/or prevent migraine and its associated symptoms. However they are not always efficacious, cost effective
or free of adverse effects. The continuous discovery of migraine susceptibility genes has proved that genetically influenced abnormalities may be involved in the pathophysiological pathway underpinning migraine. Alternative therapeutic options that are effective and inexpensive are necessary for improving the lives of millions around the world affected by migraine. It is hoped that the results described in this thesis may aid in further exploration of pharmacogenetic relationships and personalized migraine treatment possibilities.
Reference:


Olteanu, H., T. Munson, et al. (2002). "Differences in the efficiency of reductive activation of methionine synthase and exogenous electron acceptors between
the common polymorphic variants of human methionine synthase reductase." Biochemistry 41(45): 13378-85.


Appendices

Appendix A

DNA extraction Protocol by QIAGEN DNeasy Blood & Tissue Kit

1. Thaw blood

2. Pipet 100 µl QIAGEN Protease into the bottom of a 15 ml centrifuge tube.

3. Add 1 ml blood and mix briefly.

4. Add 2 ml Buffer AL and mix thoroughly by inverting the tube 15 times, followed by vigorous shaking for at least 1 min

5. Incubate at 70 °C for 10 min in Diagnostic testing Waterbath

6. Add 1 ml ethanol (96-100%) to the sample, and mix by inverting the tube 10 times, followed by additional vigorous shaking

7. Carefully transfer all of the solution from step 6 onto the QIAamp Midi column in a 15 ml centrifuge tube (provided), taking care not to moisten the rim. Close the cap and centrifuge at 1850 x g (3000 rpm) for 3 min

8. Remove the QIAamp Midi column, discard the filtrate, and place the QIAamp Midi column back into the 15 ml centrifuge tube

9. Carefully, without moistening the rim, add 2 ml Buffer AW1 to the QIAamp Midi column. Close the cap and centrifuge at 4500 x g (5000 rpm) for 1 min

10. Carefully, without moistening the rim, add 2 ml Buffer AW2 to the QIAamp Midi column. Close the cap and centrifuge at 4500 x g (5000 rpm) for 15 min

11. Place the QIAamp Midi column in a clean 15 ml centrifuge tube (provided), and discard the collection tube containing the filtrate

12. Pipet 200 µl or 300 µl Buffer AE equilibrated to room
temperature (15–25°C), directly onto the membrane of the QIAamp Midi column and close the cap. Incubate at room temperature for 5 min, and centrifuge at 4500 x g (5000 rpm) for 2 min.

13. Pipet eluate from 15ml collection tube to labelled 2ml graduated microcentrifuge tube with flat top screw cap. Discard empty collection tube into pathological waste.
Appendix B

DNA extraction Protocol by Salting out protocol.

1. Thaw blood by placing in 4˚C fridge for a few hours or quick-thawing at room temperature (RT) on bench.

2. Empty contents of blood vial by pouring blood carefully into a 50ml falcon tube. 20ml blood: Rinse blood vial by adding 5ml NKM buffer, recapping vial, and inverting tube. Empty into same 50ml tube. Repeat if necessary. 10ml blood: Rinse vial by adding 5ml NKM buffer, recapping vial, and inverting tube. Empty into same 50ml tube.

3. Add NKM buffer to bring the total volume to 47.5ml / 25ml / 15ml. Recap tube and shake vigorously.

4. Centrifuge the tube at 4800rpm for 25mins at 4˚C. Prepare a beaker with diluted (5-10%) Decon for blood waste disposal.

5. Discard the supernatant by pouring carefully into decon beaker without disturbing pellet on the bottom. Alternatively, remove supernatant by using a transfer pipette, without disturbing pellet.

   Add 10ml / 5ml / 5ml of RSB buffer to the falcon tube and break up the pellet using a transfer pipette. Ensure pellet is completely broken up/resuspended.

6. Recap tube and shake vigorously. (Multiple tubes may be inverted by clamping two racks together with hands and inverting simultaneously.) Add RSB buffer to bring the volume to 47.5ml / 25ml / 15ml and recap, shaking vigorously again.

7. Centrifuge at 4000rpm for 15 mins at 4˚C.
8. Repeat steps 5 – 7. Importantly, do not disturb white layer on top of pellet. This also contains white blood cells and will reduce yield if lost.

Thaw proteinase K (stored at -20°C in Robert’s freezer). Aliquot proteinase K if less than 1 x 10ml tube is required for each run, and re-freeze leftover proteinase K at -20°C as soon as possible.

9. Discard supernatant, and resuspend pellet by adding 2ml / 1ml / 0.5ml RSB buffer and break up pellet with transfer pipette.

10. Add 8ml / 4ml / 2ml of Lympholysis buffer and 500ul / 250ul / 125ul of proteinase K.

11. Recap, parafilm to seal falcon tube well. Place overnight in a 37°C shaking water bath.

12. Add 4ml / 2ml / 2ml sterile saturated (6M) NaCl. Invert for 15 seconds.

13. Centrifuge at 2500rpm for 15mins at 4°C.

14. Carefully decant the supernatant containing DNA (by pouring or by transfer pipette) into two 10ml yellow-cap tubes / one 10ml yellow-cap tube / one 10ml yellow-cap tube

15. Centrifuge at 2500rpm for 15mins at 4°C.

16. Carefully transfer the supernatant (by pouring or transfer pipette) into an extra 50ml falcon tube / 50ml falcon tube / 50ml falcon tube.

17. Measure the volume as accurately as possible using the marking on the tube and add two volumes of -20°C chilled absolute (100%) ethanol.

18. Gently swirl and observe the DNA strands precipitate.

19. Using an inoculating loop, transfer the DNA into one labelled 2ml tube containing 1ml 1X TE buffer (pre-prepared).

20. Parafilm the 2ml tube and allow the DNA to dissolve by incubating at 37°C overnight.
21. Discard the blood waste into the Decon beaker, cover with aluminium foil and
let sit overnight. Can be poured down the sink with copious amounts of water
the next day.

22. Vortex and quantitate using the Nanodrop or other methods (picogreen,
fluorometer) and keep a record of Nanodrop concentrations.

23. Store DNA tubes in appropriate boxes in -20°C freezer. Ethanol precipitate an
aliquot of 1X TE stock DNA for resuspension in water and use in PCRs (store
this at 4°C).
Appendix C

The Migraine Disability Assessment Test

The MIDAS (Migraine Disability Assessment) questionnaire was put together to help you measure the impact your headaches have on your life. The information on this questionnaire is also helpful for your primary care provider to determine the level of pain and disability caused by your headaches and to find the best treatment for you.

INSTRUCTIONS
Please answer the following questions about ALL of the headaches you have had over the last 3 months. Select your answer in the box next to each question. Select zero if you did not have the activity in the last 3 months.

1. On how many days in the last 3 months did you miss work or school because of your headaches?

2. How many days in the last 3 months was your productivity at work or school reduced by half or more because of your headaches? (Do not include days you counted in question 1 where you missed work or school.)

3. On how many days in the last 3 months did you not do household work (such as housework, home repairs and maintenance, shopping, caring for children and relatives) because of your headaches?

4. How many days in the last 3 months was your productivity in household work reduced by half or more because of your headaches? (Do not include days you counted in question 2 where you did not do household work.)

5. On how many days in the last 3 months did you miss family, social or leisure activities because of your headaches?

Total (Questions 1-5)

A. On how many days in the last 3 months did you have a headache? (If a headache lasted more than 1 day, count each day.)

B. On a scale of 0 - 10, on average how painful were these headaches? (where 0 = no pain at all, and 10 = pain as bad as it can be.)

Scoring: After you have filled out the questionnaire, add the total number of days from questions 1-5 (ignore A and D).

<table>
<thead>
<tr>
<th>MIDAS Grade</th>
<th>Definition</th>
<th>MIDAS Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Little or no disability</td>
<td>0-5</td>
</tr>
<tr>
<td>II</td>
<td>Mild disability</td>
<td>6-10</td>
</tr>
<tr>
<td>III</td>
<td>Moderate disability</td>
<td>11-20</td>
</tr>
<tr>
<td>IV</td>
<td>Severe disability</td>
<td>21+</td>
</tr>
</tbody>
</table>

Please give the completed form to your clinician.

This survey was developed by Richard H. Lipton, MD, Professor of Neurology, Albert Einstein College of Medicine, New York, NY, and Walter F. Stewart, MPH, PhD, Associate Professor of Epidemiology, Johns Hopkins University, Baltimore, MD.