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AN INVESTIGATION OF MIGRAINE CANDIDATE GENES AND GENOMIC SUSCEPTIBILITY REGIONS

BY

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A Thesis submitted as fulfilment for the degree of Doctor of Philosophy (PhD) in the School of Health Science, Griffith University, Gold Coast, Queensland.

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ABSTRACT

Typical migraine, comprised of migraine with aura (MA) and migraine without aura (MO), is a chronic, painful and debilitating neurovascular disease which is generally characterised by recurrent attacks of severe headache usually accompanied by nausea, vomiting, photo and phonophobia. Migraine has been shown to affect a large proportion of Caucasian populations with a recent comprehensive study indicating that around 25% of women and 8% of men suffer from the disease. Strong familial aggregation of typical migraine and an increased concordance for the disease in MZ twins over DZ twins, suggests that it has a significant genetic component. Heritability estimates are calculated to be between 40% and 60%, indicating that disease variation, in part, is explained by environmental determinants. The mode of transmission of typical migraine is not clear but is most likely multifactorial. Although the MA and MO subtypes exhibit some clinical heterogeneity, segregation analysis has suggested that there may be a common genetic aetiology for MA and MO, and a major gene contributing to typical migraine pathogenesis. This idea is substantiated by the fact that both subtypes of migraine can occur within the same family and even within the same individual, with up to 33% of sufferers experiencing both types of the disease. In addition, migraine prophylactics have been shown to result in similar effects in patients treated for both types of migraine. However, whether the two subtypes are truly separate entities or not remains unclear.

At present, the type and number of genes involved in typical migraine is not known. Despite this, several studies into Familial Hemiplegic Migraine (FHM), a very severe subtype of MA, have led to the discovery that mutations in a brain specific calcium channel subunit gene (*CACNA1A*) located on chromosome 19, cause FHM in about 50% of affected families. FHM is a rare disease and is distinguished from typical migraine by its association with hemiparesis and clear autosomal dominant mode of inheritance. However, certain clinical features are common to both FHM and typical migraine including similarities in headache characteristics and triggers. Hence, FHM genetic studies provide a valuable model for investigating the genes involved in the

more prevalent types of migraine with and without aura. For this reason the Genomics Research Centre has been conducting linkage studies utilising large Australian migraine pedigrees with a focus on the known FHM (*CACNA1A*) gene region on chromosome 19p13. Our results to date have indicated suggestive linkage to the FHM region on 19p13 in a large multigenerational pedigree (MF1) affected with typical migraine, with a maximum parametric LOD score of 1.92 ($P = 0.001$) obtained for a triplet repeat polymorphism situated in exon 47 of the *CACNA1A* gene. Expansion of this repeat was not observed, but is possible that mutations elsewhere in the *CACNA1A* gene may be responsible for migraine in this pedigree. To investigate this possibility, the current research involved sequencing two patients carrying the critical susceptibility haplotype surrounding the *CACNA1A* gene. The results of this mutation screen revealed no disease causing mutations or polymorphisms in any of the 47 exons screened. To determine whether the *CACNA1A* genomic region was implicated in typical migraine susceptibility in the general Caucasian population, 82 independent pedigrees and a large case-control group were also analysed using highly polymorphic microsatellite markers. There was no linkage or association detected in these groups and thus, it was concluded that if *CACNA1A* plays a role in typical migraine it does not confer a major effect on the disease. However, subsequent case-control studies of SNPs in the INSR gene, which is located ~15cM telomeric from *CACNA1A*, provided evidence of association to typical migraine. Thus, the INSR gene may now emerge as the new migraine susceptibility gene in this genomic region on chromosome 19.

Family linkage studies conducted by Gardner et al have implicated an additional FHM susceptibility region on chromsome 1q31. Furthermore, independent research carried out by Ducros et al. has indicated a second FHM locus at 1q21-23, which is ~ 30cM centromeric to the region reported by Gardner et al. At this stage it is not clear whether there is a single locus, or two distinct loci, on the chromosome 1q region. This research also involved a family-based linkage and association approach to investigating the FHM susceptibility region on chromosome 1q31 for involvement in typical migraine susceptibility in affected Australian pedigrees. Initial multipoint

ALLEGRO analysis provided strong evidence for linkage of Chr1q31 markers to typical migraine in a large multigenerational pedigree. The 1-LOD* unit support interval for suggestive linkage spanned ~18cM with a maximum allele sharing LOD* score of 3.36 obtained for marker D1S2782, $P = 0.00004$. Subsequent analysis of an independent sample of 82 affected pedigrees added support to the initial findings with a maximum LOD* of 1.24 ($P = 0.008$). Utilising the independent sample of 82 pedigrees we also performed a family-based association test. Results of this analysis indicated distortion of allele transmission at marker D1S249 (global $\chi^2_{(5)}$ of 15.00, $P = 0.010$) in these pedigrees. These positive linkage and association results will need further confirmation by independent researchers, but overall they provide good evidence for the existence of a typical migraine locus near these markers on Chr1q31, and reinforce the idea that an FHM gene in this genomic region may also contribute to susceptibility to the more common forms of migraine.

The serotonergic system has long been implicated in the pathophysiology of migraine. Researchers have therefore focused on the serotonin receptors and the genes that code for them when investigating this disease. Although serotonin receptor agonists have proven to be effective in the treatment of migraine, there has been little evidence of a serotonin receptor gene being associated with the disorder. However, in 1998, Ogilvie_et al reported that a VNTR in the serotonin transporter gene (SERT) showed altered allelic distributions in a Danish migraine population. In addition to serotonin, there has been renewed interest in the involvement of the dopaminergic pathways in migraine. This interest has gained impetus since the study of Peroutka et al who reported an allelic association between the dopamine receptor gene DRD2 and migraine with aura. Another dopamine related gene, the dopamine beta-hydroxylase gene (DBH), has been localised to Chr 9q34 and codes for the enzyme that catalyses the conversion of dopamine to norepinephrine. It therefore plays an important role in dopaminergic and noradrenergic neurotransmission. Serum levels of DβH enzyme have been reported to be elevated in migrainous patients during the headache phase of an attack. Also, significantly increased DβH enzyme activity has been observed in migraine patients during the headache-free interval. Thus, the DBH gene is another

good candidate for involvement in migraine pathophysiology and, to our knowledge, has not been previously implicated in this disease.

Candidate gene studies may be useful strategies for identifying genes involved in complex diseases such as migraine, especially if the gene being examined contributes only a minor effect to the overall phenotype. This research also involved a linkage and association approach to investigating neurotransmitter related migraine candidate genes. Specifically, polymorphisms within the serotonin transporter gene (*SERT*), the dopamine receptor gene (*DRD2*) and the dopamine beta-hydroxylase (*DBH*) gene were tested in unrelated Caucasian migraineurs and non-migraine control individuals. In addition, an independent sample of 82 families affected with migraine were examined. Unrelated case-control association analysis of a *DBH* intragenic dinucleotide polymorphism indicated altered allelic distribution between migraine and control groups ($\chi^2 = 16.53$, $P = 0.019$). Furthermore, the transmission/disequilibrium test (TDT) which was implemented on the family data also indicated distortion of allele transmission for the same *DBH* marker ($\chi^2 = 4.44$, $P = 0.035$). Together, these results provide evidence for allelic association of the *DBH* gene with typical migraine susceptibility (Fisher's Combined *P*-value = 0.006) and indicate that further research into the role of the *DBH* gene in migraine aetiology is warranted.

Nitric oxide (NO) is emerging as a key molecule affecting the pain associated with migraine. Since nitric oxide synthase (NOS) enzymes catalyse the synthesis of NO, the genes that code for these enzymes are good candidates for migraine molecular genetic analysis. This research involved investigating the role of a functionally relevant bi-allelic tetranucleotide polymorphism located in the promoter region of the human inducible nitric oxide synthase (*i*NOS) gene in migraine aetiology. A large group of migraine affected individuals were genotyped and compared to an age and sex matched group of unaffected controls. Results of a chi-squared analysis indicated that allele distributions for both migraine cases and controls were not significantly different ($\chi^2 = 1.93$, $P = 0.16$). These findings offer no evidence for an allelic

association of the tested *i*NOS polymorphism with the common forms of the disease and therefore do not support a role for this gene in migraine pathogenesis.

In summary, this research involved linkage and association analysis of migraine candidate genes and genomic susceptibility regions. Whilst, the known FHM gene (*CACNA1A*) was excluded for significant involvement in typical migraine the adjacent INSR gene has been associated. Migraine is genetically heterogeneous and the results of this research also provide good evidence that the DBH gene is involved in disease predisposition, whilst the *DRD2*, *SERT* and *INOS* gene were not shown to be implicated. An additional susceptibility region for typical migraine is also likely to localise to chromosome 1q31. Overall, the results presented in this thesis have contributed valuable data to the understanding of the molecular genetics of migraine with and without aura. Future research into the molecular pathophysiological mechanisms of migraine will greatly facilitate the development of more effective diagnosis and treatment strategies.

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STATEMENT OF ORIGINALITY

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

____

Rod A Lea

Publications Arising From Work Described in this Thesis and Related Publications

JOURNAL PUBLICATIONS

2002 Deborah A. Hilton, **Rod A. Lea**, John MacMillian and Lyn R. Griffiths (2002) An Analysis of Clinical Characteristics in Migraine Affected Pedigrees. *Cephalalgia* (**Accepted**).

Matthew P Johnson, **Rod A Lea**, Robert P Curtain, John C MacMillan, Lyn R Griffiths (2002). An Investigation of the *5-HT$_{2C}$* Receptor Gene as a Migraine Candidate Gene. *Am J Med Genet* (**Accepted**)

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CONFERENCE ABSTRACTS AND PRESENTATIONS

2001 **Lea R.A.,** Shepherd A.G, Curtain R.P., Brimage P and Griffiths L.R. A Typical Migraine Susceptibility Region Localises to Chromosome 1q31. The International Congress of Human Genetics, Vienna, Austria (**poster presentation**)

Lea R.A. A Typical Migraine Susceptibility Region Localises to Chromosome 1q31. 2nd Australasian Gene Mappers Meeting, Cairns (**oral presentation**)

2000 **Lea R.A.,** Nyholt D.R., Curtain R.P., Brimage P and Griffiths L.R. Linkage Analysis of FHM Susceptibility Regions on Chromosome 1 in Typical Migraine Families. American Society of Human Genetics, San Fransisco, USA. **(poster presentation)**

R.A. Lea, D.R. Nyholt, R.P. Curtain, K.L. Jordan and L.R. Griffiths. Linkage analysis of calcium channel genes in typical migraine families. *Human Genetics of Australasia Meeting*, Wellington, New Zealand (**oral presentation**)

1999 **Lea R.A,** Griffiths L.R., Nyholt D.R., Curtain R.P., Nicholson G.A., Brimage J.P. Heterogeneity of Migraine and Localisation of Susceptibility Loci to 19p13 and Xq. *Human Genome Meeting 99*. Brisbane Australia. **(poster presentation)**

1998 **Lea, R.A**., Defteros, N., Curtain, R.P., Nyholt, D.R., Brimage, P.J. and Griffiths, L.R. Direct sequencing of *CACNLIA4* gene exons in familial typical migraine. *20th Annual Conference on the Organisation and Expression of the Genome*. Lorne, Victoria, Australia. **(poster presentation)**

INTRODUCTION TO THE THESIS

> *I have a migraine again. Why me, why?..I am tired of the pain. Every day, every hour, every minute in pain, it's like loosing a part of my life. So many things to do, but can't - please pain go away. In the morning, afternoon or evening, I am wasting the life of others by not being able to function. Tired of the drugs, tired of the pain, helpless ... it's time to go to the ER again, the drugs don't work no more. Everyone put their life's on hold, again. This migraine is wasting precious moments of my life and it's taken moments of other life's, innocent casualties of this pain. I feel guilty, I feel bad, I should go to work today, I should not say that I am in pain, it might go away. If I try to ignore it it might go away. If I get busy it might go away..but it does not. I am losing my life to pain. I just want it to go away forever, go to sleep and maybe it will disappear*
>
> ***BobIrmaP@aol.com***

As this poem vividly conveys, migraine is a severe disease that affects millions of peoples lives. It is not life threatening but is definitely one of the most painful and debilitating maladies known to man. Since the beginning of human existence migraine has caused tremendous pain and suffering. Trepanation is perhaps the earliest and most extreme attempt at headache relief. Neolithic skulls dating back as early as 7000 B.C have been found to be trepanated. This procedure involved boring a hole in the skull of a sufferer and removing a piece of the cranium and was used as a treatment for the disease up until about 300 years ago (Silberstein S D, 1999).

The Greek physician Hypocrates was the first to try and describe migraine as a physiological symptom of disease in about 400 BC. He believed imbalances in natural elements affected the body’s fluids and eventually rose up to the head in a painful climax. It wasn't until about 600 years later that the word "migraine" was introduced, and in the 18th century migraine began to become accepted as a disorder of the neurovascular system (Silberstein S D, 1999).

In the 20th century there have been some substantial advances in understanding this common disease especially in the development of pharmaceuticals. However, the underlying cause of migraine and its resulting pathophysiology are still not clearly understood. Perhaps, one of the most significant findings so far has been the

discovery that migraine aetiology has a substantial genetic component. Since our genes are perhaps the essence of our physical (and maybe psychological) being, it seems only logical that further knowledge of migraine pathophysiology and how to successfully treat the disease will stem from fundamental information at the molecular genetic level. For this reason, the work described in this thesis will focus on the molecular genetics of migraine in an attempt to contribute to the existing knowledge base currently available regarding the disease.

The Objectives of the Research

Using modern and complementary gene mapping strategies, it was the overall objective of this research to investigate candidate genes and genomic regions for involvement in susceptibility to the common forms of migraine. To achieve this, blood samples from a large number of affected patients were required. These individuals were to be diagnosed as being affected with typical migraine, comprised of migraine with aura (MA) and migraine without aura (MO). Unaffected subjects were also required to be collected as a comparative control group. Extraction of DNA from all individuals collected was important for use in the migraine molecular genetic studies to be undertaken.

Prior molecular genetic research has revealed that mutations in a brain specific calcium channel subunit gene (*CACNA1A*) located on chromosome 19p13 are responsible for causing a subtype of MA known as Familial Hemiplegic Migraine (FHM) (Ophoff et al, 1996). FHM is distinguished from typical migraine by its rarity and associated hemiparaplegia. Furthermore, FHM has a clear autosomal dominant mode of inheritance, whereas typical migraine is a multifactorial disease with both genetic and environmental factors contributing. However, there is some clinical overlap between the two diseases such as similar triggers and headache types (Gardner et al, 1997). Therefore, genetic studies into FHM may be a useful model for studying the more prevalent forms of migraine. Preliminary work at the Genomics Research Centre has used large migraine affected families to localise a genomic susceptibility region to the site of the FHM (*CACNA1A*) gene region on chromosome 19p13. Whilst this original study has indicated the general position of a defective

gene, the actual gene and more importantly, the causative mutation have yet to be conclusively identified. For this reason, the research presented in this thesis was aimed at further investigating the *CACNA1A* (FHM) gene region using a family-based linkage and association strategy employing DNA markers as well as direct mutation screening techniques. FHM is genetically heterogeneous and a second susceptibility region for this disease has been localised to chromosome 1q (Gardner et al, 1997; Ducros et al, 1997). Because of the identification of this new FHM region, and the report of another calcium channel gene (*CACNA1E*) that exhibits similar sequence homology to the *CACNA1A* gene on chromosome 19p13, it was an additional primary objective of this research to test, using migraine affected families, this FHM region on chromosome 1q for involvement in typical migraine. Hence, this research investigated the chromosome 19 and chromosome 1 FHM implicated genomic regions for involvement in typical migraine.

Investigation of candidate genes is a popular approach to identifying disease susceptibility genes and provides a complementary alternative to genomic region localisation using families. Candidate gene association studies are believed to be powerful strategies especially when the risk contributed by the gene under investigation is small. This “low risk” situation is likely to be typical for complex diseases such as migraine where the effect of multiple, possibly interacting, genes combine to influence the disease. Due to their physiological importance, candidate genes for migraine have largely been selected from neurotransmitter pathways. In particular, the serotonergic and dopaminergic systems have been the focus of many international molecular genetic studies into the disease (;Peroutka et al, 1997b; Ogilvie, 1998, Del Zompo et al, 1998, Nyholt et al, 1997). One of the main aims of the research undertaken as part of this thesis was to investigate the neurotransmitter genes – serotonin trasnporter (*SERT)*, dopamine receptor 2 (*DRD2)*, dopamine beta-hydroxylase (*DBH)* that are believed to be candidates for migraine susceptibility. Furthermore, this work was aimed at testing DNA polymorphisms in the inducible nitric oxide synthase (*iNOS),* insulin receptor *(INSR)* and calcium channel α-1A subunit (*CACNA1A)* genes for association to typical migraine using samples of cases and controls as well as migraine affected pedigrees.

Significance of the Research

Migraine is a complex neurovascular disorder that is characterised by recurrent attacks of headache, which differ in intensity, frequency and duration. These headaches are usually preceded by mood swings and transient neurological disturbances and are subsequently associated with nausea and vomiting, photophobia and phonophobia. A migraine attack can be triggered by ocular factors such as bright light, allergensor chemicals found in certain foods, excessive alcohol consumption (especially red wine), physical exercise and psychological factors such as stress and mental fatigue (Blau, 1980).

A high proportion of the population suffers from severe migraine with an estimated prevalence of around 12% (Russell and Olesen, 1995). The disease incurs a heavy economic burden on society due to such factors as direct medical costs, as well as lost productivity at work and absenteeism (Australian Bureau of Statistics 1989/90). Migraine also imparts substantial collateral effects on friends and family members of sufferers. Although the primary underlying cause of migraine is still unknown, there is a high incidence of familial aggregation with approximately 50% of sufferers having a first degree relative (Russell and Olesen, 1995). This, combined with high concordance in twin studies, strongly indicates a substantial genetic component is involved (Urlich, 1999). Despite this, the number and type of genes involved is not known.

Migraine patients are currently diagnosed using criteria established by the International Headache Society (IHS) (IHS, 1988). Unfortunately, this form of diagnosis is often reliant on post hoc patient self reports and clinician opinion. Although biochemical and physiological approaches to migraine have been extensively investigated, no characteristic has been consistently recognised in affected individuals (Russell et al, 1992). Therefore, no laboratory based diagnostic test currently exists. Whilst various medications are used to treat the disorder, there is currently no completely effective pharmaceutical, and no cure. The identification of migraine susceptibility genes should contribute to a better understanding of the pathophysiology of migraine. In turn, this will aid in the development of more

reliable diagnostic tests and may lead to the development of effective pharmaceuticals that can be tailored to treat the specific symptoms of the disorder.

CHAPTER 1
GENERAL MIGRAINE BACKGROUND

1.1 Description of Migraine

In 1980, Blau described complete migraine as a syndrome that includes four main phases which provide a convenient way of classifying the many characteristics and symptoms that manifest in the disease. The phases are: the *prodrome* which occurs hours or days before the headache; the *aura* which immediately preceeds the headache; the *headache* phase itself and the final *postdrome*. Not all phases will be present in every attack, and only a portion of migraineurs experience phase 2 or auras (Blau, 1980).

1.1.1 Phase 1 (*The Prodrome*)

The prodrome of migraine begins hours or up to 2 days before the aura or headache phase and the symptoms are less dramatic than those that follow. Typical symptoms include: mood changes such as irritability, crankiness, depression; clumsiness, slurred speech, tiredness, yawning, food craving, loss of appetite, increased urination, hypersensitivity to light, sound, touch and even odour. These premonitory symptoms occur in approximately 60% of migraineurs but are often mistakenly lumped together into the aura phase and referred to as 'warning signs'. Although both phases do warn of an impending attack they are distinctly different (Blau, 1980). The first and last of the four phases have been by far the least studied, and so relatively little is known about them.

1.1.2 Phase 2 (*The Aura*)

Perhaps the most mysterious phase of migraine is the aura phase. Approximately 20% of sufferers experience the aura of migraine and this phase usually lasts for about 20 to 30 minutes. Migrainous auras, or focal cerebral disturbances, can affect all the senses: the visual, tactile (e.g. pins & needles, numbness), olfactory (e.g. imagined & recalled smells), oral (e.g. strange tastes) and aural (e.g. tinnitus). There are other characteristics too, including: poor concentration, incoherence & slurred speech,

mental confusion, distorted spatial perception, amnesia, sleepiness, poor co-ordination and sense of balance, dizziness; hallucinations, and even sweats, muscle jerks & spasms. Although auras are relatively specific to migraine, some comorbid conditions such as cerebrovascular disease and epilepsy may include related phenomena (Blau, 1980).

1.1.3 Phase 3 (*The Headache*)

The most feared phase of migraine is the headache. The headache is often severe, unilateral, and may be centred over one eye or the temple. It often throbs (in time with the pulse), and is aggravated by physical activity. As the headache begins so too, in many migraineurs, do nausea and vomiting. In addition to nausea and vomiting, a small number of migraineurs also experience diarrhoea and/or frequent and copious urination. If present, nausea, vomiting and diarrhoea make taking medication orally or by suppository difficult. During this phase migraineurs will be hypersensitive to light and/or sound and will nearly always want to lie down in a dark quiet room (Drummond, 1986). Other neurological effects that may occur during the headache phase of a migraine include impaired concentration and loss of memory, as well as anxiety and depression (Blau, 1980).

1.1.4 Phase 4 (*The Postdrome*)

The postdrome (or hangover), which may last for days, can be thought of as a time of recovery. It is often characterized by weakness, tiredness, exhaustion, and mild headache. It can be severe enough to prevent return to school or work, although some people can feel unusually refreshed and even europhoric after an attack (Blau 1980). Children tend to recover quite quickly after the headache and seem not to experience this phase to any significant degree (Silberstein and Lipton 1994).

1.2 Diagnosis of Migraine

The primary headache disorders – migraine, tension-type headache, and cluster headache – have been defined based primarily on the symptom profiles of individual patients. A number of different case definitions for these diseases have been proposed and applied. The Ad Hoc Committee on Classification of Headache described some

of the headache subtypes but failed to set definitive criteria for classifying these subtypes. In general, migraine was described as "attacks that are commonly unilateral in onset; are usually associated with anorexia and sometimes with nausea and vomiting" (Friedman et al, 1962). Further headache diagnostic criteria proposed by Waters (1970) defined migraine as consisting of any two of the following: unilateral distribution of headache, nausea or vomiting, and warning (which encompassed symptoms including aura). Research published by Bille (1962) described migraine as a paroxysmal headache separated by headache-free intervals with two or more of the following characteristics: nausea, scotomas or related phenomena, one-sided pain, and a positive family history involving at least one affected first degree relative (Bille, 1962). Overall, the definitions of migraine were rather subjective and resulted in the disease being "loosely" diagnosed. Clearly a more thorough set of universally accepted migraine diagnosis criteria was needed to allow researchers and clinicians to categorise the disease.

In 1988, a group of world leaders in the characterisation and diagnosis of migraine formed the International Headache Society (IHS) and compiled and published a consensus set of diagnostic criteria (International Headache Society, 1988). This classification system has proved to be an enormous improvement over preceding methods and is currently the gold standard (Friedman et al, 1962). The criteria are more simple, yet more informative and reliable than traditional systems and provide the clinician with the guidance to explicitly identify and define different features of a particular form of migraine (Lipton and Stewart, 1997).

The diagnostic criteria developed by the IHS require that certain attributes be present to establish a diagnosis of migraine headaches. The attributes listed in Tables 1.1 – 1.3 are the formal IHS diagnostic criteria of the two main migraine subtypes; *migraine with aura (MA)* and *migraine without aura (MO)*. Grouped together, these subtypes will be referred to as "*typical migraine*". It is these criteria that were used for all migraine diagnosis in this research.

1.2.1 Migraine Without Aura

Migraine without aura (common migraine) is characterised by at least 5 headache attacks lasting 4-72 hours. These headaches are unilateral with a pulsating quality of moderate to severe intensity. Physical activity tends to aggravate this type of migraine and the patient may experience nausea, photophobia and phonophobia (International Headache Society, 1988).

1.2.2 Migraine With Aura

Migraine with aura (classic migraine) is the term used to describe a recurring headache which is associated (usually preceded) with other *neurological* (or aura) symptoms. These symptoms almost certainly originate at the cerebral cortex or brain stem and tend to gradually develop over 5-20 minutes and usually last less than 60 minutes. Following the neurological aura symptoms, a period where no pain and/or discomfort may be experienced. However, most patients develop subsequent headache, nausea or photophobia which are characteristic of the MO symptoms. The headaches last between 4 and 72 hours as with MO (International Headache Society, 1988).

1.2.3 Other Migraine Subtypes

There are several subtypes of MA (listed in Table 1.3). *Familial hemiplegic migraine* (FHM) is a rare and severe subtype of MA and includes hemiparesis as a symptom and involves at least one first degree relative with identical attacks. Aura symptoms that clearly originate form the brain stem or from both occipital lobes is termed *basilar migraine*. A migrainous aura may also develop which is unaccompanied by headache. This is sometimes called *acephalgic* migraine. Menstrual migraine is experienced by around 25% of female MO sufferers (Rasmussen, 1993). Since 90% of MO attacks in women occur in association with menstruation, it has been suggested by the IHS that menstrual migraine may be a subtype of MO (International Headache Society, 1988). This research only investigated migraine with (MA) and without aura (MO) which are collectively referred to as “typical migraine”.

Table 1.1. Formal Classification Criteria of Migraine Without Aura (Headache Classification Committee 1988)

1.1 Migraine without aura

Previously used terms: common migraine, hemicrania simplex

Diagnostic criteria

A. At least five attacks fulfilling B through D

B. Headache lasting 4 to 72 h (untreated or unsuccessfully treated)

C. Headache has at least two of the following characteristics:
   1. Unilateral location
   2. Pulsating quality
   3. Moderate or severe intensity (inhibits or prohibits daily activities)
   4. Aggravation by walking stairs or similar routine physical activity

D. During headache at least one of the following:
   1. Nausea and/or vomiting
   2. Photophobia and phonophobia

E. At least one of the following:
   1. History, physical, and neurologic examinations do not suggest one of the disorders listed in groups 5 through 11*
   2. History and/or physical, and/or neurologic examinations do suggest such disorder but it is ruled out by appropriate investigations
   3. Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder

**Groups 5 through 11 are; 5) headache associated with head trauma, 6) headache associated with vascular disorders, 7) headache associated with non-vascular intracranial disorders, 8) headache associated with substances or their withdrawal, 9) headache associated with non-cephalic infection, 10) headache associated with metabolic disorders, and 11) headache or facial pain associated with disorder of cranium, neck eyes, ears, nose, sinuses, teeth, mouth or other facial or cranial structures, respectively.*

Table 1.2. Formal Classification Criteria of Migraine With Aura (Headache Classification Committee 1988)

1.2 Migraine with aura

Previously used terms: classic migraine; classical migraine; opthalmic, hemiparesthetic, hemiplegic, aphasic, or complicated migraine

Diagnostic criteria

A. At least five attacks fulfilling B

B. At least three of the following four characteristics:

1. One or more of the fully reversible aura symptoms indicating focal cerebral cortical and/or brainstem dysfunction
2. At least one aura symptom develops gradually over more than 4 min or two or more symptoms occur in succession
3. No aura symptom lasts more than 60 min; if more than one aura symptom in present, accepted duration is proportionally increased
4. Headache follows aura with a free interval of less than 60 min (it may also begin before or simultaneously with the aura.)

C. At least one of the following:

1. History, physical, and neurologic examinations do not suggest one of the disorders listed in groups 5 through 11
2. History and/or physical, and/or neurologic examinations do suggest such disorder. but it is ruled out by appropriate investigations
3. Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder

1.2.1 Migraine with typical aura

Diagnostic criteria

A. Fulfils criteria for 1.2 including all four criteria under B

B. One or more aura symptoms of the following types:

1. Homonymous visual disturbance
2. Unilateral paresthesia and/or numbness
3. Unilateral weakness
4. Aphasia or unclassifiable speech difficulty

Table 1.3. Migraine subtypes excerpt from International Headache Society classification of headache (Headache Classification Committee 1988)

1	**Migraine**
1.1	Migraine without aura
1.2	Migraine with aura
	1.2.1 Migraine with typical aura
	1.2.2 Migraine with prolonged aura
	1.2.3 Familial hemiplegic migraine
	1.2.4 Basilar migraine
	1.2.5 Migraine aura without headache
	1.2.6 Migraine with acute onset aura
1.3	Opthalmoplegic migraine
1.4	Retinal migraine
1.5	Childhood periodic syndromes that may be precursors to or associated with migraine
	1.5.1 Benign paroxysmal vertigo of childhood
	1.5.2 Alternating hemiplegia of childhood
1.6	Complications of migraine
	1.6.1 Status migrainosus
	1.6.2 Migrainous infarction
1.7	Migrainous disorder not fulfilling the above criteria

1.2.4 Clinical Heterogeneity of Migraine

According to The International Headache Society (IHS), migraine is clinically a heterogeneous disorder. The IHS subdivides common migraine into two main sub-categories, MA and MO. Although these subtypes are defined by separate diagnostic criteria, there has been considerable controversy as to whether MO and MA are distinguishable entities or not (Wilkinson & Blau, 1985: Olesen J, 1985: Pascual J et al, 1994). A large epidemiological survey study by Russell et al, compared the clinical characteristics of 484 migraineurs from the general Danish population. Among the results of this research, differences regarding age of onset and presence or absence of aura symptoms were found. Also, bright light was shown to be a

precipitating factor in MA, but not in MO, whereas menstruation was a precipitating factor in MO, but not likely in MA. It was concluded that these clinical differences may indicate that MO and MA are distinct entities (Russell et al, 1996). Further evidence for this argument of MA and MO being distinct lies in pathophysiological studies which have reported reduced regional blood flow in attacks of MA, whilst the same blood flow measures in MO attacks were shown to be normal (Olesen, 1981; Olesen, 1991).

The converse side of the debate is that MO and MA are manifestations of the same underlying aetiology. Perhaps the strongest argument for this "same entity" notion is that both MA and MO can occur in the same family and even within the same individual. It has been reported that up to 33% of migraine sufferers experience both subtypes of migraine (Launer et al, 1999). Furthermore, Blau (1995) has referred to striking similarities in the prodrome, pain phase, postdrome, and response to medication between MO and MA (Blau, 1995). At this stage it is still unclear whether or not MO and MA are distinct syndromes, different manifestations of the same disorder or perhaps part of a continuum. For this reason it may be most useful, at least for molecular genetic studies such as this, to consider MO and MA both as one disorder and as separate entities.

1.3 Epidemiology of Migraine

It is perhaps surprising that despite the pain and discomfort of a migraine attack many sufferers are not diagnosed or do not seek medical attention via a physician (Linet et al, 1991; Lipton et al, 1992). As a result, some characteristics of the disease, which have been described from clinical studies, are misrepresentative of the general population of migraineurs. This is due, in part, to clinical research design shortcomings such as selection and referral bias (Linet and Stewart, 1984). For this reason community (or population) based assessment of migraine is important in offering a complete picture of the disorder in the wider community (Lipton and Stewart, 1993). Epidemiology is the study of incidence, prevalence and the distribution of diseases in large populations as well as the circumstances influencing the characteristics of the disease (Beaglehole et al, 1993). Understanding the epidemiology of migraine will provide valuable insight into the scope and demographics of the disorder. This information will allow better assessment of health care delivery thus improving diagnosis and treatment. To date there has been extensive international investigation into migraine epidemiology.

1.3.1 Incidence of Migraine

The incidence of a disease is defined as the rate of onset of new cases of the disease in a specific population (Lipton and Stewart, 1997). To conduct a migraine incidence study the existing sufferers need to be excluded from the population being investigated. Non-migraineurs can then be observed to determine the rate of development of new cases (Beaglehole et al, 1993). An incidence study performed by Stewart et al (1993) investigated the age and sex-specific incidence rates of migraine with and without aura. These researchers conducted telephone interviews with over 10000 individuals and, under strict criteria ascertained information from 392 males and 1018 females aged between 12 and 29 years for inclusion in the study. A summary of the results obtained by these researchers is reproduced in Figures 1.1 and 1.2. It can be seen that the incidence of both MO and MA in males was lower and occurred at an earlier age. The highest incidence (~20/1000 person-years) was for the MO group and peaked in females at around 15 years of age (Stewart et al, 1993). The incidence of MO was higher than MA for both gender groups. The incidence of MA

and MO, shown separately by Stewart et al, have been merged here to illustrate by estimation the overall incidence of typical migraine by age of onset (Figure 1.3). It is interesting to view the differences in age specific incidence trends for typical migraine between the genders (Figure 1.4). It is quite clear that the incidence of typical migraine in males is lower than females overall but is considerably higher at around 5 years age of onset (7.5:2.5 units). However, the incidence trend of females from this group increases markedly from 5 years to peak at 15 units at ~15 years old, whereas the male incidence rate gradually decreases after about 13 years. Rozen et al, recently reported that the incidence of medically recognised migraine in the United States seems to be on the increase. These researchers measured an overall relative increase in migraine incidence between the period of 1979-1981 and 1989-1990 of 56% with the rise being most sizable for females (Rozen et al, 1999).

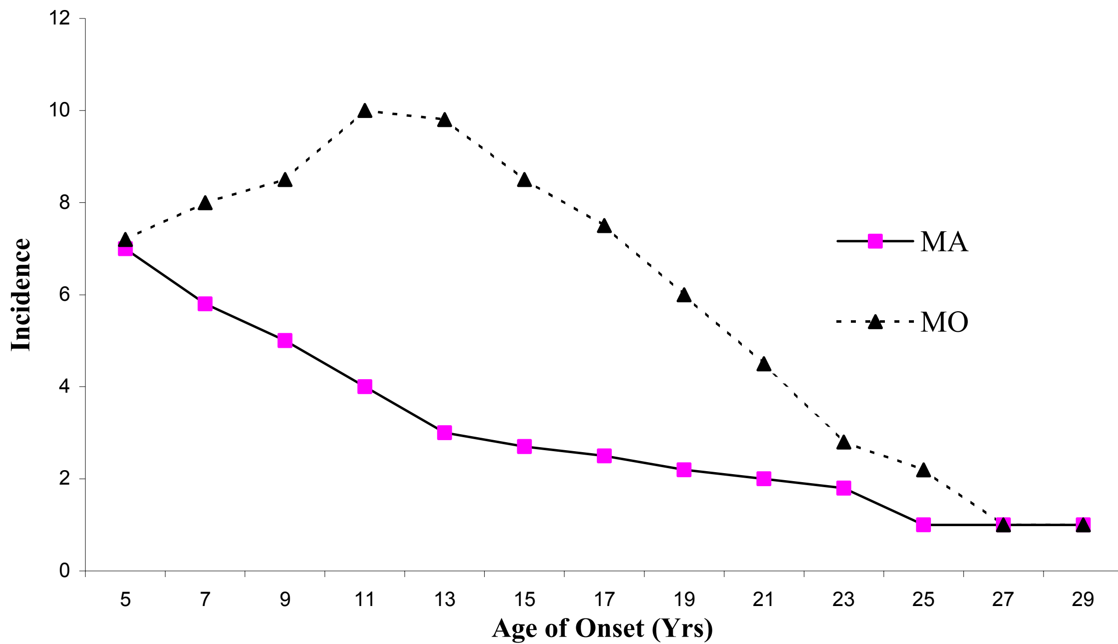


Figure 1.01. Incidence of MA and MO for males (Stewart et al, 1993)

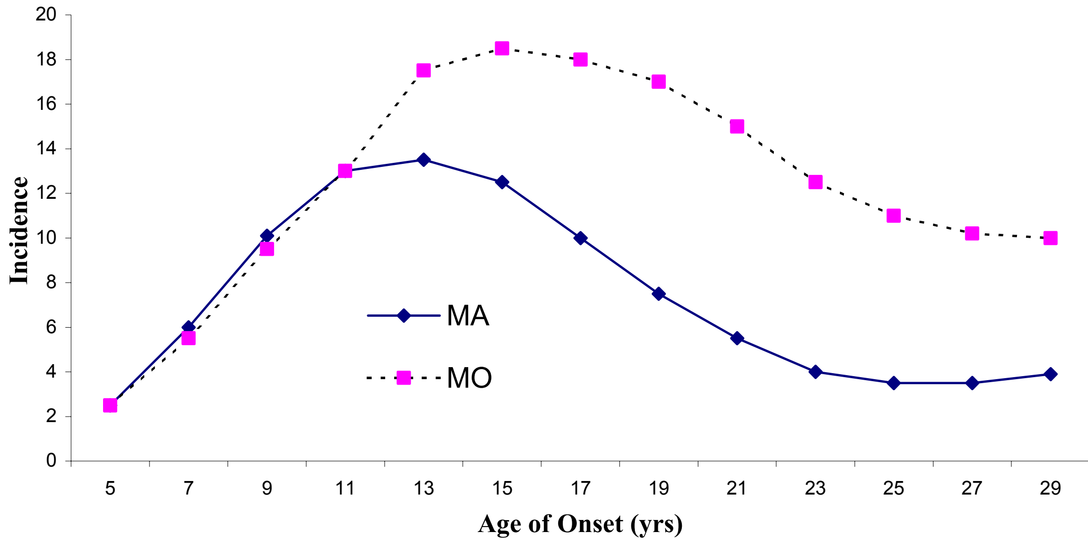


Figure 1.02. Incidence of MA and MO for females (Stewart et al, 1993)

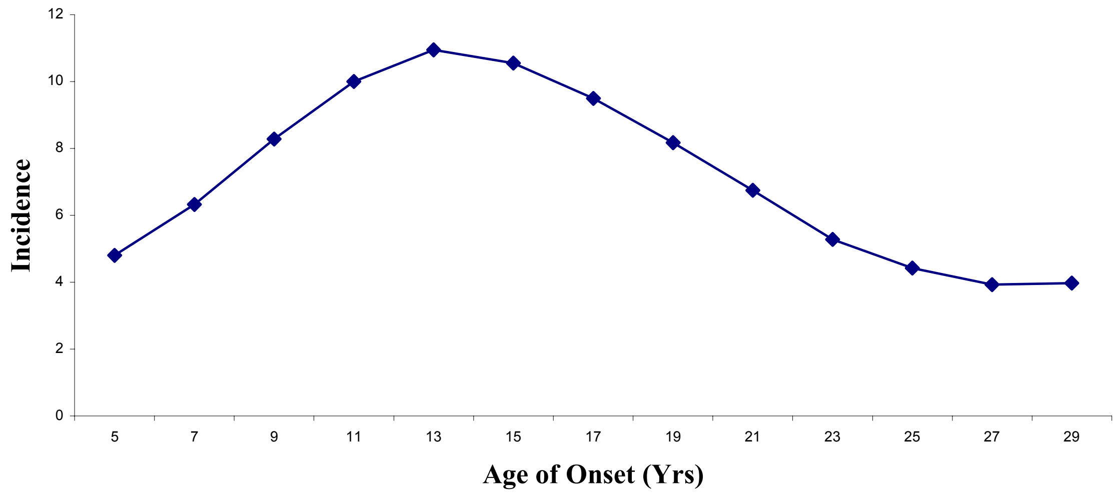


Figure 1.03. Incidence of typical migraine overall (from Stewart et al, 1993)

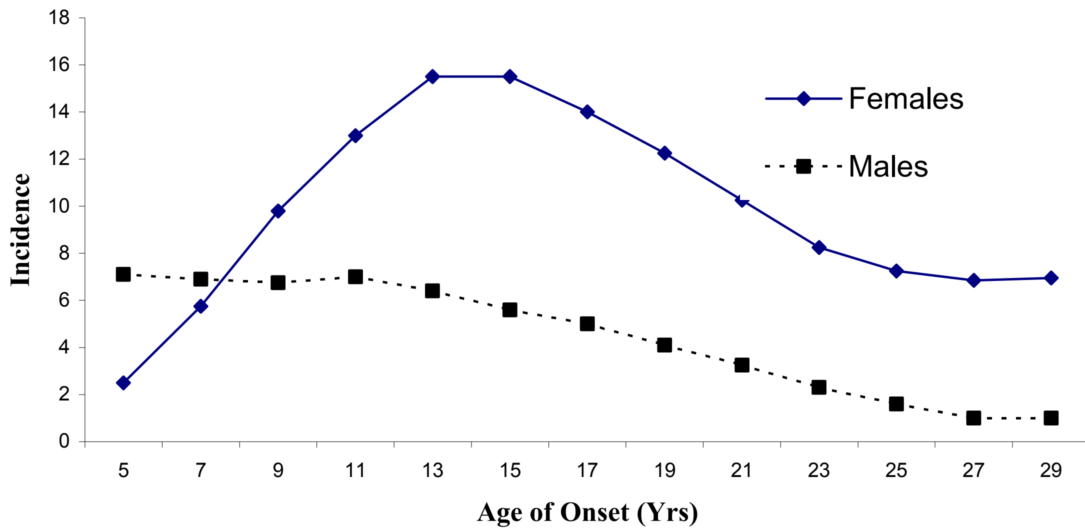


Figure 1.04. Incidence of typical migraine for males and females (Stewart et al, 1993)

1.3.2 Prevalence of Migraine

Prevalence is an important measure of the burden of a disease on a population. Accurate and reliable estimates of prevalence of migraine can facilitate health care planning and financing as well as the development of health care strategies and identification of the groups in greatest need of medical assistance (Stewart et al, 1995). Prevalence is defined simply as the number of affected individuals (in percentage units) within a defined population (at risk of the disease) measured at a specified point (or period) in time (Beaglehole et al, 1993). When referring to migraine, period prevalence estimates are more reliable than point prevalence estimates since migraine is diagnosed only if attacks have occurred recurrently over time. Lifetime period prevalence studies have been reported repeatedly in the literature but can have drawbacks such as the migraine sufferer's ability to recall information from years back about their disease. For this reason researchers are now commonly reporting the 1-year period prevalence involving patient records from the year prior to the study (Lipton and Stewart, 1988).

1.3.2.1 Migraine Prevalence in the General Population

There have been a very large number of population-based studies published reporting estimates of the prevalence of migraine (Stewart et al, 1994). Despite the extensive international investigation to date, there has been significant variation in the estimation of migraine prevalence across general (largely Caucasian) populations worldwide. This overall variation has been shown to be largely accounted for by differences in migraine case definition, but is also influenced by sample size, the method of study used by the researcher, as well as socio-demographic factors including age, gender and income distributions of the study groups (Stewart et al, 1992; Stewart et al, 1995).

The variation in prevalence estimates due to case definition was a substantial problem before the introduction and wide spread application of the IHS criteria for classifying migraine. The classification of migraine was quite subjective prior to the 1988 IHS publication. A review of population-based migraine prevalence studies was carried out by Stewart et al (1994). However, many pre-IHS studies were included in this review making accurate overall prevalence estimates difficult. An examination of these independent prevalence estimates of typical migraine prior to the development of the IHS guidelines in 1988 illustrated some marked differences in disease prevalence estimates between study populations. The overall prevalence estimates, pre-IHS guidelines, were shown to ranged from 5.4% in a Finnish study by Sillanpaa (1983) to 41.8% in a UK study by Newland et al, (1978). The overall migraine prevalence estimate averaged across all study populations was 22% with a variation measure of 10 standard deviations (Author's calculations).

Importantly, after the implementation of the IHS diagnosis guidelines, which allowed for a clearer definition of migraine cases, the 1-year prevalence estimates reported by different researchers worldwide tended to be remarkably similar across different affected populations. An up-to-date meta-analysis of migraine prevalence was performed by the Author of this thesis. This analysis included only post-IHS studies with a focus on the more reliable 1-year prevalence designs (see Table 1.4). Observation of independent post-IHS prevalence studies that determined 1-year prevalences revealed estimates that ranged from 8.15% (Breslau, 1991) to 16.25% (Launer et al, 1999) with a *mean overall prevalence around 12.5%* (Author's own

calculations). A similar reduction in variability of migraine prevalence estimates across these post-IHS study populations was also seen when the overall migraine study groups were divided into male and female subgroups. On average, the prevalence of migraine in females determined from the data was about 18.7% whilst for the male subgroups the average was 7.5%. Thus, it is clear that there is a strong female preponderance of migraine in the general population. This overrepresentation of the disease in females, which may be hormonally related, is consistent across all post-IHS studies listed in Table 1.4, with an average male to female ratio of approximately 1:3. This 1:3 ratio is known to fluctuate substantially with age (Lipton and Stewart, 1993) but tends to hold relatively constant between the ages of 35 and 45 years, which has been shown to be the age range where migraine prevalence is highest (Lipton and Stewart, 1995).

Table 1.4. Summary of Prevalence Studies of Migraine Post IHS Guidelines

Study	Year	Country	Sample Size	Age (y)	Time Frame	Migraine Prevalence (%) Male	Female	Total
Breslau et al.	1991	USA	1,006	21-30	1 year	3.4	12.9	8.15
					Life	7.0	16.3	11.65
Rasmussen et al.	1991	Denmark	740	25-64	1 year	5.9	15.3	10.6
					Life	7.8	25.2	16.5
Henry et al.	1992a	France	4,204	5-65	1 year	6.1	17.6	11.8
Henry et al.	1992 b	France	833	≥15	Life	4	12	8.1
Stewart et al.	1992	USA	20,334	12-80	1 year	6.0	17.7	11.8
Pryse-Phillips et al.	1992	Canada	1,573	≥15	Life	10	23	17
O'Brien et al.	1994	Canada	2,922	≥18	1 year	7.4	21.9	14.6
				≥18	Life	7.8	24.9	16.3
Gobel et al	1994	Germany	4061	≥18	?			11.3
Russell et al.	1995	Denmark	4,000	40	Life	11.2	23.4	17.3
Pradilla and Leon-S	1995	Columbia	1001	1-84		3.4	11.6	15
Stewart et al.	1996	USA	12,328	18-65	1 year			
		- Caucasian				8.6	20.4	14.5
		- African				7.2	16.2	11.7
		- Asian				4.8	9.2	7.0
Sanvito et al.	1996	Brazil	595	17-43	1 year	28.3	54.4	41.3
Sakai et al.	1997	Japan	4,029	>15	1 year	3.6	12.9	8.4
Launer et al	1999	Netherlands	6491	20-65	1 year	7.5	25	16.2
Hagen et al	2000	Norway	51383	≥20	1 year	8	16	12
Mattsson et al	2000	Sweden	728	40-74	1 year		18	
Wang et al	2000	Taiwan	3377	≥15		4.5	14.4	9.1
Lipton et al	2001	US	29727	≥12	1 year	6.5	18.2	12.4
Lipton et al	2002	US	4376		1 year	6	17.2	11.6
Ayatollahi et al	2002	Iran	1868	11-18	life		6.1	
Kececi and Dener	2002	Turkey	1320		life	7.9	17.1	12.5
Henry et al	2002	France	10585	≥15		4	11.2	7.9

The overall migraine prevalence estimate of 12.5% was calculated here based on summary data gathered from many independent studies conducted worldwide (Table 1.4). These studies utilised criteria published by the IHS to define migraine, and evaluated the characteristics of the disease using the more reliable 1-year period prevalence strategy. Therefore, this overall estimate of 12.5% can be considered a reasonable approximation of the prevalence of migraine in the general population. However, it should be noted that there were some apparent shortcomings in some of the studies included that may have slightly biased this resulting value. In particular, some of the study groups may have been of inadequate size to accurately represent the general population (Merikangas et al, 1990: Rasmussen et al, 1991:Henry et al, 1992b) and the age range investigated varied from specific year groups to all ages. In addition, it was assumed that the IHS criteria for diagnosing migraine was strictly adhered to by the clinicians involved in these studies, although in reality it is possible that some subjectivity influenced categorisation of the disease. A major shortcoming observed for many of these migraine prevalence studies was that the frequencies of the migraine subtypes, specifically MA and MO, were not reported. Information on these subtypes is important to genetic studies into migraine aetiology as well as to pharmacologic research.

A recent large-scale study of the prevalence and characteristics of migraine in a cohort from the Netherlands was reported by Launer et al (1999). This population-based study overcame the limitations of previous work by screening a large sample of 6491 adults for migraine. Diagnosis was based on strict IHS criteria and information was gathered on both lifetime and 1-year patient histories for both subtypes of migraine (MO and MA). An interesting finding from this study was that the co-existence of MO and MA occurred frequently, in about 33% of sufferers, and therefore has implications for future studies into the genetics of migraine (Launer et al, 1999).

1.3.2.2 Prevalence of Migraine in Ethnic Subgroups

It has been suggested that race may also be a contributing factor to the variation seen in migraine prevalence estimates across studies. There are several lines of evidence that point to an altered prevalence of the disease in different races. It has been shown that in African populations, estimates of migraine prevalence are consistently lower (Levy, 1983: Ogunyemi, 1984: Osuntokun et al, 1992) than those from Caucasian

populations. This reduced prevalence has also been observed in some Asian populations (Zhao et al, 1985: Tokio et al, 1993: Sakai and Igarashi, 1999). It was suggested that the method of study (including case definition) or cultural and environmental factors might influence the prevalence estimates. However, Stewart et al hypothesised that racial genetic differences may actually be the underlying reason for the reduced prevalence of migraine in Africans and Asians over Caucasians. To test this idea these researchers attempted to control for cultural and environmental factors by examining migraine in different race groups (Caucasian, African and Asian) living in the United States (Stewart et al, 1996). The findings of this research showed that in the US migraine prevalence is highest in Caucasians (14.5%) followed by African Americans (11.7%), and is lowest in Asian Americans (6.7%). It was concluded that although other factors contribute to race differences in migraine prevalence, race-related differences in genetic vulnerability to the disease are more likely to predominate as an explanatory factors (Stewart et al, 1996).

1.3.2.3 Migraine Prevalence in Other Demographic Groups

Migraine can be an early onset disorder and therefore occurs quite commonly in children. Studies on school aged children (15 years old or less) have shown that the prevalence of the disease can range from 3% (Raieli et al, 1995) to as high as 11% (Abu- Arefeh and Russell, 1994), with an average of ~4% reported for a large study in the United States (Stewart et al, 1992).

Migraine has long been considered a disease of the more intelligent and higher social classes but several studies into this popular idea have offered no support (Bille, 1962: Waters, 1971). In fact, epidemiological investigations of general populations have shown fairly uniform prevalence of migraine in various social groups (Pryse-Phillips et al, 1992:Rasmussen, 1992). However, there is some evidence suggesting that the prevalence of migraine is inversely correlated with household income (Stewart, 1992). That is, migraine seems to be more common in low income groups and becomes less common in groups as the household income increases (Lipton, 1993). Care should be taken when interpreting this association with migraine prevalence and socio-economic status since the relationship may be circumstantially due to poorer diet and/or lower medical care consumed by groups at the lower end of the socio-ecomomic spectrum (Lipton and Stewart, 1997).

1.4 Comorbidity of Migraine

Comorbidity is a term originally coined to refer to any two conditions observed to coexist in an individual during clinical trials (Feinstein, 1970). However, it has become common to now use comorbidity as a term to describe the relationship (or association) between two diseases observed in a population (Beaglehole et al, 1994). Comorbidity of migraine has important implications when investigating all aspects of the disease. Particularly, overlapping symptoms between two conditions can complicate the diagnosis and treatment of the disease in question (Lipton and Silberstein, 1994). Also, studying comorbidity may provide some insight into the pathogenesis of migraine. Comorbidity of migraine may occur for several reasons. Firstly, it may be that some condition actually causes migraine, or that migraine actually causes another condition. Secondly, migraine and another condition may be related (associated) due to shared environmental or genetic risk factors. And lastly, it is important to consider that detection of comorbidity may arise due to coincidence or selection bias (Lipton and Silberstein, 1994a). Population-based studies have demonstrated that migraine is comorbid with a number of neurologic and psychiatric disorders, including epilepsy, depression and anxiety disorders, (Lipton and Stewart, 1998). Arthritis, heart disease, stroke, diabetes and recently gastroenteritis have also been associated with migraine (Split and Szydlowska, 1997; Peroutka et al, 1997; Gasbarrini et al, 2000). Interestingly, a recent study estimating the risk for migraine by the presence of a defined set of comorbid conditions found that migraine patients were approximately 5 times more likely than there controls to be diagnosed with comorbid conditions. This high risk for comorbidity in migraineurs was found to significantly increase healthcare costs in this affected group compared to controls (Joish et al, 2000).

1.4.1 Migraine and Epilepsy

Comorbidity between migraine and epilepsy has long been suspected but has, until recently, been under examined. Epilepsy, like migraine is a chronic neurological disorder associated with unprovoked episodic attacks whose characteristics may include mood and behavioral changes, alterations of consciousness, focal sensory or motor symptoms, and hallucinations (Lennox and Lenox, 1960). In 1994, an

epidemiological study was conducted by Ottman and Lipton to investigate comorbidity of migraine and epilepsy in a large sample of 1948 adults diagnosed with epilepsy and 1411 of their first degree relatives. Results of this study revealed that individuals with epilepsy are at 2.4 times the risk of experiencing migraine than their non-epileptic relatives (Ottman and Lipton, 1994). This comorbidity between migraine and epilepsy may be due to a common alteration in brain state that increases the risk of both disorders (Andermann and Andermann, 1987). Alternatively, common environmental factors may cause elevated neuronal excitability or lower attack thesholds in both disorders (Welch, 1987). These shared risk factors can make differential diagnosis of these disorders difficult, although EEG recordings have been shown to assist in the differentiation of the disorders particularly during the migraine and epileptic aura phase (Lipton et al, 1994b). It has also been hypothesised that a common genetic susceptibility may contribute to the comorbidity between migraine and epilepsy. This idea was tested by Ottman and Lipton in 1996 utilising a large collection of 1967 adult probands with epilepsy. These researchers assessed the risk of migraine in relatives of probands with genetic versus non-genetic forms of epilepsy. Overall, the results of this study did not support an association between risk of epilepsy in relatives with a proband's history of migraine. Therefore, comorbidity of epilepsy and migraine does not seem to be due to a shared genetic predisposition (Ottman and Lipton, 1996).

1.4.2 Migraine and Major Depression

Psychiatric disturbances have been observed in patients with migraine and described by clinicians for many years. However, these observations of association between migraine and psychiatric disorders in clinical samples may have suffered from potential bias. Several recent epidemiological studies have tested this proposed comorbidity between migraine and psychiatric disorders by randomly sampling the general population and utilising well constructed diagnostic criteria for both disorders.

Major depression is a type of affective psychiatric disorder that has been demonstrated to co-exist with migraine (Breslau et al, 1994). Depression can range from mild expression of symptoms of dysphoria to a severe and incapacitating disorder that may be life threatening in its most severe form. Depression, like

migraine, is a continuum of disorders and therefore requires specific definition upon diagnosis. A major depressive episode is defined in the DSM-III-R standards and is characterised by the presence of symptoms including dysphoria, loss of interest or pleasure, weight loss or weight gain, insomnia, fatigue and suicide ideation or attempts (American Psychiatric Association, 1987). After implementing careful diagnosis strategies of both depression (using DSM-III-R standards) and migraine (using IHS criteria), Breslau and colleagues examined the relationship of these disorders in an epidemiological study of a cohort of young adults from the USA and Switzerland. The results of this investigation strongly substantiated the previous clinical observations that migraine and major depression are comorbid disorders (Breslau et at, 1994). A follow up study also performed by Breslau et al, set out to examine the co-involvement of suicide attempts in the comorbidity of migraine and depression. These new results demonstrated that there was a consistent trend toward higher psychiatric comorbidity in migraine with aura than in migraine without aura. Interestingly, persons with migraine had a higher rate of suicide attempts than controls with an odds ratio, adjusted for coexisting depression, of 3.0 (95% CI, 1.4-6.6) for migraine with aura (Breslau et al, 1991).

1.5 Social and Economic Impact of Migraine

The significance of migraine as a public health problem is frequently overlooked. This is most likely due to the episodic nature of the disease as well as the lack of mortality. However, not only can migraines be extremely painful but they are also often incapacitating and therefore can have considerable impact on a sufferers social life and work occupation. Collectively this pain and incapacitation can impact heavily on society. The socio-economic burden includes costs associated with healthcare utilization as well as costs associated with lost productivity in the workplace due to reduced efficiency or absenteeism (Rasmussen, 1999).

Migraines can strike an individual at an early age and may persist through that person's valuable school years, most productive work years and well into retirement. A population study of school children conducted by Abu-Arefeh and Russell (1994) found that children suffering from migraine were absent from school about twice as frequently as normal control children (Abu-Arefeh, 1994). Thus from the early years

of life migraine, causing a significant reduction in school attendance, may impact on the education of, not only individuals, but also communities as a whole.

Migraine has been shown to be an enormous public health problem in Australia. An Australian Bureau of Statistics: National Health Survey was conducted in 1989/90 and reported a number of alarming statistics describing migraine in this country. In particular, it was revealed that over 7 million migraine attacks nationwide were experienced and on average 1.4 days were lost for each migraine attack. This equates to approximately 1.8 million days lost per year due to absenteeism and reduced productivity (Australian Bureau of Statistics, 1989/90). Results of the national health survey also gave estimates on the cost of the disease to the Australian community. Specifically, figures show that the cost of reduced productivity and absenteeism were estimated at ~$250 million, whilst the breakdown medical costs were ~$22 million for medical consultation; ~$10 million on migraine drug therapy; ~$2.7 million on hospital bed costs; ~$650000 on X-ray costs and about $16 million on other health profession services and time spent seeking and being treated. Overall, the findings of this Australian survey estimated the total cost of migraine to the country over the period of 1989/90 was greater than $300 million (Australian Bureau of Statistics, 1989/90). These figures can be considered conservative given the likelihood of a large number of undiagnosed individuals living in Australia. A recent evaluation of the burden of migraine in the United States found that migraineurs required about 4 bed rest days for men and 6 days for women, annually. This results in a total of ~112 million bedridden days for American migraineurs each year, an enormous impact even considering the 15 fold difference in population size. In addition, this study showed that migraine costs American employers about $13 billion a year because of reduced productivity and absenteeism, and annual direct medical costs for migraine care were about $1 billion (Hu et al, 1999).

Considerable benefits can be gained by strategies that set out to reduce the amount of lost productivity and absenteeism caused by migraine. It is important to realise that the greatest impact of all is on the sufferer's quality of life. As well as the pain and debilitation associated with a migraine attack, many patients live in fear of the next attack (Rasmussen et al, 1999). This can confine their lifestyles and may disrupt the ability of them, as well as friends and family, to meet social obligations. Clearly more

attention needs to be focused on understanding the cause of this disease so that better prevention and treatment strategies can be developed and implemented.

1.6 The Pathophysiology of Migraine

1.6.1 Factors that Trigger a Migraine

Knowledge of the factors that set off a migraine attack is a crucial step towards designing the best prevention strategies for individual patients as well as understanding the pathophysiology of the disease (Blau, 1992). It has been hypothesised that migraine is a threshold-based disease where this set-point is determined by genetic susceptibility. Exposure to specific factors may than breach the threshold in the predisposed individual, thus triggering a migraine attack (Hargreaves and Shepheard, 1999). The internal and environmental triggers that can induce migraine are many and varied. Some of the more common precipitating factors included hormonal fluctuations (in females), fatigue, relaxation after a stressful period, changes in weather, substance abuse and certain foods (Ferrari, 1998)

1.6.2 Current Concepts of Migraine Pathophysiology

Up until about 10 years ago, the physiological events that occur prior to and during a migraine were theorised to be vascular problems whereby the headache was specifically caused by blood vessel dilation. However, recent biochemical and pharmacological evidence does not completely support this idea, making the overall mechanisms of the disease still largely speculative. The two main hypotheses now existing are – the *vascular* theory and the *neurogenic* theory.

The notion of the vascular theory is that, whilst the head pain of migraine is caused by vasodilation of the external carotid artery by vasoactive polypeptides, the aura of migraine is caused by intracerebral vasocontriction (Wolff and Tunis, 1952). It was thought that these occurrences might explain the throbbing characteristic of the headache pain as well as the variable localisations of the pain. However, there are several shortcomings to this theory. Firstly, it does not attempt to explain the features of the prodrome phase of an attack. Secondly, the vascular theory does not account for the fact that some drugs used to treat migraine have no effect on blood vessels.

Finally, this theory is not supported by blood flow studies (Silberstein, 1992). The *neurogenic* hypothesis of migraine states that the disease is caused by significant brain dysfunction. Contrary to the vascular theory, the neurogenic hypothesis is supported by evidence from blood flow studies, which indicate a decrease in cerebral blood flow in migraine with aura and no blood flow alteration in migraine without aura (Olesen and Edvinsson, 1988).

In MA, the aura is characterised by visual and sometimes speech or motor disturbances and a phenomenon called cortical spreading depression (CSD) has been predominantly implicated as the cause of these neurological changes (Lauritzen, 1994). CSD is defined as a wave of depolarisation that propagates across the brain cortex at 2-3 mm/min and is associated with transient depression of spontaneous and evoked neuronal activity (Lauritzen, 1994). The depression wave lasts several minutes preceded by a front of neuronal excitation. During CSD there is dramatic failure of brain ion homeostasis and efflux of excitory amino acids from nerve cells (Silberstein, 1992).

Although the central processes involved in the initiation of a migraine attack are still not well understood, more is known about the pathophysiology of migraine head pain. The trigeminovascular system (comprised of the cerebral vasculature and associated sensory nerve fibres) has been extensively implicated in the pathophysiology of migraine head pain. The brain has a sparse sensory innervation and it is the capsule structures (meninges) that are the most significant pain producing intracranial tissues (Moskowitz, 1984). It is the ophthalmic division of the trigeminal nerve that innervates the meninges. These fibers provide a pathway for pain signal transmission from meningeal blood vessels into the brain where headache pain is registered (Goadsby, 1997; Moskowitz, 1994).

1.6.3 Serotonin (5-HT) and Migraine

The involvement of serotonin in migraine has been known for many years and is based on several important observations. Firstly, urinary excretion of 5-hydroxyindoleacetic acid, the main metabolite of serotonin, was observed to be increased in association with migraine attacks (Curran et al, 1965). Secondly, platelet

5-HT was found to drop rapidly during the onset of migrainous attack (Anthony et al, 1967). And thirdly, intravenous injection of 5-HT aborts either reserpine-induced or spontaneous headache (Kimball et al, 1960). These early findings offered the suggestion that serotonin levels decrease during a migraine attack and when replenished intravenously, attacks are relieved. Unfortunately, the use of 5-HT as a migraine abortive medicine was associated with significant adverse events such as nausea, faintness and paraesthesiae (Goadsby 2000). Nevertheless, this provided pharmacologists with a mechanism to target (serotonin receptor and transporter proteins) for development of more effective anti-migraine compounds (Ferrari, 1998).

1.6.4 The Role of Nitric Oxide in Migraine

Nitric oxide (NO) is a small messenger molecule that may play an important role in the initiation and sustenance of a migraine headache (Ferrari, 1998). It has been demonstrated that migraineurs injected intravenously with the NO donor nitroglycerine showed greater dilation of the middle cerebral artery and were more likely to experience migraine-like headache than controls (Thomsen and Olesen, 1998). Thus, it is hypothesised that NO released from either blood vessels, perivascular nerve endings, or brain tissue is a trigger molecule involved in initiating migraine pain. NO synthesis is carried out by a family of enzymes known as nitric oxide synthases (NOS) and the genes that code for these enzymes are therefore plausible candidates for studying the molecular genetics of migraine.

1.6.5 Dopamine and Migraine

Most scientific research studies into the biochemical changes that take place in migraineurs have to date focused on the serotonergic system. However, it has also been suggested that the monoamines including dopamine may play a role in disease pathophysiology (Peroutka, 1997). Initial evidence for this idea was published many years ago by Sicuteri (1977). He reported that severe attack-related symptoms such as nausea, vomiting and blood pressure changes are mediated by the dopaminergic system. Sicuteri also observed that migraine patients are hypersensitive to dopamine agonists and proposed that the headache and accompanying autonomic and gastrointestinal symptoms of migraine could be the result of hypersensitivity of monoaminergic receptors in certain regions of the brain (Sicuteri, 1977). It was also

acknowledged by Lance (1981) that since nausea usually precedes the headache of migraine, then alterations in brainstem dopaminergic neurotransmission must be part of the attack (Lance, 1981). A variety of dopamine receptor antagonists are effective in the acute treatment of migraine although the actions of these compounds have been shown to be indirectly involved in anti-migraine response (Peroutka, 1997a). However, the finding of Peroutka in 1997 that a DNA variant in the dopamine receptor gene (DRD2) is associated with migraine with aura has enhanced interest in the role of dopamine in migraine, particularly at the molecular genetic level (Peroutka et al, 1997b).

1.6.6 Progress into the Pathogenetics of Migraine

At present the underlying cause of migraine is not known. However, some valuable insight has been gained from the discovery that mutations in the calcium channel subunit gene (*CACNA1A*) cause Familial Hemiplegic Migraine (FHM) in ~50% of affected families (Ophoff et al, 1996). For FHM, the missense mutations in the *CACNA1A* gene predict amino acid changes which in turn alter the pore forming and voltage sensor regions of the subunit protein (Ophoff et al, 1996; Ducros et al, 1999). Research has suggested that changes in the calcium channel subunit cause variation in channel activity and ultimately result in brain dysfunction. In particular, Hans et al, have provided evidence that *CACNA1A* mutations alter calcium channel expression and kinetics that can result in gain and loss of function (Hans et al, 1999). The $\alpha_{1\text{-}A}$ subunit calcium channel belongs to the voltage-dependent P/Q type ion channel family. Interesting, P/Q type channels are involved in CSD and release of neurotransmittors such as serotonin (Codignola et al, 1993). Hence, the *CACNA1A* gene is a strong candidate for the more common forms of migraine. However, unlike the clear autosomal dominant mode of inheritance of FHM, the common forms of migraine (MO and MA) are genetically more complex and are also influenced by a substantial environmental component. Thus, despite the *CACNA1A* breakthrough with FHM, dissecting the molecular genetics of typical migraine may prove substantially more formidable.

1.7 Prophylactic Treatment of Migraine

Drugs available for the management of migraine, either; modify the release of transmitters involved in pain transmission, block sensitization of nerve fibers or affect inflammatory mechanisms involving the blood vessel wall. Prescription of individual treatment is largely governed by the level of pain intensity and disability, associated symptoms, the presence of comorbid illness and the patient's previous history of response to headache medication. A variety of acute and preventative migraine medications are available, which affect a broad range of pharmacological targets and thus produce a widely variable inter-patient response.

As an initial treatment for those patients who respond favourably, the non-specific nonsteroidal anti-inflammatory drugs (NSAIDS) or combination analgesics such as aspirin and acetominiphen and caffeine are generally recommended (Ophoff et al, 2001). Alternatively, migraine specific agents are available for acute management, such as the triptans or ergotamines, which are generally recommended for treatment of moderate to severe migraine (Ferrari, 1998). For preventative migraine treatment, beta-blockers (e.g. propranolol), Ca^{2+}-channel blockers (e.g. flunarizine), or tricyclic antidepressants (e.g. amitriptyline) are considered most effective. Finally, dopamine antagonist antiemetics, such as metoclopramide and prochlorperazine, are sometimes recommended for use as an adjunct therapy in the treatment of head pain and nausea associated with the disorder (Peroutka et al, 1997a).

CHAPTER 2
RESEARCH BACKGROUND

2.1 Heredity and Molecular Genetics

The science of genetics began in the mid 1800s with Gregor Mendel's studies of inheritance. The Austrian Monk's breakthrough experiments on the heritable characteristics of pea plants provided an important understanding of how "genes" (and related traits) are transmitted from parent to offspring. It was later discovered that genes reside on genetic "super structures" called chromosomes that are contained within the nucleus of cells. A normal diploid cell has 44 autosomes and 2 sex chromosomes. The autosomes are comprised of 22 homologous pairs (chromosomes 1-22) where each set is inherited from one parent. The sex chromosomal makeup is different for each of the genders with normal females possessing two copies of an X chromosome and normal males possessing and X and a Y chromosome. It is the segregation, independent assortment and crossing over of these chromosomes during the process of meiosis that leads to the genetic and therefore phenotypic variability seen between individuals (Marieb, 1998). In order to quantify and explain the genetic variation in populations and follow the inheritance of genetic traits in families an understanding of the genetic elements on the molecular level is required.

Molecular genetics is a relatively new field of biological science that really took off with the discovery by Watson and Crick in 1953 that the chemical structure of each of the 23 pairs of chromosomes in the nucleus of every somatic cell is composed of very long strands of deoxyribonucleic acid (DNA) (Watson and Crick, 1953). These strands, normally bound together lengthwise by hydrogen bonds, are twisted around each to form a characteristic double helix structure (Figure 2.01). Each strand of DNA is a very long molecule comprised of a linear sequence of subunits called nucleotides. These nucleotides have a sugar (deoxyribose), a nitrogeneous base, and a phosphate group. There are four different nitrogeneous bases called adenine (A), guanine (G), cytosine (C) and thymine (T) which are able to pair up as A-T and G-C base pairs to form the double stranded helix (Figure 2.01)(Snustad and Simmons,

2000). It is the sequence variability introduced by having four different bases that allows the DNA molecule to store information. The biological data held within the DNA is, in simple terms, the instructions that can be translated into many different amino acids and ultimately structural and functional proteins.

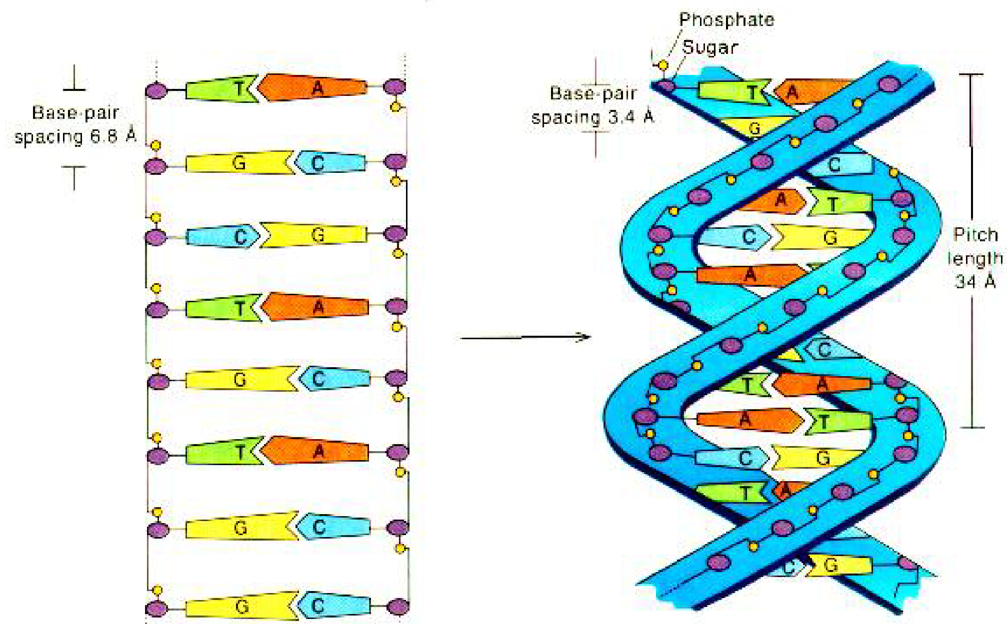


Figure 2.01. A diagram illustrating the helical structure and base pairing of the DNA molecule (from www.dnapics.com)

The human genome is comprised of approximately 3 billion base pairs of DNA. Only about 5% of the human genome contains coding DNA and some chromosomes have a higher density of these genes than others. However, it is now known that the vast amount of non-coding DNA (often referred to as "junk") contains important regulatory sequences and repetitive elements that provide useful markers as well as pseudogenes and other valuable intronic sequences (Snustad and Simmons, 2000). Massive worldwide efforts, by private and public consortia, have now developed a substantial working draft sequence of the human genome, and based on a physical map of markers have established the location of many of the estimated 30000-40000 protein coding genes contained within. This knowledge has provided researchers with the tools to map the genes involved in genetic disorders and to date many genes following a "simple" mode of inheritance, and subsequent disease causing mutations, have been identified. However, one of the most difficult problems now facing genetic

researchers is localising the genes contributing to diseases that have a “complex” pattern of inheritance, such as in the cases of diabetes, cancer, mental illness and migraine.

2.2 Understanding the Genetics of Complex Diseases

2.2.1 Identifying a Genetic Component in Complex Disease

An individual’s genetic makeup will, to some extent, determine their susceptibility to almost any disease. Whilst some diseases such as Tay-Sachs disease are entirely due to an individual’s genetics, common diseases at the other end of the spectrum are attributable both to the presence of predisposing gene variants and to the influence of environmental factors acting on those individuals who are genetically predisposed. The main approach that researchers utilise for assessing the influence of genetics on a particular disease involves studies on affected families. In particular, familial aggregation studies and twin studies are typically employed.

2.2.1.1 Familial Aggregation

A popular approach to identifying the influence of genes on a disease is to examine whether the disease prevalence in genetically related family members of affected individuals is greater than the prevalence of the disease in the general population. In these types of studies it is important that disease affected probands (also called index cases or propositi) are ascertained in an unbiased way and that appropriate controls are also recruited for comparison. Methods for obtaining information about disease rates include (1) questioning the proband directly, (2) questioning relatives directly, (3) verifying disease status by a review of medical records, and (4) examining (diagnosing) relatives directly (Russell and Olesen, 1995).

A widely used statistic that is employed to describe the excess risk of disease to an affected family member is the “relative risk” statistic. This is often written as λ_R where the R denotes the type of relation: for example λ_O and λ_S are risks to offspring and siblings, respectively (Lander and Schork, 1994). The relative risk is easily interpreted because it quantifies the amount of risk to family members compared to the general population. Simply, the relative risk statistic is determined by calculating

the ratio of the disease prevalence in relatives of affected probands to the prevalence in control individuals. Statistically, the relative risk can be calculated according to the following equation;

$$\textbf{Relative Risk} \quad \frac{\textbf{\textit{Prob}(Relative is affected|Proband is affected)}}{\textbf{\textit{Prob} (Random member of the population is affected)}}$$

A relative risk significantly greater than 1 indicates that the disease is more prevalent in the relatives of affected probands than in the general population (Beaglehole et al, 1993). For example, a relative risk of 3.5 means that relatives of affected individuals are 3.5 times more likely to be affected with the disease than the risk to the general population. Therefore, from this risk estimate the magnitude of the genetic contribution to the disease can be inferred. The lower the risk usually means the more genetically complex the disease (Lander and Schork, 1994). Familial aggregation studies, which examine the risk to relatives, are a good initial step for establishing genetic involvement in a disease, however they have one considerable limitation. Since families have environment in common as well as genetics, an elevated risk to relatives of affected individuals may in fact be due to common environmental conditions rather than shared genetics. To assist in elucidating whether genes or environmental factors are the source of the familial aggregation of a disease, twin and adoption studies that measure concordance rates and heritability can be performed.

2.2.1.2 Twin Studies and Heritability

An individual's phenotype for a particular trait may be influenced by several gene loci, and environmental factors that may modify gene expression. The extent to which variance among individuals are due to genetics and the extent due to environmental differences can be assessed by employing variance component (or partition) analysis (Snustad and Simmons, 2000).

Heritability is defined as the proportion of a population's phenotypic variation attributable to genetic factors. Heritability reflects the relative contribution of genes and environment to variation in a specific trait. A heritability value of 1 indicates that

all of the variation in the phenotype is due to genetic differences. That is, the trait is 100% genetically determined. If the heritibility is less than 1 than a portion of the total phenotypic variability is accounted for by environmental (or non-genetic) factors (Sham, 1998). Knowing the extent to which genes affect variation in disease is very useful when conducting disease gene mapping studies.

Studying twins is a classic and often very useful method for investigating the relative importance of genetic factors contributing to a disorder, or heritability. Many twin studies have been performed to date examining disease concordance rates in monozygotic (MZ) and dyzygotic (DZ) twin pairs. MZ (identical) twins share 100% of their genes whilst DZ (fraternal) twins share approximately 50% (as is the case with non-twin siblings). Thus, twin pair concordance rates can be used to estimate the heritability of a disease. If the concordance rate in MZ twins is 100% and ~50% in DZ twins than the disease is completely due to genetic factors. Furthermore, the larger the difference in concordance rates between MZ and DZ twins the larger the heritability (Snustad and Simmons, 1998).

2.2.2 Genetic Factors Contributing to Disease Complexity

The term “complex trait” typically refers to any phenotype that does not exhibit classic Mendelian (recessive or dominant) inheritance attributable to a single gene locus (Lander and Schork, 1994). There are many reasons why a disease can have a complex genetic aetiology. For instance the same genotype may give rise to different phenotypes due to the effects of chance, environment, or interactions with other genes. Alternatively, different genotypes (multiple genes) can result in the same phenotype (Terwilliger and Weiss, 2000). Figure 2.02 shows a simplified model of factors involved in predisposition of a "complex trait".

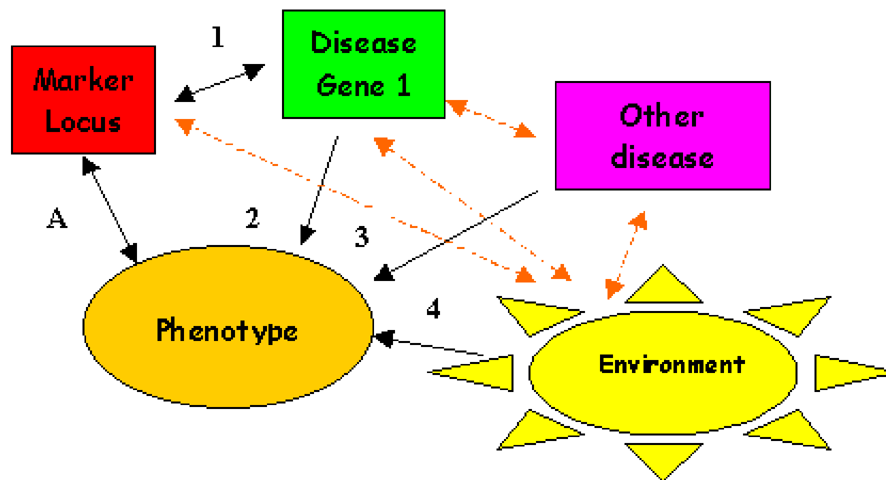


Figure 2.02. This is a simplified model of the factors predisposing individuals to a complex disease phenotype. Most current gene mapping strategies test indirectly whether there is a correlation between some marker locus and a disease phenotype (path A). From this we seek to infer a direct (causative) correlation of the disease gene and the phenotype (path 2) assuming a marker locus and disease gene correlation (path 1). Factors such as other disease genes and environmental contributions dramatically increase the complexity (paths 3 & 4), not to mention interactions between any or all of these factors (shown as red dotted lines)(Terwilliger and Weiss, 2000)

Some of the specific reasons why a disease may be classed as genetically complex include the following; A) incomplete penetrance ie: an individual has the disease susceptibility allele but does not become affected, or the onset of the disease may be extremely late, B) phenocopy ie: an individual does not have the susceptibility allele but still becomes affected with the disease, C) polygenic inheritance ie: the effects of several genes may add together or interact to predispose an individual to disease and D) heterogeneity ie: different alleles (in the same or different genes) can result in the same disease phenotype. Figures 2.03 illustrate the forms of allelic "geneity" that can exist in populations.

Model 1. Allelic Homogeneity

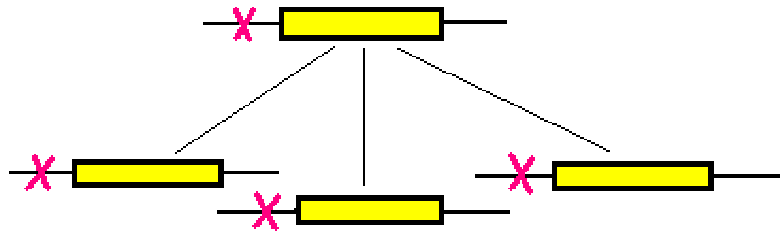


Figure 2.03a: In this model, all disease-predisposing alleles at a given locus are identical by descent in the population, having originated from the same ancestor. In this situation there is expected to be a conserved haplotype around the disease allele, which is shared by all carriers in the population many generations later.

Model 2. Allelic Heterogeneity

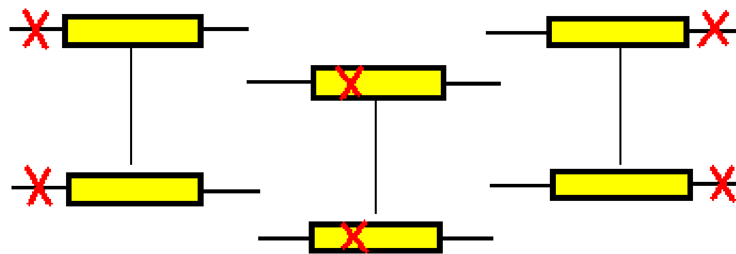


Figure 2.03b: The model shows allelic heterogeneity, in which multiple different allelic variants can each predispose to the phenotype. For this reason, there will be an assortment of haplotypes and therefore less allelic association.

Model 3. Locus Heterogeneity

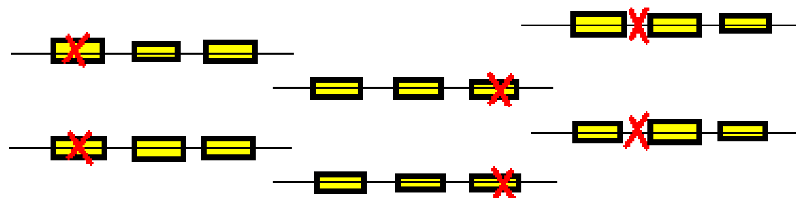


Figure 2.03c: This model shows locus heterogeneity, the situation where multiple alleles in multiple genes can predispose to the phenotype.

2.2.3 Developing a Disease Inheritance Model

Once it is established that genetic factors play a role in the aetiology of a disease, it can be helpful to attempt to determine the fashion with which the disease is being inherited. There are many analysis strategies for testing the mode of inheritance of a disease (Cantor and Rotter 1991). Each of these analytical methods depends upon the suspected genetic model of the disease. For example, segregation analyses are often useful for determining the mode of inheritance of single gene disorders. Common complex diseases are often multifactorial and transmission probabilities may be useful key parameters for analysis of this type of disease (Cantor and Rotter 1991). Notably, many researchers today are opting to avoid the thorny and often fruitless issue of complex disease inheritance by performing "model-free" genotype/phenotype correlation analyses.

2.3 Techniques for Mapping Complex Disease Genes

The modern molecular biologist is fortunate to have a extensive factory of "tools" for studying the genetics of complex disease. These "tools" include valuable information about the properties of DNA, architecture of genes and a realistic working map of the human genome. Furthermore, advanced laboratory techniques such as high throughput DNA extraction, PCR and fragment detection methods have made it possible to efficiently collect large amounts of genetic data from disease and control populations. Finally, there is a continual array of powerful statistical and computing solutions being made available for extracting meaningful information from this genetic data.

2.3.1 Variation in the Genome

With the exception of identical twins, every individual has a unique genetic makeup. Even though ~99.9% of our genomes are identical this still leaves over 3000 Mb of differences between individuals. It is precisely these differences that account for the heritable variation among individuals, including susceptibility to disease. In particular, it is the occurrence of changes in DNA called mutations as well as recombination that "shuffles up" the genetic material, that introduces diversity into a population (Sham, 1998).

There are several different types of mutations. The simplest example is the replacement of one base pair for another, called a nucleotide substitution. Other types of mutations include the deletion or insertion of one or more base pairs, DNA sequence repeats, or even the translocation of a segment of DNA from one chromosomal region to another. If a mutation actually changes the sequence of amino acids it is said to be a missense mutation, although the effect of a missense mutation will depend on the characteristic properties of the resulting protein product (Russell, 1992). Not all genetic variations are transcribed into proteins. Variants that accumulate within the non-coding DNA appear to be, in evolutionary terms, selectively neutral. However, these non-coding sequence differences (called DNA "morphisms") have turned out to very useful tools for flagging the position of genes in the genome (Gray et al, 2000).

2.3.1.1 DNA Markers

There are two main classes of DNA variants that have been used as "markers" for mapping genes involved in human disease. These are, Single Nucleotide Polymorphisms (SNPs, pronounced snips) and Short Tandem Repeat (STR) polymorphisms (or microsatellites).

In the 1980s Restriction Fragment Length Polymorphisms (RFLPs) were the most widely used types of DNA markers for molecular genetic studies. These single nucleotide dimorphisms are originally generated by mutation in sequences between individuals. Some of these mutations may, by chance, result in a change in an enzyme restriction site. These are sites that can be recognised and cleaved by restriction enzymes. RFLPs can be detected by specific probes in conjunction with the southern blotting technique and autoradiography (Snustad and Simmons, 2000). However, since RFLPs are biallelic there informativeness in family-based inheritance studies is limited and thus they are not very useful in initial searches for genomic susceptibility regions involved in complex disease.

Not until the discovery of short tandem repeats did family-based genetic analysis make a substantial advance. First came minisatellites that cluster at the distal ends of human chromosomes, then variable number tandem repeats (VNTRs) and most recently, in the early 1990s, microsatellites were found (Weissenbach et al, 1992).

Satellite DNA is so named because its high % of GC base content causes distinct "satellite" bands to be formed when it is centrifuged (Russell, 1992). Microsatellites are particular types of satellite DNA with sequences of two or three nucleotides repeated many times, for example $(AC)_n$, where n is a variable number usually between 10 and 100 units. These repeated sequences are abundant in the human genome with approximately 1 occurring every 1 million bases (~1cM)(Gray et al, 2000).

Microsatellites (or STRs) are generally uniformly distributed throughout the human genome (unlike minisatellites) and they can be readily detected by PCR amplification. Many are highly polymorphic in their number of base repeat units and are therefore very informative in linkage studies in families. Figure 2.04 illustrates microsatellite variablity. An extensive linkage map of the human genome has been compiled based on highly informative microsatellite loci. This map consists of over 2000 markers that are closely spaced (5-20cM) and cover the entire genome (Weissenbach J. et al, 1992).

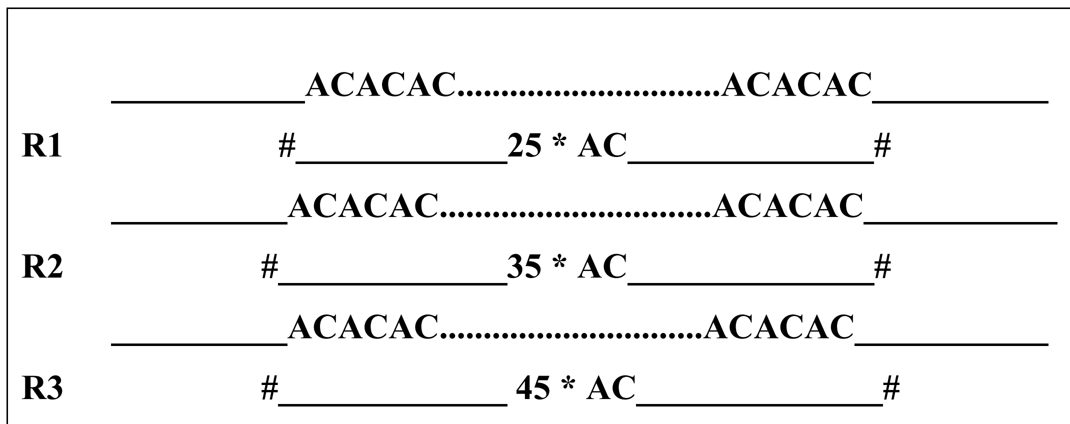


Figure 2.04. A schematic of microsatellite dinucleotide repeat variablity with R1, R2 and R3 representing three alleles of differing numbers of AC repeat units (25, 35 and 45, respectively).

Due to their being highly polymorphic, relatively common and evenly distributed in the human genome, STR markers have proven to be useful tools for performing family-based linkage analyses to search for genomic regions involved in complex diseases. However, the genomic regions identified using these markers often span genetically broad distances (due to a limited number of recombination events in

families) and thus only offer a coarse indication for the location of the disease susceptibility gene(s). In an attempt to narrow implicated regions and even to target specific susceptibility genes, researchers have begun testing for linkage disequilibrium (LD) in population-based case-control cohorts.

LD analysis measures the extent that alleles of tightly linked genetic markers are associated with each other (as haplotypes). Due to the breakdown of haplotypes over many generations LD in populations often extends over very short distances. This allows more indicative fine maps to be constructed, though many markers are often required. STRs are usually too sparsely separated to be used in unrelated case-control LD studies. Single nucleotide polymorphisms (SNPs) offer a useful alternative. Recently, a genome-wide map of SNPs was released into the public domain by an international SNP consortium (The International SNP Map Working Group, 2001). This map, comprised of ~1.5 million SNPs, revealed that SNPs are spaced, on average, one every 2 kilobases. Thus, even though relatively few SNPS can be detected by RFLP analysis, they are much more abundant than STRs in the human genome. Furthermore, SNPs are considered more stable than STRs (due to lower mutation rates). Also of importance is the fact that SNPs can often confer a direct functional effect on a disease phenotype, whereas STRs are nearly always indirectly associated with disease through an adjacent functional variant. With the development of new high throughput detection methods, SNPs are emerging as the marker of choice for studies of complex diseases particularly when genomic regions have already been identified using STRs in families (Gray et al, 2000). This research involved work using both microsatellites and SNPs in families and unrelated populations affected with migraine.

2.3.2 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a technique for selectively and repeatedly replicating defined sequences of DNA (Russell, 1992) and was an integral technology for this research. In PCR, the reaction mixture contains the DNA sequence to be amplified, a pair of oligonucleotide primers that flank the target segment, four separate building block nucleotides, a special DNA polymerase (*Taq*) which extends the primers, and various buffers to optimise the reaction conditions (Russell, 1992).

In this research, oligonucleotide primers (single stranded nucleotide sequences) that amplify specific microsatellite and SNP DNA marker fragments as well as exonic gene fragments were used for PCR.

The oligonucleotide primers are essential components of PCR. These segments of about 20 base pairs in length are important in the initiation of DNA synthesis and amplification. Because primers are actually produced from knowledge of the terminal sequences of the target segment they are highly specific strands of DNA (Nuovo GJ, 1992). Amplification is assisted by the enzyme DNA polymerase. Since the process of PCR involves a denaturation of double stranded DNA at high temperature, most enzymes would be destroyed if exposed to the high temperatures even for a brief period of time. To avoid this problem DNA polymerase was extracted from a thermophilic bacterium called *Thermus aquaticus* which lives in hot springs. This polymerase termed *Taq* polymerase can withstand the high temperatures required for denaturation (Nuovo GJ, 1992).

The PCR process begins with the DNA being denatured into two single strands by incubating the reaction solution at around 95°C. If the temperature were allowed to decrease to room temperature the single strands would renature preventing primer annealing. The temperature is therefore dropped to around 55°C. This temperature favours the primers that bind to specific sequences flanking the target DNA. The temperature is then raised slightly to around 70°C. At this temperature the *Taq* polymerase may locate the region where the primer has hybridized to the single target strand. In the presence of the dNTPs the *Taq* can extend the primers and rapidly synthesise new target DNA (Nuovo, 1992).

These three steps constitute one cycle of amplification. Consequently, from one initial double stranded segment of DNA (the target sequence), two identical segments are produced. If the cycles were to be repeated again then two more segments would result from each of the original segments. The geometric growth accomplished by PCR allows millions of copies of the original target segment to be produced in only a few hours . In fact 30 cycles (usually the maximum performed) would result in 1 billion copies of the original DNA sequence. Figure 2.05 illustrates the process of

PCR. This resulting quantity of PCR product is quite easily detected and observed using a variety of methods.

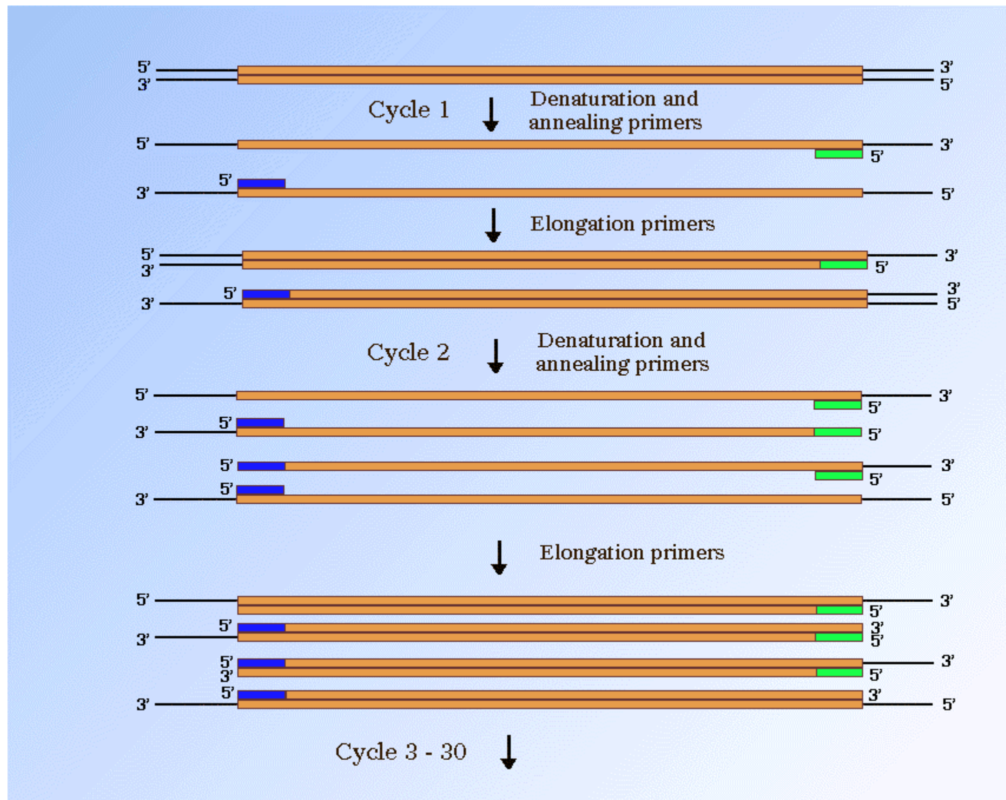


Figure 2.05. An illustration of the process of denaturation, elongation and amplification of a target DNA segment that comprises PCR.

2.3.3 PCR Fragment Separation and Detection

Once amplified by PCR, a DNA fragment of a certain size can be separated using a number of specific technologies. Underlying most of the current applications for PCR fragment separation is the concept of gel electrophoresis usually with gels composed of agarose or polyacryamide. The basis of electrophoresis lies in the fact that DNA molecules have a slightly negative charge. By placing the DNA fragments produced by PCR in a gel medium and then introducing an electric field, the DNA fragments will travel through the porous gel matix from negative to positive at varying speeds. Fragments will separate according to their shape and size, with smaller fragments moving faster and thus further in a given time (Figure 2.06). Denaturing gels can be used if extra sensitivity is desired, because single-stranded (ss) DNA folds in a shape that is determined by its nucleotide sequence, these different folded structures will result in increased separation sensitivity. The ssDNA will also travel faster than

double stranded DNA through the gel, therefore reducing the overall fractionation time (Nuovo, 1992). Following electrophoresis the PCR fragments can be visualised using UV light (Figure 2.06), X-ray radiation or fluorescent techniques depending on the type of DNA chemistry, gel composition. Since traditional slab gel electrophoresis techniques are labor intensive they are not suitable for analysis of large numbers of different PCR products.

Gene mapping studies of complex genetic diseases usually involve testing 100s-1000s of DNA samples. In addition, DNA markers that result in fragments (alleles) that differ by just 2 base pairs (ie. dinucleotide STRs) are difficult to unambiguously distinguish on regular slab gels. To overcome these limitations new automated fluorescence-based capillary electrophoresis instrumentation has been developed. This new technology was used extensively in this research.

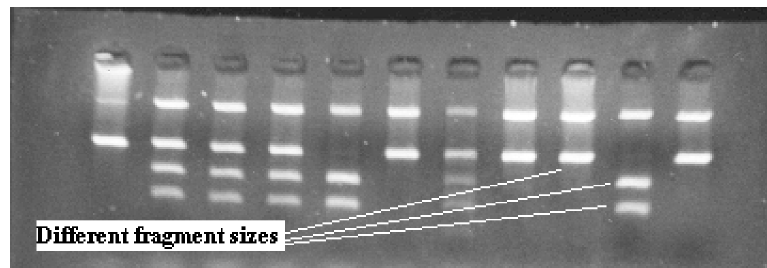


Figure 2.06. An example of a typical "slab" gel electrophoresis photo illustrating different sized fragments fractionated on an agarose gel. By diffusion law, smallest fragments (bottom) travel further in a given time.

2.3.3.1 Fluorescence and Automated DNA Fragment detection

One of the initial limitations of using microsatellite markers for linkage studies was the difficulty in sizing the PCR products that vary between individual DNA samples. These variations, representing alleles, may differ by only two nucleotides. Therefore, distinguishing heterozygote genotypes with alleles two base pairs apart from homozygotes with alleles of the same size was difficult (Ziegle et al, 1992).

In 1992, Ziegle *et al*. overcame this problem by applying fluorescent-based DNA sizing technology to genotyping microsatellite markers. This concept involves the use of fluorescent dyes that are attached to microsatellite specific primers. These primers are incorporated in the PCR to amplify target segments (microsatellite alleles) which carry the fluorescent label. A laser beam excites the fluorescently labelled PCR products as they pass through it during electrophoresis (Figure 2.07). The particular emission frequency of each dye is transferred to a computer. The computer software can determine the size of the fragments by reference to a fragment of known size (size standard) (Ziegle et al, 1992). The current research incorporated this technology by use of an *Applied Biosystems* 310 Genetic Analyser with GENESCAN® software. This instrument allows genotyping DNA markers using automated capillary electrophoresis.

Capillary electrophoresis (CE) is a family of related techniques that employ narrow capillaries (20 -200µm in diameter) to perform high efficiency separations of both large and small molecules (McLaughlin et al, 1991). These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary. CE offers several advantages over traditional slab gel electrophoresis. Firstly, it uses modern detector technology such that the electrophoretogram often resembles a chromatogram. Also, CE requires minute amounts of sample and limited quantities of reagents. Finally, this method is easily automated for precise quantitative analysis and ease of use (McLaughlin et al, 1991).

The basic instrumental configuration for CE is relatively simple. The system is composed of a fused-silica capillary with an optical viewing window, a controllable high voltage power supply, two electrode assemblies, two buffer reservoirs, and an ultraviolet (UV) detector. The operational procedure is the same as described above (Figure 2.08). Modern automated systems are able to quantitate fragments several hundred nucleotides in length even if they differ by only one nucleotide. Furthermore, because there are four different dyes available for labelling, several microsatellite alleles can be run in the same lane of a gel (ie. pooled) (McLaughlin, 1991). This form of multiplexing can reduce genotyping time considerably. After the

genotypes of all individuals in the affected population have been determined statistical testing of the data can be undertaken.

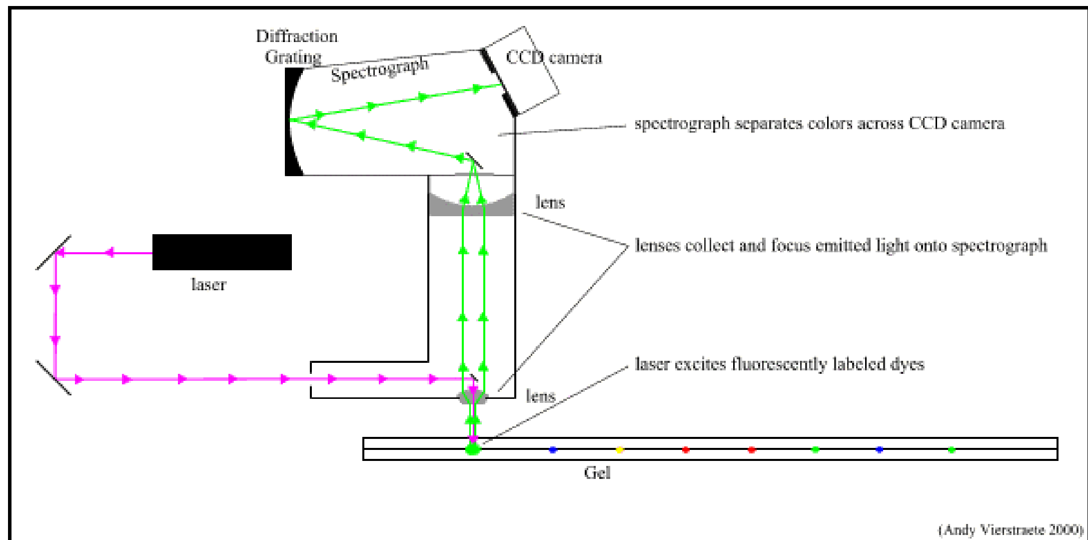


Figure 2.07. This is a schematic illustrating fluorescence excitability arising from lens condensation of a laser beam on the capillary gel containing labelled DNA fragments.

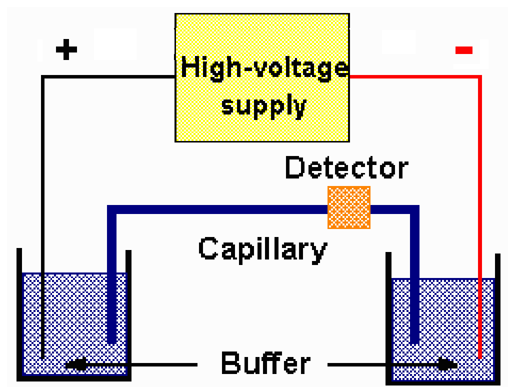


Figure 2.08..This diagram shows the buffers and charge differential that allows fragment electrophoresis through a capillary system

2.4 Study Designs and Data Analysis Strategies

Studies investigating the molecular genetics of complex disease have to date utilised two main research approaches. Firstly, inheritance studies involving *linkage* analysis of families affected with the disease have proven successful in the identification of many disease susceptibility regions. Unfortunately, the genomic regions implicated in these types of studies are usually genetically broad and therefore may harbour hundreds of genes, dozens of which may be considered candidates for disease involvement. Furthermore, many genes have not yet been identified, or characterised enough to be properly examined for disease involvement. This is despite the immense physical mapping efforts currently being undertaken worldwide.

A second popular strategy involves directly testing alterations in specific candidate genes for *association* to the disease or disease influencing allele. Basically, this is achieved by sampling the general population for affected cases and unaffected controls and than comparing the distribution of the particular gene variant between the two groups. A newer derivative of the standard unrelated case-control design is the family-based transmission disequilibrium test (TDT) that protects against false positive results due to population stratification. Collectively, these allelic association designs have so far yielded evidence for the existence of "risk genes" in many complex traits.

2.4.1 Linkage Analysis: The Traditional LOD Score Method

Traditional linkage (or LOD score) analysis is commonly used in gene mapping studies, particularly when the disease follows a simple Mendelian inheritance pattern. It proposes to detect correlations in the inheritance pattern of phenotypes and genotypes observed in a pedigree (Figure 2.09).

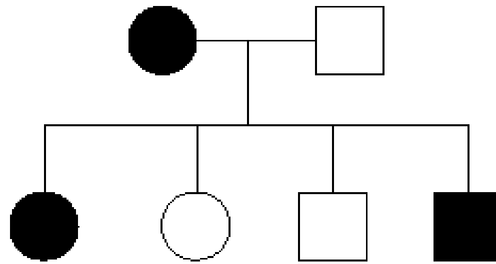
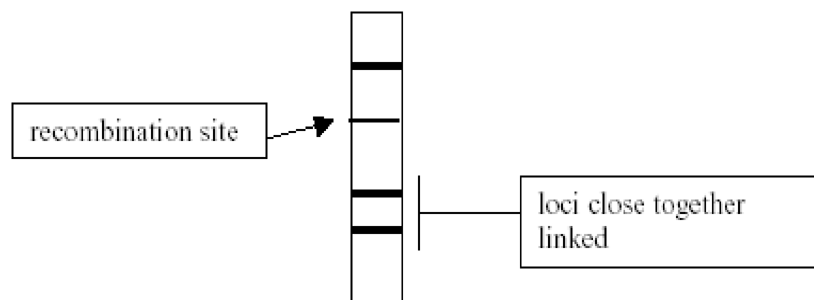


Figure 2.09. Linkage analysis involves determining a model of disease transmission to explain the inheritance of the disease in a pedigree.

Essentially, traditional linkage analysis compares the likelihood that a disease locus will be at a specified position relative to the marker(s), given a particular disease model (Lander and Schork, 1994). Disease models are typically defined by parameters such as mode of inheritance (eg. X-linked recessive), penetrance, and disease gene frequencies. The pivotal idea in this strategy is to limit the location of the disease gene to a small region of a single chromosome. The rate at which two alleles cosegregate is related to the distance between them (Ott, 1994). This rate is the probability, of a cross-over event (or recombination) occurring between the two loci, where a recombination fraction (Ø) of 0 equals 0% recombinants and 0.5 equals 50% recombinants (Figure 2.10).



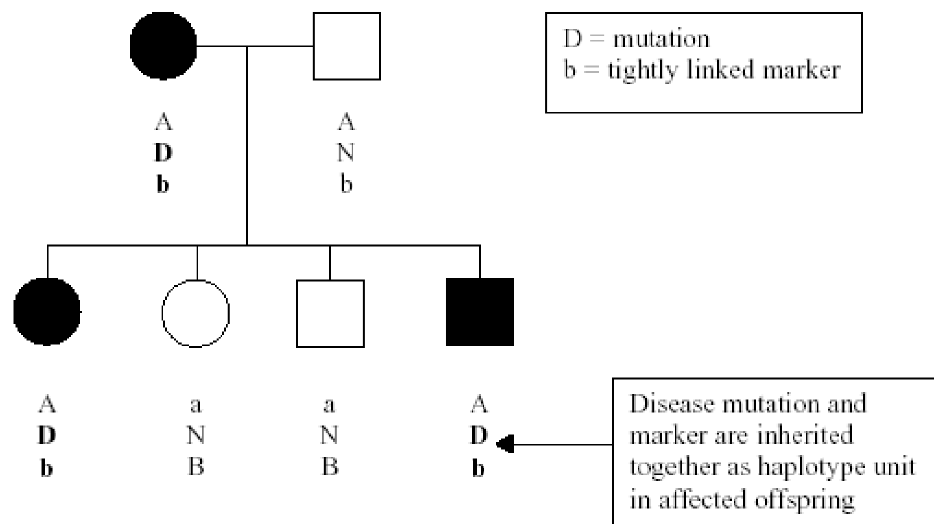


Figure 2.10. Recombination events allow only those loci that are closely linked to cosegregate in pedigrees. LOD score analysis allows likelihoods of linkage at set recombination fractions to be determined.

The LOD statistic is the logarithm-of-odds that is based on the likelihood ratio of the probabilities of two alternatives L_{Ha}/L_{Ho}. L_{Ho} is the likelihood under the null hypothesis of no linkage (ie. Ø equals 0.5 indicating that the loci are not linked and probably reside on different chromosomes, or are further apart than 50cM) and L_{Ha} is the likelihood of the alternate hypothesis of linkage (ie. Ø < 0.5 and the loci are linked). The LOD score equation is written as;

$$LOD(\theta) = \log_{10} \frac{LHa(\theta < 0.5)}{LHo(\theta = 0.5)} \text{ (Morton, 1955)}$$

The LOD score is calculated for several values of θ, for example θ = 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5. Where the LOD score reaches its maximum, the most likely value for θ is located. This method is called the Maximum Likelihood Estimation (MLE) method. Calculation of this likelihood ratio (or LOD score) can be extremely complicated in some cases and requires the use of computer analysis programs (Terwilliger and Ott, 1994). The FASTLINK and GENEHUNTER-PLUS programs were employed for the traditional linkage analysis conducted in this research.

Many recent studies have compared multiple markers (multipoint analysis) rather than a single marker (two point analysis) to a disease locus. This can increase the likelihood of detecting linkage to a locus underlying a disease trait especially if the disorder is genetically heterogeneous (different defective loci causing the disease). There are two main reasons why multipoint analysis is more powerful than single-point. Firstly, since all markers are homozygous in a proportion of the population, it is inevitable that some gametes in a large sample will be uninformative for linkage when only one marker is genotyped. Using several adjacent markers can reduce this loss of information. That is, it becomes unlikely for an individual not to have at least one heterozygous marker in the region. Secondly, unless the single marker is directly adjacent to the disease locus (ie $\theta = 0$), it can only provide information about cross-overs on one side of the disease locus. By testing two (or more) markers flanking the disease locus, recombination in both directions can be assessed (Ott, 1994; Sham, 1998)

Both singlepoint and multipoint linkage analyses are robust to genetic model misspecification. For instance, the power of the linkage test is not sensitive to incorrect gene allele frequencies and is only slightly affected by poor estimates of penetrance. However, misspecification of the degree of dominance (mode of transmission) of a disease can cause serious errors in results, the primary affect being artificial reporting of the recombination fraction *(∅)* (Commenges, 1994). For complex diseases such as migraine, asthma and schizophenia the mode of transmission (a test parameter) is often difficult to define. For this reason confirmatory model-free (non-parametric) tests can also be employed in complex disease studies.

2.4.2 Model-Free Allele Sharing Analysis in Affected Relatives

Model-based testing strategies can be very powerful when the "model" of the trait is known with some surety. However, when the disease model parameters are wrong classical linkage analysis is quite sensitive and prone to false positive outcomes (Nyholt, 2000). Furthermore, linkage analysis relies on assessment of recombination events between specific loci that are most frequent in large multigenerational pedigrees. Multiplex families that are informative for linkage at certain loci are often difficult to ascertain especially for late onset diseases such as hypertension. For these

reasons, alternative methods for mapping genes involved in complex diseases have be developed. One such alternative is to measure allele-sharing among affected relatives in families suffering from a disease. These allele-sharing studies have become very popular in the last ten years and can offer certain advantages over the traditional model-based linkage analysis method. By utilising allele-sharing gene mapping studies the power of measuring the segregation of alleles and disease phenotype through the generations is lost (Lander and Schork, 1995). Instead, if there is a disease causing mutation in a specific chromosomal region in a high proportion of families, it might be expected that affected relatives from the same family will share alleles at a marker locus more often than is expected by chance alone (excess allele-sharing) (Risch, 1990a). Large pedigrees are not necessary for studies involving allele-sharing and the analyses are independent of whether the disease is dominant, recessive, or more complex. Since some (but not all) of the assumed parameters about the underlying genetic model can be avoided by allele-sharing techniques they are usually referred to as "non-parametric" or "model-free" methods (Nyholt, 2000).

2.4.2.1 The Affected Sibling Pair (ASP) Method

Analysis of sibling pairs affected with a disease is one of the oldest methods of hunting for genetic linkage (Risch, 1997). The Affected-SibPair (ASP) test is the most common allele-sharing technique used by gene mapping researchers. At a particular locus, a pair of siblings can have 0, 1 or 2 parental alleles in common. That is, sib-pairs can share 0,1 or 2 alleles identically-by-descent (IBD). Figure 2.11 illustrates an allele (1) that is shared IBD between the two affected siblings in the pedigree. When there is no linkage between a certain locus and a disease, and under the assumption of random mating, siblings are expected to share 0, 1 and 2 alleles IBD in the ratio of 25%, 50% and 25%, respectively. Thus, the alternate hypothesis that linkage exists between a disease and a marker locus is easily tested by comparing observed IBD sharing distributions in a sample of ASPs to the expected 1:2:1 ratio (Sham, 1998). Detecting a deviation of the observed frequencies from expected is achieved by the standard χ^2 goodness-of-fit test with 2 degrees of freedom defined by;

$$\chi^2 = \sum_{i=0}^{2} \frac{(Observed - Expected)^2}{Expected}$$

A sib-pair family is said to be fully informative if the IBD sharing status can be unambiguously determined from the genotype data. Figure 2.12 illustrates such a pedigree where all four parental alleles can be distinguished.

Because some families will not always be heterozygous for different genotypes there will often be a loss of information due to the "fully informative" family restriction. To deal with this problem, likelihood methods have been introduced to provide an extrapolation to the problem of incomplete inheritance information (Risch, 1990b, 1990c).

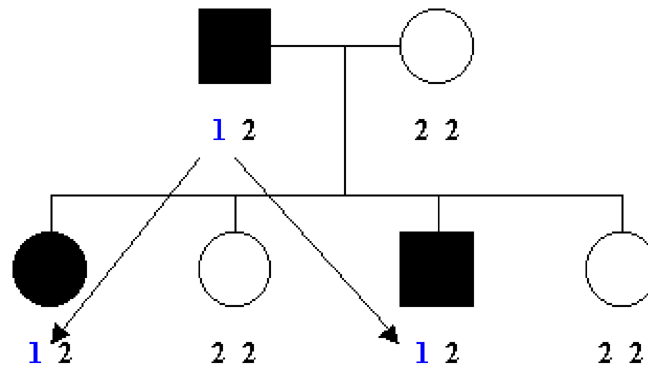


Figure 2.11. This pedigree diagram shows inheritance of allele 1 identical-by-descent in two affected siblings

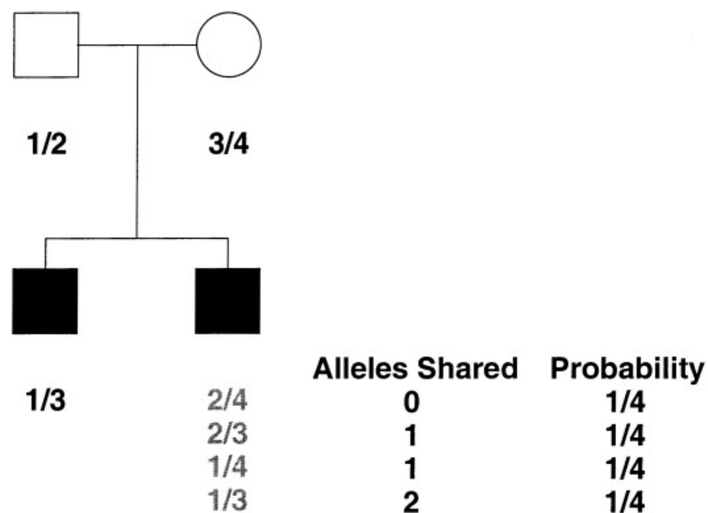


Figure 2.12. A situation where inheritance of all 4 parental alleles can be unambiguously distinguished and sharing probabilities determined (from Nyholt, 2000).

2.4.2.2 Affected Relative Pair (ARP) Methods

Complex disease studies involving pairs of affected *siblings* are one of the most popular study designs for assessing allele sharing at a marker locus. This method of analysis is particularly useful for middle-onset diseases (such as hypertension and prostate cancer) where it is difficult to ascertain large pedigrees with multiple generations of affected individuals. Migraine is a prevalent, early onset disease (median age of ~18 years) for which extended pedigrees are more readily available for molecular genetic studies. An ASP analysis could plausibly be applied to a sample of extended pedigrees by first decomposing these large pedigrees into nuclear families. However, this "pedigree breakdown" strategy would be inefficient because it is wasteful of a large amount of valuable inheritance information contained within the extended pedigree structure. For this reason a number of analytical/statistical methods have been developed to measure the extent of allele sharing among affected *relative pairs* (ARPs) from extended multiplex pedigrees (Curtis and Sham, 1994: Davis et al, 1996: Kruglyak et al, 1996).

The SimIBD Program

To correctly deal with the allele sharing in extended pedigrees, Weeks and Harby developed the affected-pedigree-member (APM) method. This method tests whether identical-by-state (IBS) sharing between ALL affected relative pairs is significantly increased compared to that expected under the null hypothesis (Weeks and Harby, 1995). However, the APM is very susceptible to misspecification of marker allele frequencies. Also, the APM assesses IBS allele sharing in affected family members and ignores valuable IBD data. To address these shortcomings Davis et al (1996) proposed a much more powerful extension of APM call the SimIBD program. This new method utilises conditional simulation techniques to determine an empirical *P*-value and is robust against allele frequency misspecification. Furthermore, the SimIBD program measures IBD sharing between all affected relatives and is more powerful for detecting linkage in general pedigrees of arbitrary size (Davis et al, 1996).

The SimIBD program begins by computing a "similarity" statistic using the genotype data from the pedigree. This sharing statistic is calculated by first examining two affected relatives (excluding parent-child pairs), and determining the likelihood that

the pair shares alleles either IBS or IBD. When the probability of IBS/IBD status is not certain between ARPs, approximate values are computed using a recursive algorithm. This algorithm works by tracking all IBD sharing information from the lower generations back up through the pedigree until a common founder is identified. The resulting likelihoods are than summed over all pairs of ARPs, and across all pedigrees, to obtain the overall similarity statistic. An empirical *P*-value, and thus significance, for this statistic is determined based on a simulated null distribution (Davis et al, 1996).

The GENEHUNTER Program

A major disadvantage of the SimIBD methods described above is the restriction to allele sharing measurements of a single marker locus. As is the case with traditional parametric linkage analysis, it is widely accepted that genotypes from multiple adjacent markers capture more (model-free allele-sharing) information then single marker data. However, since ARP analysis does not rely on information about recombination, but rather allele sharing, multipoint analysis may only be beneficial when the markers are closely spaced (≤ 10cM). This than becomes an analysis of excess *haplotype* sharing. In 1996, Kruglyak et al released a new (and now well-known) computer program called GENEHUNTER (GH). As well as computing traditional parametic LOD scores, the methods implemented in GH combine to simultaneously perform allele-sharing analysis of multipoint data in extended pedigrees. The allele-sharing statistics and corresponding *P*-values ultimately produced by GH are based on the counting of inheritance vectors and calculation of the inheritance vector distribution (Kruglyak et al, 1996).

The Inheritance Vector

If a nuclear pedigree with two siblings has full inheritence information for a particular marker. That is, the alleles of the four grandparents can be unambiguously distinguished; a set of all possible genotype configurations can be constructed. Once this is established the grand parental alleles can be given a binary code. Those alleles originating from either grandfather (grandpaternal alleles) are coded "0" whilst alleles from either grandmother (grandmaternal alleles) are coded "1". Conversion of the possible genotypes into the descendent-specific binary codes will result in a four "bit" inheritence vector (Figure 2.13). Data in this form is amenable to computer programs.

An inheritence vector of "0000" means that both siblings have inherited the grandpaternal allele from both parents whilst "1111" indicates that both siblings have inherited the grandmaternal allele from both parents. Alteratively, an inheritence vector of "0011" indicates that one sibling has inherited one grandpaternal alleles from both parents and the other sibling has inherited one grandmaternal allele from both parents. Thus, the inheritance vector completely specifies which of the distinct grandparental (founder) alleles are inherited by each non-founder (Figure 2.13). Through calculation of the inheritance-vector distribution conditioned on observed genotype data, the probability distribution is concentrated on certain inheritance vectors. That is, the likely IBD inheritance patterns are determined (Lander and Green, 1987; Nyholt, 2000)

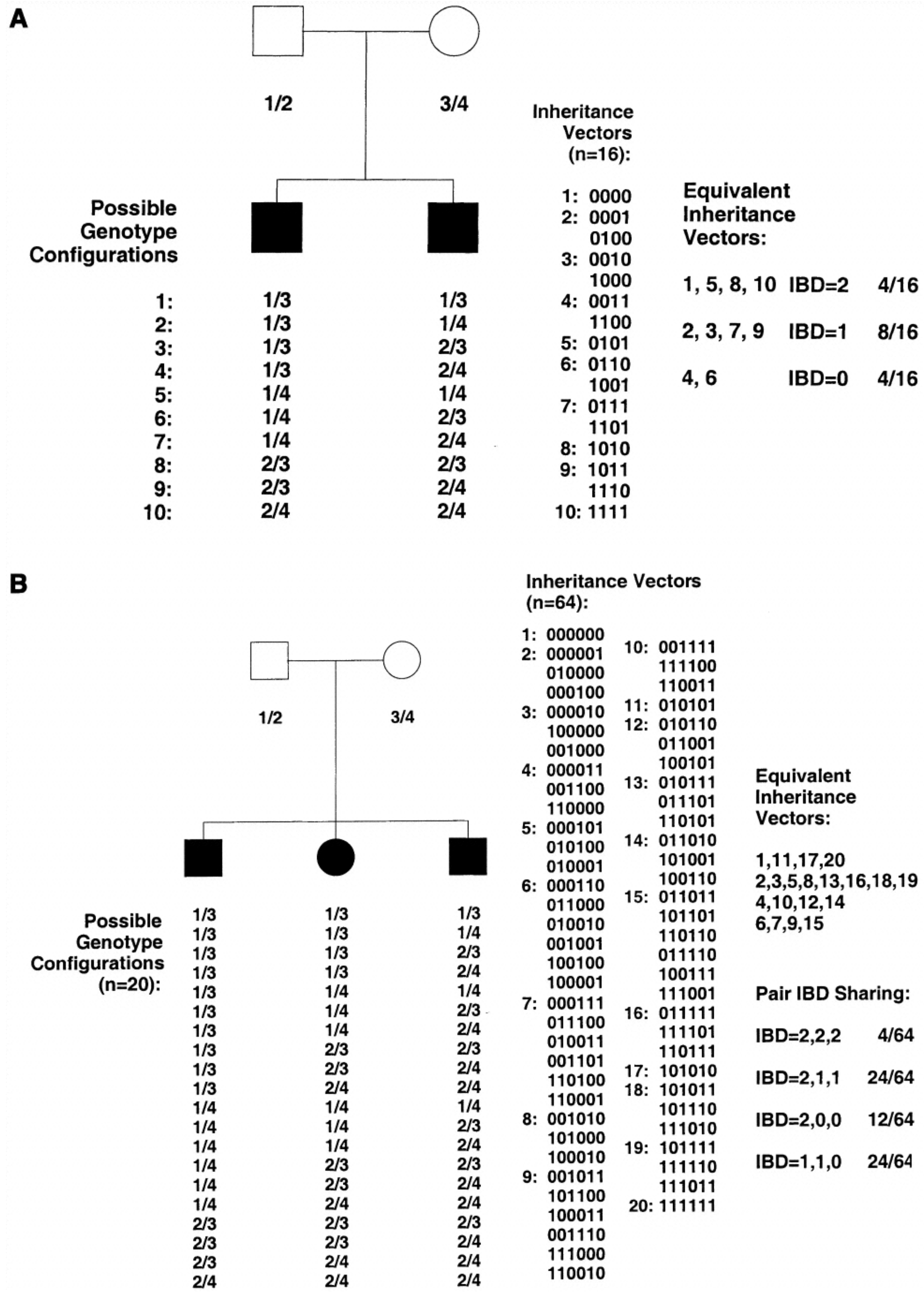


Figure 2.13. Determination of inheritance vectors from possible genotype configurations (from Nyholt, 2000).

The GENEHUNTER Linkage (NPL) Statistic

Following determination of the inheritance vectors, GH sets about calculating the Non-Parametric Linkage (NPL) statistic based on one, of two possible scoring functions. These scoring functions (S_{pairs} & S_{all}) allow the allele-sharing among affecteds in the pedigree(s) to be compared to random segregation expectations (Whittemore and Halpern 1994). The S_{pairs} statistic is simply the number of pairs of alleles shared IBD among distinct affected pedigree members, given a particular inheritance vector. However, it would be much more beneficial to detect sharing of a specific allele IBD among more than just a pair of affected individuals within a pedigree (ie. ≥ 3 affecteds). The S_{all} statistic and resulting NPL score are designed to harness the allele sharing associated with a given inheritance vector determined from more distant affected relatives. This statistic applies extra weight to families where three or more affected individuals are sharing the same allele IBD. For either the S_{pairs} or S_{all} approach GH than calculates a standardised Z score as;

$$Z(v) = \frac{[S(v) - \mu]}{\sigma}$$

where S(v) is the score statistic for a particular inheritance vector. Under the null hypothesis, and when many pedigrees of similar size and structure are analysed, the Z score is normally distributed with a mean of 0 and variance of 1 (Kruglyak et al, 1996). These scores can than be added across pedigrees to give a Z_{total} (or NPL score) as;

$$Z = \sum_{i=1}^{m} \gamma_i Z_i$$

where m is the no. of pedigrees, Z_i denotes the normalised score for the ith pedigree (as above) and γ_i are weighting factors which usually equal 1 (Kruglyak et al, 1996).

When many families of the same size and structure are analysed such as for a large ASP study the significance of the NPL is determined from the standard normal distribution. However, when there are only a small number of families, or the

families differ in size and structure GH calculates an exact probability distribution of the global NPL score. It does this by enumerating (counting) all possible inheritance vectors for each pedigree. In the common case where there is missing genotype data in the pedigrees, GH uses the so-called perfect-data approximation method to determine significance. However, the *P*-values derived for NPL scores under this method are known to be overly conservative with the degree of conservativeness dependent on the amount of missing data, marker heterozygosity and spacing (Kruglyak et al, 1996; Nyholt, 2000). The issue of conservativeness of the NPL statistic was overcome by Kong and Cox (1997) who developed a new one parameter likelihood based allele sharing model. This new approach is implemented in the computer programs GENEHUNTER-PLUS (GHP) and ALLEGRO (Kong and Cox, 1997; Gudbjartsson et al, 2000). A model-free LOD score (LOD*) is produced when a single parameter alternative model is introduced. The free parameter (δ) know represents the degree of allele sharing where $\delta = 0$ indicating the null hypothesis and $\delta > 0$ is the alternative. This ultimately renders the LOD* score asymptotically distributed as;

$$LOD^*(x) = \frac{Z_{lr}^2(x)}{2\ln(10)}$$

where Z_{lr} (x) denotes the likelihood ratio allele sharing statistic Z_{lr} at a particular marker position (x). This new model-free allele sharing LOD* is less conservative than the original NPL score of Kruglyak et al, and has the attractive property of being interpreted in the same manner as the traditional model-based LOD score (Kong and Cox, 1997).

Weighting Factors

The GHP and ALLEGRO programs allow the user to specify several different pedigree weighting schemes. Firstly, the weighting factor implemented in GH can be used to assign an equal weight to all pedigrees in the analysis. Secondly, weights can be set to coincide proportionally with the standard deviation of the particular scoring function specified. This option is most meaningful when the S_{pairs} scoring function is specified. For this type of analysis choosing standard deviation (s) > 0 corresponds to putting more weight on larger families (with more affecteds), whilst seting s = 1

means that all affected pairs are weighted equally. As a compromise, it has been proposed that the standard deviation should be set to 0.5 and this is the default option for ALLEGRO (McPeek, 1997). The final option provides the user with the flexibility to give families individual weightings (Gudbjartsson et al, 2000).

Inheritance Information

In pedigree analysis it is desirable to determine the total inheritance information that has been extracted at each marker locus being tested. Information content provides a measure of how closely a study approaches the goal of completely determining the inheritance outcome and it indicates regions that may require additional marker typing. Inheritance information content in ALLEGRO is assessed using the entropy-based methods applied in the GH program. Unlike traditional linkage information methods which rely on the disease and marker phenotype information, entropy-based content mapping is concerned only with marker information (Kruglyak et al, 1996).

Recombination Counting and Haplotype Reconstruction

The Hidden Markov Models (HMM) approach used by GH can also be applied to the estimation of the number of recombination events between two markers. By computing this GH allows the user to detect possible genotyping errors that may cause artificial inflation of the linkage statistics.

Haplotyping is an important ingredient in localising disease genes in linkage analysis. ALLEGRO reconstructs haplotypes approximately by utilising one inheritence vector path (which are set as inheritance vectors) at each locus. The haplotype reconstruction is obtained by first calculating the most likely inheritence vector path, using the Viterbi algorithm (Viterbi 1967). This algorithm is an application of dynamic programming to hidden Markov models. Having obtained this, the most likely alleles are assigned for each vector in the path by using the corresponding founder graphs. Thus, producing the desired haplotype. The haplotype produced is the same as that given by GH, but the algorithm in ALLEGRO takes advantage of both the founder reduction and founder couple reduction, whereas GH works with full inheritance vectors (Kruglyak et al, 1996; Gudbjartsson et al, 2000).

2.4.2.3 Statistical Significance in Pedigree Analysis

Traditionally, in human genetic linkage studies a LOD score of 3 has been considered statistically significant and equivalent to a probability of 0.001. A LOD score of 3 indicates that the observed data is 1000 times more likely to occur under the model-specific hypothesis of linkage than under the null hypothesis of no linkage. However, calculations performed by Lander and Kruglyak (1995) have shown that, for LOD score analysis in human pedigrees, in which the total meiotic crossover rate between genotypes compared is set to one, an exact genome-wide significance threshold of $P = 0.05$ is achieved at a point-wise P value of $4.9 * 10^{-5}$ (Table 2.01). This is equivalent to a LOD score of ~3.3 (Kruglyak and Lander, 1995; Nyholt, 2000). It has also been proposed that proclamation of *suggestive* linkage occur only after a point-wise LOD score of 1.86 ($P = 1.7 * 10-3$) has been achieved. Furthemore, *nominal* evidence for linkage of potentially interesting regions is obtained at the convential P-value of 0.05 (LOD = 0.59) (Kruglyak and Lander, 1995). Table 2.01 lists appropriate significance thresholds for common study designs and statistical tests using human samples.

Table 2.01. Required LOD-Score Values for Common Significance Thresholds (from Nyholt, 2000)

Significance Threshold	LOD/MLS	MLS_{PT}	X-MLS_{PT}	MMLS/het
$P < .05$	.59	.74	1.18	1.09
$P < .01$	1.18	1.38	1.90	1.71
$P < .005$	1.44	1.66	2.21	1.99
$P < .001$	2.07	2.32	2.93	2.63
$P < 7.4 \times 10^{-4}$	2.19	2.45	3.06	2.75
$P < 2.2 \times 10^{-5}$[a]	3.63	3.93	4.62	4.20
$P < 3 \times 10^{-7}$[b]	5.3	5.76	6.52	5.99

[a]Significant linkage threshold when allele-sharing methods in human sib pairs are used (Lander and Kruglyak 1995).
[b]Highly significant linkage threshold when allele-sharing methods in human sib pairs are used (Lander and Kruglyak 1995).

2.4.3 The Candidate Gene Approach

The candidate gene approach is a direct (and therefore powerful) method for studying the molecular genetics of complex disease. A gene may be a candidate either because it is lying in a genomic region detected in a systematic screening after linkage/allele sharing analysis or because it is functionally related to the disease and thus may represent a risk factor. For example, genes of the HLA complex are currently candidates for diseases with an immune component and apolipoprotein genes are candidates for cardiovascular diseases. The issue underlying the candidate gene strategy is that firstly, information concerning the role of a given candidate gene in disease must be obtained and secondly, the risk that an individual will develop the disease according to the available information must be estimated (Clerget- Darpoux 1998).

Once a particular candidate gene is identified for investigation, it is most desirable to test an intragenic coding variant that specifically changes an amino acid sequence and preferably is associated with some altered biological function. Such an ideal candidate gene test variant is not always available. Thus, anonymous DNA markers such as SNPs, that are presumably located adjacent to the hypothesised trait-influencing mutation, can by used to indirectly examine the gene for involvement in the disease. This strategy relies strongly on the presence of linkage disequilibrium between marker alleles.

2.4.4 Allelic Association (linkage Disequilibrium)

Linkage disequilibrium (LD) is a form of allelic association that occurs in a population when an allele at one marker (or mutation) is associated with an allele at an adjacent (linked) marker. As an example, suppose that a mutation is introduced into a population at a certain point in time. Then, the mutant allele and the alleles of the adjacent markers (at specific θ) form a haplotype and are in complete LD. In other words, individuals who carry the mutant allele also possess the alleles of the extended haplotype. Importantly, the mutation may introduce a new disease into the population. If the mutation is relatively new than the haplotype containing the mutation will span a genetically broad distance, but after many generations representing many recombination events, the size of the haplotype (or disease

susceptibility DNA segment) will be reduced (Sham 1998). In other words, LD at a certain recombination fraction will decay over time (generations) (Figure 2.14). This means that only alleles of markers in close proximity to the mutation will be in LD for a long period of time. It is this phenomenon that assists population geneticists in localising disease susceptibility genes and more specifically pinpointing the disease causing mutations. If LD (or allelic association) is detected in a population of disease affected individuals than it can be inferred that a mutation directly involved in the disease is located nearby (Ott, 1994; Sham 1998).

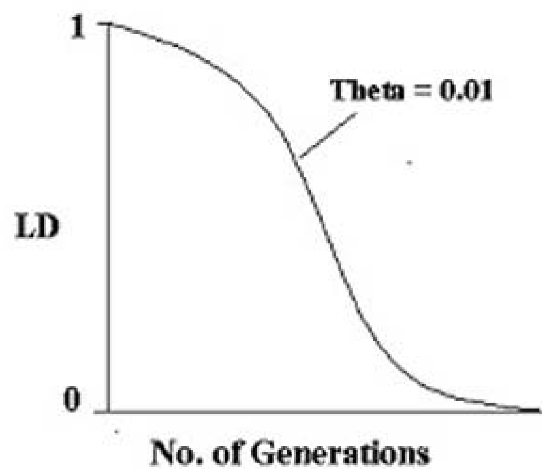


Figure 2.14. This diagram illustrates the LD decay trend of over time (many generations)

The magnitude of the LD that may exist between alleles at two adjacent markers is measured most commonly by the D' coefficient (Lewontin, 1971). A D' coefficent of 1 indicates complete LD, or absolute correlation between alleles at each marker. Coversely, a D' of 0 indicates linkage equilibrium and therefore the adjacent alleles are completely independent of one another. Significance of the D' statistic is estimated using the well known χ^2 statistic (Sham, 1998). Linkage disequilibrium analysis is usually assessed using either of two association study designs; the traditional population-based case-control test for association and the more recent family-based transmission disequilibrium test design (Terwilliger, 1995).

2.4.4.1 Population-Based (Unrelated Case-Control) Association Studies

Population-based association studies are a very popular design for disease gene mapping research. This is largely because they do not rely on familial inheritance patterns but rather comparison of case-control groups that preferably differ only by the disease state. These test groups are comprised of a sample of unrelated (and therefore independent) individuals affected with the disease and a comparative sample of unrelated unaffected controls (Figure 2.15).

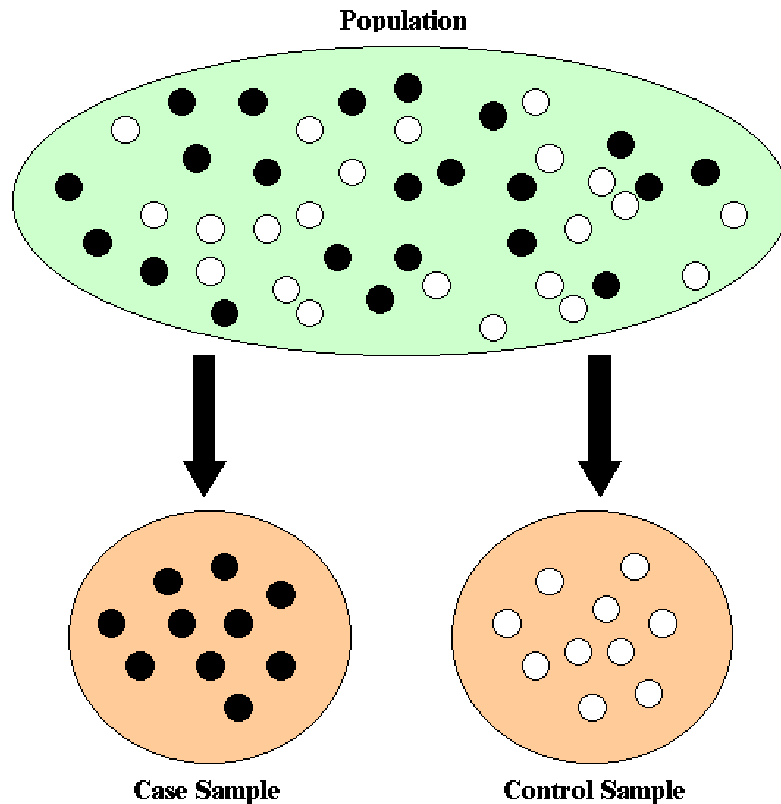


Figure 2.15. Association studies compare allelic and genotypic frequencies between a case and control group sampled from the general population.

An allele (or genotype) is said to be associated with the disease if the frequency is significantly altered among the affected group compared to controls. The magnitude of deviation between frequency distributions is measure using standard contingency table (Table 2.02) analysis and the chi-squared statistic;

Table 2.02. Example of a Contingency Table

	Case	Control	Total
A	80 (0.67)	20 (0.29)	100
a	40 (0.33)	50 (0.71)	90
Total	120	70	190

$$\chi^2 = \sum_{i=0}^{2} \frac{(Observed - Expected)^2}{Expected}$$

Preferably, the case and control groups used in association studies should be matched for variables such as age, sex and ethnicity. Failure to do so can lead to false positive associations between a genetic marker and disease. In fact, even careful matching of groups does not always safeguard against admixture, heterogeneity and stratification (Spielman and Ewens, 1996). This possible underlying population sub-structure is a major draw back for this type of study design. To overcome this problem the family-based transmission disequilibrium test was proposed by Spielman et al, 1993.

2.4.4.2 Family-Based (TDT) Association Studies

The Transmission Disequilibrium Test (TDT) has become a widely used study design for investigating candidate gene allelic association (LD) in complex diseases. Unlike the unrelated case-control design, the TDT is a valid test of allelic association when data is available from parents and one or more affected offspring (Spielman et al, 1993). The TDT is similar to unrelated case-control association analysis in that is involves simple allele counting and subsequent comparison by chi-squared statistical analysis. However, the control alleles in TDT analysis are those that are not transmitted from the parent to the affected child. To distinguish allelic transmission the source parent needs to be heterozygous at the test marker (Figure 2.16). An allelic association is said to exist when there is a significant distortion of alleles transmitted versus alleles untransmitted.

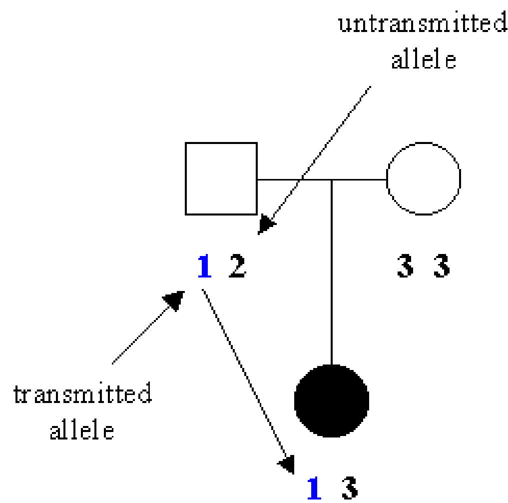


Figure 2.16. A parent-child triad illustrating alleles transmitted and untransmitted from a heterozygous.

The classic transmission/disequilibrium test (TDT), was designed for a specific pedigree structure (one genotyped proband and two genotyped parents per pedigree). However, the migraine pedigrees studied in this research had variable structure, often extending over several generations. Therefore, a family-based association test (FBAT) was employed. This test utilizes data from nuclear families, sibships, or a combination of the two to test for linkage and linkage disequilibrium between disease and genotypes. The test for linkage is valid when multiple affected members per pedigree are used, and the power to detect linkage is increased if there is an existing association. The test for association is valid if at least one affected member per pedigree is used (the genotypes of all the affected members can be included) or if the empirical variance is used to account for correlation between transmissions in families when linkage is present (Laird, 2000). The FBAT test allows the user to test two null hypotheses; 1. H_o = no linkage and no association, H_a: both linkage and association 2. H_o = no association, H_a: association in the presence (or independent) of linkage. It is assumed that there are N nuclear families, and n offspring in each family. The *S* statistic is the additive combination of offspring phenotypes and alleles at a specific marker.

$$S = \sum_{ij} T_{ij} X_{ij}$$

where; $\sum_{ij}$ = summing over all pedigrees and all individuals
i = pedigree
j = individual
T_{ij} = a phenotype function of each individual in each pedigree
1 = affected, 0 = unaffected or unknown
nb. only affected offspring contribute to S
X_{ij} = a variable which counts a certain allele for each offspring
nb. the specific value of X_{ij} depends on the genetic model under consideration for multiple alleles, X_{ij} is a vector quantity

Therefore: *S* is the total number of a certain allele among affected offspring for a particular marker (same as TDT statistic)

The distribution of the *S* statistic is generated by treating the offspring genotype data as random and conditioning on the phenotypes of the parental genotypes. When the marker is multiallelic, a χ^2 test is performed, with the number of df equal to the number of alleles. A significant default test statistic produced by the FBAT program is evidence for the alternative hypothesis of both linkage and association. To elucidate whether the significance is arising from either the linkage or association an additional analysis can be performed. By computing the empirical variance of the allele count variable (X_{ij}) , the correlation between transmissions in families when linkage is present can be accounted for (Laird et al, 2000).

2.4.5 Hardy-Weinberg Equilibrium

In 1908, Hardy and Weinberg independently published papers describing the mathematical relationship between genotypes and alleles representing a particular genetic trait. This relationship is called the Hardy-Weinberg Equilibrium (HWE) Law and allows prediction of genotypes from information about the alleles (Hardy, 1908). In this research the HWE law was employed as a statistical control of systematic genotyping errors for all markers. For biallelic markers, the HWE equation is taken directly from the well-known binomial expansion table (Table 2.03), where p and q denote alleles 1 and 2, respectively. Since alleles 1 and 2 make up the total sample space there frequencies (ie. the frequencies of p and q) will sum to 1. Indeed, it is these frequency values that are used to predict the marker genotypes expected under HWE. Once the expected genotype frequency distribution is established a chi-

squared goodness-of-fit test can be applied to determine how well the observed genotype distribution fits the expected (Hardy 1908). A *P*-value of < 0.05 indicates a significant deviation of the observed distribution from the expected and that Hardy-Weinberg DISequilibrium exists. As can be seen from Table 2.03, the expansion (HWE) equations become more complex as the *nomial* order (or the number of alleles) increases. For example for a triallelic marker the HWE calculation includes p, q and r for alleles 1, 2 and 3, respectively. The corresponding genotype combinations are represented by the terms p^2 = 1 1, q^2 = 2 2, r^2 = 3 3, 2pq = 1 2, 2qr = 1 3 and 2pr = 2 3. So the extent of the HWE equation for a triplet repeat marker that has ~22 alleles is vast. Hence, to perform HWE for multiallelic markers a computer program called HWE is used (Guo and Thompson, 1992).

Table 2.03 . Expansion equations for HWE

alleles	Equations
2	$p^2 + 2pq + q^2$ (*HWE equation for 2 alleles*)
3	$p^2 + q^2 + r^2 + 2pq + 2qr + 2pr$ (*HWE equation for 3 alleles*)

2.5 Summary of Research Methodology

The mapping of genes conferring susceptibility to complex traits is an intractable task, particularly when the genetic component of the disease is low and the number of risk genes comprising the genetic component is high. This is often the case with common diseases especially those of a neurological nature such as migraine. Therefore, to increase the likelihood of localising and identifying disease susceptibility genes several main points should be considered. Firstly, thorough and reliable relative risk and heritability estimates should be obtained from large epidemiology studies before any molecular analysis is performed. Also, it is important to attempt to reduce the clinical heterogeneity in the study samples by careful diagnosis and subsequent phenotyping of more definitive sub-classes of the disease. Furthermore, genetic heterogeneity can be partially reduced by utilsing families where possible. In addition, it is important to be selective with the candidate markers chosen for testing. Those conferring a functional effect are ideal. And finally, researchers should adopt multi-pronged study designs which incorporate the complementary strategies of linkage and association analysis, as well as emerging techniques such as microarray analysis.

CHAPTER 3
MIGRAINE GENETICS BACKGROUND

3.1 Genetic Epidemiology of Migraine

3.1.1 Positive Family History

A family history of a disease is usually the first clue that there may be an inherited genetic component involved in the aetiology of a disorder. Transmission of migraine from parents to children was reported as early as the seventeenth century (Willis, 1682). Since then, numerous studies have reported a positive family history of migraine. The positive family history varies between 37% and 91% in probands with migraine (Friedman et al, 1954; Vahlquist, 1955, Rasmussen and Olesen, 1992) and between 5% and 26% in probands who had never had migraine (Childs and Sweetnam, 1961; Vilatela, 1992). The large difference between family history percentages of migrainous probands and non-migrainous probands may be real. However, it may also be explained by differences in migraine assessment of relatives by the proband. The relatives that were included in the family history reports changed from first degree, first and second degree to unspecified. It is also possible that the high prevalence of migraine in the general population (~12%) may be responsible for the observed positive family histories of the disease simply by chance (Russell and Olesen, 1993). Therefore, a positive family history may not be a useful indicator of increased familial risk of migraine. Specific information about parents may be more useful. Of the probands with migraine, 22% to 91% had one or two affected parents and the mothers were reported to be affected twice to eight times more frequently than the fathers (Russell, 1997). Whilst positive family history of a disease, particularly regarding affected parents may suggest a genetic background, there are factors that can confound such an inference and thus closer investigation is typically sought.

3.1.2 The Relative Risk of Migraine

The aetiology and pathogenesis of migraine remains largely unknown. Previous family studies of the disease have suggested a genetic component based on frequent familial occurrence (Russell, 1997). However, the substantial prevalence of migraine in the general population (~12%) may, by chance alone, explain this frequent familial clustering. A more reliable study design is to assess familial aggregation of the disorder utilising the relative risk measure. This can be estimated by comparing the prevalence of affected relatives of probands against the prevalence of the disorder in the general population (Beaglehole, 1992) (see Chapter 2).

Estimates of familial risk of migraine have varied considerably across studies, ranging from 1.5 – 19.3. This large variation is mainly due to methodological differences (Russell, 1997). Nevertheless, several large studies which have examined the occurrence of migraine in families have reported fairly consistent results. In 1993, Russell and Olesen published results of an epidemiological study investigating the familial occurrence of migraine in Denmark (Russell and Olesen, 1993). The objective of the study was to determine the frequency and relative risk of MO and MA in first degree relatives and spouses of patients with MO and MA. In total, 193 migraine probands (121 MO and 72 MA) were selected because their diagnosis fitted the IHS criteria. Compared with the general population first degree relatives of probands with MO were shown to have a 3-fold increase of MO. Also, first degree relatives of probands with MA had a two-fold increase in both MO and MA. Overall, this study provided some evidence that the common forms of migraine are genetically determined (Russell and Olesen, 1993). However, it was recognised by the authors themselves that several methodological shortcomings of their research may have biased the results. For instance, the study relied on referral clinics as a source population for identifying migraine probands. Those who seek care in referral centres tend to have more severe attacks and more complex symptom profiles (Vilatela, 1992). An important critisism raised by Russell and Olesen was that all information attained was through probands and not first degree relatives. It has been noted that migraine affected probands may tend to over-report migraine in there family members (Smith, 1988). Another limitation was that the sample size used was also quite small.

To overcome the problem of lack of direct interview of relatives and small sample size, the same researchers performed a follow-up study on a much larger group of migraine sufferers (n = 378), and their first-degree relatives and spouses (Russell and Olesen, 1995). In addition to the diagnosis of probands, all spouses and first degree relatives were also carefully diagnosed by telephone interview. The results of this subsequent study indicated that, compared with the general population, the first degree relatives of probands with MO had ~2 times the risk of having MO and ~1.5 times the risk of MA. However, first degree relatives of MA probands had a striking 4-fold increase in risk of MA whilst no increase risk of MO. Interestingly, spouses of probands with MO also conferred a ~1.5-fold increase rsik of MO over the general population. These findings suggested a different aetiology may exist for MO and MA, with MO possibly due to both genetic and environmental factors and MA probably exclusively genetic (Russell and Olesen, 1995).

An independent migraine relative risk study was conducted in the USA by Stewart et al in 1997. This study was relatively small (no. of probands = 73) compared to the previous work of Russell et al (1995). Nevertheless, Stewart and Colleagues designed their study wisely by including carefully matched controls with each of the 73 affected probands. Moreover, they addressed the issue of migraine severity and gender when assessing relative risk in the cohort. The results of this study provided evidence that first degree relatives of MO and MA affected probands are approximately 50% more likely to be affected with migraine (either MO or MA) than the general population. It was also shown that the relative risk of first degree relatives of male migraine probands may be as high as 4 (Stewart et al, 1997). These estimates of migraine relative risk are lower than previous reports . This may be explained by some previous studies overestimating the degree of familial aggregation due to the shortcomings already mentioned.

Table 3.1 summarises the migraine relative risk results of the 3 studies reviewed here. Considered together, these data indicate that the risk of migraine for a first degree relative of a proband with MO only is between 1.44 – 2.9. If the proband has MA the risk of either subtype of migraine to a first degree relative ranges from 1.24 – 3.79. Overall, it can be concluded that if a proband suffers from migraine, their parents,

siblings or children are approximately twice as likely as the general population to also be affected by the disease.

Table 3.01. Summary of migraine relative risk studies

Disease in probands	Disease in 1st° relatives	Familial Relative Risk	
		Estimated	95% CI
MO Only			
Russell et al. 1993	**MO**	2.9	2.2 – 3.8
	MA	not calculated	
Russell & Olesen, 1995	MO	1.86	1.56 – 2.16
	MA	1.44	1.03 – 1.85
Stewart et al. 1997	MO	1.43	0.83 – 2.47
	MA	2.36	0.87 – 6.28
MA Only			
Russell et al. 1993	MO	2.0	1.5 – 2.8
	MA	2.2	1.6 – 3.2
Russell & Olesen, 1995	MO	1.02	0.77 – 1.26
	MA	3.79	3.21 – 4.38
Stewart et al. 1997	MO	1.41	0.71 – 2.77
	MA	1.24	0.28 – 5.47
Both MA & MO			
Russell & Olesen, 1995*	MO	1.64	0.94 – 2.33
	MA	2.17	0.98 – 3.38
Stewart et al. 1997	MO	1.42	0.86 – 2.35
	MA	1.95	0.78 – 4.93

3.1.3 Migraine Twin Studies and Heritibility

Studying concordance in twins is a classic and often very useful method for investigating the relative importance of genetic factors contributing to a disorder. Migraine twin data, which has been published from studies conducted after the release of the strict IHS diagnostic criteria have been summarised in Table 3.2. The table shows the comparison of concordance rates between MZ and DZ twins where migraine subtype is unspecified, and for MO and MA affected twins only.

Table 3.02. A summary of migraine twin (heritibility) studies

	Monozygotic Twin Pairs			Dizygotic Twin Pairs		
	Concordant	Discordant	%Concordance	Concordant	Discordant	%Concordance
Migraine Subtype Unspecified						
Honkasalo et al. (1995)	57	257	18	58	594	9
Larsson et al. (1995)	219	479	31	268	1190	18
Merikangas et al. (1994)			34			19
MO Only						
Gervil et al. (1999)	38	99	28	47	210	18
MA Only						
Urlich et al. (1999)	26	51	34	16	118	12
Overall (Combined)	340	886	28	389	2112	16

In each of the studies displayed in Table 3.2 the concordance is significantly higher in MZ twins than in DZ twins ($P<0.05$), with an average concordance rate in MZ twins of 28%, and 16% in DZ twins. These data indicate a strong genetic component in migraine but also show that there is no simple inheritance of the disease since concordance is far less than 100% in MZ twins. An interesting finding by Ziegler et al (1993) was that MZ twins who were separated at birth and raised apart not only were concordant for the occurrence of migraine but also for the age of onset of the attacks (Ziegler, 1993).

In addition to calculating concordance rates, a large Swedish twin study conducted by Larsson et al, also included a structural modelling analysis. This type of testing is aimed primarily at describing individual differences in the migraine phenotype by partitioning these differences (total variance) into the genetic (heritable) and environmental effects contributing to the disease (Urlich et al, 1998). The results of this model-fitting analysis indicated that heritability estimates were greater in women than men for lifetime migraine (58% vs 44%), thus making it plausible that genetic effects for migraine to some extent may reflect genetic influence on hormone factors (Larsson et al, 1995). Utilising similar statistical methods Honkasalo et al, estimated the heritability of migraine to between 0.4 and 0.5, although no remarkable gender

differences were observed (Honkasalo et al, 1995). Examining a twin population affected with MA only, Urlich reported a heritability of 0.65, also with no gender differences. In summary, it can be concluded that the observations in twins strongly support a genetic component of migraine with heritability estimates ranging from 0.4 –0.65.

3.2 Inheritance of Migraine

Once it has been establishing that a disease is (at least partly) genetically inherited, it is an important progression to attempt to determine the transmission pattern by which it is passed through generations. The mode of transmisison of migraine has been extensively investigated for many years but to this day it is a somewhat contentious issue. Table 3.3 (taken from Nyholt, 1998c) lists some of the proposed modes of migraine inheritance.

Table 3.03 Summary of Migraine Inheritance Studies (Reproduced from Nyholt 1997c)

Study	Year	Mode of Inheritance
Migraine unspecified		
Allan	1928	Dominant inheritance
Goodell et al.	1954	Recessive gene with a penetrance of about 70%
Dalsgaard-Nielsen	1965	Additive effect of numerous genes, but dominant gene cannot be excluded
Barolin and Sperlich	1969	Autosomal recessive
Baier*	1985	Polygenic inheritance
Devoto et al.	1986	Suggested possible heterogeneity (after the rejection of both simple autosomal dominant and recessive transmission)
Honkasalo et al.*	1995	Multifactorial inheritance
Kalfakis et al.*	1996	Multifactorial inheritance (but the contribution of a major gene could not be excluded)
Migraine without aura		
D'amico	1991	"Sex-limited" transmission
Russell et al.	1993	Most likely multifactorial, but dominant inheritance could not be excluded
Mochi et al.	1993	Autosomal recessive (reduced penetrance)
Russell et al.	1995	Multifactorial inheritance
Migraine with aura		
Russell et al.	1993	Genetic transmission - mode undetermined
Mochi et al.	1993	Autosomal recessive
Russell et al.	1995	Multifactorial inheritance
Ulrich et al.	1997	Multifactorial inheritance

*MO and MA were considered together for inheritance study.

The earlier studies listed in Table 3.3 are based mainly on simple inspection of the pedigrees. Three of the studies included a classic segregation analysis (Devoto et al, 1986:Mochi et al, 1993:Kalfakis et al, 1996). The first of these studies was based on a questionnaire and included 128 probands with migraine. The result suggested the existence of a possible genetic heterogeneity after the rejection of both simple autosomal dominant and recessive transmission (Devoto et al, 1986). The second segregation analysis involved 46 probands, 34 with MO and 12 with MA. An analysis of the pedigrees in this study excluded maternal and X-linked transmission, whilst the segregation analysis indicated an autosomal recessive pattern of inheritance for both MO and MA (Mochi et al, 1993). The third segregation analysis of migraine was conducted by Kalfakis et al, in 1996. The results of this study suggested that multifactorial inheritance seemed to be the most probable mode of genetic transmission of migraine (Kalfakis et al, 1996). Several independent studies have been performed testing this hypothesis of multifactorial inheritance. A Danish study including an analysis for multifactorial inheritance found that this was likely in MO, but dominant inheritance could not be excluded. The mode of inheritance in MA could not be determined (Russell & Olesen, 1993). A follow-up study performed by the same researchers confirmed the original findings for MO inheritance and concluded that MA was also inherited in a multifactorial fashion (Russell et al, 1995). Independent research by Urlich et al, considered other modes of transmission for MA only. Mitochondrial and X-linked inheritance were excluded because of paternal transmission and the female preponderance was too low to explain sex-influenced inheritance. These researchers also concluded that MA most likely has a multifactorial inheritance even in high risk families with the disease (Urlich et al, 1997).

Studies into the mode of inheritance of migraine have produced some conflicting results but considering the high prevalence of both MO and MA, a single gene would not be expected to cause either type of migraine. Based on the more powerful of the genetic studies shown in Table 3.3 it seems to be most likely that the disease is multifactorial, however, autosomal dominant inheritance with reduced penetrance could not be excluded (Russell & Olesen, 1992). Furthermore, a number of important linkage analysis findings have been recently reported regarding the common forms of

migraine. These indicate that an X-chromosomal inheritance factor may also be involved (Nyholt et al, 1998b, Nyholt et al, 2000).

3.3 Molecular Genetics of Migraine

3.3.1 Migraine Linkage Studies

Genetic linkage studies have long been a powerful design for mapping the chromosomal location of a disease gene. Families affected with the disease provide a valuable resource for this method of susceptibility gene localisation.

3.3.1.1 Chromosome X Linkage Studies

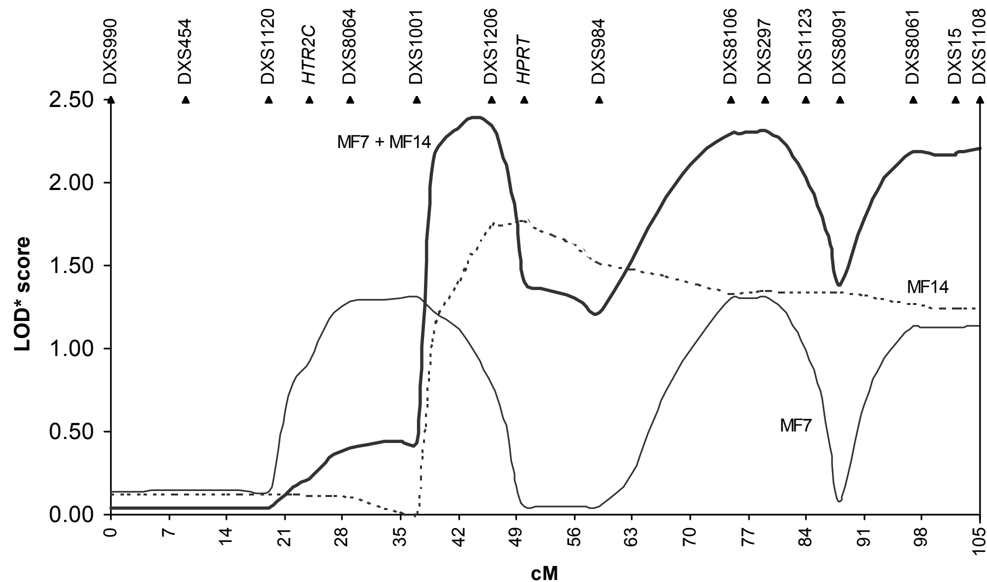
Molecular genetic studies conducted at the Genomics Research Centre (GRC) have been utilising large multigenerational Australian families affected with typical migraine in conjunction with current DNA technologies and linkage analysis strategies in an effort to map the genes influencing susceptibility to the disease. Whilst, the mode of inheritence of migraine is still not clear, the unequal gender distribution for both MO and MA (female preponderance of 3:1) may indicate X-linked involvement. (Launer et al. 1999; Rassmussen and Olesen 1992; Rozen et al. 1999).

The GRC research group set out to test the hypothesis of an X-chromosomal inheritance factor in the molecular genetics of migraine. Initial investigations involved a linkage study utilising 3 large Australian Migraine families and included 28 microsatellite markers spanning the entire X chromosome (Figure 3.02). For this study, the strategy analysing the marker and pedigree (phenotype) information for linkage was two pronged. Using the GENEHUNTER computer program (Kruglyak et al. 1996), both traditional parametric and non-parametric tests were performed. The non-parametric method is often called model-free analysis since the calculation of the excess allele-sharing statistic, called a Non-Parametric Linkage (NPL) score is not reliant directly on specification of a disease model. This approach may be beneficial when the mode of inheritance is not clear, as is the case with migraine. The parametric method requires disease model information and the conservative model proposed by Horvatta et al. was used (Horvatta et al, 1994). Parametric testing

produces a tradional LOD score with a LOD score greater than 2 conventionally interpreted as significant for X linkage.

The results of the parametric analysis provided some evidence of linkage of markers telemeric on Xq in one of the three families tested (LOD = 1.48 for MF 14 at the DXS1123 marker locus). Non-parametric analysis indicated signficant excess allele sharing to Xq markers in two of the families analysed (P = 0.031 and P = 0.012). Overall analysis of the data from all three pedigrees gave significant evidence in support of linkage and heterogeneity (HLOD = 3.1). These results provided conclusive evidence that migraine was a genetically heterogeneous disorder and suggested that a region on chromosome Xq may be linked to the disorder in two large pedigrees (Nyholt et al. 1998b).

Figure 3.01. This graph shows the GENEHUNTER-PLUS LOD* score curves for MF7 and MF14 for markers spanning chromosome Xq24-28



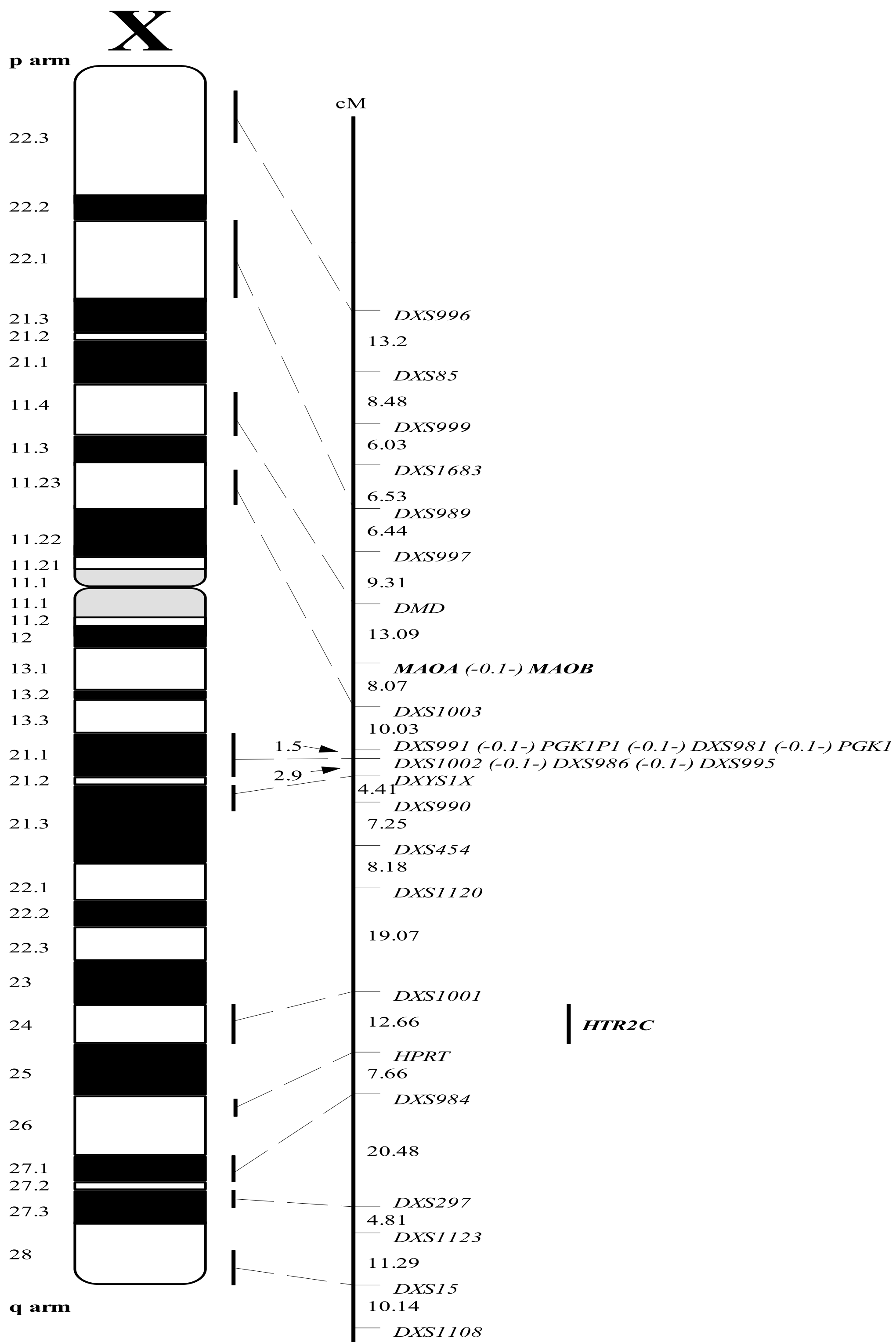


Figure 3.02. This is an ideogram illustrating chromosome Xq markers tested by Nyholt, 2000. Distances are in cM.

To further investigate the findings of the original study, the two migraine pedigrees previously showing evidence for linkage were re-examined utilising a dense map (~5cM) of DNA markers spanning the implicated genomic region on chromosome Xq (Nyholt et al. 2000). This time a modified version of the GENEHUNTER program (called GENEHUNTER-PLUS) was used to analyse the pedigree and genotype data. This newer program performs a typical GENEHUNTER analysis but also incorporates additional allele sharing models to calculate the non-parametric LOD score (LOD*). Particularly, the LOD* scores were calculated using the exponential model which provides a better fit to data consisting of a small number of pedigrees with extreme IBD sharing (Kong and Cox 1997).

The multipoint non-parametric linkage analysis results of this study are reproduced in Figure 3.01. Peak LOD* scores were obtained for MF7 and MF14, at marker DXS1001 (LOD* = 1.32, $P = 0.0069$) and *HPRT* (LOD* = 1.77, $P = 0.0021$), respectively. Importantly, a combined analysis of the data from both pedigrees produced a *significant* result with a LOD* score of 2.388 ($P = 0.0005$) equidistant between markers DXS1001 and *HPRT* (Nyholt et al. 2000). Haplotype analysis of the markers in the linkage region was then performed. Critical recombination events were observed between the two pedigrees which allowed the distinction of three shared regions between markers DXS1001 – DXS1206, DXS984 – DXS1123 and DXS8091 – qter. These regions translated to a physical distance of 5 Mb, 11 Mb and 6.5 Mb, respectively (Nyholt et al. 2000). Overall, these findings provided good evidence for the presence of a typical migraine susceptibility locus on chromosome Xq24-28. Obviously, this is a very large genomic region and the distinct peaks shown in Figure 3.01 may indicate multiple predisposing genes or the extended signal may be the result of a single locus. Thus further investigation using additional migraine pedigrees would be required to elucidate how this region is involved in migraine.

3.3.1.2 Chromosome 19 Linkage Studies

A genomic region on chromosome 19p13 has also been targeted as a potential typical migraine susceptibility region by several international research groups (May et al. 1995, Horvatta et al. 1995; Monari et al. 1997; Nyholt et al. 1998a; Terwindt et al. 2001). This interest was sparked in 1993, when a team of French researchers, led by Tournier-Lasserve, mapped CADASIL to the P arm of chromosome 19 using

microsatellite markers (Tournier-Lasserve, 1993). Genetic linkage analysis was conducted on two unrelated families affected by CADASIL in the absence of any biochemical defect. CADASIL (Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is a disorder characterised, in the absence of hypertension, by recurrent ischaemic strokes leading to dementia. Significant linkage was shown between the disease and the microsatellite markers tested on chromosome 19p13. The best estimate for the location of the affected gene was within a 2cM interval bracketed by markers D19S199 and D19S226 (Tournier-Lasserve, 1993). This information led Chabriat *et al* (1996) to directly sequence the *NOTCH3* gene, located in the same region, and discover that a mutation in this gene was the cause of CADASIL.

Whilst investigating CADASIL, these scientists noticed that some patients had recurrent attacks of hemiplegic migraine. FHM is a rare and extremely debilating subtype of *migraine with aura*. It was therefore hypothesised that the same gene could be involved in the aetiology of both CADASIL and FHM (Tournier-Lasserve, 1993). Linkage analysis was then performed on two large FHM pedigrees and the loci responsible for both diseases were subsequently allocated to within a 30cM interval on chromosome 19p13. These, and other results provided strong evidence that FHM and CADASIL were genetically homogeneous disorders.

Locus heterogeneity occurs when disease alleles at two or more independently acting, chromosomal positions could each cause the same disease. In another study carried out on 5 unrelated families affected with FHM, it was found that three showed linkage to chromosome 19 microsatellite markers, whilst two gave no evidence of linkage. These results confirmed the localisation of an FHM gene to chromsome 19 but also indicated that locus heterogeneity is likely to exist (Ophoff et al, 1994). In 1994, Joutel *et al* assessed 9 large pedigrees and using two-point and multipoint LOD score analysis they provided strong evidence for genetic heterogeneity in FHM. Further evidence of heterogeneity came when two independent studies mapped a second FHM locus to chromosome 1q. (Gardner et al, 1997:Ducros et al, 1997)

The FHM breakthrough came in 1996 when Ophoff *et al* sequenced a large brain specific calcium channel gene (*CACNA1A*) located in the region linked to FHM in

affected families. These researchers showed that the FHM phenotype in some families was caused by single missense mutations in four exons within *CACNA1A* (Ophoff et al, 1996). Furthermore, two other mutations were shown to cause episodic ataxia (EA) in some individuals. Subsequent studies performed on families suffering from another neurological disorder called spinocerebullar ataxia type 6 (SCA6) found that a *CACNA1A* intragenic CAG repeat polymorphism was undergoing expansion and affecting the disease (Zhuchenko, 1997). These neurological disorders, as well as diseases caused by other ion channels, have since been termed "channelopathies".

Using FHM as a model for typical migraine, May *et al*, (from the Dutch Migraine Group) tested the involvement of the FHM susceptibility region on chromosome 19p13 utilising 28 small families affected with the common forms of MO and MA. Sibpair analysis was performed which included assessing the sharing of highly informative microsatellite markers between pairs of affected siblings. In particular, this type of analysis measures the observed sharing of parental marker alleles (IBD) by affected siblings compared with the expectations based on Mendelian laws. Under these expectations, 25% of sibling pairs share alleles from one of their parents, 50% share alleles from both parents and 25% share no parental alleles. This study found that siblings shared the same allele for marker D19S394 slightly more frequently than was expected by chance (P = 0.04) (May et al, 1995). Although, these results were marginally significant, they offered the suggestion that the underlying genetics of FHM and migraine with and without aura are perhaps partly the same. We tested this hypothesis by performing an entire chromosome scan concentrating on markers spanning the region on 19p13 (Figure 3.03) (Nyholt et al, 1998a).

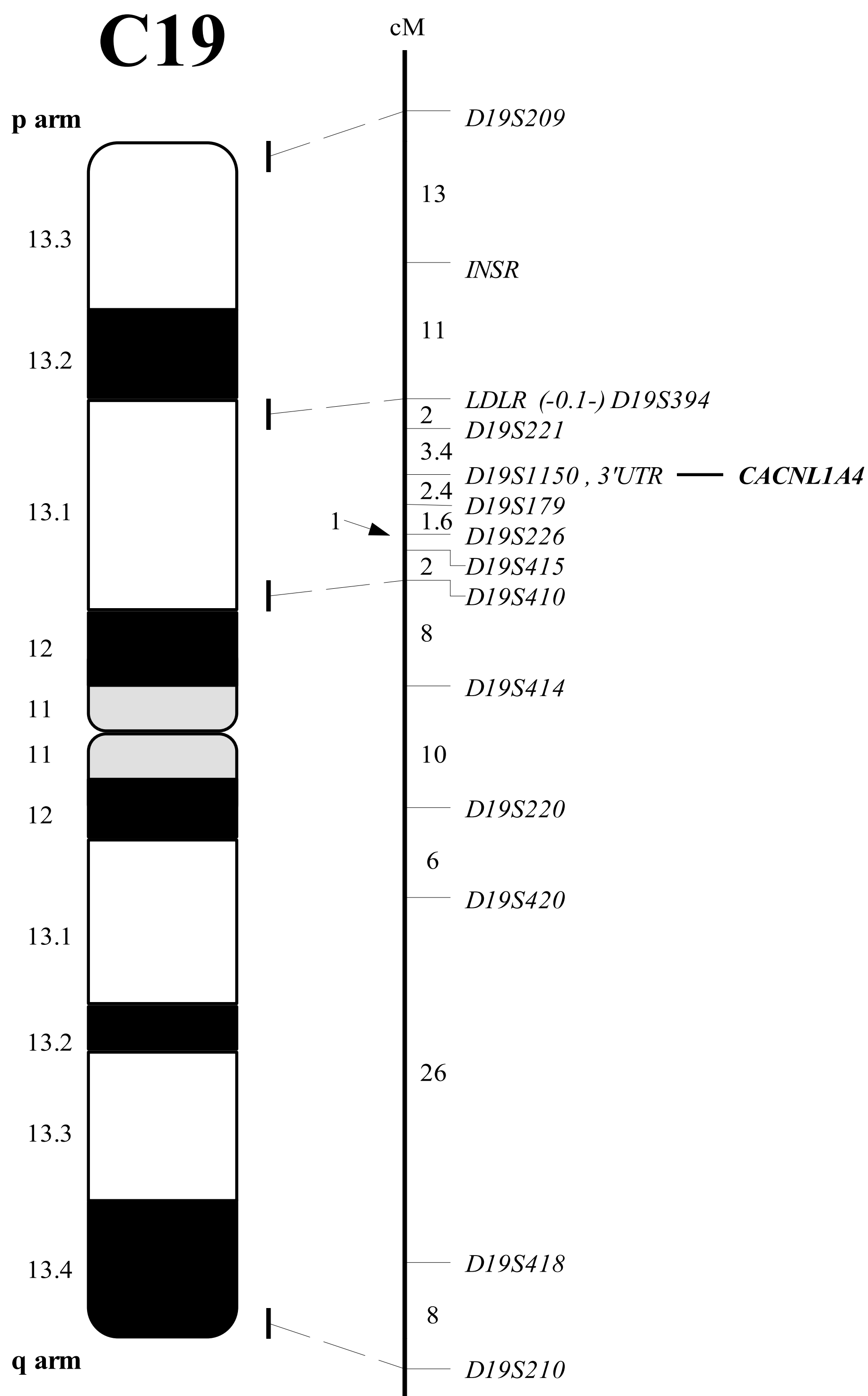


Figure 3.03. This is an ideogram illustrating chromosome 19 markers tested by Nyholt et al (1998a). Distances are in cM

Multigenerational pedigrees can offer greater power for detecting linkage given the likelihood of a larger number of informative meioses (Terwilliger and Ott, 1995). The non-parametric linkage results of this study (calculated by the GENEHUNTER program) indicated significant excess allele-sharing of markers spanning 12.6cM across the FHM region in one of the pedigrees (MF1) (NPL = 6.64, P = 0.0026). Interestingly, two *CACNA1A* intragenic markers, including a CAG triplet repeat polymorphism located in exon 47 near the 3'UTR of the gene, were among those markers linked to migraine in this pedigree (Figure 4.04). Additionally, a maximum parametric LOD score of 1.92 was obtained for the CAG triplet repeat marker (Nyholt et al. 1998a). Overall, these results provided good evidence for a migraine susceptibility gene in this area. Moreover, these findings strongly implicate the *CACNA1A* gene as a candidate for typical migraine.

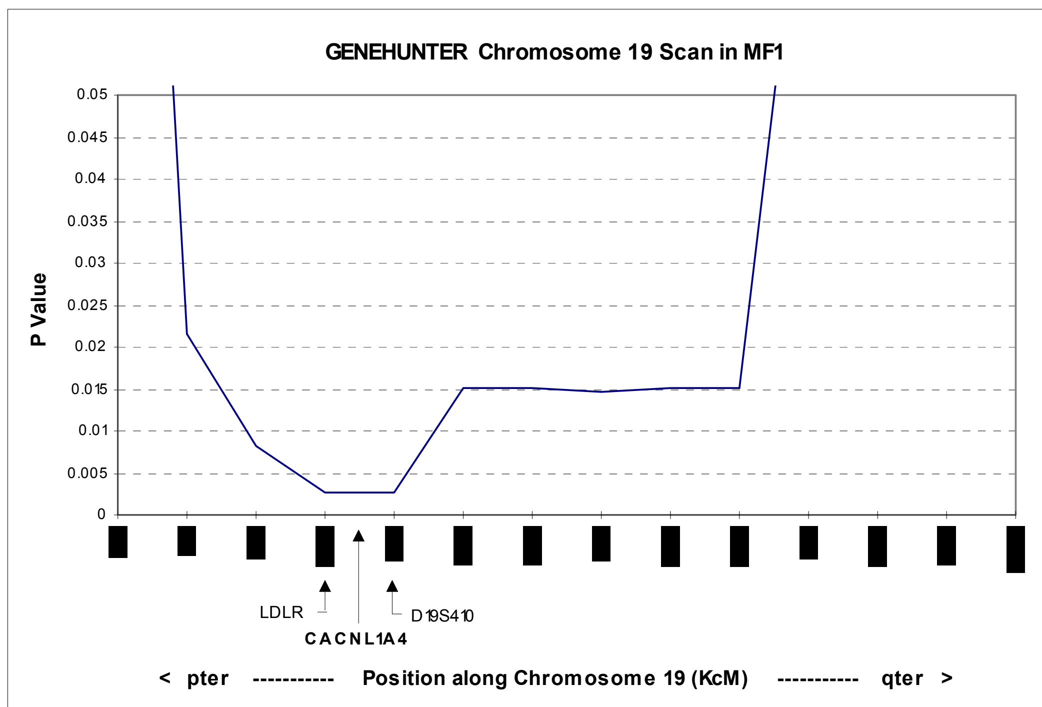


Figure 3.04. This graph shows the GENEHUNTER- *P*-value curves for MF1 for markers spanning chromosome 19

Recently the Dutch Migraine Group published new results of a migraine affected sibpair study (Terwindt et al. 2001). This work involved examination of 189 affected siblings from 36 extended families with typical migraine. These researchers reported that sibling pairs with any form of migraine had inherited the same 19p13 *CACNA1A*-

containing region significantly more frequently than by chance (Maximum multipoint LOD score = 1.22). However, this result was almost exclusively dependent on the increased sharing found in siblings with MA (Maximum multipoint LOD = 1.44) (Terwindt et al. 2001).

In conclusion, the complexity of the common forms of migraine make it difficult to diagnose and even more difficult to trace genetically. Studies have indicated that both genetic and environmental factors probably cause *migraine without aura* whilst *migraine with aura* is mostly the result of a defective chromosomal region(s). Several studies have reported that the FHM region on chromosome 19p13 also shows evidence for linkage to the common forms of migraine (May et al. 1995; Nyholt et al. 1998; Terwindt et al. 2001). One of the main objectives of the research presented in this thesis was to further investigate this migraine susceptibility region on 19p13 with particular attention focused on the *CACNA1A* candidate gene.

3.3.1.3 Chromosome 1 Linkage Studies and FHM

FHM linkage studies have proven that this type of migraine is genetically heterogeneous since only about 50% of affected pedigrees tested show linkage to chromosome 19p13 (Ophoff et al. 1996). Therefore, at least one other gene elsewhere in the genome is involved in the aetiology of this disorder. Gardner et al ascertained a four generational pedigree affected with autosomal dominant FHM. Initial linkage analysis of this pedigree using markers spanning the chromosome 19p13 region provided no evidence for linkage to the disease in this family (Gardner et al. 1997). These researchers than decided to investigate other candidate gene regions for linkage to FHM. They focused their attention on markers spanning a section of chromosome 1q given the presence of several ion channel genes in this region. The entire family was genotyped for 12 markers spanning a 44 cM region on 1q31. Parametric linkage analyses were carried out using accepted FHM model parameters (Joutel et al. 1993). Maximum two-point LOD scores of 3.51 and 3.21 were obtained for markers D1S2782 and D1S249, respectively. Multipoint analysis also indicated significant LOD scores across all markers tested, peaking near markers D1S249 and D1S2782 (LOD score = 3.328)(Gardner et al. 1997). In conclusion, the results of this study indicated a new locus for FHM localises to a 44cM region on chromosome 1q31. This genomic region on chromosome 1q is of particular interest because it has been

reported that the region contains another neuronal calcium channel gene. This gene is the calcium channel α_{1E} subunit gene (*CACNA1E*), thought to be associated with R- (resistant) or possibly T- (transient) type calcium channels (Diriong et al. 1995). Hence the *CACNA1E* gene is also an excellent FHM candidate gene.

At around the same time that Gardner et al published their FHM findings, Ducros et al. published a report that also examined the long arm of the chromosome 1 for linkage with non-chromosome-19-linked FHM families. Of four large French families one pedigree showed significant linkage to two microsatellite markers *D1S2635* (Z_{max} = 3.33 at θ = 0.05) and *D1S2705* (Z_{max} = 3.64 at θ = 0.05). Interestingly, the Ducros et al. study revealed linkage almost 30 cM centromeric to the Gardner et al. study (Figure 3.05). This may suggest the existence of a second locus for FHM on chromosome 1q21-q23.

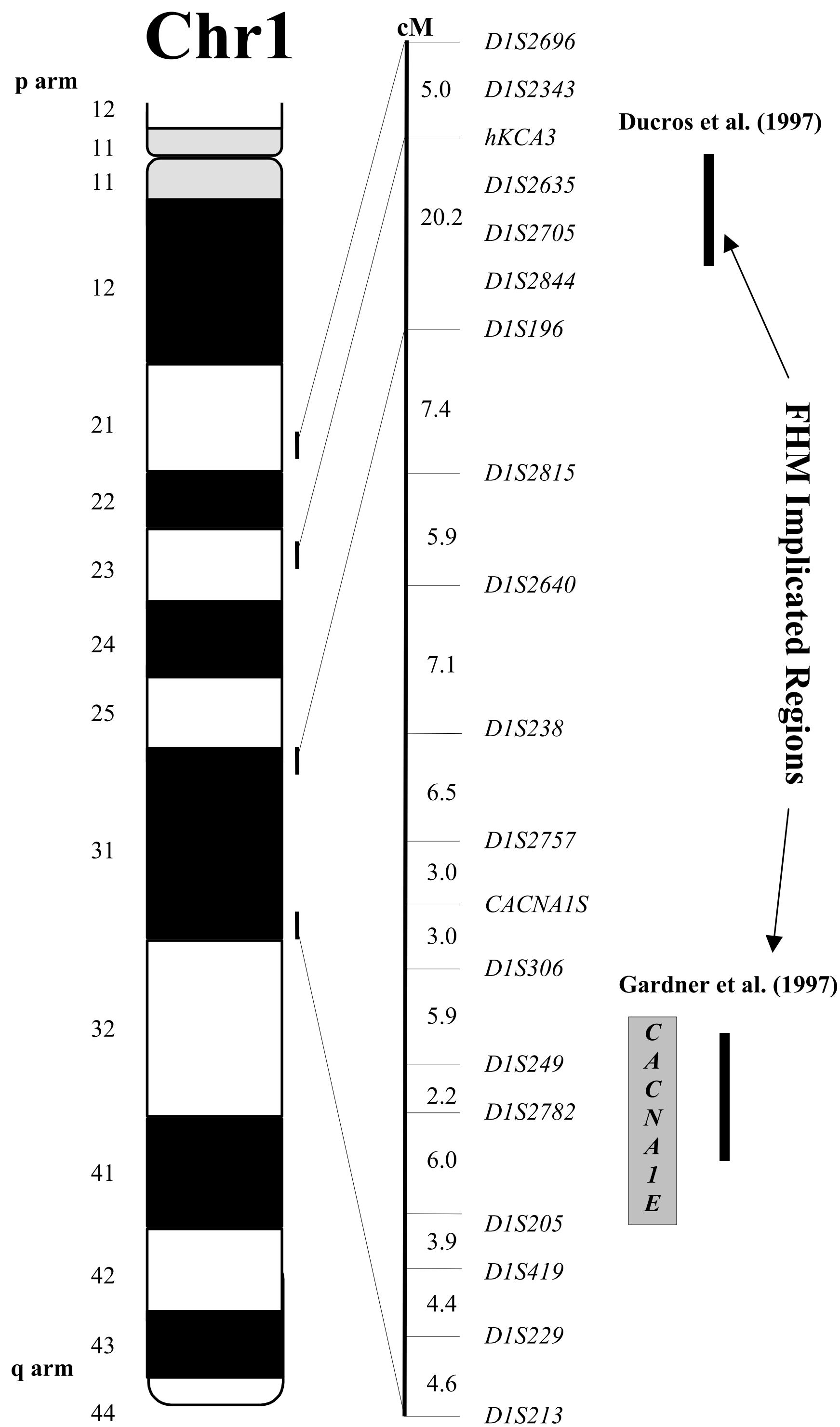


Figure 3.05. This an ideogram illustrating chromosome 1q markers tested for linkage to FHM by Gardner et al (1997) and Ducros et al (1997).

However, multipoint analysis using the same markers used by Gardner et al. in the Ducros et al. pedigrees would be needed to further investigate the possibility of two different FHM loci existing on chromosome 1q. An additional aim of the research undertaken as part of this thesis was to investigate the new FHM linkage results reported by Gardner et al and Ducros et al for involvement in the more common forms of typical migraine (MO and MA).

In summary, linkage studies of families affected with FHM have lead to the discovery that the *CACNA1A* gene on chromosome 19p13 is responsible for the disease in some pedigrees. Further linkage studies have also provided some evidence for involvement of this genomic region in the more common forms of migraine with and without aura. Subsequent FHM studies have also linked a disease susceptibility region to chromosome 1q, although the number of FHM loci at this region is not yet known. Interestingly, investigation of the X chromosome has also revealed the presence of a typical migraine susceptibility region on Xq24-28.

3.3.2 Association Studies of Migraine Candidate Genes

A gene may be a candidate for involvement in disease either because it is lying in a region detected in a systematic screening of the genome after linkage analysis or because it is hypothesised to be functionally related to the disease and thus may represent a plausible risk factor. For example, genes of the HLA complex are currently candidates for diseases with an immune component and apolipoprotein genes are candidates for cardiovascular diseases. Thus, the issue underlying the candidate gene strategy is that firstly, information concerning the role of a given candidate gene must be obtained and secondly, the risk that an individual will develop the disease according to the available information must be estimated (Clerget-Darpoux 1998). The genotypes of the candidate gene are not always observable. Indirect information on a genetic marker situated at or near the candidate gene locus may then be utilised.

Linkage studies involving affected families can be a useful approach in marking the general genomic region that a disease susceptibility gene may be located, but association studies, which are often used as a complementary strategy to linkage

studies, can offer a more direct method of disease gene identification. Traditional association studies in molecular genetics are aimed at comparing the frequency of alleles of a candidate gene marker (or a marker in very close proximity to the gene) between appropriately matched groups of disease affected patients and unaffected control subjects. These are often called case-control studies. The differences in allele frequencies between case and control groups are typically measured using the standard χ^2 statistic (or some similar derivative). If a statistically significant deviation is observed then the marker alleles, if they are functionally relevant, are said to be associated with the disease. If the marker alleles are not functionally relevant they are said to be associated (or in linkage disequilibrium) with the disease alleles. This usually implies that the mutation affecting the disease is in very close proximity to the marker being tested. A summary of all migraine association studies published to date is shown in Table 3.04.

One of the first migraine genetic association studies was published by Pardo *et al* in 1995, who examined 112 unrelated migraine patients with a random sample of healthy control volunteers. These individuals were genotyped for markers that were previously localised in or near eleven genes on different chromosomes. The genes tested in this study were; red cell antigens: ABO and Rh, red cell enzymes: Esterase D, Acid phosphatase I, Phosphoglucomatase I and Adenosine deaminase, serum proteins: Haptoglobin, Transferrin, Alpha-1-antitrypsin and Group-specific component and a DNA polymorphism (D1S80) near the pMCT 118 gene. These researchers chose these particular genes to investigate due to reports that they are proteins or enzyme loci related with the aetiology of migraine (Pardo et al. 1995). Of these eleven markers, nine markers showed no evidence of association to migraine in this cohort. Two markers however, a blood group component located on chromosome 4 and esterase D located on chromosome 13, showed a significant association to migraine even after correction for multiple testing (corrected *P* values of 0.001 and 0.002, respectively). However, these results should be viewed with some caution. Firstly, the correction for multiple comparison was based only on the number of markers tested. Many phenotype comparisons were also performed. Had the correction factor included these extra phenotypic comparisons as well as the number of alleles at each marker locus, the significance would be far less convincing. In addition, the high odds ratios obtained for both ESD and GC alleles (7.33 and 10.71,

respectively) are more than likely due to the very low numbers of subjects in each group with the particular risk phenotype (less than 11). Nevertheless, the conclusions reached in this study were that each of the markers found to be associated with migraine were more likely to be in LD with alleles of other gene markers. Since there is no reason to suppose a role for ESD in migraine pathogenesis it was suggested that the 5HT2a receptor gene located nearby on chromosome 13 may be a more plausible candidate that is in LD with the ESD locus (Pardo et al, 1995).

3.3.2.1 Setononin-Related Genes and Migraine

The serotonergic system has long been implicated in the pathogenesis of migraine. Abnormalities in this system have been observed before and during migraine attacks and have been the target of several therapeutic developments including sumatriptan, a highly selective serotonin agonist (Tfelt-Hansen, 1993). Antagonists of 5HT receptors, such as ergotamine, methysergide and amitriptyline are also antimigraine drugs (Fozard and Gray, 1989). Dysfunctional genes in the serotonergic system are therefore excellent candidates to mediate susceptibility to typical migraine and for this reason markers in them are useful for association studies into the molecular genetics of the disorder.

Following the suggestion of Pardo et al. that the 5HT receptor gene (HTR2A) located on chromosome 13 may be driving the association they found with the ESD gene marker, Nyholt *et al*. tested markers within, and nearby, the 5HT2A gene. In particular, both linkage and association analyses were performed on an RFLP within the gene and a highly polymorphic microsatellite marker closely linked to the gene. The association and linkage analyses involved 96 cases and 91 unrelated controls, as well as 3 large migraine pedigrees, respectively. No significant association between the migraine group and either DNA marker was observed ($P > 0.05$). These association results were subsequently replicated by Buchwalder et al. 1996. Performing linkage analysis on the pedigree data it was also shown by Nyholt et al. that both markers were significantly excluded from linkage to the disease (LOD < -2). Thus, the results from this research excluded the 5HT2A gene for involvement with common migraine (Nyholt et al. 1996). An independent linkage analysis that examined migaine in Italian families confirmed that 5HT2A was not involved in typical migraine susceptibility (LOD < -2) (Monari et al. 1997).

Another serotonin receptor gene, HTR2C (previously termed 5HT2C) has been marked as a potential migraine candidate gene. A case-control study was carried out by Burnet et al. to test the hypothesis that alterations in this gene located on the X-chromsome may confer an increased risk of being affected with typical migraine. A common polymorphism within the HTR2C gene at codon 23 in which serine (Ser) is substituted for cysteine (Cys) was the target of the study. This variant has previously been associated with response to the antipsychotic drug cloapine (Sodhi et al. 1995), and bipolar disorder (Gutierrez et al. 1996). The statistical analyses revealed no differences nor trends in allele or genotype frequencies in the 242 migraineurs when compared to the 129 controls ($P > 0.05$). Neither did the frequencies vary significantly in migraineurs with and without aura, or if men and women were analysed separately. Therefore, these data did not provide any evidence that this marker is associated with migraine in this population (Burnet et al. 1997).

Prior to the publication of the Burnet et al (1997) study, family linkage analysis results at GRC had already implicated markers in the HTR2C gene region on chromosome Xq24 in migraine involvement. These results gave us good reason to suspect that this gene may be involved in predisposing to the disease. Therefore, as part of the objective of the present research a comprehensive investigation of the HTR2C gene was undertaken which included further linkage, association and mutation screening studies.

The Serotonin Transporter Gene and Migraine

There is considerable co-morbidity of migraine with depression (Breslau et al. 1991; Merikangas et al. 1993). By inference, the pathophysiology and specific treatment of depression with selective serotonin re-uptake inhibitors supports the idea that an overlap of mechanisms involving serotonergic function may be involved in both conditions. The human serotonin transporter protein actively mediates the reuptake of serotonin into the presynaptic terminal in the brain and thus plays an important role in the regulation of synaptic serotonin levels (Kelsoe et al. 1996). The serotonin transporter gene (HTT or SERT), located on chromosome 17q11.1 – 17q12 (Lesch et al 1994), has been shown to be associated with susceptibility to major affective disorder (Ogilvie et al 1996). Therefore, the *SERT* gene is also a candidate for migraine susceptibility.

Located within intron 2 of the *SERT* gene is a variable number tandom repeat (VNTR) of 17bp units. This polymorphism was tested for association with the common forms of MO and MA in a Danish cohort of 266 migraineurs (173 with MO, 94 with MA and 18 with both MO and MA) and 133 unaffected controls (Ogilvie et al 1997). Three alleles of 9, 10 and 12 copies of the repetitive element were identified in this DNA set. These alleles were designated (STin2.9, STin2.10 and STin2.12, respectively). A comparison of the distribution of the allele frequencies between all affected groups (MO, MA and MO/MA) against the control group was carried out. It was revealed that there were no significant differences between any affected groups and controls for allele frequencies. These researchers also assessed the genotype distribution between groups for this marker. The MO group was found to have an over-representation of genotypes with two 12 repeat alleles (STin2.12) (P = 0.031). and a reduction of genotypes containing one 10 repeat (STin2.10) compared to controls (P = 0.017). A similar pattern was also shown in the MA group, but also a trend towards an increase in genotypes containing the nine repeat allele of the VNTR (STin2.9). Genotypes containing this allele were present in 6.4% of the MA group compared to only 2.3% of controls. Overall, these researchers concluded that these results supported the view that susceptibility to MO and MA has a genetic component and that these disorders are distinct. The major conclusion was that the genetic susceptibility to migraine in some cases is associated with a locus at or near the serotonin transporter gene (Ogilvie et al. 1997). However, the conclusions of this work should be treated with caution for several reasons. Firstly, contrary to the title of the article, there was no evidence of an alteration in allelic distributions between any affected groups and controls (P>0.05). Secondly, the genotype distributions were only found to differ significantly when many allele combinations were compared. Thirdly, no effort was made to adjust the significance level according to the number of comparisons made. Considering the smallest P-value obtained in this study was 0.011, it is highly likely that correction for multiple comparisons would render the this value non-significant. Prior to the release of the *SERT* results by Ogilvie et al, a similar migraine association study was being conducted by the Author of this thesis. Results are presented in Chapter 7.

3.3.2.2 Dopamine-Related Genes and Migraine

Abnormalities have been observed in the serotonergic system before and during migraine attacks, but clinical and pharmacologic evidence also supports the hypothesis that dopamine is also involved in the pathogenesis of the disease (Sicuteri F 1977). Studies have reported alterations in dopaminergic neurotransmission in migraineurs (Piccini et al. 1995;Fanciullacci et al. 1980). Antagonists to the dopamine receptor *DRD2* have been shown to be effective in the acute treatment of migraine (Fisher 1995). Therefore, the gene encoding the dopamine receptor *DRD2* may also be involved in migraine pathogenesis. To test this, Peroutka et al, performed genetic association using 129 migraineurs (77 MO and 52 MA) and 121 unaffected controls. In the study, an *Nco*I RFLP polymorphism in the *DRD2* was tested. The major finding of the study was that susceptibility to MA is modified by *DRD2 Nco*I alleles, with MA individuals (n = 52) having an increased frequency (0.84) of the *DRD2 Nco*I C allele ($\chi^2 = 6.47$; $P = 0.005$, df = 1), compared with control individuals (n = 121; C allele frequency = 0.71). Some of the shortcomings suffered by the Ogilvie et al study on the *SERT* gene are also noteworthy here, including the possibility of a false positive finding due to population stratification. Spurious associations are a thorny problem for association studies based on the tradional case-control design. If there is sub-structure in the unrelated population being examined then studies may reveal evidence for association to the tested marker even if it is unlinked to the disease locus (Sham 1998). One way of overcoming this problem of hidden population stratification is to compare affected cases with their relatives rather than unrelated controls (Sham 1998).

The results of another independent investigation into the role of dopamine receptor genes (*DRD2, DRD3* and *DRD4*) and migraine were published shortly after those of Peroutka et al. This association study was a family-based design that employed the transmission disquilibrium test (TDT) using a Sardinian sample of migraine families (Del Zompo et al. 1997). The TDT analysis used in this study compared the number of alleles transmitted from heterozygous parents to affected offspring to the number of alleles nontransmitted. The nontransmitted parental alleles serve as ethnically matched controls and thus this test is a valid test for association of between marker and disease that is robust against stratification. Interestingly, the results of this study

also implicated the *DRD2* gene in migraine aetiology, although a different intragenic marker was tested. In particular, a significant transmission distortion of allele 1 was observed in a subset of 22 triads (P = 0.02). However, unlike Peroutka et al. who found an association between *DRD2* alleles and MA, Del Zompo reported an association between *DRD2* alleles and MO in their pedigrees. (Peroutka et al. 1997; Del Zompo et al. 1997). The interpretation of the findings from these two studies is unclear but the independent association of two different *DRD2* polymorphisms with MO and MA may indicate that a common dopaminergic disturbance is influencing both types of typical migraine. In an attempt to offer further confirmation that the *DRD2* gene may be involved in the common forms of migraine, an additional association study was performed as part of this thesis research incorporating both population and family-based designs.

Table 3.04 Summary of Migraine Candidate Gene Studies

Dopamine-Related Candidate Gene Studies in Migraine

Authors	Gene	Type of marker tested	Study design	Type of migraine examined	Sample size: Affecteds cases	Sample size: Affecteds families	Sample size: Controls	Conclusion (significance)•
Peroutka et al, 1997	DRD2	RFLP *(Nco*I)	case-control	MA/+MO	129		121	**MA - allelic association (*P* < 0.005)**
Del Zompo et al, 1998	DRD2	microsatellite	TDT	MO only		50		**MO - allelic association (*P* < 0.01)**
	DRD3	RFLP (*Bal*I)						not significant
	DRD4	VNTR						not significant
Dichgans et al, 1998	DRD2	RFLP (*NcoI*)	allele frequency comparison in cases	MA+MO	102		no controls	
Lea et al, 2000	DRD2	RFLP (*NcoI*)	case-control, TDT, linkage	MA+MO	177	82	182	not significant
	DBH(1)	Dinucleotide			142		136	**MA/MO - allelic association (*P* < 0.01)**
	DBH(2)	Ins/Del			142		136	not significant
Maude et al, 2001	DRD2	Ins/Del -141C	case-control	MA+MO	200		464	not significant
Shepherd et al, 2002	DRD1	RFLP (*Bsp*)	case-control	MA+MO	262		262	not significant
	DRD3	RFLP (*BalI*)			252		252	not significant
	DRD5	microsatellite			273		273	not significant

DRD* = dopamine receptor subtype genes

DBH = Dopamine Beta-Hydroxylase

TDT = Transmission Disequilibrium Test

Serotonin-Related Candidate Gene Studies in Migraine

Authors	Gene	Type of marker tested	Study design	Type of migraine examined	Sample size			Conclusion (significance)•
					Affecteds		Controls	
					cases	families		
Buchwalder et al, 1996	5-HT2A 5-HT2C	microsatellites	linkage,sequencing	MA+MO		18		not significant, no mutation
Nyholt et al, 1996	5-HT2A	RFLP *(Msp*I)	linkage,case-control	MA+MO	96	3	91	not significant
Burnett et al, 1997	5-HT2C	RFLP *(Hinf*I)	case-control	MA+MO	242		129	not significant
Monari et al, 1997	5-HT1D 5-HT1B 5-HT2A SERT	microsatellites	linkage	MA+MO		14		not significant
Olgivie et al, 1997	Serotonin transporter	VNTR	case-control	MA+MO	266		133	**MA/MO - genotypic & allelic association (P<0.05)**
Lea et al, 2000	Serotonin transporter	VNTR	linkage,case-control,TDT	MA+MO	148	82	141	not significant
Yilmaz et al, 2001	Serotonin transporter	VNTR	case-control	MA+MO	52		80	**MA/MO - allelic association (P < 0.05)**
Emin Erdal et al, 2001	5-HT2A	RFLP *(Msp*I)	case-control	MA+MO	61		44	**MA/MO - genotypic association (P < 0.01)**

5-HT* = serotonin receptor subtype genes

TDT = Transmission Disequilibrium

Other Migraine Candidate Gene Studies

Authors	Gene	Type of marker tested	Study design	Type of migraine examined	Sample size			Conclusion (significance)
					Affecteds			
					Cases	Families	Controls	
Pardo et al, 1995	ABO Rh Acid Phosphatase Phosphoglucomutase 1 Adenosine Deaminase Haptoglobin Transferrin Alpha-1-antitrypsin D1S80 esterase-D GC 1F-1F	markers in or near candidate genes	case-control	MA+MO	112		Healthy Controls	not significant **P<0.05 allelic association** **P<0.05 allelic association**
Griffiths et al, 1997	endothelium NOS	microsatellite	linkage,case-control	MA+MO	91	4	85	not significant
Monari et al, 1997	calcium channel subunit (CACNA1B) calcium channel subunit (CACNA1A)	microsatellites	linkage (pedigrees)	MA+MO		14		not significant
Paterna et al. 2000	Angiotensin-converting enzyme (ACE)	ACE-SNP	case-control	MO	302		201	**MO frequency - genotypic (P<0.05)**
Kowa et al, 2000	Methylenetetrahydrofolate	C677T mutation	case-control	MA+MO	74		261	**MA/MO - genotypic association (P<0.05)**
Lea et al, 2001	Cytochrome P450 2D6 Inducible NOS (iNOS)	mutation tetra-repeat		MA+MO	261		252	not significant
Terwindt et al, 2001	calcium channel subunit (CACNA1A)	microsatellites	linkage (sibpairs)	MA+MO		189 sibs		**Excess allele-sharing (P<0.05)**
Lea et al, 2001	calcium channel subunit (CACNA1A)	microsatellites	linkage,case-control,TDT, sequencing	MA+MO	177	81	182	not significant
McCarthy et al, 2002	Insulin Receptor (INSR)	SNPs	case-controls	MA+MO	>1000		>1000	**MA/MO - allelic association (P<0.05)**

3.3.3 Genetic Heterogeneity of Migraine

Genetic heterogeneity typically refers to a situation in which any number of genetic causes can act independently to produce an identical disease phenotype. There are several types of heterogeneity. One form of genetic heterogeneity is allelic heterogeneity. This is a situation in which multiple separate disease alleles at the same locus can each cause the same disease phenotype. An example of this is cystic fibrosis, for which a number of different mutated alleles have been isolated, each of which can contribute to the CF phenotype (Ott 2000). Allelic heterogeneity is most easily detected using an association, rather than linkage, approach. The independent genetic association studies reported Peroutka et al. and Del Zompo et al, that found evidence of association of alleles at two adjacent markers in the *DRD2* gene to migraine, may indicate the presence of allelic heterogeneity at this gene locus.

Locus heterogeneity is a form of genetic heterogeneity that is of particular significance in linkage analysis situations. In this case disease alleles at two or more independently acting loci could each be responsible for the same disease phenotype (Sham 1998). Familial hemiplegic migraine is a rare type of migraine that has been shown to display locus heterogeneity. The first FHM region was localised to chromosome 19p13 (Joutel 1993) and the defective gene (*CACNA1A*) subsequently identified. However, it was found that not all families investigated demonstrated linkage to this genomic region. In fact, only 50% of pedigrees examined gave any evidence that this chromosome 19 locus was involved in FHM (Ophoff et al. 1996). Further investigation of another candidate gene region revealed the presence of a second FHM locus on chromosome 1q (Gardner et al. 1997). This genetic heterogeneity for FHM clearly illustrates that more than one gene is affecting FHM. It has been hypothesised that a biochemical pathway involving ion channels is being disrupted by defective genes. Where a mutation(s) at either locus can prevent the normal end product of the pathway (maybe serotonin) from being synthesised or released, causing the FHM phenotype (Ferrari 2000).

Locus heterogeneity can be further subdivided into diseases with multiple genetic causes, even with different modes of inheritance. When the mode of inheritance is the same for different genes underlying a disease a special type of linkage analysis that

tests for heterogeneity can be carried out (Terwilliger and Ott 1995). Utilising such tests on data from large Australian pedigrees affected with typical migraine, it has been conclusively demonstrated that this disease is genetically heterogeneous (Nyholt et al. 1997). To date, there is strong evidence suggesting that typical migraine susceptibility regions lie on chromosome 19p13 (at the FHM locus) and chromosome Xq24-28 (Nyholt et al. 1997; Terwindt et al. 2001; Nyholt et al. 2000). Furthermore, multiple positive associations have been reported implicating several different candidate genes in the aetiology of migraine (Pardo et al. 1995, Peroutka et al. 1997, Ogilvie et al. 1997). In conclusion, migraine is a genetically heterogeneous disorder. Although the type and number of genes involved is still not known it is probable that many genes are involved.

CHAPTER 4
METHODS AND MATERIALS

4.1 Study Designs

The objective of this research was to investigate the molecular genetics of migraine in an Australian Caucasian cohort with a focus on genomic susceptibility regions and specific candidate genes. The genetic nature of the disorder is complex and at present there is no single best method for localising and identifying the particular genes that predispose individuals. However, to increase the chance of identifying these genetic risk factors, this study utilised several different, yet complementary, research designs. The studies presented here involved allelic association, linkage/allele sharing, and mutation screening methods, incorporating DNA marker testing and direct sequence determination. These methods and techniques were applied using a large number of migraine affected pedigrees and unrelated case-control populations from Australia. The DNA examined was extracted from whole blood samples using standard techniques. All subjects were diagnosed with MA or MO based on the criteria specified by the International Headache Society. DNA microsatellite markers spanning migraine susceptibility regions on chromosome 1q and chromosome 19p were genotyped in the migraine pedigrees. In addition, markers within the candidate genes - *CACNA1A*, *INSR*, *DRD2*, *DBH*, *SERT* and *iNOS* were tested. All marker alleles were amplified by PCR and discrimination of fragments determined using RFLP and electrophoretic techniques. The resulting genotypic data were analysed using powerful computational and statistical methods such as GENEHUNTER and CLUMP to determine if any of the investigated loci contributed to migraine susceptibility.

4.2 Subject Ascertainment and Diagnosis

4.2.1 Diagnosis

All subjects who participated in this study gave informed consent and were of Caucasian origin residing mainly on the east coast of Australia. The subjects were recruited through a combination of local and national media coverage and migraine support groups. Migraine affected individuals were diagnosed as having either migraine with aura or migraine without aura through interview by an experienced clinical neurologist (Dr Peter Brimage and Professor John McMillan) and from semi-structured questionnaires prepared using strict IHS guidelines. The migraine questionnaire is shown in Appendix I. Those patients diagnosed as experiencing both types of migraine (MA and MO) were phenotyped as suffering from MA. Collectively, migraineurs with MA and/or MO were referred to as being affected with typical migraine.

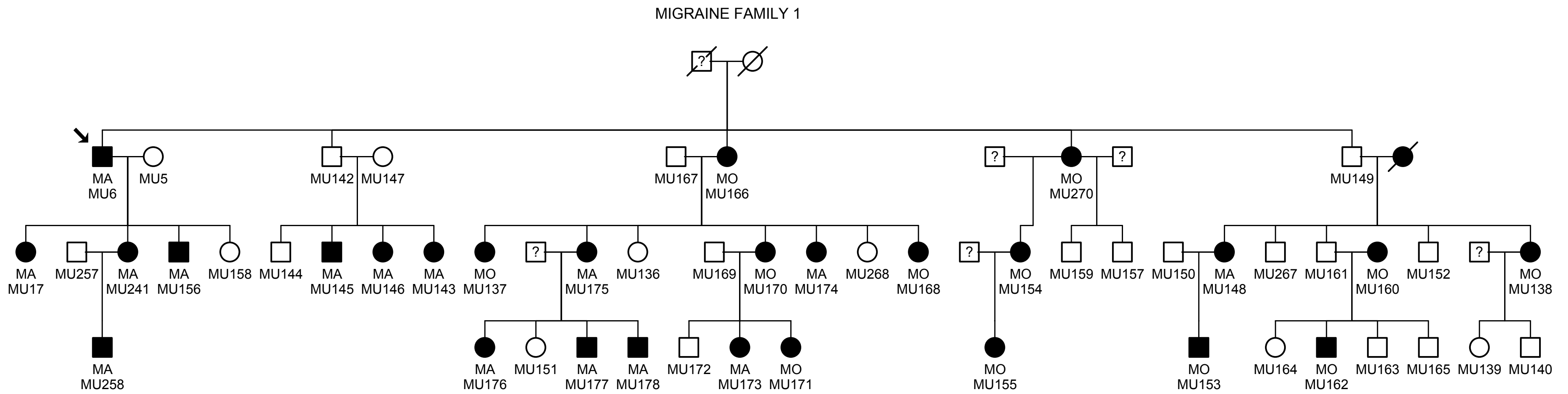
4.2.2 Unrelated Case-Control Samples

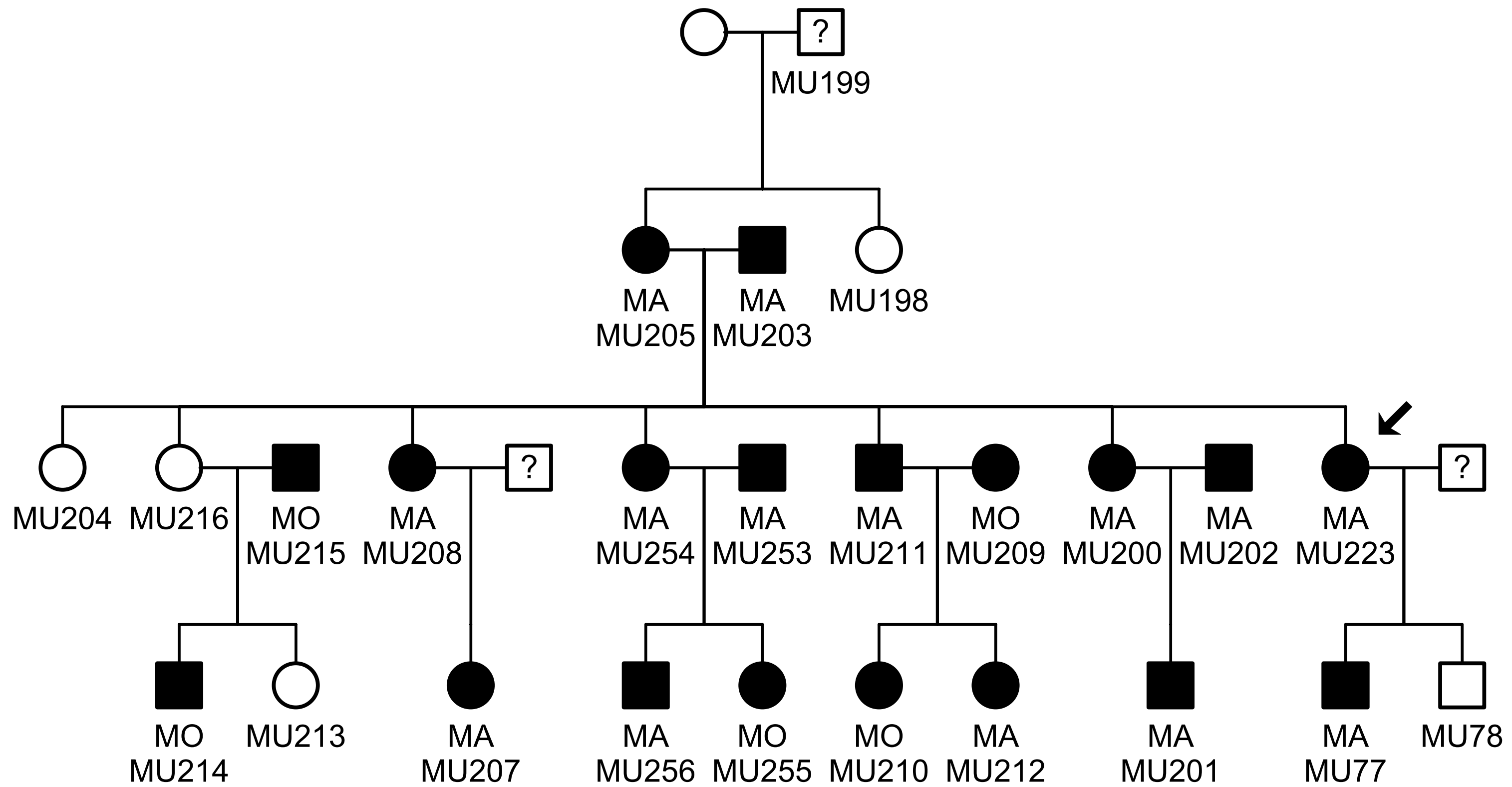
Migraine case-control association studies have been ongoing at the Genomics Research Centre (GRC) for approximately 8 years (Griffiths et al, 1996; Nyholt et al, 1997, Nyholt, 1998c). Over this time, the number of subjects included in the case-control groups has been continually increasing and is now balanced at 270 cases and matched controls. Of the migraine affected groups used in this study, ~90% of patients had a known family history of the disease, or at least one first-degree relative suffering from migraine. Clinically, the affected group had a median age of onset of ~19yrs, average headache duration of ~20 hours with a frequency of approximately ~30 per year. This case group was comprised of individuals diagnosed as clearly having MA or MO. The proportion of MA affected individuals in the case group varied between 55% - 60%. The unaffected control group was recruited from the same geographical location (East Coast of Australia) as the affected group, had <10% of individuals reporting a positive family history of migraine and was carefully matched for age, gender and ethnicity, thus reducing the possibility of spurious results due to population stratification.

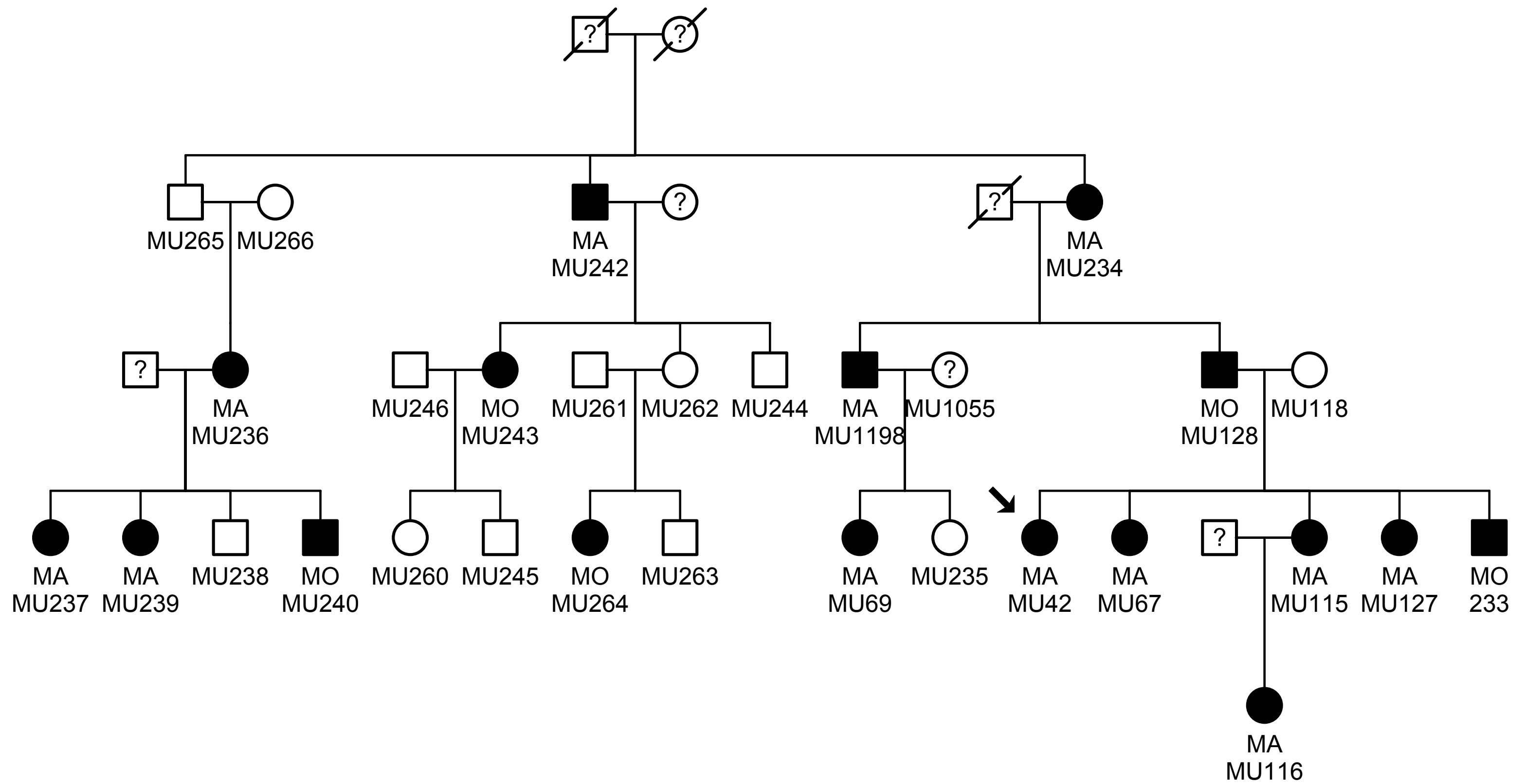
4.2.3 Migraine Pedigrees

Three large multigenerational pedigrees were previously collected and utilised in migraine linkage studies at GRC (Nyholt et al, 1998a; Nyholt et al, 1998b). These three pedigrees included 106 individuals for whom DNA was available (as indicate by the sample number preceded by MU). A total of 63 individuals were classified as being affected with typical migraine after being clinically diagnosed as either migraine with aura (n=41) or migraine without aura (n=22). DNA was available for 27 affected and 24 unaffected individuals in MF1; 19 affected and 6 unaffected individuals in MF7; 17 affected and 13 unaffected individuals in MF14. The overall male to female ratio in these pedigrees was 1:2 (Figure 4.01).

Figure 4.01. The three large multigenerational pedigrees - MF1, MF7, and MF14 investigated in this research. Blackened symbols indicate subjects affected with MO or MA. MU* indicates individuals from which blood (and DNA) was available.




MIGRAINE FAMILY 7
?
MU199
MA
MU205
MA
MU203
MU198
MU204
MU216
MO
MU215
MA
MU208
?
MA
MU254
MA
MU253
MA
MU211
MO
MU209
MA
MU200
MA
MU202
MA
MU223
?
MO
MU214
MU213
MA
MU207
MA
MU256
MO
MU255
MO
MU210
MA
MU212
MA
MU201
MA
MU77
MU78


MIGRAINE FAMILY 14
?
?
MU265
MU266
MA
MU242
?
?
MA
MU234
?
MA
MU236
MU246
MO
MU243
MU261
MU262
MU244
MA
MU1198
?
MU1055
MO
MU128
MU118
MA
MU237
MA
MU239
MU238
MO
MU240
MU260
MU245
MO
MU264
MU263
MA
MU69
MU235
MA
MU42
MA
MU67
?
MA
MU115
MA
MU127
MO
233
MA
MU116

As well as investigating the large families MF1, 7 and 14, this research also involved ascertainment of DNA from 296 subjects comprising 82 unrelated families affected with migraine (shown in Appendix II). Of the 296 subjects ~70% were diagnosed as being affected with MA. These pedigrees were comprised of 252 affected relative pairs excluding parent-child pairs (Table 4.01). All families had at least two affected individuals and at least one parent was available for genotyping in all but 10 pedigrees.

Table 4.01. Affected Relative Pairs of a Sample of 82 typical migraine Pedigrees

Category	No. of individuals with Typical Migraine
Total Families	82
Affected relative pairs	
Sibling pairs	97
Half-sibling pairs	4
Uncle (aunt)-nephew (niece) pairs	90
Grandparent-grandchild pairs	24
Great Grand Parents-grandchild pairs	3
Cousin pairs (first + second)	19
Great uncle (great aunt)-nephew (niece) pairs	15
Subtotal	252

* All possible combinations of pairs formed by all affecteds within each family (excluding parent-child pairs)

4.3 DNA Extraction and Quantitation

4.3.1 White Blood Cell Isolation

To prepare the DNA for analysis 10ml of blood from each subject (contained in EDTA collection tubes) was thawed (from -70°C). The samples were shaken and transferred to 50ml centrifuge tubes. The original test tubes were washed out with NKM buffer (0.14M NaCl-30mM KCl-3mM $MgCl_2$) and centrifuge tubes increased to 25ml volume. Each sample was then vigorously shaken and centrifuged at 4800rpm for 25mins at room temperature. The supernatant was discarded from each tube. Care was taken to ensure that the white cell layer on top of the packed red blood cells remained intact. 10ml of cold RSB buffer (10mM Tris pH 7.5-10mM NaCl-3mM $MgCl_2$) was added to lyse the remaining red blood cells. The samples were mixed well and increased to a final volume of 25ml with RSB buffer. Centrifugation was performed at RT for 15mins at 4000rpm. The supernatant was removed and the WBC pellet resuspended in 1ml of RSB. 4ml of *lympholysis* and 250µl of *proteinase K* was added to each sample. These ingredients lyse the WBCs. All solutions were placed in a water bath set at 37°C and shaken vigorously overnight.

4.3.2 Extraction of Genomic DNA

Following overnight digestion of cell lysates from 10ml of anticoagulated blood, 2ml of sterile saturated NaCl (approx. 6M) was added to each sample and mixed for 15secs. The samples were then centrifuged at RT for 15mins at 2500rpm. The supernatant containing the DNA was carefully decanted and placed into 10ml capped tubes. Centrifugation was repeated to pellet any transferred proteins and the supernatant was again decanted. The volume of supernatant was accurately measured and approximately two volumes of absolute ethanol were added to each tube. The samples were gently swirled and precipitation of DNA strands observed. Using a plastic rod the DNA was transferred into a clean tube containing 2ml of TE buffer pH 8 (10mM Tris-Cl-EDTA). The DNA was then dissolved in the TE buffer at 37°C for 4-6 hrs.

4.3.3 Ethanol Precipitation and Quantitation of DNA

After the DNA was fully dissolved, 250µl of the DNA solution was added to 500µl of absolute ethanol. 190µl of 10M-ammonium acetate was then added to the solution. to remove residual triphosphates. To allow precipitation to take place the samples were stored at -20°C for 30mins. Centrifugation was then performed at 11000rpm for 15mins at 4°C and the supernatants were carefully removed from each sample. The pellet was than resuspended in 1ml of sterile water. The DNA was quantitated by determining the optical density (OD) at 260nm with an OD of 1.0 being equivalent to 50µg/ml of dsDNA.

4.4 Primer Preparation

Oligonucleotide primer sequences for all DNA fragments to be amplified were obtained from previously published sources (see results chapters for sequences). Primers were purchased from either *Research Genetics* (200µl supplied at 20µM in 1 ×TE, pH8), *Perkin Elmer*, *Bresatec* companies.

A purification step was often required in order to remove organic by-products produced during their synthesis. A rapid method of oligonucleotide purification, through n-butanol, was used: 100µl of oligonucleotide solution in 30% ammonium hydroxide were vortexed vigorously in a 1.5ml eppendorf tube with 1000µl n-butanol (ACS reagent grade) for 15 seconds, then centrifuged for 1 minute at 11,000 rpm. The single H_2O-containing n-butanol phase was removed and discarded. The pellet was then resuspended in 100µl of H_2O and the n-butanol extraction was repeated to achieve complete removal of contaminants. Following n-butanol extraction, the pellet was dried under vacuum and resuspended in 100µl of H_2O. The concentration was determined by reading the optical density at 260nm, where an OD of 1.0 is equivalent to 40µg/ml.

4.5 PCR Amplification

All DNA fragments were amplified using a *PERKIN ELMER GeneAmp PCR SYSTEM 9600* thermal cycler (Applied Biosystems, Foster City, CA). In general, the PCR reactions were carried out in a final volume of approximately 10-15µl containing approximately 25ng of DNA, 5µM primer mix, 2.5mM dNTPs, 1.75mM $MgCl_2$, 10 x buffer II, 5 units/µl of *Taq* polymerase and sterile water.

The PCR reactions were typically performed under the assumed following thermal cycle conditions. Firstly, the mixture was heated to 94°C and held for 5mins. Then 30 cycles of denaturation, annealing and extension at 94°C for 15secs, 55°C for 15secs and 72°C for 30 secs respectively was implemented. Finally, each typical reaction was held at 72°C for 10mins and then allowed to cool to 4°C. PCR conditions specific for each analysis fragment are shown in the respective results chapters.

One of the objectives of this project was to genotype all subjects as efficiently as possible. A multiplex PCR allows simultaneous amplification of multiple markers. This can greatly reduce the amount of individual reactions necessary for PCR analysis saving valuable laboratory reagents and time. Although it is common practice for markers to be amplified individually, the small size of the amplified segments (100-300 bp) suggested that theoretically several loci could be easily amplified simultaneously by multiplex PCR and then analysed with precise allele identification by Gene Scanning. Edwards et al (1991) demonstrated successful amplification of 8 and 9 genetic-site multiplexes for the hypoxanthine phosphoribosyltransferase and dystrophin genes (Edwards et al, 1991).

The results of the multiplex experiment indicated that, although not consistently, a 6-plex PCR reaction could be achieved, and 2-3-plex reactions amplified fragments approximately 50% of the time. However, this failure rate was too high for multiplexing to be of benefit in this research. Even the substitution of Ampli*Taq* with the more efficient Ampli*Taq* Gold polymerase failed to substantially increase the proportion of successful multiplex reactions.

There are several main reasons for the non-amplification of all primers in a single PCR reaction. Firstly, the presence of impurities in the template DNA solution may have prevented primer annealing. Also, slight variations in the concentration of reaction components may occur during mixing. This can effect one or more chemical interactions (Nuovo, 1992). In addition, it is known that primers may be competitive during multiplexing. Therefore, more effective primers will consume more reaction ingredients like dNTPs, thus amplifying at a greater rate and intensity. By amplifying primers by individual optimisation these problems were avoided and the number of successful reactions was greatly increased (>90%). Although multiplex PCR was not utilised in this study further development of these working protocols would offer valuable advantages to the speed at which microsatellites alleles, in particular, are sized and genotyped. DNA pooling techniques may also be of great benefit.

The PCR products for the microsatellite markers used in this research differed in length between individuals. This is because individuals have a different number (n) of repeat units. Each of these different fragment lengths represents an allele. The exact size of these alleles and therefore the genotype of the subjects for the particular marker were determined (sized) by GENESCAN® analysis using an automated ABI 310 Genetic Analyser.

4.6 Agarose Gel Electrophoresis (AGE)

Following PCR, certain marker fragments, such as the *DRD2* and *INSR* SNPs, required digestion with specific restriction enzymes (see results chapters). To discriminate alleles such fragments were electrophoresed through 2% agarose slab gels (*Promega*). Typically, the gel contained 1x TAE (40mM Tris-acetate pH 8.0 2mM EDTA), and 5ul EtBr (Ethidium bromide). There was 2µl of loading dye (37% sucrose, 0.05% bromophenol blue, 50mM EDTA pH 8.0) added to ~10µl of each sample prior to electrophoresis, which was carried out in a *Pharmacia* Gel Electrophoresis Apparatus at 100 volts, for approximately half to one hour at room temperature. EtBr stained bands in agarose gels were visualised on a *Pharmacia* Macrovue UV light box. They were photographed using a *Polaroid* camera with *Polaroid* Type 667 film. Visual verification of the expected PCR products was

performed to optimise PCR conditions of all microsatellite and sequencing fragments before genotyping or sequencing via fluorescence-based electrophoresis on the ABI 310 or ABI 377, respectively.

4.7 Gene Scan Analysis of Microsatellite Markers

All microsatellite PCR products were fractionated on an ABI-310 DNA Genetic Analyser (Figure 4.03) that features fluorescence detection and fragment analysis software (Applied Biosystems, Foster City, CA). This instrument is an automated system capable of determining base sequence or size and quantity of DNA fragments. The system consists of a capillary electrophoresis component and associated GENESCAN® analysis software for allele calling. For the ABI 310 Genetic Analyser, 0.5µl of internal size standards and 1µl of each sample were combined with 12µl of deionised formamide. When multiple (usually ≤ 4) samples were pooled the amount of formamide was adjusted. Samples were then heated to 95°C for 4 minutes immediately prior to automated loading of 1.5µl from this mix.

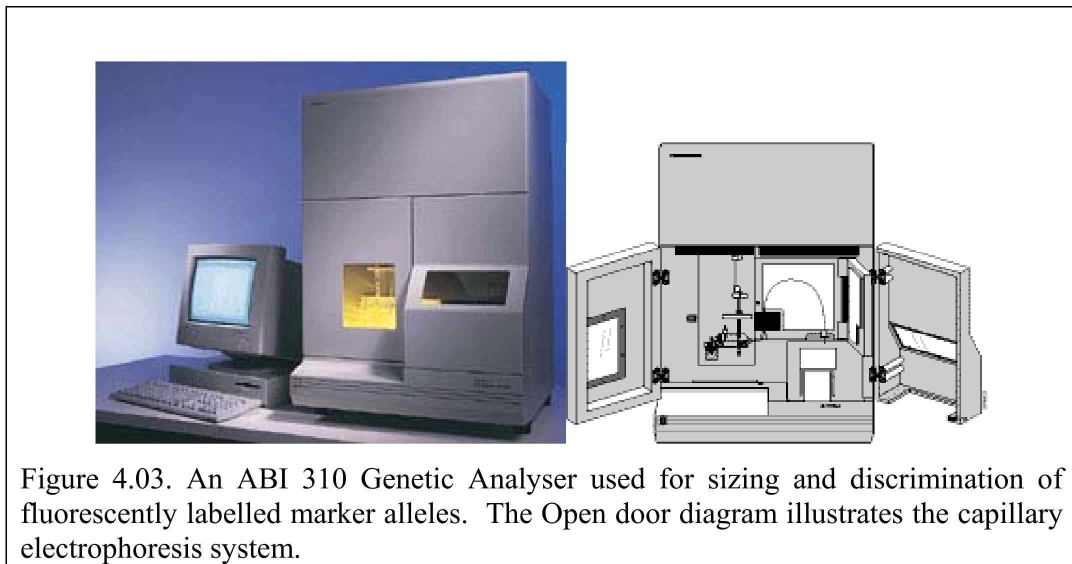


Figure 4.03. An ABI 310 Genetic Analyser used for sizing and discrimination of fluorescently labelled marker alleles. The Open door diagram illustrates the capillary electrophoresis system.

The size of PCR products was determined by reference to internal lane size standards by applying the method of Carrano et al. (1989). The GENESCAN® software (*ABI Prism*) automatically recognises the expected fragment size distribution of the size standards that were labelled with the TAMRA (red) dye. A calibration curve based on

the standard size fragments was used to estimate the sizes of PCR products labelled with either the HEX (yellow) dye, the FAM (blue) dye and the TET (green) dye. Results were presented in a spreadsheet format. Gel band fluorescence intensities were also displayed as peaks distributed across a user-selected DNA fragment size range. These graphical displays are referred to as electrophoretograms (Figure 4.4).

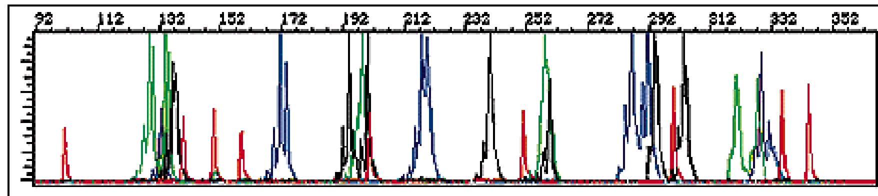


Figure 4.04. Electropherogram showing the resolving power of multiple PCR products labelled with different dyes. The resolution is 1 nt from 20-250 bp and 2 nt from 250-350 bp.

This allowed separate analysis of the data obtained from each gel lane/capillary run for the PCR products expected in a particular size range and dye colour combination. Scores (allele sizes in nucleotide bases) were assigned by the GENOTYPER® software and manual user interaction to distinguish major peaks from background artefact peaks for each allele (Carrano et al. 1989; Ziegle et al. 1992).

Artifact (stutter) peaks were evident in all electrophoretograms even those of high peak resolution. This phenomenon is seen when minor peaks, of lower fluorescence intensity, lag the major peaks by 1 or 2 bp. These smaller sized fragments are due to polymerase skipping (deletion of nucleotides within the core of the microsatellite repeat) (Ziegle et al, 1992). When heterozygote alleles differ by only 2 nucleotides, it is expected that the major peak of the smaller allele will be greater in signal intensity (ie., peak height) because it overlaps with the skipping product of the larger allele (Ginot, 1996).

Slippage of *Taq* polymerase (skipping) during PCR also complicates the migration profile of the microsatellite marker by its ability to add an extra base to the end of the amplified fragments in a template dependent manner (Ginot, 1996). This characteristic of *Taq* generates spurious peaks known as shadow peaks that are 1 bp longer than the true allele peaks. This complicated pattern is relatively easy to

recognise by an experienced eye, but may mislead or confuse the allele sizing software. Ginot et al (1995) were able to reduce most allele calling errors by eliminating the extra base added by *Taq* polymerase. By manually treating the PCR sample with T4 polymerase, which cleaves the extra base, these researchers reduced allele calling error by GENESCAN software, from ~5% to ~1% (Ginot et al, 1995). This procedure, although corrective, would be both more time consuming and expensive, and was therefore not included in this research. Therefore, all automated allele calling was manually checked for quality control.

Prior to running samples on the ABI 310 a sample sheet was prepared that contained information about the samples to be analysed (Figure 4.05). An injection list was also preset with important electrophoresis parameters. The injection time, which is proportional to the amount of PCR product sampled, was usually set between 5-15secs. The run time was set between 20-30mins (Figure 4.06). The genotypes detected by the GENETIC ANALYSER software were either heterozygous or homozygous. Successful amplification and detection yielded one or two allele peaks with the associated PCR stutter peaks. The number of allele peaks depended on whether the individual tested was a heterozygote or homozygote.

Sample Sheet "dbh cont (SS)-26/8/98 5.24 PM"

GeneScan™ Sample Sheet

#	Sample Name	Color	Std	Pres	Sample Info	Comments
A1	N3	B		☒	dbh controls	
		G		☒		
		Y		☒		
		R	◆	☒		
A2	5	B		☒		
		G		☒		
		Y		☒		
		R	◆	☒		
A3	6	B		☒		
		G		☒		
		Y		☒		
		R	◆	☒		
A4	9	B		☒		
		G		☒		
		Y		☒		
		R	◆	☒		
A5	10	B		☒		
		G		☒		
		Y		☒		
		R	◆	☒		
A6	16	B		☒		
		G		☒		
		Y		☒		
		R	◆	☒		
A7	18	B		☒		
		G		☒		
		Y		☒		
		R	◆	☒		
A8	21	B		☒		

Figure 4.05. An example sample sheet used for Gene Scan analysis

Injection List-1/8/01 2.35 PM

Sample Sheet: 96 SAMPLES-A... Skip Pause Cancel

Length to Detector: 30 cm Operator:

Inj.#	Tube & Sample Name	Module	Inj. Secs	Inj. kV	Run kV	Run °C	Run Time	Matrix file	Auto Anlz	Analysis Parameters	Size Standard
26	C2 - C2	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
27	C3 - C3	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
28	C4 - C4	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
29	C5 - C5	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
30	C6 - C6	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
31	C7 - C7	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
32	C8 - C8	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
33	C9 - C9	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
34	C10 - C10	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
35	C11 - C11	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
36	C12 - C12	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
37	D1 - D1	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
38	D2 - D2	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
-->	D3 - D3	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/
40	D4 - D4	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/1
41	D5 - D5	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/1
42	D6 - D6	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/1
43	D7 - D7	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/1
44	D8 - D8	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/1
45	D9 - D9	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/1
46	D10 - D10	GS STR POP4 (2.5 mL	10	15.0	15.0	60	23	GS FAMHEXTET	☒	1/11/99	350tamra 18/1

Figure 4.06. An example injection list used for Gene Scan analysis

4.8 DNA Sequence Analysis

Direct DNA sequence analysis was performed in an attempt to detect disease-causing mutations within the coding region of the *CACNA1A* gene on chromosome 19p13. Specific oligo nucleotide primers were employed for amplification of *CACNA1A* exonic fragments (see Chapter 5 for a list of primer sequences). Before sequencing assays could be carried out the PCR product was purified using a high pure PCR product purification kit.

Following PCR product purification the sequence reactions are performed. These are carried out with Ampli*Taq* ™ DNA Polymerase FS dye terminator cycle sequencing chemistry using the 'ABIPRISM BigDyε™ Terminator Cycle Sequencing Ready Reaction Kit' according to the manufacturer's protocol (Perkin Elmer Applied Biosystems Division, Foster City, CA). This kit contains the four ddNTPs with different fluorescence labels (BigDyε™ Terminators). 2µl PCR product and 3pmol of the sequencing primer are used in a 20µl sequencing reaction. The sequencing reaction mixes were subjected to 25 cycles (95°C for 30secs, 50°C for 15secs and 60°C for 4mins) in a Perkin Elmer thermal cycler 9600.

The resulting sequencing reaction products underwent electrophoresis using an automated ABI 377 sequencer (Applied Biosystems, Foster City, CA). Specifically, a 0,2mm thick 5% polyacrylamide (acrylamide/bisacrylamide 29:1) - 7 M urea gel was used [gel composition: 21.0 g urea, 8.4 ml 30% acrylamide, 6.0 ml TBE buffer (10x TBE-buffer: 108.0 g Tris base, 55.0 g boric acid, 7.4 g Na^{2}EDTA), 15 µl TEMED, 360 µl 10% Ammonium Persulfate (0,1 g Ammonium Persulfate in 1 ml bidistilled water)]. Electrophoresis was run at constant 48 watt for 8h. Data collection is initiated immediately after starting the electrophoresis on the ABI 377. Following data collection sequence analysis was performed using the SEQUENCHER and EDITVIEW programs. The sequence data was provided in two forms for mutation detection analysis. These were as chromatograms and as standard letter sequence strings. The test sequence was aligned with a known control sequence obtained from the NCBI database. The SEQUENCHER program highlights any base differences detected between the control and test sequence. These differences are further assessed by viewing the underlying chromatogram produced by the EDITVIEW program (Figure 4.07).

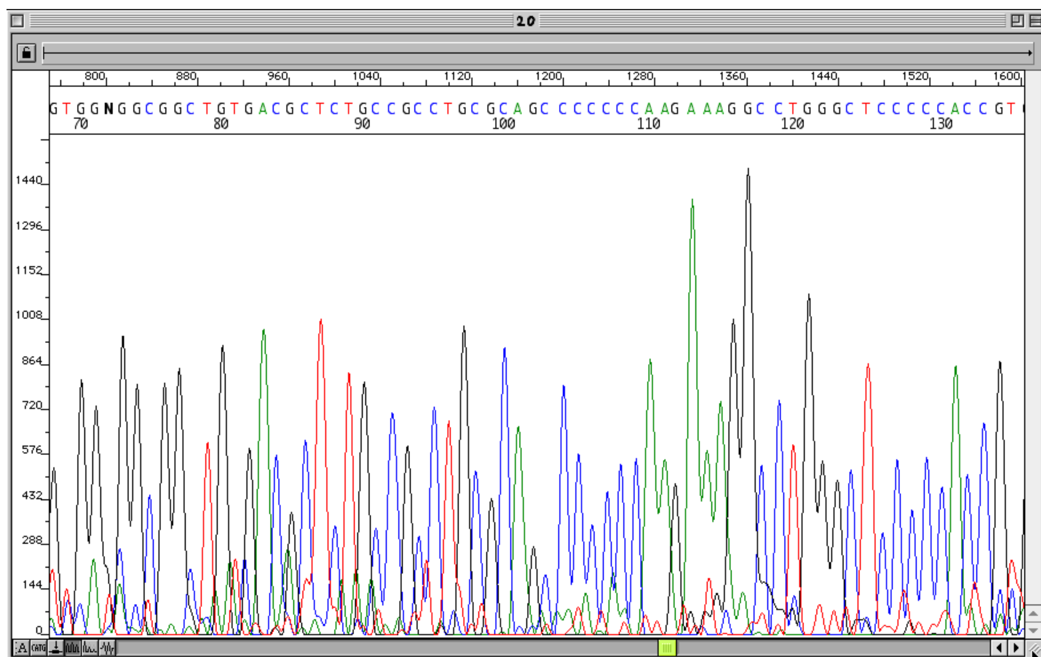


Figure 4.07. An example sequence electrophoretogram produced by the program SEQUENCHER

4.9 Statistical Data Analysis

4.9.1 Computer Programs and Systems

Most statistical genetic analysis programs utilised in this study were downloaded via the internet either from the program Authors website or using links published on the Rockefeller University Statistical Genetics department internet site; http://linkage.rockefeller.edu/soft/list.html. The Statistical Package for the Social Sciences (SPSS) program and Microsoft Excel Spreadsheet software were also employed for data management and certain statistical analyses. The CYRILLIC (v2) program was used for all pedigree drawing and haplotype forming.

The programs HWE, PEDMANAGER, SPLINK, SimIBD, FASTLINK and TRANSMIT were installed and executed on the Australian National Genome Information Services' (ANGIS) Sun Microsystem SPARC station (www.angis.org.au). The platform for this hardware was a UNIX operating system (SUNOS 5.7). The GENEHUNTER-PLUS and ALLEGRO programs were used for large pedigree analysis and involved intensive computations that required large amounts of random access memory (RAM) often exceeding that provided on the ANGIS system. Thus, these programs were installed and run on a Compaq Deskpro Pentium III running LINUX Redhat (6.5) with 512MB of RAM and 5Gb of swapspace. The programs (SPSS, EXCEL, CYRILLIC, FBAT) and (CLUMP) were run on the same PC but under a Windows 95 and DOS environment, respectively.

4.9.1.1 Pedigree Data Management and Error Checking

All pedigree analysis required the compilation of raw genotype data into standard linkage pedigree files as described in following section (4.9.2). The Microsoft Excel program was utilised to prepare these raw data files as seen in Table 4.02. Following input of the raw marker genotype data and pedigree structure information, the "micorsoft.xls" files were save as standard "text only" (.txt) files and subsequently transferred (as an input file) to the PEDMANAGER program (Whitehead Institute of Biomedical Research, 1995). This program was designed to ease the transition of raw data from spreadsheet format to linkage style format ready for analysis. PEDMANAGER also has the added benefit of being able to calculate marker allele

frequencies and determine Mendelian inheritance errors in the raw data. A typical PEDMANAGER output showing the existence of likely genotyping errors for a marker in a single pedigree is shown in Table 4.03. Useful pedigree statistics calculated by PEDMANAGER are also provided at the bottom the Table. All inheritance errors identified by PEDMANAGER were manually re- checked from raw GENESCAN electrophoretograms. Calling errors and failed genotypes were corrected where possible and null genotypes due to failed PCR were repeated.

Table 4.03. Output from the PEDMANAGER Program

```
*********************************************************************
  Output from:                            Fri Dec 14 12:33:18 2001
         PEDMANAGER - check, preprocess, and draw pedigrees
                         (version 0.9)
*********************************************************************

'photo' is on: file is 'chr1.error'

pedmngr:2> load chr1a.txt
=====================================================================
Non-Mendelian inheritance in pedigree 3 at marker 15
can't assign child 12's alleles

parents' IDs: 6 & 7
parents' genotypes:          0   0, and   6   6
parents' forced/(assigned):  5   0, and   6   6

child   10's alleles:   3   6
child   11's alleles:   5   6
child   12's alleles:   2   5
child   13's alleles:   5   6
child   14's alleles:   5   6
=====================================================================
91 pedigrees checked -> 297 Mendelian-inheritance errors
             ==========PEDIGREE STATS==========

91 pedigrees loaded --> genotype data for 31 markers

Average pedigree size: 6.7 (614 individuals total)
        Smallest pedigree 4 individuals (1 pedigree)
        Largest pedigree 28 individuals (20 pedigree)

Number of individuals missing genotypes at all markers: 183 (29.8%)
   for the remaining individuals 21.3% of the genotypes are missing
             ==================================
```

4.9.2 Model-Based Traditional Linkage Analysis

Traditional LOD score analysis was performed in large pedigrees affected with migraine using the FASTLINK (Lathrop et al. 1985) and GENEHUNTER (Kruglyak et al, 1996) programs. Since this type of analysis is model-based a pre-specified set of migraine disease model parameters was required. The parameters used are listed in Table 4.04 and are based on conservative estimates of 70% penetrance and a disease gene prevalance of ~12-14% as used by Horvatta (1994). As input, these programs require standard LINKAGE files. These files include a *pedigree* data file (Table 4.02) containing information on pedigree structure, the affection status of the subjects and marker genotypes, and a *locus* data file (Table 4.05), which contains marker map distances and disease and marker allele frequencies (Terwilliger and Ott, 1995).

Table 4.04. Values Used for Parametric Linkage Analyses*

Parameter	Value
Mode of Inheritance	Dominant – Autosomal
Disease Frequency (population prevalence)	12-14% (0.12 – 0.14)
Disease Penetrance	70%-80% (0.7-0.8)
Phenocopy Rate	0.7% (0.007)
Mutation Rate	either zero (0) or 0.000001
Number of Liability Classes	one (1)

* All analyses assumed equal male and female recombination (i.e., $\theta_m = \theta_f$)

Table 4.02. An example of a pedigree file used for parametric linkage analyses

Family	Individual	Father	Mother	Gender	Affection	Marker
2	1	0	0	1	2	7 8
2	2	0	0	2	1	0 0
2	3	1	2	1	2	6 8
2	4	1	2	1	2	6 7
3	1	0	0	2	0	0 0
3	2	0	0	1	1	0 0
3	3	2	1	2	2	7 7
3	4	2	1	2	2	1 7

Table 4.05. An example of a locus file used for parametric linkage analyses

```
Example 'locus' file
2 0 0 5   << NO. OF LOCI, RISK LOCUS, SEXLINKED (IF 1) PROGRAM
0 0.0 0.0 0  << MUT LOCUS, MUT MALE, MUT FEM, HAP FREQ (IF 1)
1  2  << LOCI NAMES AND ORDER
1  2  << AFFECTION, NO. OF ALLELES
0.88  0.12  << GENE FREQUENCIES (I.E. 12% DISEASE FREQUENCY)
1  << NO. OF LIABILITY CLASSES
0.007  0.7000  0.7000  << PENETRANCES
3  10  << ALLELE NUMBERS, NO. OF ALLELES
0.1  0.1  0.1  0.1  0.1  0.1  0.1  0.1  0.1  0.1 << FREQUENCIES
0 0  << SEX DIFFERENCE, INTERFERENCE (IF 1 OR 2)
0.01  << RECOMBINATION VALUES
1 0.1 0.5  << REC VARIED, INCREMENT, FINISHING VALUE
```

4.9.3 Model-Free Allele-Sharing Analysis

4.9.3.1 SPLINK Analysis

In this research, ASP analysis was performed on markers spanning the *CACNA1A* gene on chromosome 19p13. From the migraine pedigrees shown in Figure 4.02, there were a total of 97 sibpair comparisons available for analysis of these marker loci. SPLINK (SibPair LINKage) is a program developed for linkage analysis using affected sib pairs (ASPs) (Clayton, 1995). The maximum likelihood algorithms built into the SPLINK were used to estimate the probability that two affected siblings share 0, 1 or 2 alleles IBD in the migraine affected pedigrees. SPLINK input consists of a LINKAGE format pedigree file (Table 4.02) (Clayton 1995). The SPLINK program has a number of run options which are specified using flags when invoking the program. The SPLINK analyses were run by entering the following commands in the UNIX environment.

```
splink <pedfile.pre –mf –l1 -wt>outputfile.out
```

where;
1. The *pedfile.pre* is the genotype datafile for the pedigrees being tested
2. The *–mf* flag instructs the program to use multiple nuclear families from a single extended pedigree
3. The *–l1* flag indicates that there is only 1 marker being tested
4. The *–wt* flag weights the nuclear families according to the number of siblings in a sibship

Risch (1990) has demonstrated that the no. of sibpairs required to achieve a given statistical power is a function of the magnitude of the genetic effect, measured as

sibling relative risk (λs). The λs for migraine has been estimated at between approximately 1.5 and 4 (Russell and Olesen, 1995; Stewart et al, 1997). Therefore, assuming a conservative and realistic λs of ~2 for migraine, and using the 97 sibpairs available, the power of the ASP analysis to detect a gene conferring a modest susceptibility to the disease was estimated at <80%. This power was less than the acceptable level (80%) and therefore more powerful affected relative methods were also employed.

4.9.3.2 SimIBD Analysis

The SimIBD program was used in this research to perform allele-sharing analysis in relative pairs affected with migraine. This software package requires two LINKAGE-format input files, a pedigree file (after processing with the MAKEPED program) and a locus file. As part of the output SimIBD provides pedigree statistics as well as simulation replicates, weighted *Z* scores with corresponding *P*-values (Table 4.06).

Table 4.06. Output File for SimIBD. The global *P*-value for this analysis is shown in bold at the bottom of the table

```
SIMIBD  V2.1
Last modified:  January 17, 1998

Pedigree file:  c1a.ped
Locus data file:  c1.dat

Weighting function is f(p) = 1/sqrt(p)

              Families  Total   Genotyped   Genotyped   Genotyped
    Marker      Used    People  Affecteds  Unaffecteds  Unknowns
         4        73     372       165          32           2

   Observed   Null Distribution   Bootstraps
  Replicates     Replicates        performed
         100         500            500000

Affected Comparison
                          -- Summary --
  Family   ZObs    # Aff.  Weight  Weighted ZObs  PValue   Range
  -------------------------------------------------------------
       2         0     3  0.707107            0    0.913  0.087
       3   21.1717     6  0.447214      9.46826    0.398  0
       4   18.0821     5       0.5      9.04103     0.51  0
      13         0     3  0.707107            0      0.5  0.5
      15   36.2198     7  0.408248      14.7867    0.502  0
      17   4.59552     3  0.707107      3.24953     0.43  5.55112e-17

   TOTAL                              191.944  0.82458  1.38778e-17
```

4.9.3.3 GENEHUNTER-PLUS and ALLEGRO Programs

The GENEHUNTER-PLUS (GHP) (Kong and Cox, 1998) & ALLEGRO (Gudbjartsson et al, 2000) programs are extensions of the well-known GENEHUNTER software package (Kruglyak et al, 1996). These programs were used extensively in this research to perform allele sharing and haplotype analysis in the migraine pedigrees.

GENEHUNTER-PLUS and ALLEGRO Input

Both GENEHUNTER-PLUS and ALLEGRO require a standard LINKAGE format pedigree and locus file as well as a batch file for input. The batch files contain information about the types of analysis to be performed. An example GENEHUNTER-PLUS batch file is shown in Table 4.07. This file indicates to the program that a model-based and model-free multipoint analysis should be performed. Following GENEHUNTER-PLUS analysis the Allele Sharing Model (ASM) auxillary program can be run to fit either the "linear" or "exponential" allele-sharing models when calculating LOD scores (Kong and Cox, 1998). The standard GENEHUNTER-PLUS analysis will produce two output files, "nullprobs.dat" and "probs.dat", containing information about the distribution of the test statistic; one under the null hypothesis, and the other conditional on the position and marker data for each pedigree. The ASM program expects the presence of those two files in the directory in which it is being run.

The ASM program is invoked using the following commands:

```
1) asm LIN
2) asm LIN GRID dmin dmax numintervals
3) asm EXP
4) asm EXP GRID dmin dmax numintervals
```

Table 4.07. Input batch file for GENEHUNTER-PLUS. The bold commands instruct GHP to perform a multipoint model-free and model-based analysis using the S_{pairs} scoring function

```
photo ghm.out
load markers chr19.dat
use
single point off
count recs off
haplotype off
discard off
max bits 20
skip large off
analysis both
score pairs
postscript on
off end 0.0
increment step 2
scan pedigrees chr19.pre
total stat
```

ALLEGRO has the added advantage of being able to perform allele sharing analysis for several different allele sharing models, simultaneously (Gudbjartsson et al, 2000). An example ALLEGRO batch file is shown in Table 4.08.

Table 4.08. Input Batch File for ALLEGRO**.** These commands instruct GHP to perform a multipoint and singlepoint model-free and model-based analysis using the exponential allele sharing model and S_{pairs} and S_{all} scoring functions

```
% Read input in LINKAGE format from pre-file pre and dat-
file dat
PREFILE allegro-4.pre
DATFILE allegro-4.dat

% Linkage analysis to be performed
MODEL mpt par het param.mpt
MODEL spt par het param.spt
MODEL mpt exp pairs equal exppairs.mpt
MODEL spt exp pairs equal exppairs.spt
MODEL mpt exp all equal expall.mpt
MODEL spt exp all equal expall.spt
MODEL mpt exp robdom equal robdom.mpt
MODEL mpt lin all power:1 linall.1.mpt
```

GENEHUNTER-PLUS and ALLEGRO Output

The typical output file format from the allele sharing analysis performed by the GENEHUNTER-PLUS and ASM program includes NPL scores, marker information maps and allele sharing LOD* scores as is shown Table 4.09. Similarly, the output for ALLEGRO has 6 columns (Table 4.10). The first column is the marker location in cM. All other columns contain statistical quantities. The 'LOD' column is the allele sharing LOD* score (as produced in GHP), 'dhat' is the maximum likelihood estimator of δ in the allele sharing model, 'NPL' is the Non-Parametric Linkage score, 'Zlr' is Zlr = sign(dhat) and info is the measure of information. Note that as the LOD score is asymptotically distributed as χ^2_1, Z_{lr} is asymptotically normal with mean zero and variance one. A negative dhat means that there is less sharing observed than would be expected under the null hypothesis of no difference in allele sharing and may indicate genotyping error (Gudbjartsson et al, 2000).

Table 4.09. Output File for GENEHUNTER-PLUS

```
npl:17> total stat
Totalling pedigrees: ..................................
position LOD_score   NPL_score  p-value     information
loc1     -6.657604    -1.35962 0.928025      0.634724
loc2     -3.981240    -0.94544 0.841553      0.408595
loc3     -6.202892    -1.20556 0.901292      0.610355
loc4     -4.784698     0.12208 0.440616      0.660173

0.00000e+00  -7.33333e-02  -3.23224e-01   2.26861e-02  -5.00000e+00
1.12493e+00  -5.82595e-02  -2.33470e-01   1.18364e-02  -5.00000e+00
2.24987e+00  -8.49649e-02  -4.60137e-01   4.59758e-02  -5.00000e+00
2.29992e+00  -8.08298e-02  -4.38984e-01   4.18457e-02  -5.00000e+00
2.34997e+00  -7.67764e-02  -4.16918e-01   3.77447e-02  -5.00000e+00
5.54580e+00   3.26555e-02   2.76918e-02   1.66516e-04   2.00591e-02
8.74164e+00   6.95067e-02   5.16507e-02   5.79305e-04   3.08519e-02
1.05022e+01   7.16503e-02   5.29228e-02   6.08191e-04   3.12605e-02
1.22628e+01   7.38828e-02   5.42318e-02   6.38648e-04   3.18251e-02
1.48968e+01   1.41532e-01   9.00342e-02   1.76023e-03   4.28704e-02

where;
column 1:  location (cM)
column 2:  the (weighted) NPL score
column 3:  Zlr (Zlr = sign(dhat) * sqrt(2.0 * ln(10.0) * LOD))
column 4:  maximized LOD* for the allele-sharing model selected (LOD)
column 5:  delta which produced the maximized lodscore (dhat)
```

Table 4.10. Output File for ALLEGRO

location	LOD	dhat	NPL	Zlr	info	marker
0.000	0.3702	-0.2388	-1.0650	-1.3057	0.8022	M1
3.196	0.5834	-0.3216	-1.2608	-1.6391	0.7260	-
6.392	0.8038	-0.3720	-1.5028	-1.9240	0.7663	M2
9.531	0.8655	-0.4202	-1.3358	-1.9965	0.6625	-
12.670	0.6624	-0.3846	-0.9697	-1.7465	0.5945	M3
13.795	0.6934	-0.4198	-0.9138	-1.7869	0.5317	-
14.920	0.6802	-0.4316	-0.8715	-1.7699	0.4962	M4
18.116	0.7517	-0.4290	-1.0979	-1.8606	0.5543	-
21.311	0.5687	-0.3142	-1.2544	-1.6183	0.7385	M5
23.342	2.1461	0.1302	0.1997	3.1437	0.6774	-
25.372	0.8653	-0.4672	-1.3253	-1.9962	0.5486	M6
27.675	0.8209	-0.5306	-0.5057	-1.9443	0.4167	-
29.978	0.9370	-0.6068	-1.1519	-2.0772	0.3785	M7
32.390	0.9526	-0.6879	-1.0592	-2.0944	0.3126	-
34.803	0.9682	-0.7824	-0.9747	-2.1116	0.2587	M8

4.9.4 Association Analysis Using The CLUMP Program

A population-based association analysis was also implemented on unrelated groups of migraineurs and unaffected controls matched for age, sex and ethnicity. The allele frequency distributions for each of the markers in case and control groups were compared using the standard χ^2 test as implemented in the program SPSS. In addition, the CLUMP program (Curtis and Sham, 1995), which is useful for association testing when markers produce sparse contingency tables, was used for the multiallelic DBH-1 polymorphism (see Chapter 7). The T1 statistic produced by the CLUMP program, results from a test for the null hypothesis of no difference in observed allele distribution between cases and controls, whilst significance is assessed using Monte Carlo simulations (Curtis and Sham, 1995). The CLUMP program was run over 10000 simulations to estimate the *P*-value for this analysis. Table 4.11 shows input required by CLUMP. This data is for a 5-allele marker genotyped in 121 cases and 139 controls with simulations run over 1000 simulations. Output for the T1 and T4 statistics is shown in Table 4.12.

Table 4.11 Input for the CLUMP program.

```
CLUMP v1.6
Enter number of columns (must be two rows): 5
2 34 45 3 37
Enter 5 values for first row:
  2  34  45   3  37   First row total = 121
Enter 5 values for second row:
1 45 23 5 65
  1  45  23   5  65   Second row total = 139

Enter number of simulations to perform: 1000
Enter random number seed: 1
Number of simulations = 1000
Random number seed = 1
```

Table 4.12 Output results produced by CLUMP.

```
Normal chi-square (T1) was 15.999431
This was reached 1 times in 1000 simulations (p=0.001000)
(p value from chi-squared distribution, 4 df, p=0.003050)

Chi-square from 2x2 table clumped to produce maximum (T4) was
15.171444
This was reached 2 times in 1000 simulations (p=0.002000)

Achieved by clumping together columns:
1 3
```

4.9.5 Family-Based Association Analysis (The TRANSMIT and FBAT Programs)

The TRANSMIT and FBAT programs were used in this research to test for allelic association between markers and disease in the migraine pedigrees. Both programs require standard LINKAGE-format pedigree files as input. TRANSMIT tests for association between genetic marker and disease by examining the transmission of markers from parents to affected offspring (Clayton, 1995). The program can deal with transmission of multi-locus haplotypes, even if phase is unknown, and parental genotypes may be also unknown (Clayton, 1995). The tests are based on a score vector that is averaged over all possible configurations of parental haplotypes and transmissions consistent with the observed data. Data from unaffected siblings (or siblings whose disease status is unknown) may be used to narrow down the range of possible parental genotypes which need to be considered. The program produces the following asymptotic chi-squared tests: 1. For each haplotype or allele, a test on 1-df

for excess transmission of that haplotype. 2. A global test for association on H-1 df, where H is the number of haplotypes for which transmission data are available (Table 4.13)

Table 4.13. Output produced by the TRANSMIT program

Haplotype (1df)	Observed	Expected	Var(O-E)	Chisq
1	2	2.2359	0.91811	0.060624
2	11	8.793	2.9839	1.6324
3	55	56.071	17.198	0.066722
4	24	20.599	7.994	1.4466
5	22	24.76	9.8001	0.77705
6	23	20.307	6.5103	1.1143
7	32	33.28	13.302	0.1231
8	42	46.005	15.494	1.0354
9	16	15.043	5.8246	0.15712
10	5	4.8273	1.7744	0.016815
11	0	0.078638	-0.027622	0

The FBAT program determines, from the data, an *S* statistic that is the linear combination of offspring genotypes and phenotypes. The distribution of the *S* statistic is generated by treating the offspring genotype data as random and conditioning on the phenotypes and parental genotypes (Laird et al, 2000). When the marker is biallelic, a *Z* statistic and its corresponding *P* value is calculated. When the marker is multiallelic, a 2 test is performed, with number of df equal to the number of alleles (Table 4.14). The empirical variance option is useful when the marker in question is known to be linked to a gene underlying a disease trait. In this case the null hypothesis is no association.

Table 4.14. Output produced by the FBAT program

```
load fbat-4.ped
read in: 8 markers from 75 pedigrees (116 nuclear families,399 persons)
fbat
trait affection; model additive; test bi-allelic; minsize 10; p 1.000000
```

Marker	Allele	afreq	fam#	S	E(S)	Var(S)	Z	P
2757	3	0.237	31	32.000	32.000	13.292	0.000	1.000000
2757	4	0.075	17	18.000	15.750	6.821	0.862	0.388953
2757	5	0.116	18	12.000	12.750	6.021	-0.306	0.759867
2757	6	0.107	14	14.000	12.233	5.479	0.755	0.450394
2757	7	0.116	24	24.000	25.433	10.412	-0.444	0.656900
2757	8	0.182	28	23.000	27.333	11.956	-1.253	0.210115
2757	9	0.066	14	11.000	10.500	4.083	0.247	0.804571

4.9.6 Linkage Disequilibrium Analysis (The EH Program)

Linkage disequilibrium (LD) is a form of allelic association that occurs in a population when an allele at one marker is associated with an allele at an adjacent (linked) marker (Lewontin, 1971). In this research LD between adjacent markers was calculated using the EH (Estimate Haplotypes) program (Xie and Ott, 1993). For this analysis, H_0 is the null hypothesis of no association whilst H_A is the alternate hypothesis of allelic association between markers. Table 4.15 below shows the output of a typical EH analysis. For this particular analysis a tri-allelic microsatellite is tested for LD with a biallelic SNP. As can be seen for the bolded HA in Table 4.15, the Likelihood ratio χ^2 of 6.29 for 2 degrees of freedom is marginally significant (P = 0.0429). Thus, in this case there is weak evidence for allelic association between the two markers. Lewontins D' coefficient is also calculated (Lewontin, 1971). This is a measure of the strength of LD (allelic association) between markers. The observed haplotype frequencies listed in Table 4.15 are used to calculate the resulting D' coefficient (a D' coefficient of 1 indicates complete LD).

Table 4.15. LD Output from the EH Program

```
Estimates of Gene Frequencies (Assuming Independence)
----\-----------------------------------
locus \allele     1       2       3
---------\------------------------------
  1  |         0.8955  0.1045         snp
  2  |         0.0424  0.6949  0.2627      microsatellite
----------------------------------------
# of Typed Individuals: 177

There are 6 Possible Haplotypes of These 2 Loci.
They are Listed Below, with their Estimated Frequencies:
----------------------------------------------------
| Allele   Allele  |      Haplotype Frequency      |
|   at       at    |                               |
| Locus 1  Locus 2 |  Independent   w/Association  |
----------------------------------------------------
   1        1          0.037944     0.042280
   1        2          0.622283     0.599894
   1        3          0.235253     0.253305
   2        1          0.004429     0.000092
   2        2          0.072632     0.095021
   2        3          0.027459     0.009406
----------------------------------------------------
                                      df   Ln(L)      Chi-
square
-------------------------------------------------------------
------
H0: No Association                     3   -305.66      0.00
HA: Allelic Associations Allowed       5   -302.51      6.29
```

4.9.7 Hardy-Weinberg Equilibrium (HWE) Analysis

The HWE equations were primarily used in this research as a statistical control against systematic genotyping error that may have been incurred in the laboratory. The main alternative hypotheses were that the marker genotype distributions of the affected (migraine) groups are significantly different than in unaffected controls. If this hypothesis is actually true, than we would expect that the migraine affected distribution may NOT be in HWE. Thus, HWE checks were performed only using genetic data from the control group.

To perform HWE calculations for multiallelic markers a computer program called HWE was used (Guo and Thompson, 1992). This program not only computes the expected genotype frequencies for multiallelic markers but also uses Fisher's Exact methods to determine the significance of the HWE test. This overcomes the problem of inaccurate chi-squared *P*-values due to sparse genotype contingency tables.

Input for the HWE program consists of a first line which contained the number of alleles (n); then, the next n lines which contain the genotype data, arranged conforming to Figure 4.08; the last line contained; 1) the length of dememorisation period, 2) the number of batches, and 3) the size of each batch (Guo & Thompson 1992). For HWE analysis of marker genotypes in this research; appropriate values of 1000, 100 and 5000 were specified for the dememorisation period, batch number and batch size, respectively (Guo & Thompson 1992). A significance level of $P < 0.01$ was used for evidence of Hardy-Weinberg disequilibrium (Guo & Thompson 1992, Nyholt, 1998c).

A_1	f_{11}			
A_2	f_{12}	f_{22}		
•	• •	• •	• •	
A_n	f_{n1}	f_{n2}	• •	f_{nn}
	A_1	A_2	• •	A_n

Figure 4.08. HWE genotype data format: where f_{ij} ($1 \le j \le i \le n$) is the observed count of genotype A_iA_j (Guo & Thompson 1992, Nyholt, 1998c).

4.9.8 Power of Association Studies

Power is an important consideration in any study involving hypothesis (probability) testing. An analysis where the conclusions are based on a probability distribution requires that the chance of falsely rejecting the null hypothesis (or committing a type I errors) be specified. Similarly, the chance of incorrectly accepting the null when the alternate hypothesis is true (Type II error) also needs to be considered. The power of a study is a function of Type II error. Specifically, power is the probability complement of the type II error rate (ie: power = 1 - type II error). In other words, power can be defined as correctly rejecting the null hypothesis when the alternate is true (Zar, 1998). For association studies, power is being able to detect an association as significant when one actually exists. In genetic association studies power is largely governed by the size of the effect that a risk allele is thought to have on the disease. This is often measured as relative risk, which is correlated to the magnitude of the differences in the risk allele (gene) frequencies between cases and controls. In turn the size of the sample required to detect this genetic effect, or proportional difference, can be determined. Figure 4.09 illustrates power estimate curves for varying significance levels based on marker allele frequency in controls. Other parameters required for accurate power estimation include penetrance and the distance a marker is away from the trait-influencing allele (Schork, 2002). Typically, 80% a priori power is considered adequate for genetic case-control studies, and this value for power was set as minimum in this research .

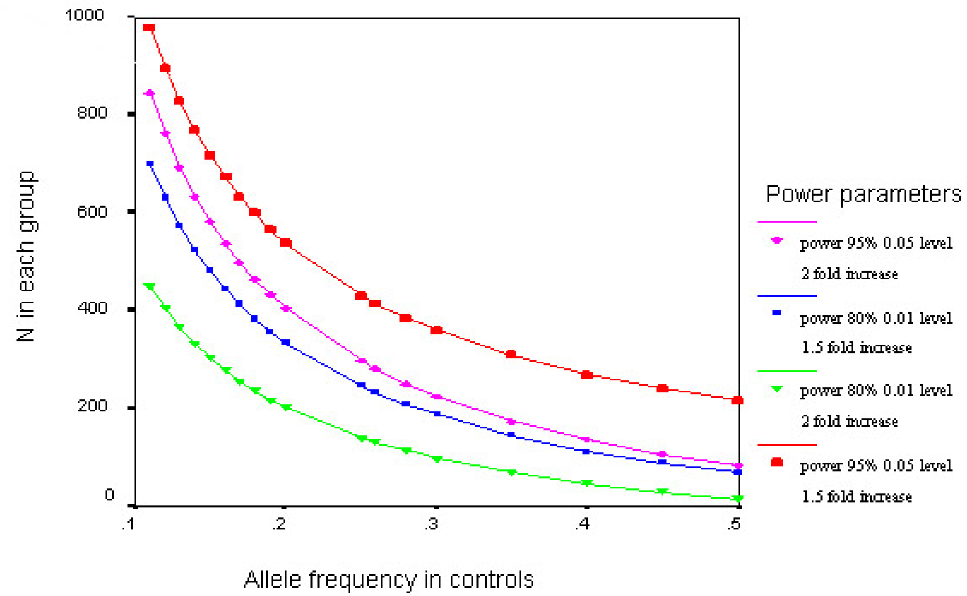


Figure 4.09. Power estimate chart for case control genetic association studies. Curves allow determination of approximate sample size required for case and control group different significant levels (0.01 – 0.05). Calculations are based on marker allele frequencies in controls.

CHAPTER 5
AN INVESTIGATION OF A MIGRAINE SUSCEPTIBILITY REGION ON CHROMOSOME 19P13

5.1 A Study of the *CACNA1A* Gene as a Candidate for Typical Migraine

5.1.1 Introduction

Typical migraine, comprised of migraine with aura (MA) and migraine without aura (MO), is a complex, often painful neurological disorder. It is characterised by severe recurrent headache, vomiting, photophobia and phonophobia, with the frequency and duration of symptoms shown to vary widely between affected persons. Classification of typical migraine is based on a set of diagnostic criteria as established by the International Headache Society (Headache Classification Committee of the International Headache Society, 1988). The disorder is prevalent, with a recent comprehensive study by Launer et al. indicating that around 25% of women and 8% of men suffer from the disease (Launer et al., 2000). At present, the aetiology of a migraine attack is only partly understood. Environmental influences play a role but family studies suggest that defective genes may be primarily involved in disease causation. The mode of transmission of typical migraine in families is still unclear but it is widely believed to be multifactorial (Russell and Olesen, 1993), although a role for a major susceptibility gene cannot be excluded (Mochi et al., 1993).

5.1.1.1 Calcium Channels

Calcium channels have many functions in the human body: they mediate the entry of Ca^{2+} ions into excitable cells and are involved in muscle contraction, as well as hormone and neurotransmitter release by a variety of calcium directed processes (Jen J, 1999). The voltage-gated calcium channels are heteromers, containing protein

subunits of about 30 to 230 kd that form calcium-selective pores across the cell membrane, thus allowing calcium to enter the cell in response to membrane depolarisation (Dunlap et al, 1995). The subunits have different functions, and isoforms of these subunits give rise to distinct channel subtypes. The α_1 subunit consists of four homologous domains of six transmembrane spanning segments. These segments form the transmembrane pore that expresses most of the channels known drug-binding sites (Gurnett and Campbell, 1996).

Mutations in the *CACNA1A* gene are known to cause several neurological and muscular diseases such as Episodic Ataxia Type 2 (EA-2), Spinocerebellar Ataxia Type 6 (SCA-6) and Familial Hemiplegic Migraine (FHM). Mutations in the genes that code for other voltage-gated calcium channel subunits can also result in diseases such as Malignant Hypothermia Syndrome (Table 5.01). Collectively, these calcium channel disorders are termed "calcium channelopathies".

Table 5.01. Voltage-Gated Calcium Channelopathies

Subunit	Gene	Locus	Disease
α_{1F}	*CACNA1F*	Xp11-21	Congenital Stationary Night Blindness
α_{1S}	*CACNA1S*	1q32	Hypokalemic Periodic Paralysis Malignant Hypothermia Syndrome
α_{1A}	*CACNA1A*	19p13	Familial Hemiplegic Migraine Episodic Ataxia Type 2 Spinocerebullar Ataxia Type-6

5.1.1.2 The *CACNA1A* Gene and Familial Hemiplegic Migraine

In recent years there has been immense research interest focusing on the molecular genetics underlying the pathophysiology of migraine. Particular attention has been paid to localising and identifying disease susceptibility genes through studies of genomic regions known to harbour genes of potential pathophysiological importance. However, due to the clinical variability of migraine, the identification of causative genes has been difficult. Despite this, substantial progress was made in 1996, when Familial Hemiplegic Migraine (FHM), a rare and very severe migraine subtype, was

shown to be caused by deleterious mutations in the voltage-gated calcium channel α_1 subunit gene, *CACNA1A* located on chromosome 19p13 (Ophoff et al, 1996). Several polymorphisms including a $(CA)_n$-repeat (D19S1150), a $(CAG)_n$-repeat in the 3' untranslated region and a nucleotide substitution predicting an amino acid change in exon 11 were also revealed within the gene (Ophoff et al., 1996). It has since been demonstrated that a variety of other mutations in this gene are associated with FHM in chromosome 19p13 linked pedigrees (Table 5.02). Figure 5.1 illustrates topographically the domains and segments of the *CACNA1A* protein with known FHM mutation sites.

Table 5.02. *CACNA1A* Mutations in FHM Patients

Original Publishing Authors	Location	Domain	Nucleotide Change		Consequence		
Ophoff et al, 1996	exon 4	I S4	nt 850	G→A	Arg→Gln	R192Q	gain of Sfcl site
	exon 16	P-Segment	nt 2272	C→T	Thr→Met	T666M	
	exon 17	II S6	nt 2416	T→C	Val→Ala	V714A	gain of Bbvl site
	exon 36	IV S6	nt 5706	A→C	Ile→Leu	I1811L	gain of MnII site
Ducros et al, 1999	exon 17	II S6	nt 2420	C→A	Asp→Glu	D715E	
Battistini et al, 1999	exon 13	II S4	nt 2023	G→A	Gln→Arg	G583A	loss of BanII site
Jen et al, 1999	exon 29		nt 4914	C→T			early stop codon
Vahedi et al, 2000	exon 25	III S5		A→G	Tyr→Cys	T1385C	

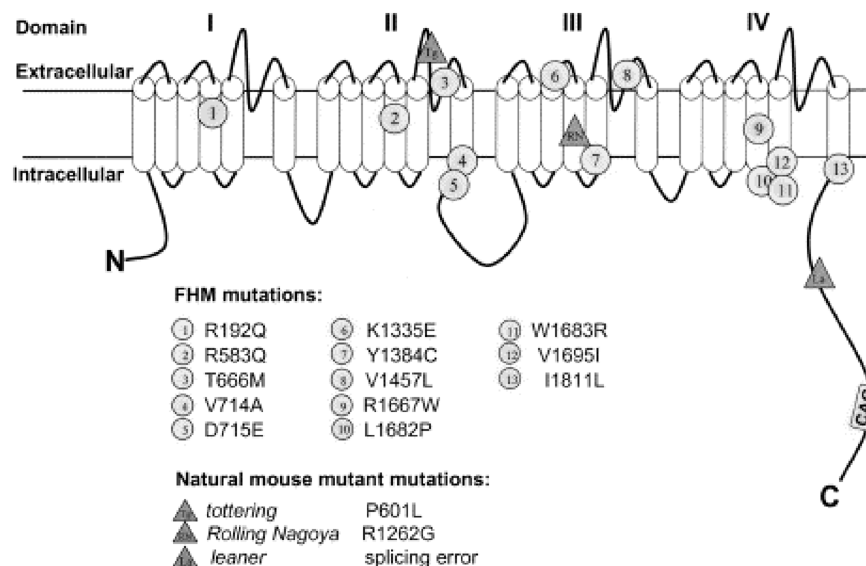


Figure 5.01. Topology of the CACNA1A subunit protein showing positions of known FHM mutations

It is now thought that the mutations in the *CACNA1A* gene lead to channel loss or gain of function and altered channel properties (Kraus et al, 1998: Guida et al, 2001:). These disturbances may in turn cause abnormally increased or decreased calcium entry, which could have profound influence on various calcium-dependent signalling pathways including neurotransmitter release (Jen J, 1999)(see Figure 5.2).

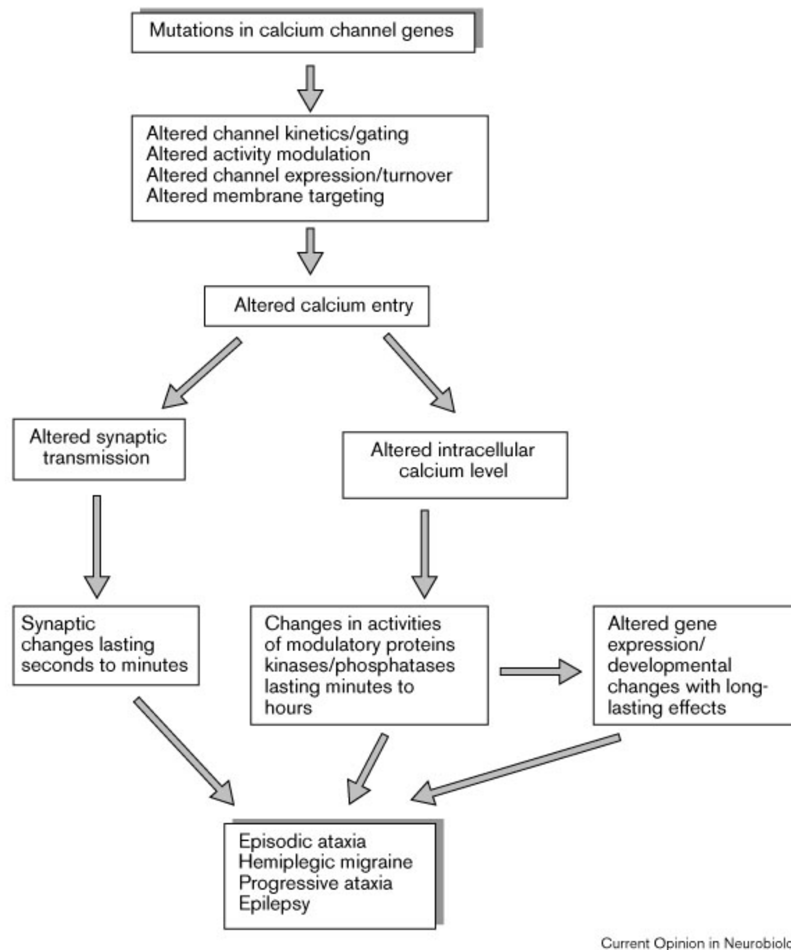


Figure 5.02. Hypothesised functional consequences of calcium channel mutations (Jen J, 1999)

5.1.1.3 The Chromosome 19p13 Region and Typical Migraine

There has been some evidence indicating that the FHM locus on chromosome 19p13 may also contribute to migraine with and without aura (May et al., 1995). Thus, FHM, MO and MA may be part of a migraine continuum with a common genetic aetiology. Using FHM as a genetic model for studying typical migraine, we have

previously performed linkage analysis on 4 large Australian families affected with typical migraine using markers spanning the putative FHM locus on Chr19p13, including the two highly variable repeat polymorphisms located within the *CACNA1A* gene (see Figure 5.3) (Nyholt et al., 1998b). Multipoint results of this previous study indicated significant excess allele sharing in one family across a 12.6cM region containing the FHM locus (max. NPL Z score = 6.64; P = 0.0026) with a peak parametric LOD score of 1.92 (P = 0.001) obtained at the CAG triplet repeat in *CACNA1A* (Nyholt et al., 1998). Adding further support to the hypothesis that *CACNA1A* also causes typical migraine, Terwindt et al. have reported that the *CACNA1A* (FHM) mutation I1811L was also detected in a patient with MA (Terwindt et al, 1998). Based on this evidence, the present study was focused on examining the *CACNA1A* gene as a candidate for typical migraine. In particular this study describes a comprehensive investigation of the *CACNA1A* gene in our chromosome 19-linked Australian typical migraine pedigree (MF1). Direct DNA sequencing was undertaken to screen all 47 *CACNA1A* exons in two typical migraine individuals from this pedigree who share the critical susceptibility haplotype surrounding the gene. Furthermore, we utilised an additional 82 Australian typical migraine pedigrees, as well as unrelated groups of typical migraine cases and unaffected controls, to test whether polymorphisms within, and flanking, the *CACNA1A* gene were linked and/or associated with typical migraine in this general Caucasian population.

5.1.2 Materials and Methods

5.1.2.1 Study Subjects

This research complies with Australian ethics standards and was approved by the Griffith University ethics committee. All subjects who participated in this study resided along the eastern coastal region of Australia and were recruited through the Genomics Research Centres patient clinic through ascertainment by physician or migraine support group referral and local media advertising. All subjects gave informed consent, were of Caucasian origin, and were diagnosed for typical migraine by questionnaire and a clinical neurologist according to the criteria specified by the International Headache Society (Headache Classification Committee of the International Headache Society, 1988). Under the hypothesis of a common genetic etiology, all individuals with MA, MO, or both were grouped together and classified

(phenotyped) as being affected with typical migraine, as well as being analysed separately as MA and MO subgroups, where appropriate.

Patients Screened for CACNA1A Mutations

DNA from two affected patients belonging to the large multi-generational family (MF1) previously showing linkage to 19p13 was screened for mutations in all 47 exons in the *CACNA1A* gene. These individuals were chosen specifically because they carried the *CACNA1A* susceptibility haplotype shared among affecteds in this pedigree MF1. These patients are shown as individuals II:1 and II:6 in Figure 5.4. A summary of the clinical characteristics of these two patients is shown in Table 5.03.

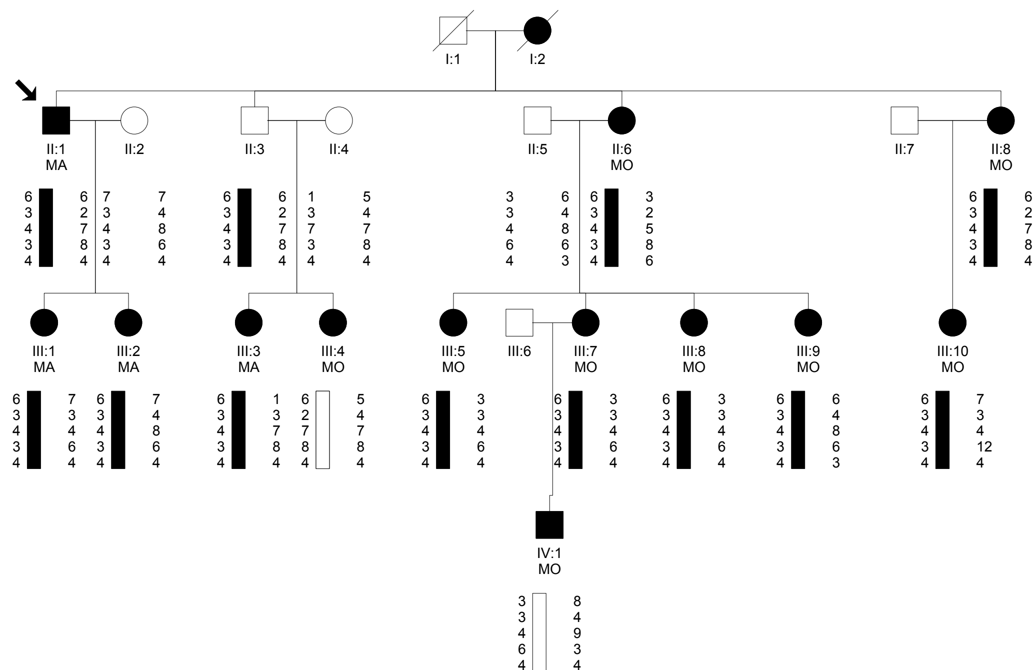


Figure 5.4. MF1 showing segregation of typical migraine susceptibility haplotypes for chromosome 19p13 microsatellite markers (D19S221, $(CAG)_n$, D19S1150, D19S226, and D19S179 from top to bottom). Cases of phenocopy can be seen for individuals III:4 and IV:1, respectively. Mutation screening for 47 exons in the CACNA1A gene was performed for individuals II:1 and II:6.

Table 5.03. Summary of Clinical Characteristics of MF1 Migraine Patients Screened for Mutations in CACNA1A Gene

Patient No.	Sex	Sub Diagnosis	Age of onset (y)	Highest attack Frequency	Average Duration	Pain/Symptoms	Pulsating	Obvious Triggers
Mu06	M	MA	21	1/mth	24hrs	pain left side head, nausea	Yes	physical activity, red, wine, chocolate
Mu166	F	MO	18	4/yr	4hrs	pain top of head and neck	No	----

Eighty-two independent pedigrees affected with typical migraine

Additional DNA belonging to 296 subjects (263 affected) from 82 unrelated families affected with typical migraine was ascertained for genotyping. These pedigrees were comprised of 252 affected relative pairs excluding parent-child pairs. All families had at least two affected individuals and at least one parent was available for genotyping in all but 10 pedigrees. We estimated the power to detect a defective gene contributing a modest effect to the disease in this sample to be greater than 80% based on an overall typical migraine relative risk of ~ 2.5.

Unrelated Case-Control Groups

A further 347 DNA samples from unrelated individuals were utilised. This cohort was comprised of 177 individuals diagnosed as clearly having MA or MO, while 182 were unaffected controls. Of the typical migraine-affected group, ~80% of patients had a known family history of the disease, or at least one first-degree relative suffering from migraine. Clinically, the affected group had a median age of onset of ~22 years, average headache duration of ~18 hours, with an attack frequency of ~28 per year. The unaffected control group was recruited from the same geographical location (east coast of Australia) as the affected group and was carefully matched for age, gender, and ethnicity, thus reducing the possibility of spurious results due to population stratification. It was estimated that sample groups of this size would have > 85% power to detect an allelic association conferring a twofold increase in risk to the disease.

5.1.2.2 DNA Analysis

DNA Markers and Genotyping

This study utilised highly polymorphic markers within, and flanking the *CACNA1A* gene on chromosome 19p13. Specifically, three dinucleotide markers D19S221, D19S1150 (intragenic) and D19S179 and one trinucleotide (CAG) repeat polymorphism in the 3' untranslated region of the *CACNA1A* gene were tested. The marker map with physical distances (Mb) shown in parentheses is D1S221-(0.8)-CAG-(0.2)-D1S1150-(1.1)-D1S179 (Trettel et al, 2000). Primers for each marker were labelled with fluorescent dyes and PCR amplification performed under standard conditions. Table 5.04 lists the primer sequences for these markers. The resulting fragments were then sized by capillary electrophoresis using an ABI-310 Genetic Analyser™. All data were accurately genotyped using GENOTYPER™ software.

Table 5.04. Primer Sequences for the CACNA1A Gene Region Markers on Chromosome 19p13 (*intragenic markers)

Gene	Marker Locus	Variant	Primer Name	Primer Sequence (5'→ 3')
CACNA1A	*D19S221*		AFM224ye9a	GCAAGACTCTGACTCAACAAAA
			AFM224ye9m	CATAGAGATCAATGGCATGAAA
	*Exon 47**	(CAG)n	3'UTR –F	CCTTTGTTTCAATTTTCGTGTAG
			3'UTR –R	TGGGGCCTGGGTACCTCCGA
	*D19S1150**	(CA)n	p858 –F	GGAGAAGCATAGAAAAGCCA
			p858 –R	CCTGTTGAAAACTCCTGACC
	D19S226	(CA)n	AFM256yc9a	CCAGCAGATTTTGGTGTTGTCTA
			AFM256yc9m	GGTCCAGGATTTGAACTAAAGCA

Mutation Analysis

Genomic DNA was used as a template to generate both forward and reverse exonic fragments for automated direct sequencing analysis. In particular, primers specific for

all 47 *CACNA1A* gene exons (Ophoff et al., 1996: Yue et al., 1997) were used to perform PCR amplification according to standard procedures. The complete coding sequence of CACNA1A is shown in Table 5.05 with known FHM mutations highlighted. Exons containing known FHM mutations are highlighted. The exon/intron organisation of the human *CACNA1A* gene and exon-specific primer pairs used for sequencing are shown in Table 5.06. All resulting PCR fragments were prepared for sequencing using QIAGEN™ sequencing kits and fractionated using an ABI-373 automated DNA sequencer™. Editview™ and Sequencher™ software was used to assess and analyse all sequence data.

Table 5.05. CACNA1A (7446bp) Coding Sequence (Acc No. X99897)

```
   1 cgcaccctcc ttccgcccct ccttctccgg ggtcagccag gaagatgtcc cgagctgcta
  61 tccccggctc ggcccgggca gccgccttct gagcccccga cccgagcgcc gagccgccgc
 121 gcgatgggct gggccgtgga gcgtctccgc agtcgtagct ccagccgccg cgctcccagc
 181 cccggcagcc tcagcatcag cggcggcggc ggcggcggcg gcgtcttccg catcgttcgc
 241 cgcagcgtaa cccggagccc tttgctcttt gcagaatggc ccgcttcgga gacgagatgc
 301 cggcccgcta cgggggagga ggctccgggg cagccgccgg ggtggtcgtg ggcagcggag
 361 gcgggcgagg agccgggggc agccggcagg gcgggcagcc cggggcgcaa aggatgtaca
 421 agcagtcaat ggcgcagaga gcgcggacca tggcactcta caaccccatc cccgtccgac
 481 agaactgcct cacggttaac cggtctctct tcctcttcag cgaagacaac gtggtgagaa
 541 aatacgccaa aaagatcacc gaatggcctc cctttgaata tatgatttta gccaccatca
 601 tagcgaattg catcgtcctc gcactggagc agcatctgcc tgatgatgac aagaccccga
 661 tgtctgaacg gctggatgac acagaaccat acttcattgg aatttttgt ttcgaggctg
 721 gaattaaaat cattgccctt gggtttgcct tccacaaagg ctcctacttg aggaatggct
 781 ggaatgtcat ggactttgtg gtggtgctaa cgggcatctt ggcgacagtt gggacggagt
 841 ttgacctacg gacgctgagg gcagttcgag tgctgcggcc gctcaagctg gtgtctggaa
 901 tcccaagttt acaagtcgtc ctgaagtcga tcatgaaggc gatgatccct ttgctgcaga
 961 tcggcctcct cctatttttt gcaatcctta tttttgcaat catagggtta gaattttata
1021 tgggaaaatt tcataccacc tgctttgaag aggggacaga tgacattcag ggtgagtctc
1081 cggctccatg tgggacagaa gagcccgccc gcacctgccc caatgggacc aaatgtcagc
1141 cctactggga agggcccaac aacgggatca ctcagttcga caacatcctg tttgcagtgc
1201 tgactgtttt ccagtgcata accatggaag ggtggactga tctcctctac aatagcaacg
1261 atgcctcagg gaacacttgg aactggttgt acttcatccc cctcatcatc atcggctcct
1321 tttttatgct gaaccttgtg ctgggtgtgc tgtcagggga gtttgccaaa gaaagggaac
1381 gggtggagaa ccggcgggct tttctgaagc tgaggcggca acaacagatt gaacgtgagc
1441 tcaatgggta catggaatgg atctcaaaag cagaagaggt gatcctcgcc gaggatgaaa
1501 ctgacgggga gcagaggcat ccctttgatg gagctctgcg gagaaccacc ataaagaaaa
1561 gcaagacaga tttgctcaac cccgaagagg ctgaggatca gctggctgat atagcctctg
1621 tgggttctcc cttcgcccga gccagcatta aaagtgccaa gctggagaac tcgacctttt
1681 ttcacaaaaa ggagaggagg atgcgtttct acatccgccg catggtcaaa actcaggcct
1741 tctactggac tgtactcagt ttggtagctc tcaacacgct gtgtgttgct attgttcact
1801 acaaccagcc cgagtggctc tccgacttcc tttactatgc agaattcatt ttcttaggac
1861 tctttatgtc cgaaatgttt ataaaaatgt acgggcttgg gacgcggcct tacttccact
1921 cttccttcaa ctgctttgac tgtggggtta tcattgggag catcttcgag gtcatctggg
1981 ctgtcataaa acctggcaca tcctttggaa tcagcgtgtt acgagccctc aggttattgc
2041 gtattttcaa agtcacaaag tactgggcat ctctcagaaa cctggtcgtc tctctcctca
2101 actccatgaa gtccatcatc agcctgttgt ttctcctttt cctgttcatt gtcgtcttcg
2161 ccctttttggg aatgcaactc ttcggcggcc agtttaattt cgatgaaggg actcctccca
2221 ccaacttcga tacttttcca gcagcaataa tgacggtgtt tcagatcctg acgggcgaag
2281 actggaacga ggtcatgtac gacgggatca agtctcaggg gggcgtgcag ggcggcatgg
2341 tgttctccat ctatttcatt gtactgacgc tctttgggaa ctacaccctc ctgaatgtgt
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2461 aggaagaaga agcagcgaac cagaaacttg ccctacagaa agccaaggag gtggcagaag
2521 tgagtcctct gtccgcggcc aacatgtcta tagctgtgaa agagcaacag aagaatcaaa
2581 agccagccaa gtccgtgtgg gagcaggcga acagtgagat gccaaagcag aacttgctgg
2641 ccagccggga ggccctgtat aacgaaatgg acccggacga gcgctggaag gctgcctaca
2701 cgcggcacct gcggccagac atgaagacgc acttggaccg gccgctggtg gtggacccgc
2761 aggagaaccg caacaacaac accaacaaga gccgggcggc cgagcccacc gtggaccagc
2821 gcctcggcca gcagcgcgcc gaggacttcc tcaggaaaca ggcccgctac cacgatcggg
2881 cccgggaccc cagcggctcg gcgggcctgg acgcacggag gccctgggcg ggaagccagg
2941 aggccgagct gagccgggag gacccctacg gccgcgagtc ggaccaccac gcccgggagg
3001 gcagcctgga gcaacccggg ttctgggagg gcgaggccga gcgaggcaag gccggggacc
3061 cccaccggag gcacgtgcac cggcaggggg gcagcaggga gagccgcagc gggtccccgc
3121 gcacgggcgc ggacggggag catcgacgtc atcgcgcgca ccgcaggccc ggggaggagg
3181 gtccggagga caaggcggag cggagggcgc ggcaccgcga gggcagccgg ccggcccggg
3241 gcggcgaggg cgagggcgag ggtcccgacg gggcgagcg caggagaagg caccggcatg
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3361 ggaggaaaga gaaccagggc tccggggtcc ctgtgtcggg ccccaacctg tcaaccaccc
3421 ggccaatcca gcaggacctg ggccgccaag acccacccct ggcagaggat attgacaaca
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3541 ccggcctgcc ccagagccca gccaagatgg gaaacagcac cgaccccggc cccatgctgg
3601 ccatccctgc catggccacc aacccccaga acgccgccag ccgccggacg cccaacaacc
3661 cggggaaccc atccaatccc ggccccccca agacccccga gaatagcctt atcgtcacca
3721 accccagcgg cacccagacc aattcagcta agactgccag gaaacccgac cacaccacag
3781 tggacatccc cccagcctgc ccacccccc tcaaccacac cgtcgtacaa gtgaacaaaa
3841 acgccaaccc agacccactg ccaaaaaaag aggaagagaa gaaggaggag gaggaagaag
3901 acgaccgtgg ggaagacggc cctaagccaa tgcctcccta tagctccatg ttcatcctgt
3961 ccacgaccaa ccccttcgc cgcctgtgcc attacatcct gaacctgcgc tactttgaga
4021 tgtgcatcct catggtcatt gccatgagca gcatcgccct ggccgccgag gaccctgtgc
4081 agcccaacgc acctcggaac aacgtgctgc gatactttga ctacgttttt acaggcgtct
4141 ttacctttga gatggtgatc aagatgattg acctggggct cgtcctgcat cagggtgcct
4201 acttccgtga cctctggaat attctcgact tcatagtggt cagtggggcc ctggtagcct
4261 ttgccttcac tggcaatagc aaaggaaaag acatcaacac gattaaatcc ctccgagtcc
4321 tccgggtgct acgacctctt aaaaccatca agcggctgcc aaagctcaag gctgtgtttg
4381 actgtgtggt gaactcactt aaaaacgtct tcaacatcct catcgtctac atgctattca
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4561 aggtgaaggc gcgagaccgg gagtggaaga agtatgaatt ccattacgac aatgtgctgt
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5101 tcttctctct ggaatgtgtg ctgaaagcca tggcttttgg gattctgaat tatttccgcg
5161 atgcctggaa catcttcgac tttgtgactg ttctgggcag catcaccgat atcctcgtga
5221 ctgagtttgg gaataacttc atcaacctga gctttctccg cctcttccga gctgcccggc
5281 tcatcaaact tctccgtcag ggttacacca tccgcattct tctctggacc tttgtgcagt
5341 ccttcaaggc cctgccttat gtctgtctgc tgatcgccat gctcttcttc atctatgcca
5401 tcattgggat gcaggtgttt ggtaacattg gcatcgacgt ggaggacgag gacagtgatg
5461 aagatgagtt ccaaatcact gagcacaata acttccggac cttcttccag gccctcatgc
5521 ttctcttccg gagtgccacc ggggaagctt ggcacaacat catgctttcc tgcctcagcg
5581 ggaaaccgtg tgataagaac tctggcatcc tgactcgaga gtgtggcaat gaatttgctt
5641 atttttactt tgtttccttc atcttcctct gctcgtttct gatgctgaat ctctttgtcg
5701 ccgtcatcat ggacaacttt gagtacctca cccgagactc ctccatcctg ggcccccacc
5761 acctggatga gtacgtgcgt gtctgggccg agtatgaccc cgcagcttgc ggtcggattc
5821 attataagga tatgtacagt ttattacgag taatatctcc ccctctcggc ttaggcaaga
5881 aatgtcctca tagggttgct tgcaagcggc ttctgcggat ggacctgccc gtcgcagatg
5941 acaacaccgt ccacttcaat tccaccctca tggctctgat ccgcacagcc ctggacatca
6001 agattgccaa gggaggagcc gacaaacagc agatggacgc tgagctgcgg aaggagatga
6061 tggcgatttg gcccaatctg tcccagaaga cgctagacct gctggtcaca cctcacaagt
6121 ccacggacct caccgtgggg aagatctacg cagccatgat gatcatggag tactaccggc
6181 agagcaaggc caagaagctg caggccatgc gcgaggagca ggaccggaca cccctcatgt
6241 tccagcgcat ggagcccccg tccccaacgc aggaaggggg acctggccag aacgccctcc
6301 cctccaccca gctggaccca ggaggagccc tgatggctca cgaaagcggc ctcaaggaga
6361 gcccgtcctg ggtgacccag cgtgcccagg agatgttcca gaagacgggc acatggagtc
6421 cggaacaagg ccccctacc gacatgccca acagccagcc taactctcag tccgtggaga
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6541 agggccgggc tgcctccatg ccccgcctcc ctgcagagaa ccagaggaga aggggccggc
6601 cacgtgggaa taacctcagt accatctcag acaccagccc catgaagcgt tcagcctccg
6661 [illegible]
6481 [illegible]
6721 agaaccagcg gcaccaccag cggcgccgcg accgcagcca ccgcgcctct gagcgctccc
6781 tgggccgcta caccgatgtg gacacaggct tggggacaga cctgagcatg accacccaat
6841 ccggggacct gccgtcgaag gagcgggacc aggagcgggg ccggcccaag gatcggaagc
6901 atcgacagca ccaccaccac caccaccacc accaccatcc cccgcccccc gacaaggacc
6961 gctatgccca ggaacggccg gaccacggcc gggcacgggc tcgggaccag cgctggtccc
7021 gctcgcccag cgagggccga gagcacatgg cgcaccggca gtagttccgt aagtggaagc
7081 ccagccccct caacatctgg taccagcact ccgcggcggg gccgccgcca gctcccccag
7141 acccccctcca ccccccggcc acacgtgtcc tattcccctg tgatccgtaa ggccggcggc
7201 tcggggcccc cgcagcagca gcagcagcag cagcagcagc agcaggcggt ggccaggccg
7261 gccgggcggc caccagcggc cctcggaggt acccaggccc cacggccgag cctctggccg
7321 gagatcggcg cccacggggg gccacagcag cggccgcacg cccaggatgg agaggcgggt
7381 ccaggcccgg cccggagcga gtctccaggg cctggtcgac acggcggggc ccggctggcg
7441 gcagtc
```

*highlighted are the known FHM mutations listed in Table 5.02

Table 5.06. Exon/Intron Organisation of the Human CACNA1A Gene and Exon-Specific Primer Pairs*

Exon	cDNA	Exon Size	Forward Primer	Reverse Primer	PCR Size
1	UTR-568	500	TCTCCGCAGTCGTAGCTCCA	GGTTGTAGAGTGCCATGGTC	320
			CGCAAAGGATGTACAAGCAG	ATTCCCAAGCCTCCAGGGTAG	370
2	569-674	106	CACCTCCAACACCCTTCTTT	TCTGTGCCCTGCTCCACTC	240
3	675-814	140	ACGCTGACCTTGCCTTCTCT	CAACCAAAAGCCTCGTAATC	230
4	815-906	92	AAAACCCACCCTCTGTTCTC	TTGTCAGGGTCGGAAACTCA	160
5	907-1059	153	CTTGGTGGCGGGGTTT	CTGCCTAATCCTCCCAAGAG	290
6	1060-1253	194	TCCCTTCCCTTTTGTAGATG	GTGGGGCTGTGTTGTCCTT	350
7	1254-1357	104	GACAGAGCCACAAGAGAACC	AGCAAAGAGGAGTGAGTGGG	250
8	1358-1473	116	ATACTCTGGCTTTTCTATGC	GCATGACTCTCTTTGTACTC	230
9	1474-1530	57	GCAGAGAATGGGGGTGG	CTGAGGTGGGTTTAGAGCAG	180
10	1531-1623	93	GGGTAACGTCTTTTTCTCTTGC	ATGTCTCTTGGGCGATAGGT	200
11	1624-1833	210	ATTTCTTCTGAAGGAACAGC	GGAGGGATCAGGGAGTTGGC	310
12	1834-1946	113	CAAGCCTAACCTCCTCTCTG	TCATTCCAGGCAAGAGCTG	200
13	1947-2051	105	ATTTGGAGGGAGGAGTTTGG	TCACTTTCCCAACTTTCTGG	310
14	2052-2191	140	CAGAAAGTTGGGAAAGTAGC	TTGAATTCCTGTGAAGGAC	250
15	2192-2264	73	CTTGGAGATGAGATACTGAGC	CAGGCACTTTCATCTGTGAC	200
16	2265-2382	118	TCCACAGCTGCATCTCCAAG	ACCCTCCCTTGAGCCCCT	270
17	2383-2450	68	CAGTGGTTGCTTTTCCTGAC	TTGCCAGAGAAACATTCTCC	130
18	2451-2557	107	TGAACAAAGATTCCACGTGC	TTCAGGAGCCAGGGTAGCATC	170
19	2558-3367	810	TAGCAATGCTCTAAGTCCTG	TGTTTCCTGAGGAAGTCCTC	320
			CGCAGGAGAACCGCAACAA	GCGATGACGTCGATGCTC	450
20	3368-3831	464	GGTTCTTTTTCATTCACTTGC	TTTCCTGGCAGTCTTAGCTG	430
			GAGAATAGCCTTATCGTCAC	CAGTGATGTGAGAGCAGAG	200
21	3832-3973	142	TGGGAAATTGTGGAGGGAGC	TGACTTCCGCCACCCTGGTG	250
22	3974-4103	130	AGCCTGTGGTCTGAGTGGAC	TAGGAAGGGGTGTGCTCTGTG	210
23	4104-4163	60	ATCCACTGCTCTCTTGCTTT	GTGGTTCTCACTTATAATCTGC	170
24	4164-4270	107	TGGCCTCATTGGCTTCCCTGC	AAGAGGAAACCCTTGCGAAG	250
25	4271-4370	100	CTACCCAACCTGACCTCTGC	ACATGATAACCCTGACAGTC	220
26	4371-4531	161	CTCATGCTCTCTGTCAACTC	TGGTTCCAATGGGAATGTGC	250
27	4532-4669	138	CTGCTTCCCAAGCAGTCTAG	TCCTGGATAGATTTCCAGTC	300
28	4670-4871	202	AGTTTTTAAAGGACAGATGG	TTTCCCTGCCCCATTCCTTTGC	280
29	4872-5036	165	CTCTGCCGCTCTCACCACTG	TTTATCATCAGGTAGAGGCAGG	250
30	5037-5147	111	TTCCAAGCCCATAGCTGTAGC	TGACCCTGCTACTCCTGCTTC	180
31	5148-5231	84	ACTGTGCCTCTAACATGCAC	AAGTGCTGGCTCAAGCAG	250
32	5232-5348	117	TCTGTGAGTGGTGACAGCTC	GTCACCTGTCTTCTCAGC	240
33	5349-5414	66	TGGAAGGACTCTGGCACGTG	GGAGGCTCTGGGAACCTTAG	250
34	5415-5530	116	AGAAGCCACTGGAGGAATGGC	ATTATCAGAGCAGGTCCCCTTC	250
35	5531-5681	151	TCCGAGTCTCTGATTTCTCC	AGACGGCCCTCACAGTGTC	210
36	5682-5809	128	TTCATTCCCTCGGTCTCTGC	CTGACTGAACCTGTGAGAC	350
37	5810-5906	97	GTTCTCTGTTCCCATTCCCG[▽]	AACCCAGTGCCTGGACGTC[▽]	208[▽]
38	5907-6012	106	ATGCCTGGGAATGACTGC	TGTCACGCCTGTCTGTGC	200
39	6013-6120	108	TGACACCCAGGCAGGCAG	TCTGTCCTGGTGGATTGGATC	200
40	6121-6221	101	TTGGTGAGCTCACCGTGT	TTCCCGTGGTGACATGCAAGC	200
41	6222-6331	110	GTCCACACACTGCTCTCTGC	ACACTCCACCTCCCTGGC	320
42	6332-6470	139	GCCAGGGAGGTGGAGTGT	GGTTCCTTCCACCGCAAC	550
43	6471-6584	114	CAACTCCCCAATGGCTC	CCTACCCAGTGCAGAGTGAGG	350
44	6585-6620	36	TCTGTGTGCACCATCCCATG	AAGGATTGGGCTCCATGGAG	200
45	6621-6807	187	GTTGGTGCTAGCTGCTGAC	CTTTCTTCTTCCTTAGTGTC	330
46	6808-7061	254	GTGTGCTGTCTGACCCTCAC	AGCCTGGGGTCACTTGCAGC	320
47	7062-3'UTR	350	CCTTTGTTTCAATTTTCGTGTAG	TGGGGCCTGGGTACCTCCGA	280

Size of Exons and PCR products are given in base pairs.

* Ophoff et al. (1996) ▽ Trettel et al. (1999)

5.1.2.3 Statistical Analysis

We employed the complementary strategies of family-based linkage and association analyses, as well as cross-sectional case-control approach to test for involvement of the *CACNA1A* gene in typical migraine at the population level. Traditional linkage analysis can be a powerful technique for mapping disease loci but may have shortcomings when the disease model is unclear. Therefore, in addition to performing model-based analysis using the FASTLINK program, model-free analyses were also carried out using the GENEHUNTER-PLUS (modification of the GENEHUNTER) and SimIBD programs which can be robust to disease model misspecification (Kruglyak et al., 1996: Kong and Cox, 1997: Davis et al., 1996). Model-free linkage analyses were implemented on pairs of affected relatives using the SPLINK program as well as the scoring functions of the GENEHUNTER-PLUS package and the 1/sqrt (p) weighting function of the SimIBD program. Each of these programs measured identity-by-descent (IBD) allele sharing at a given marker locus between all pairs of affected siblings and extended relatives and they report a LOD score (LOD*) and/or a probability of linkage (Clayton, 1997; Kong and Cox, 1997: Davis et al., 1996). In addition, the TRANSMIT program (Clayton, 1997) was used to perform the TDT on the data from nuclear families. The TDT compares alleles transmitted from a heterozygous parent to an affected offspring and can overcome the problem of population stratification by using parental alleles as internal controls (Spielman et al., 1993). The TRANSMIT program has the added features of being able to deal with datasets where parental genotypes are missing (Clayton, 1997).

A population-based cross-sectional association analysis was also implemented on an unrelated group of migraineurs and unaffected controls matched for age, gender and ethnicity. The allele frequency distributions for each of the two intragenic markers tested in case and control groups were compared using the standard χ^2 test. In addition, the CLUMP program that is useful for association testing when markers produce sparse contingency tables was used. The T1 statistic produced by CLUMP, results from a test for the null hypothesis of no difference in observed allele distribution between cases and controls, with significance being assessed using Monte Carlo simulations (Sham and Curtis, 1995). The CLUMP program was run over 5000 simulations to estimate the *P*-value for this analysis.

5.1.3 Results of the *CACNA1A* Gene Study

5.1.3.1 Mutation Screening

The DNA examined for mutations in the *CACNA1A* gene came from two individuals belonging to a large family affected with typical migraine. Individuals from this family were seen as particularly significant candidates for mutation screening of this gene because the *CACNA1A* intragenic markers D19S1150 and the $(CAG)_n$ repeat showed linkage to the disease in this pedigree (Nyholt et al., 1998). Importantly, the disease phenotype and a five-marker haplotype containing alleles from these *CACNA1A* polymorphisms was shown to cosegregate in the family including the 2 affected individuals screened for mutations (Figure 5.4). All 47 exons of the *CACNA1A* gene were tested for mutations using direct sequencing methods with particular attention focused on the exons containing the known FHM mutations previously identified (Table 5.05).

Results of this work did not reveal any mutations within any of the protein coding segments examined in these migraine patients. The three *CACNA1A* nucleotide substitution polymorphisms described previously by Ophoff et al. were not present in either individual, including the A454T polymorphism in exon 11 that predicts an amino acid change. An example of a sequence electrophoretogram produced from this work is displayed in Figure 5.5.

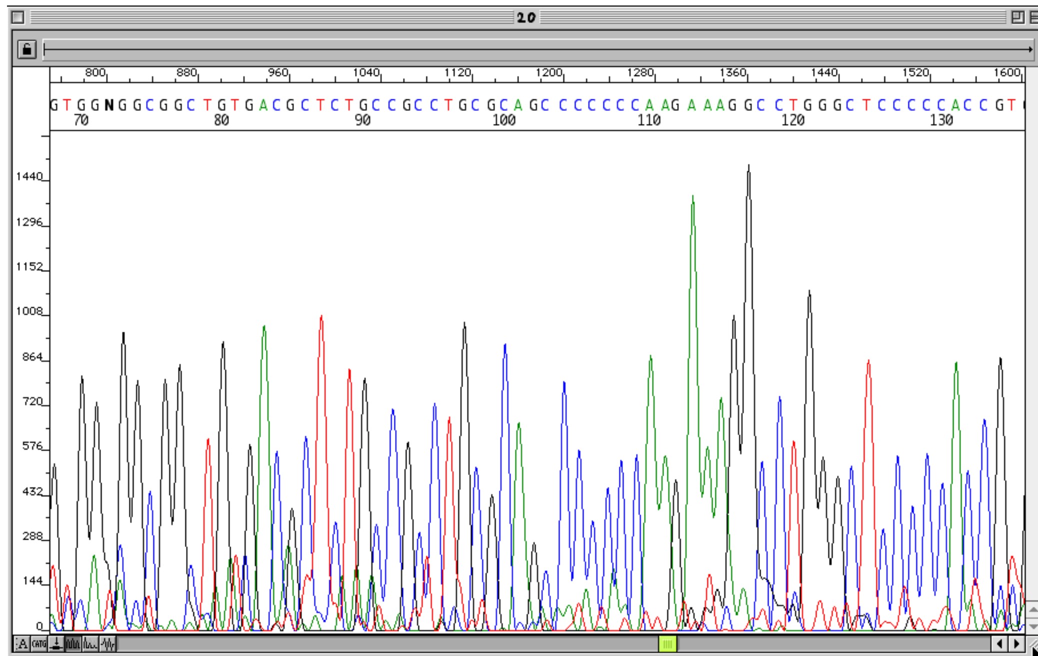

Figure 5.05. An electrophoretogram produced by the SEQUENCHER software. This sequence represents part of the *CACNA1A* exonic gene sequence examined in this mutation screen.

The CACNA1A gene also contains a coding triplet repeat known to be expanded and in some families with SCA6 (Zhuckenko et al., 1997). Observations of alleles for the $(CAG)_n$ repeat polymorphism in this migraine family however did not reveal a higher than normal level of repeats and therefore expansion was not indicated (Nyholt et al., 1998). The CAG repeat number was found to vary between 7 and 13 units in this family with normal levels of between 4 and 14 units previously reported (Ophoff et al., 1996; Riess et al., 1997; Zhuckenko et al., 1997).

5.1.3.2 Linkage and Association

Linkage analysis of MF1 was initially performed to include several additional markers (D1S1150) as well as correcting the 5' – 3' direction of the *CACNA1A* gene based on recent fine physical map results published by Trettel et al. 2000. The latest GENEHUNTER-PLUS multipoint results for this pedigree were not significantly altered from the original published information (Nyholt et al., 1998)(see Figure 5.6), with the model-free (non-parametric) linkage peak still producing a minimum *P*-value of 0.006.

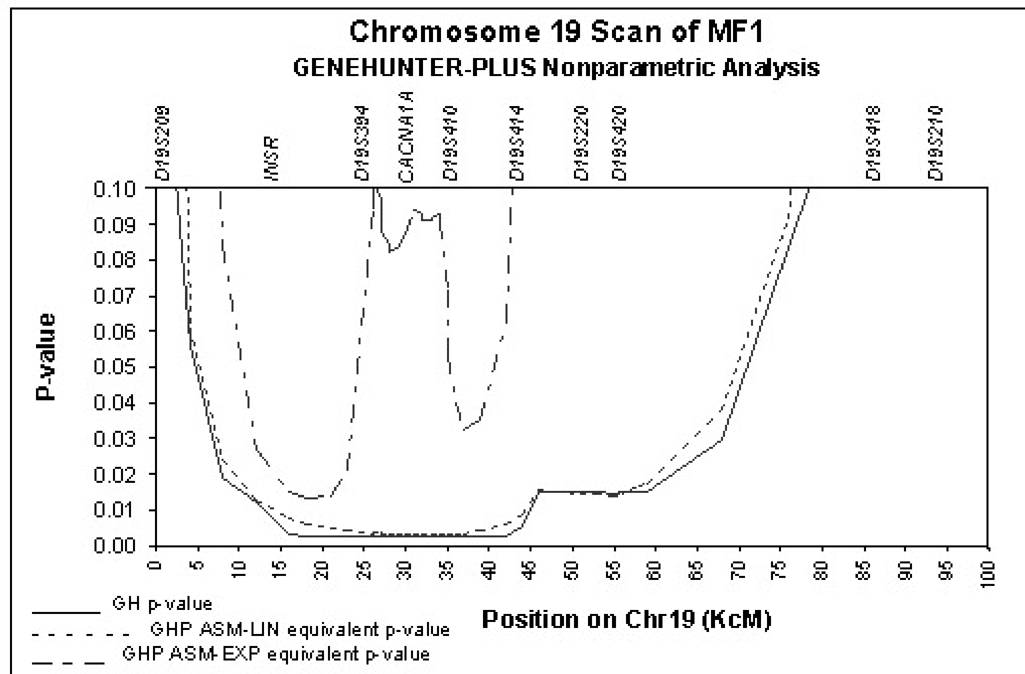

Figure 5.06 This graph shows the statistical curves produced by the GENEHUNTER-PLUS. In particular, these results illustrate the evidence of excess allele sharing ($P<0.05$) of chromosome 19p13 markers in MF1 for various allele-sharing models (ASM).

Affected relative pair allele sharing analysis was then performed on 82 independent pedigrees affected with typical migraine. The two markers flanking the *CACNA1A* gene (D19S179 and D1S221) and the two intragenic markers (D19S1150 and CAG repeat) were tested in these pedigrees. The traditional LOD score (FASTLINK) analysis is presented Table 5.07. Intermarker linkage analysis in these pedigrees shows strong significant linkage between adjacent marker loci independent of a disease locus (LOD > 8, $\theta = 0.1$). However, under the assumed dominant disease model with reduced penetrance these results clearly show exclusion of linkage of the disease locus to markers D19S221 and the *CACNA1A*(CAG)$_n$ at a $\theta = 0.01$ (LOD <-2)

Table 5.07. Model-based LOD score analysis results for markers D19S221, *CACNA1A*-CAG_n and D19S179 and the hypothesised migraine locus

Between Markers *d19s221 and cag* as well as *cag and d19s179*

Order	0.0	0.01	0.05	0.1
D19S221-CAGn	-infini	**11.77**	12.82	11.74
CAGn-D19S1179	-infini	**8.64**	8.91	8.20

Between Markers and Disease Locus

Locus Order	0.0	0.01	0.05	0.1
migraine-D19S221	-2.44	**-2.09**	-1.04	-0.27
migraine-CAG	-2.89	**-2.57**	-1.61	-0.84
migraine-D19S179	-0.82	-0.63	-0.07	0.31

Table 5.08. Results of Model-Free Analyses of an Additional 82 typical migraine pedigrees

Microsatellite Marker	SPLINK (*P*-Values)	GHP LOD* Scores	SimIBD *P*-Values	TRANSMIT (Global χ^2)
D19S221	*P* = 0.581	0.019	*P* = 0.952	9.293, 9 df, (*P* = 0.411)
$(cag)_n$	*P* = 0.356	0.002	*P* = 0.967	6.446, 8 df, (*P* = 0.597)
D1S1150	*P* = 0.674	0.005	*P* = 0.561	8.244, 10 df, (*P* = 0.605)
D19S179	*P* = 0.454	0.011	*P* = 0.450	7.243, 7 df,(*P* = 0.404)

The SPLINK, GENEHUNTER-PLUS and SimIBD results are displayed in Table 5.08. It can be seen that there is no overall evidence for excess IBD allele sharing in pairs of affected family members at any of the markers utilised in this analysis. Similarly, the TDT analysis for allelic association carried out on these pedigrees was negative for all 4 markers tested (Table 5.08). Further population-based association testing using independent groups of unrelated cases and controls was also performed. Comparison of allelic distributions for both intragenic markers (D19S1150 and CAG repeat) did not provide any evidence for alteration between all case and control groups (Tables 5.09 and 5.10). All Monte Carlo simulations performed by CLUMP yielded a *P*-value greater the 0.05 (Table 5.11).

Table 5.09. *CACNA1A* $(CAG)_n$ Triplet Repeat Polymorphism

(CAG)n Allele No.

	1	2	3	4	5	6	7	8	9	10	11	12	Total Alleles (Genotypes)
MA	0	0	11	2	2	1	74	30	54	7	0	1	182 (91)
MO	2	0	20	1	1	1	48	19	42	4	0	0	138 (69)
Total Migraine	2	0	31	3	3	2	122	49	96	11	0	1	320 (160)
Control	1	0	31	1	1	1	111	66	112	10	2	0	336 (168)

Table 5.10. *CACNA1A* Intragenic Dinucleotide Marker (D19S1150)

D19S1150 Allele No.

	1	2	3	4	5	6	7	8	9	10	11	12	Total Alleles (Genotypes)
MA	0	1	0	5	4	48	8	12	24	46	2	12	162 (81)
MO	1	0	3	5	9	33	11	13	25	33	1	14	148 (74)
Total Migraine	1	1	3	10	13	81	19	25	49	79	3	26	310 (155)
Control	0	1	0	6	6	75	10	20	41	60	5	14	238 (119)

Table 5.11. CLUMP Analysis Comparing all Migraine Groups against Controls

Group	(CAG)n	D19S1150
Migraine (combined)	$\chi^2 = 9.59$, $P = 0.51$	$\chi^2 = 9.27$, $P = 0.63$
MA	$\chi^2 = 11.08$ $P = 0.35$	$\chi^2 = 2.01$, $P = 0.99$
MO	$\chi^2 = 8.88$ $P = 0.46$	$\chi^2 = 17.93$, $P = 0.061$

5.1.4 Discussion

The *CACNA1A* gene encodes a brain specific P/Q-type calcium channel α-1 subunit. This protein plays an important role as a voltage sensor and forms an ion-conducting pore of the channel. Many different mutations in this gene have been shown to cause the neurological disorders, FHM and Episodic Ataxia type 2, in unrelated families (Ophoff et al., 1996: others). To our knowledge there has been only one report of a *CACNA1A* mutation being associated with a typical migraine patient (specifically suffering MA). This patient was from a predominantly FHM pedigree and may represent a case of non-penetrant FHM (Terwindt et al., 1998). A study by Kim et al

failed to find any *CACNA1A* mutations after screening all 47 exons in 9 propositi suffering from familial migraine with vertigo (Kim et al., 1998). However, it should be noted that the authors of this paper provided no evidence that the disease in these 9 patients was linked to chromosome 19p13. Research in our laboratory has demonstrated linkage of typical migraine to the genomic region containing the *CACNA1A* gene in a large Australian family. In agreement with the results of Kim et al., direct sequence analysis of affected patients sharing the susceptibility haplotype in this family did not show any mutations in the 47 exons of the *CACNA1A* gene. Another type of inherited ataxia, spinocerebellar ataxia type 6 (SCA6), has been associated with CAG-expanded variants of *CACNA1A* (Riess et al., 1997: Zhuckenko et al., 1997). Our results also did not show that a CAG repeat expansion in this family was involved in the disease (Nyholt et al., 1998).

5.1.5 Summary of *CACNA1A* Gene Study

In conclusion, the findings of this investigation indicate that the *CACNA1A* mutations seen in some FHM families do not cause typical migraine in a large Australian pedigree linked to the same genomic region. In addition, linkage and association studies in a large case-control population and in multiple migraine pedigrees did not support a role for this gene in typical migraine. However, there are other genes located in the 19p13 region that could be considered as potential candidates for typical migraine. Hence we are continuing our search for mutations within candidate genes predominantly in our chromosome 19-linked pedigree.

5.2 Single Nucleotide Polymorphism (SNP) Alleles in the Insulin Receptor (*INSR*) Gene are Associated with Typical Migraine

5.2.1 Introduction

Our comprehensive study of the FHM gene (*CACNA1A*) failed to yield any positive results when examined for mutations, linkage and association in typical migraine populations. Although the *CACNA1A* gene cannot be completely ruled out as contributing to the overall genetic risk of the common forms of migraine in the general population, it is more likely that variations within an adjacent gene(s) on 19p13 may confer a more substantial risk to the disease. Figure 5.3 is an ideogram of the chromosome 19p13 region and illustrates the positions of other migraine candidate genes (*PRKCSH*, *LDLR* and *INSR*) relative to the *CACNA1A* gene. The idea of an adjacent gene locus being involved in typical migraine is well supported by a recent migraine linkage study, which incorporated haplotype analysis, of 16 pedigrees affected with MA. Results of this work provide very strong evidence that a MA susceptibility gene on chromosome 19p13 actually localises to a region distinct from (and telomeric to) the FHM (*CACNA1A* gene) locus (Jones et al, 2001). Using a multipoint non-parametric linkage analysis approach Jones et al, obtained a LOD score of 4.28 near D19S592. Using an affecteds-only model of linkage, they also observed a LOD score of 4.79 near D19S592. They were than able to identify five recombination breakpoints between the interval on chromosome 19p13 co-segregating with migraine with aura and FHM gene (*CACNA1A*). These data indicate that chromosome 19p13 contains a locus which contributes to the genetic susceptibility of migraine with aura yet is *distinct* from the FHM locus (Jones et al, 2001).

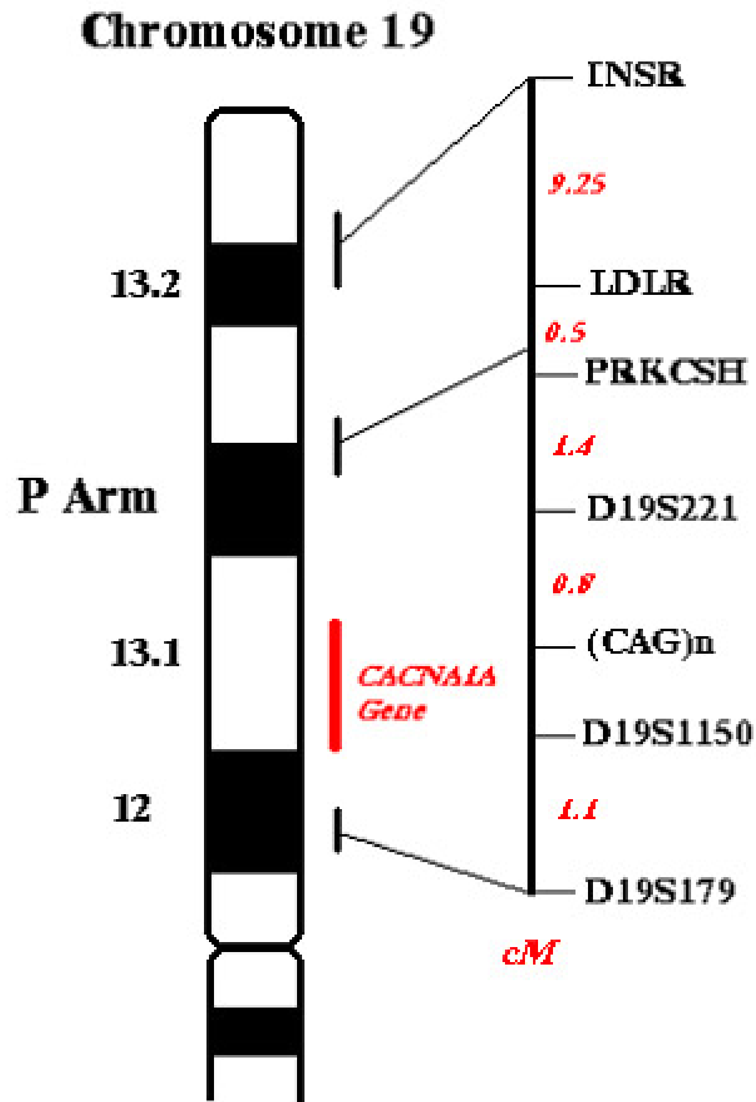

Figure 5.03. An ideogram of the chromosome 19p13 region showing relative locations of microsatellite markers and migraine candidate genes

Encouraged by the chromosome 19p13 linkage study of Jones et al, that indicated a locus distinct from the *CACNA1A* gene may actually be involved with MA susceptibility, McCarthy et al, focused on the gene encoding the insulin receptor (*INSR*) which resides ~12cM telomeric to the *CACNA1A* gene and localises under the strong linkage peak reported by Jones et al. (Jones et al, 2001). This gene may be a candidate for migraine especially since there is some evidence of co-morbidity of migraine and non-insulin dependent diabetes (Split and Szydlowska, 1997). Thus, the

experiment conducted by McCarthy et al, assessed the involvement of the INSR gene in the common forms of migraine by using a SNP-based measurement of LD in very large migraine case-control populations. This work initially involved comprehensive physical mapping efforts to identify useful SNPs spanning the INSR gene region (see Figure 5.7). SNPs were chosen as the marker of choice in this study primarily because of their abundance in the region, with the average genomic SNP frequency estimated at >1/kb. The SNPs were isolated within the INSR gene locus at a minimum frequency of 1 every 50kb, by re-sequencing in 12 Caucasian DNA samples. These variants were identified from a variety of sources, including random genomic sub-clones, inter-*Alu* products, clone-end sequence, gene polymorphism screens, *in silico* analysis of gene sequence, and public databases. Following this identification process, a total of 27 SNPs covering the INSR gene region were selected for genotyping for the purposes of developing an LD map between adjacent SNPs (McCarthy et al, 2001). The results of this analysis revealed 12 SNPs that were in strong LD with at least one other adjacent SNP. Subsequent association analysis using very large Caucasian case and control groups (n>750) was carried out on these, and 4 additional SNPs in coding regions, of the INSR gene. The INSR gene spans 120kb of genomic DNA containing 4.9kb of coding sequence in 22 exons (Seino S. *et al.*, 1989). A schematic of the genes organisation with identified SNPs is shown in Figure 5.8). A comparison of SNP allele frequencies between cases and controls provided evidence that 5 SNPs within INSR were associated with migraine in these populations (McCarthy et al, 2001). Specifically, SNPs 274, 273, 84, 90, and 81 produced χ^2 related P values less than 0.05. Although, none of the SNP alleles associated with migraine in this study changed an amino acid, the location of SNP 274 in exon 17, was only 3 nucleotides away from a splice site, suggesting a possible functional role in splice regulation (McCarthy et al, 2001). Furthermore, this study did not reveal any significant effect of the SNPs on insulin binding to mononuclear cells or *INSR* mRNA levels in whole blood cells, or *INSR* -mediated effects on clinical parameters. Nevertheless these results provide insights into further investigational paths to be explored to understand the possible role of this receptor (McCarthy et al, 2001).

Figure 5.7. Minimum tiling physical map of the chromosome 19p13.3/2 migraine locus. The gap between PAC 55382 and BAC 85258 is estimated to be no greater than 20kb, based on radiation hybrid mapping. No clones could be identified which spanned this gap. The locations of known genes in the locus are indicated under the map. The SNPs assayed in the primary association screen are displayed in bold font (McCarthy et al, 2001).

Figure 5.8. Location of SNPs across the *INSR* gene. The 22 *INSR* exons are located across 120kb of genomic sequence. The identifiers and locations of the 16 INSR SNPs genotyped on the migraine case/control populations are presented on top. The triangles indicate the locations of all INSR SNPs identified in this study, including the 16 genotyped SNPs (McCarthy et al, 2001).

Interestingly, the linkage peak obtained from our initial studies of MF1 spanned a genetically broad distance on chromosome 19p13 and encompassed the *INSR* gene as well as *CACNA1A*. Therefore, we decided to test the 5 associated SNPs reported by McCarthy et al in our typical migraine case-control samples in the hope of replicating their positive results. A general transcription factor (*GTF2*) located ~500kb telomeric to the *INSR* gene was also examined by testing a SNP located within this gene (see Figure 5.7).

5.2.2 Methods

5.2.2.1 Study Subjects

All subjects who participated in this study gave informed consent, were of Caucasian origin and were diagnosed for migraine based on the operational set of diagnostic criteria specified by the IHS. As in the *CACNA1A* study, typical migraine individuals were diagnosed as having either migraine with aura (MA) or migraine without aura (MO) through interview by a clinical neurologist and from questionnaires prepared using strict IHS guidelines. This study however, involved a larger sample of cases and controls. In total, 492 DNA samples from unrelated individuals were analysed (237 unaffected controls and 255 migraineurs). Of the migraine affected group, ~90% of patients had a known family history of the disease, or at least one first degree relative suffering from typical migraine. Clinically, the affected group had a median age of onset of 19yrs, average headache duration of 20 hours with a frequency of approximately 30 per year. This group was comprised of 145 individuals diagnosed as clearly having MA, whilst 110 were diagnosed as having MO. Again, the unaffected control group was recruited from the same geographical location (East Coast of Australia) as the affected group and was carefully matched for age, gender and ethnicity, thus reducing the possibility of spurious results due to population stratification

5.2.2.2 DNA and Genotyping

Genomic DNA was extracted from whole venous blood samples by a standard SDS-Proteinase K (Blin and Stafford, 1976) incorporating a salting out procedure (Miller et al., 1988) and used as a template to generate PCR products for genotyping. The SNPs

tested in this study included 5 from the *INSR* gene and 1 from the *GTF2*. Primers specific for these SNPs were employed to perform PCR (Table 5.12). PCR amplification was performed as per the following conditions. A total volume of 15µl was prepared containing 30ng of DNA, 1 unit of *Taq* polymerase, 1.75mM $MgCl_2$, 5mM dNTPs and buffer. Samples were then subjected to thermal cycling conditions of 1 cycle at 94°C for 4min, 35 cycles of 94°C for 1min, 60°C for 1min, and 1 cycle of 72°C for 2min. The PCR products underwent RFLP digest using the enzymes listed in Table 5.12. Resulting fragments were then discriminated on 2-5% agarose gel and genotyped for statistical analysis.

Table 5.12. Primer sequences for INSR gene SNPs

Marker	Physical Location	Primer Sequence	Restriction Enzyme
SNP 274	Exon 17- INSR	F-GTGTTGCCACGTCAGTCAGTC R-AACGTTGTCGTACCGGTGTCA	*Eco*II
SNP 273		F- GGTCGTAACGTACGTTCTCTG R-CACAACCGTCACGTCCCGTCC	*Bg*l11
SNP 84	Intron15 - INSR	F-GACGGCTACGTAGGGCCTAA R-CAACCTGTTGTACGTCGTACG	*Nco*I
SNP 90	Intron 14 - INSR	F-GAGGTATGCGACTGGGACTCAG R-GTAATCCCCAATGTTGGAGGAG	*Bg*l11
SNP 81	Intron 2 - INSR	F-CGCTAACGTTGCGTCCGTCCG R-GCTTCTCGGCTGCTAAAACTA	*Nco*I
SNP 86	440kb distal to INSR	F-GCATGCATGCGCTACGTCAGT R-CAGATTACGATCGAGCATCGT	*Taq*I

5.2.2.3 Data Analysis

Allele and genotype frequencies were compared using standard χ^2 (association) contingency table analysis implemented in the SPSS program. Hardy-Weinberg equilibrium calculations were performed as a statistical control for genotyping errors. The extent of LD between SNPs was assessed using Lewontin's D' coefficient with significance based on standard χ^2 as implemented in the EH program (Xie and Ott, 1993). Power estimates indicated that if any of these polymorphisms were to confer a two-fold increase in relative risk of typical migraine, the case and control groups used

in this study were of sufficient size to have 95% power to detect an allelic association and 85% power to detect a genotypic association at the 0.05 significance level. Significance is presented as raw *P*-values, although multiple comparisons were performed and need to be considered when interpreting these results. Allele sharing analysis of SNP 84 and 86 was performed in MF1 using the GENEHUNTER-PLUS program.

5.2.3 Results of the *INSR* Gene Study

Initial polymorphism testing in this study was performed using a marker (SNP 84) from the *INSR* gene that was previously shown to be associated with migraine (McCarthy et al, 2001). Although INSR was the focus of this replication study, an additional marker (SNP 86) located in the adjacent gene, GTF2 was also examined. This marker was tested since alleles of the SNP were also found to be marginally positive for association in the migraine case-control groups used by McCarthy et al (2001).

5.2.3.1 Contingency Table Analysis

The SNP 84 and SNP 86 genotype and allele frequencies for total case-control populations are displayed in Tables 5.13 and 5.14, respectively. HWE testing indicated that observed genotype frequencies for both SNP 84 and SNP 86 were not significantly different from expected values. Statistical comparison was also performed on distributions of data from these SNPs incorporating genetic migraine enriched, as well as matched population sets. The first enriched analysis was carried out on only those migraineurs possessing a positive family history of the disease (Tables 5.15 and 5.16). Secondly, all migraine affected individuals who did not match to a sex and age unaffected control were removed from the analysis (Tables 5.17 and 5.18). These chi-squared analyses are shown in Tables 5.13a – 5.18a.

The results of this initial marker analysis revealed a positive association for SNP 84 in the *INSR* gene. At an α level of 0.05 there was a significant difference in allele frequencies seen between the MO group and controls (χ^2 = 5.77, P = 0.016). The observed difference is largely due to over-representation (24%: 16%) of the rarer allele (SNP 84-A2) in the affected MO group. Analysis of this marker in the enriched

and matched populations also showed similar results. Allele and genotype frequencies in the SNP 86 marker of the adjacent GTF2 gene were not significant different in any of the analyses carried out.

Table 5.13. Distribution of SNP 84 Genotype and Allele Frequencies of INSR gene in Migraine ***(Total)**** and Control Groups

Group	*SNP 84 Genotypes*			N *alleles*	SNP 84 Alleles	
	A1/A1	*A1/A2*	*A2/A2*		A1	A2
Migraine (combined)	167 (66%)	77 (30%)	11 (4%)	510	411 (81%)	99 (19%)
MA	102 (70%)	39 (27%)	4 (3%)	290	243 (84%)	47 (16%)
MO	65 (59%)	38 (35%)	7 (6%)	220	168 (76%)	52 (24%)
Control	167 (70%)	64 (27%)	6 (3%)	474	398 (84%)	76 (16%)

* all migraineurs used

Table 5.13a. Chi-squared (χ^2) Analysis of all Migraine Groups against Controls

Group	Frequency Comparison	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 2.01$, $P = 0.37$	$\chi^2 = 1.92$, $P = 0.17$
MA	$\chi^2 = 0.02$, $P = 0.99$	$\chi^2 = 0.00$, $P = 0.95$
MO	$\chi^2 = 5.85$, $P = 0.054$	$\chi^2 = 5.77$, $\mathbf{P = 0.016}$*

* All results are presented as raw *P*-values, ie: they have not been corrected for multiple comparisons.

Table 5.14. Distribution of SNP 86 Polymorphism Genotype and Allele Frequencies of INSR gene in Migraine ***(Total)**** and Control Groups

Group	*SNP 86 Genotypes*			N *alleles*	SNP 86 Alleles	
	A1/A1	*A1/A2*	*A2/A2*		A1	A2
Migraine (combined)	182 (66%)	79 (29%)	13 (5%)	548	443 (81%)	105 (19%)
MA	108 (66%)	48 (29%)	8 (5%)	328	264 (80%)	64 (20%)
MO	74 (67%)	31 (28%)	5 (5%)	220	179 (81%)	41 (19%)
Control	162 (66%)	72 (29%)	12 (5%)	492	396 (80%)	96 (20%)

* all migraineurs used

Table 5.14a. Chi-squared (χ^2) Analysis of all Migraine Groups against Controls for SNP 86

Group	Frequency Comparison	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 0.02$, $P = 0.99$	$\chi^2 = 0.02$, $P = 0.89$
MA	$\chi^2 = 0.00$, $P = 1.00$	$\chi^2 = 0.00$, $P = 1.00$
MO	$\chi^2 = 0.07$, $P = 0.96$	$\chi^2 = 0.08$, $P = 0.78$

Table 5.15. Distribution of SNP 84 Genotype and Allele Frequencies of INSR gene in Migraineurs *(with family history of migraine only)** and Control Groups

Group	*SNP 84 Genotypes*			N *alleles*	SNP 84 Alleles	
	A1/A1	*A1/A2*	*A2/A2*		A1	A2
Migraine (combined)	135 (66%)	62 (30%)	8 (4%)	410	332 (81%)	78 (19%)
MA	83 (71%)	30 (26%)	3 (3%)	232	196 (84%)	36 (16%)
MO	52 (58%)	32 (36%)	5 (6%)	178	136 (76%)	42 (24%)
Control	167 (70%)	64 (27%)	6 (3%)	474	398 (84%)	76 (16%)

* Migraineurs without a family history (or who do not have information on this category) have been removed from the analysis due to the possible environmental (non-genetic) occurence of the disease.

Table 5.15a. Chi-squared (χ^2) Analysis of all Migraine Groups against Controls for INSB

Group	Frequency Comparison	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 1.40$, $P = 0.50$	$\chi^2 = 1.37$, $P = 0.24$
MA	$\chi^2 = 0.05$, $P = 0.97$	$\chi^2 = 0.03$, $P = 0.86$
MO	$\chi^2 = 4.98$, $P = 0.083$	$\chi^2 = 4.99$, $\mathbf{P = 0.025}$*****

Table 5.16. Distribution of INSB Polymorphism Genotype and Allele Frequencies of INSR gene in Migraineurs *(with a sex/age matched control only)** and Control Groups

Group	*INSB Genotypes*			N *alleles*	INSB Alleles	
	A1/A1	*A1/A2*	*A2/A2*		A1	A2
Migraine (combined)	141 (65%)	67 (31%)	10 (4%)	436	349 (80%)	87 (19%)
MA	83 (70%)	33 (28%)	3 (2%)	238	199 (84%)	39 (16%)
MO	58 (59%)	34 (34%)	7 (7%)	198	150 (76%)	48 (24%)
Control	154 (71%)	58 (27%)	6 (2%)	436	366 (84%)	70 (16%)

* Migraineurs without a sex and aged matched control have been removed from the analysis to reduce the possibly of population stratification.

Table 5.16a. Chi-squared (χ^2) Analysis of all Migraine Groups against Controls for INSB

Group	Frequency Comparison	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 2.22$, $P = 0.33$	$\chi^2 = 2.24$, $P = 0.13$
MA	$\chi^2 = 4.88$, $P = 0.088$	$\chi^2 = 0.01$, $P = 0.91$
MO	$\chi^2 = 5.98$, $P = 0.050$	**$\chi^2 = 6.03$, $P = 0.014$***

Table 5.17**.** Distribution of Rap74 Polymorphism Genotype and Allele Frequencies of INSR gene in Migraineurs *(with family history of migraine only)** and Control Groups

Group	*Rap74 Genotypes*			N *alleles*	Rap74 Alleles	
	A1/A1	*A1/A2*	*A2/A2*		A1	A2
Migraine (combined)	150 (67%)	65 (29%)	10 (4%)	450	365 (81%)	85 (19%)
MA	89 (66%)	40 (30%)	6 (4%)	270	218 (81%)	52 (19%)
MO	61 (68%)	25 (28%)	4 (4%)	180	147 (82%)	33 (18%)
Control	162 (66%)	72 (29%)	12 (5%)	492	396 (80%)	96 (20%)

* Migraineurs without a family history (or who do not have information on this category) have been removed from the analysis due to the possible environmental (non-genetic) occurence of the disease.

Table 5.17a. Chi-squared (χ^2) Analysis of all Migraine Groups against Controls for Rap74

Group	Frequency Comparison	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 0.06$, $P = 0.97$	$\chi^2 = 0.06$, $P = 0.81$
MA	$\chi^2 = 0.04$, $P = 0.98$	$\chi^2 = 0.01$, $P = 0.93$
MO	$\chi^2 = 0.11$, $P = 0.94$	$\chi^2 = 0.12$, $P = 0.73$

There was no significant difference between allele frequencies for any migraine group and controls.

Table 5.18. Distribution of Rap74 Polymorphism Genotype and Allele Frequencies of INSR gene in Migraine *(with a sex/age matched control only)** and Control Groups

Group	*Rap74 Genotypes*			N *alleles*	Rap74 Alleles	
	A1/A1	*A1/A2*	*A2/A2*		A1	A2
Migraine (combined)	146 (65%)	69 (31%)	10 (4%)	450	361 (80%)	89 (20%)
MA	89 (65%)	43 (31%)	5 (4%)	274	221 (81%)	53 (19%)
MO	57 (65%)	26 (30%)	5 (5%)	176	140 (80%)	36 (20%)
Control	150 (67%)	65 (29%)	10 (4%)	450	365 (81%)	85 (19%)

* Migraineurs without a sex and aged matched control have been removed from the analysis to reduce the possibly of population stratification.

Table 5.18a. Chi-squared (χ^2) Analysis of all Migraine Groups against Controls for Rap74

Group	Frequency Comparison	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 0.17$, $P = 0.92$	$\chi^2 = 0.11$, $P = 0.74$
MA	$\chi^2 = 0.35$, $P = 0.84$	$\chi^2 = 0.02$, $P = 0.88$
MO	$\chi^2 = 0.25$, $P = 0.88$	$\chi^2 = 0.02$, $P = 0.66$

There was no significant difference of allele frequencies between any migraine group and controls.

5.2.3.2 Linkage Disequilibrium Analysis Between SNP 84 and SNP 86

In concurrence with McCarthy et al., the initial markers we tested revealed a significant allelic association between SNP 84 and migraine and lack of allelic association between alleles at SNP 86 and migraine. As with the study of McCarthy et al, we also performed LD analysis between the SNPs tested in this replication study using the EH program. To conduct LD analysis, estimation of the possible haplotype frequencies formed between the two markers is required. The results of the LD analysis for SNP 84 and SNP 86 are shown in Table 5.19. The likelihood ratio chi-squared for H1 in Table 5.19 indicates that there is no evidence of LD between these two markers independent of the disease (χ^2 = 0.01, df = 2, P = 0.97).

Table 5.19. Linkage Disequilibrium Results for INSR SNPs

```
There are 4 Possible Haplotypes of These 2 Loci.
They are Listed Below, with their Estimated Frequencies:
-----------------------------------------------------------------------
|         Allele   Allele  |         Haplotype   Frequency           |
| Haplo     at       at    |                                         |
|         Marker1  Marker2 |  Independent   Ind-Disease   w/Asso.   |
-----------------------------------------------------------------------
    1       1        1          0.582214      0.582910      0.588196
    2       1        2          0.127053      0.126344      0.116740
    3       2        1          0.140149      0.139478      0.151733
    4       2        2          0.030584      0.031268      0.023331
---------------------------------------------------------------------
                                      #DF   Ln(L)     Chi-square
---------------------------------------------------------------------
H0: No Association                     2   -713.86      0.00
H1: Markers Asso., Indep. of Disease   3   -713.86      0.01
```

Family-based allele sharing analysis of SNPs 84 and 86 was also carried out using our chromosome 19p13 linked pedigree, MF1. The results of the GENEHUNTER results provided no evidence of linkage or excess allele sharing for either of these SNPs in MF1. It should be noted that being biallelic these markers were not likely to provide enough inheritance information to be useful for this type of analysis. This is evident in the bolded information column of Table 5.20

Table 5.20. Genehunter Results for SNP 84 and SNP 86

Position	Parametric LOD Score	NPL Score	P-Value	Information
SNP 86	-0.4364	-0.1602	0.335	**0.176**
SNP 84	-0.4181	-0.1873	0.369	**0.401**

5.2.3.3 Follow-up Analysis of 4 additional SNPs in INSR Gene

In an attempt to substantiate the positive association results produced in this study at SNP 84 in the INSR gene, and add further support to results determined previously, we decided to test the 4 other SNPs that produced significant results in the study by McCarthy et al. Thus we tested SNPs 273, 274, 90 & 81 in our independent case-control populations for association to migraine. We also examined LD between alleles at these SNPs.

We were able to obtain genotypic data on three of the four SNPs examined. The SNP 81 PCR and RFLP assay produced fragments that were unable to be discriminated using the electrophoresis methods adopted for this study. This was due to double cleavage of the PCR product by the chosen enzyme *Nco*I, resulting in many DNA segments, several of which were too similar in size to be distinguished.

The genotype and allele frequencies observed for the other markers SNP 90, 274 and 273 are shown in Tables 5.21 – 5.23. The test for HWE indicated no alteration of genotypes from expected distributions. Chi-squared analysis of all three SNPs revealed no significant difference in genotype or allele frequency ($P > 0.05$). LD analysis of all INSR markers examined in this study indicated strong and highly significant LD between SNPs 274 and 273 which are located in the exon 17 coding region of the INSR gene (D' = 0.67, $P = 0.0001$). Alleles of these SNPs were not found to be in LD with either of the other two markers. Similarly, there was no signficant LD detected between alleles of SNP 84 and SNP 90, and any of the other markers ($P > 0.05$). The lack of LD between SNP 84 and any of the adjacent SNPs tested in this study may partially explain the failure to also detect a co-association between alleles at these SNPs and migraine (or haplotype association). In addition, the negative association produced for SNP 274, 273 and 90 and migraine conflicts with the positive results of McCarthy et al. This disparity may be the result of lack of statistical power using our considerably smaller samples.

Tables 5.21 a & b: Association Results of **SNP 90** Marker

Group	SNP 90 *Genotypes*			N *(Genotypes)*	*SNP 90* Alleles	
	1/1	*1/2*	*2/2*		1	2
Migraine (combined)	85 (70%)	27 (46%)	3 (3%)	121	203 (84%)	39 (16%)
MA	43	17	1	61	103	19
MO	42	16	2	60	100	20
Control	87 (67%)	39 (30%)	3 (3%)	129	213 (83%)	45 (17%)

Group	Standard χ^2 Results	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 0.27$, $P = 0.87$	$\chi^2 = 0.16$, $P = 0.69$
MA	$\chi^2 = 0.23$, $P = 0.89$	$\chi^2 = 0.21$, $P = 0.65$
MO	$\chi^2 = 0.38$, $P = 0.83$	$\chi^2 = 0.03$, $P = 0.85$

Tables 5.22 a & b: Association Results of **SNP 274** Marker

Group	Exon 17a *Genotypes*			N *(Genotypes)*	*Exon17a* Alleles	
	1/1	*1/2*	*2/2*		1	2
Migraine (combined)	107 (65%)	53 (32%)	5 (3%)	165	267 (81%)	63 (19%)
MA	64	26	3	93	154	32
MO	43	27	2	72	113	31
Control	108 (67%)	43 (27%)	11 (6%)	162	259 (80%)	65(20%)

Group	Standard χ^2 Results	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 3.27$, $P = 0.2$	$\chi^2 = 0.1$, $P = 0.75$
MA	$\chi^2 = 1.45$, $P = 0.48$	$\chi^2 = 0.63$, $P = 0.43$
MO	$\chi^2 = 3.82$, $P = 0.15$	$\chi^2 = 0.13$, $\mathbf{P} = 0.72$

Tables 5.23 a & b: Association Results of **SNP 273** Marker

Group	Exon 17b *Genotypes*			N *(Genotypes)*	*Exon17b* Alleles	
	1/1	*1/2*	*2/2*		1	2
Migraine (combined)	119 (86%)	20 (14%)	0	139	258 (93%)	20 (7%)
MA	67	10	0	77	144	10
MO	52	10	0	62	114	10
Control	139 (85%)	22 (13%)	3 (2%)	164	300 (91%)	28(9%)

Group	Standard χ^2 Results	
	Genotypes	Alleles
Migraine (combined)	$\chi^2 = 2.6$, $P = 0.27$	$\chi^2 = 0.37$, $P = 0.54$
MA	$\chi^2 = 1.45$, $P = 0.49$	$\chi^2 = 0.6$, $P = 0.44$
MO	$\chi^2 = 1.37$, $P = 0.5$	$\chi^2 = 0.03$, $\boldsymbol{P =} 0.87$

Summary of the *CACNA1A* and *INSR* Gene study

This study was primarily aimed at investigating genes localising to the migraine susceptibility region on chromosome 19p13. Initially, we comprehensively analysed the FHM gene (*CACNA1A*) for linkage, association and mutations using migraine pedigrees and case-control groups. The results of our study interpreted with the linkage/haplotype exclusion work of Jones et al, indicate the *CACNA1A* gene is not likely to confer a substantial risk to typical migraine in the general population. We then decided to examine variations in the INSR gene, also located on chromosome 19p13, for involvement in susceptibility to typical migraine. In particular, SNPs were analysed using our migraine case-control population collected in Australia. The SNPs were chosen for testing due to their being previously associated with migraine in a large, independent case-control cohort (McCarthy et al, 2001). It was the objective of the present study to attempt to replicate these positive *INSR* allelic associations. The comparison of allele frequency distributions indicated that the rare allele at one SNP within the INSR gene (SNP 84) was significantly over-represented in migraineurs. These results concur with those of McCarthy et al, and hence provide compelling evidence suggesting that alterations at the *INSR* locus confer susceptibility to migraine in the general population. Further work is now required to determine whether the *INSR* gene on chromosome 19p13 will emerge as a major migraine susceptibility gene and if so what role this gene and it's upstream receptor protein will play in the pathophysiology of the disease.

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CHAPTER 6
LOCALISATION OF A MIGRAINE SUSCEPTIBILITY REGION TO CHROMOSOME 1Q31

6.1 Introduction

All types of migraine are genetically heterogeneous even the most genetic form, FHM. In fact, the *CACNA1A* gene, located on chromosome 19p13, is responsible for causing FHM in only 50% of affected families. Family linkage studies conducted by Gardner et al have implicated an additional FHM susceptibility region on chromosome 1q31 (Gardner et al. 1997). Furthermore, independent research carried out by Ducros et al. has indicated a second FHM locus at 1q21-23, which is ~ 30cM centromeric to the region reported by Gardner et al (Ducros et al. 1997). At this stage it is not clear whether there is a single locus, or two distinct loci, on the chromosome 1q region. Of particular interest though, is the report of another neuronal calcium channel α-1 subunit gene, *CACNA1E* in the region of 1q31 (Diriong et al. 1995). This gene, thought to be associated with R (resistant) or T (transient) type calcium channels has high sequence identity to *CACNA1A* (~85%), and is therefore an excellent candidate for FHM, and also a plausible candidate for involvement in typical migraine in some pedigrees. Considering the hypothesis that FHM and typical migraine, in part, may be caused by a common defective gene(s), and that the *CACNA1E* gene may be a good candidate for disease susceptibility, we decided to also test the FHM susceptibility region on chromosome 1q for involvement in the more prevalent and genetically complex typical migraine disorder. Hence, this study employed a family-based linkage and association approach to test pedigrees affected with typical migraine (MO and MA), specifically utilising markers located within the FHM genomic region on chromosome 1q31, the region reported to contain the *CACNA1E* gene (Diriong et al. 1995). (See Figure 6.01)

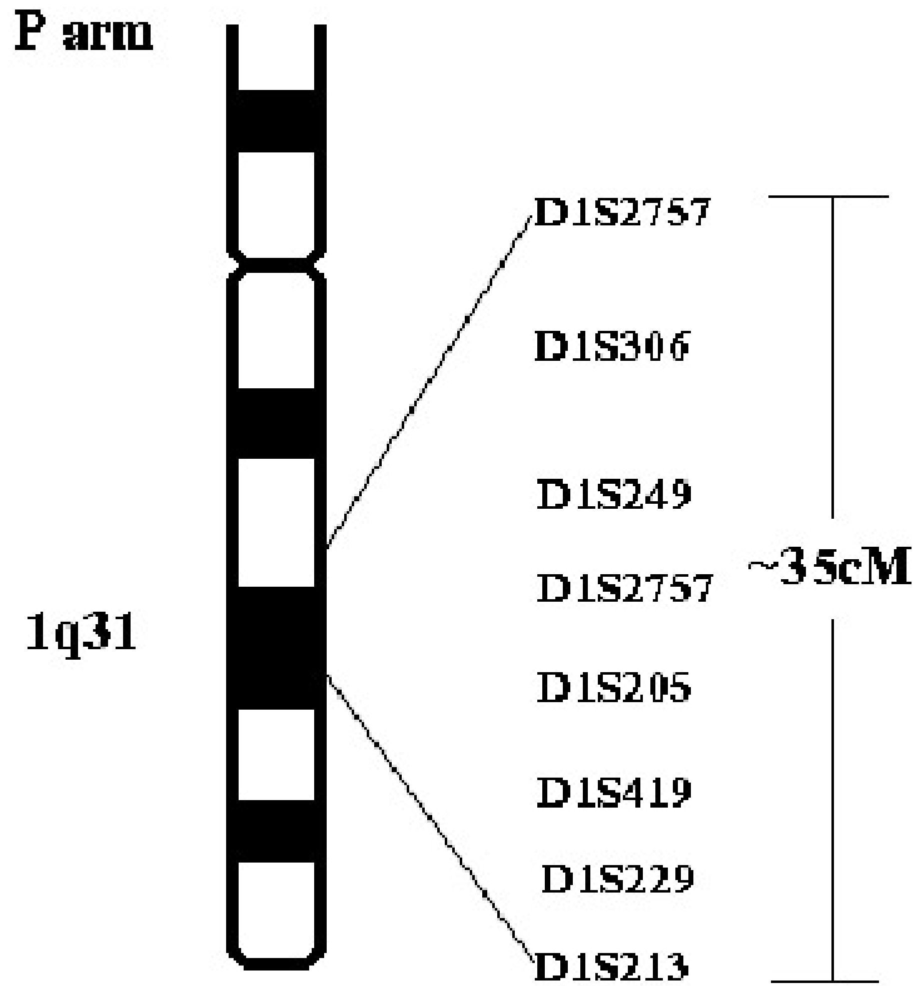

Figure 6.01. The FHM susceptibility region reported by Gardner et al (1997) on chromosome 1q31 showing all markers tested. Markers D1S249 and D1S2757 were tested in the Gardner study and found to be significantly linked to FHM (Gardner et al, 1997).

6.2 Materials and Methods

6.2.1 Patients and Families

All individuals were of Caucasian origin and gave informed consent before participating in the research. Sufferers of typical migraine were shown to exhibit phenotypic variation that included differences in age of onset, frequency and severity of attacks, environmental triggers and medication response. However, all affected individuals were diagnosed as having either migraine with aura (MA) or migraine without aura (MO), based on criteria specified by the International Headache Society (IHS) (MA = criteria 1.2.1 and MO = criteria 1.1) and through interview by a clinical neurologist (Headache Classification Committee of the International Headache Society 1988). As with previous studies, all individuals with MA and MO were grouped together and phenotyped as being affected with typical migraine, as well as being analysed separately as affected with MA only.

Our initial chromosome 1 investigations focused on 3 previously published typical migraine pedigrees (MF1, MF7, MF14) (Nyholt et al. 1998a; Nyholt et al. 1998b). These large multigenerational families consisted of 123 members in total (106 DNA samples available), 63 of which were affected with MA, MO or both. In addition to the 3 large families, DNA from 296 subjects (263 migraineurs) from an independent sample of 82 additional families affected with typical migraine was ascertained for genotyping. These pedigrees have been described previously and relative pair counts are shown in (Table 6.01).

Table 6.01. Affected Relative Pairs of a Sample of 82 typical migraine Pedigrees	
Category	No. of individuals with Typical Migraine
Total Families	82
Affected relative pairs	
Sibling pairs	97
Half-sibling pairs	4
Uncle (aunt)-nephew (niece) pairs	90
Grandparent-grandchild pairs	24
Great Grand Parents-grandchild pairs	3
Cousin pairs (first + second)	19
Great uncle (great aunt)-nephew (niece) pairs	15
Subtotal	252

* All possible combinations of pairs formed by all affecteds within each family (excluding parent-child pairs)

6.2.2 Markers and Genotyping

Genomic DNA was extracted from blood samples using a standard SDS-proteinase K method (Blin and Stafford 1976), incorporating a salting out procedure (Miller et al. 1988). In total, 8 dinucleotide repeat markers were selected for testing in this study. Microsatellite information including primer sequences, marker spacing (cM) and order was obtained from Genethon, Genome Database and NCBI. The entire map had an average marker spacing of 4cM and including recombination fraction distances (in parentheses) was; D1S2757-(0.06)-D1S306-(0.059)-D1S249-(0.022)-D1S2782-(0.06)-D1S205-(0.039)-D1S419-(0.044)-D1S229-(0.046)-D1S213 (see Figure 6.01). The forward primer for each marker was labelled with either FAM, TET or HEX fluorescent dyes. Amplification of all markers was performed using standard PCR conditions as follows; a total volume of 15µl was prepared containing 30ng of DNA, 1 unit of *Taq* polymerase, 1.75mM $MgCl_2$, 5mM dNTPs and buffer. Samples were then subjected to thermal cycling conditions of 1 cycle at 94°C for 4min, 35 cycles of 94°C for 1min, 60°C for 1min, and 1 cycle of 72°C for 2min. All PCR products were pooled (multiplexed), where possible, then fractionated by capillary electrophoreses and genotyped using an ABI 310 GENETIC ANALYSER and GENOTYPER software, respectively (Perkin Elmer). Figure 6.02 illustrates an example electrophoretogram of multiplex GENESCAN analysis generated in this study.

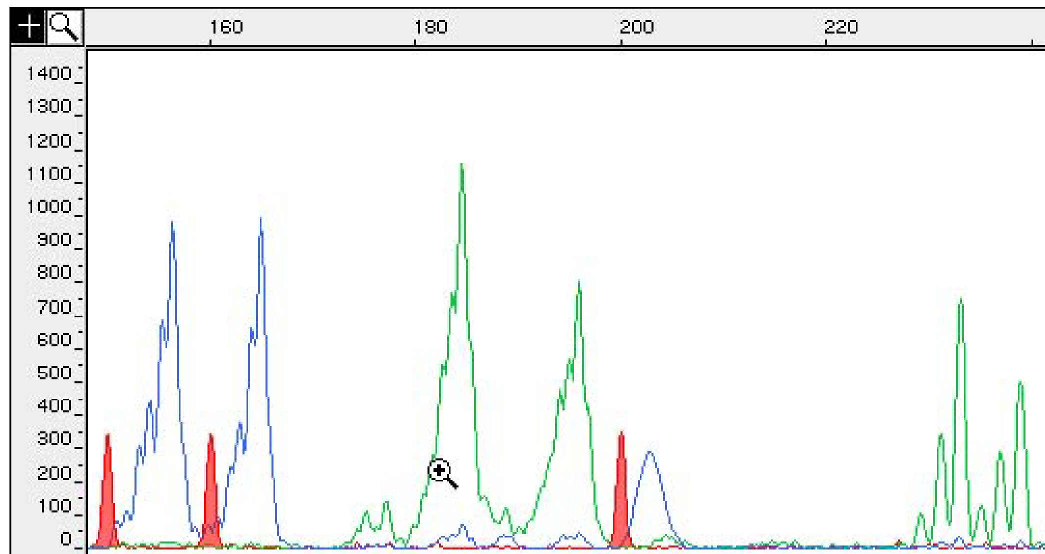

Figure 6.02. An electrophoretogram illustrating a multiplex Gene Scan analysis. Three microsatellite markers are in the PCR product pool. Two markers are labelled with TET (green) dyes and one marker is labelled with FAM (blue) dye. Each marker is heterozygous and the size range is from 150 to 250 bp.

6.2.3 Statistical Analysis

As with the research described in Chapter 5, this study employed the complementary strategies of family-based linkage and association analyses using Australian pedigrees affected with typical migraine. An estimate of 85% power to detect linkage, in the three large pedigrees initially examined, was previously calculated using the SIMLINK program (Nyholt, 1998c). The overall relative risk for typical migraine was estimated to be ~2.5 based on a migraine family study by Russell et al (Russell and Olesen, 1995). Therefore, the independent population of 82 additional pedigrees tested in this study should have ~80% power to detect linkage assuming a recombination fraction of zero between disease and marker loci (Risch. 1998). Non-mendelian inheritance errors in pedigrees were checked and allele frequencies for all microsatellite markers calculated using the PEDMANAGER program. Individuals that suffered from either MA or MO were analysed collectively as being affected with typical migraine.

6.2.3.4 Linkage and Allele Sharing Analysis

For the model-based calculations of this disease phenotype, a conservative disease model assuming an autosomal dominant mode of inheritance with 70% penetrance, a phenocopy rate of 0.7% and disease gene frequency of 12% was specified. Given the

high prevalence of MA in our affected pedigrees (~70%) we also chose to consider individuals who suffered from MA as a separate phenotype, treating individuals with MO as unaffected for this analysis. Estimated disease model parameters for the MA phenotype were 90% penetrance with a 1% phenocopy rate and 6% disease gene frequency. Traditional two-point linkage analysis was initially performed on the 3 large pedigrees using the FASTLINK program (Lathrop et al. 1985). This program calculates LOD scores for pedigrees of arbituary size but is not practical for multipoint analysis of many loci. For all multipoint analysis the ALLEGRO computer program was used (Gudbjartsson et al, 2000), which is an extension of the well-known GENEHUNTER programs (Kruglyak et al. 1996; Kong and Cox 1997). In addition to providing improvements in computational algorithms, ALLEGRO also allows the user to perform multiple analyses, incorporating various allele sharing and disease models, simultaneously (Gudbjartsson et al. 2000). The model-free analyses performed by ALLEGRO incorporated the $S_{pairs\ and}$ S_{all} scoring functions,which are said to perform well over all disease models (McPeek, 1999). LOD scores were calculated by fitting an exponential allele-sharing model which may be more suited to datasets with small numbers of pedigrees, or where pedigrees are very different in size (Kong and Cox, 1997). Like GENEHUNTER, ALLEGRO is restricted by the size of the pedigree to be analysed and therefore our large pedigrees required some trimming starting with unaffected members at the base. The significance levels for the linkage analysis were set according to the guidelines suggested by Lander and Kruglyak (1995) with a allele sharing LOD* score of 3.6 indicative of significant linkage. A LOD* of 2.2 is suggestive of linkage (Lander and Kruglyak, 1995). Reconstruction of the most likely haplotypes and recombination points was calculated approximately by the method applied in ALLEGRO.

6.2.3.5 Family-Based Association Analysis

The Family Based Association Test (FBAT) was performed for the independent sample of 82 migraine affected pedigrees. The FBAT is a unified approach for assessing association between marker and disease alleles. Unlike the classic transmission disequilibrium test (TDT) that was designed for specific pedigree structures (triads), the FBAT utilises data from nuclear families, sibships, or a combination of the two to test for linkage and linkage disequilibrium. The test for linkage is valid when multiple affected members in each pedigree are used, and the

power to detect linkage in this situation is increased when there is association. The test for association is valid if at least one affected member from each pedigree is used, or if the empirical variance is used to account for any correlation between transmissions in families when linkage is present (Laird et al, 2000). Considering that four markers were tested for association assuming two different phenotypes (MA/MO & MA) the significance level for the FBAT analysis was set at $0.05/8 \approx 0.005$, and 0.05 set as marginal evidence for association.

In addition, the TRANSMIT program was used to perform the transmission disequilibrium test (TDT) for allelic association on the data from the 82 additional typical migraine pedigrees. The TDT compares alleles transmitted from a heterozygous parent to an affected offspring (Spielman et al. 1993). By using non-transmitted parental alleles as internal controls, this method of association testing can overcome the problem of hidden genetic heterogeneity due to population stratification, thus reducing the chance of a false positive association. The TRANSMIT program has the added features of being able to deal with datasets where parental genotypes are missing (Clayton, 1997).

6.3 Results

6.3.1 Linkage and Allele Sharing Analysis

Three multigenerational pedigrees (MF1, MF7 and MF14) affected with typical migraine were initially utilised for investigating the FHM susceptibility region on chromosome 1q31. Traditional model-based linkage analysis of typical migraine was first performed on these untrimmed families using the FASTLINK Program. Pairwise LOD scores for typical migraine were calculated for the 8 markers at fixed recombination fractions (θ). These results showed a maximum positive LOD score of 1.32 ($P = 0.007$) at $\theta = 0.00$ for marker D1S2782 in MF14. LOD scores obtained for the other two pedigrees (MF1 and MF7) were negative for all markers tested. Multipoint analysis, incorporating all 8 markers spanning 33 cM on chromosome 1q31 was then performed on affected individuals from these pedigrees using the ALLEGRO program. Information content for these pedigrees is shown in (Table 6.02). The output from the model-based analysis indicated a maximum parametric

LOD score of 2.65 (P = 0.0002) at marker D1S2782 for MF14 when testing the MA disease phenotype (Figure 6.03). The results of the model-free analysis also showed significant excess allele-sharing in this pedigree, with a maximum allele sharing LOD* score of 2.04 (P = 0.001) for typical migraine and 3.36 (P = 0.00004) for MA at marker D1S2782 (Figure 6.04). Given the evidence for linkage of these markers to the disease in MF14, haplotypes were reconstructed. Cosegregation of marker haplotypes with the disease in this pedigree are displayed in Figure 6.05. The complex nature of typical migraine is illustrated in MF14 with cases of non (or incomplete) penetrance and phenocopy evident in individuals II:1 and IV:3, IV:10, respectively. Importantly, haplotyping shows key recombination events in affected individuals II:6 (D1S306×D1S249) and IV:1 (D1S2782×D1S205) that narrow a critical genomic region to a 14.1cM distance between markers D1S306 and D1S205 in this pedigree. Table 6.03 shows the clinical characteristics of pedigree MF14.

Table 6.03. Clinical Characteristics of Patients in Migraine Family 14 (MF14)

Patient No.	Diagnosis	Age of Onset	Longest Attack	Attack Freq/mth	Symptoms	Triggers
II:3	MA	50	12	<1/mth	vomiting,dizziness	stress
II:6	MA	15	3hrs	3-4/mth	vomiting,light/sound sensitive	
III:2	MO/MA	20	48hrs	<1/mth	visual,vomiting, light sound sensitivity	chocolate, cheese
III:7	MO	22	3hrs	3-4/mth	dizziness	stress
IV:1	MA	12	48hrs	<1/mth	visual,dizziness, vomiting	Crohn's disease episodes
IV:2	MO	12	24hrs	<1/mth	nausea,light/sound sensitivity	preservatives
IV:3	MO	20	12hrs	3-4/mth	nausea/vomiting	contraceptive pill
IV:4	MA	2	48hrs	>4/mth	visual,vomiting	oranges
IV:5	MA	15	12hrs	>4/mth	visual,speech,nausea	pregnancy
IV:6	MA	17	4hrs	1-2/mth	vomiting,visual,light/sound sensitive	menses
IV:8	MA	21	72hrs	>4/mth	vomiting,visual,light/sound sensitive	relaxation
IV:9	MA	30	12hrs	>4/mth	visual,numbness	alcohol
IV:10	MO	12	12hrs	1-2/mth	light/sound sensitivity	exercise and red wine
V:1	MA	4	12hrs	1-2/mth	vomiting,speech	menses

Table 6.02. Chromosome 1q31 Microsatellite Marker Information

Microsatellite Marker	No. of Alleles	Heterozygosity	Cumulative Marker Distance (cM)	Information Content
D1S2757	11	0.85	0	0.99
				0.88
D1S306	9	0.62	6	0.89
				0.69
D1S249	14	0.88	11.9	0.91
				0.86
D1S2782	10	0.82	14.1	0.96
				0.80
D1S205	13	0.80	20.1	0.96
				0.71
D1S419	14	0.85	24	0.98
				0.62
D1S229	8	0.77	28.4	0.97
				0.97
D1S213	11	0.86	33	0.98

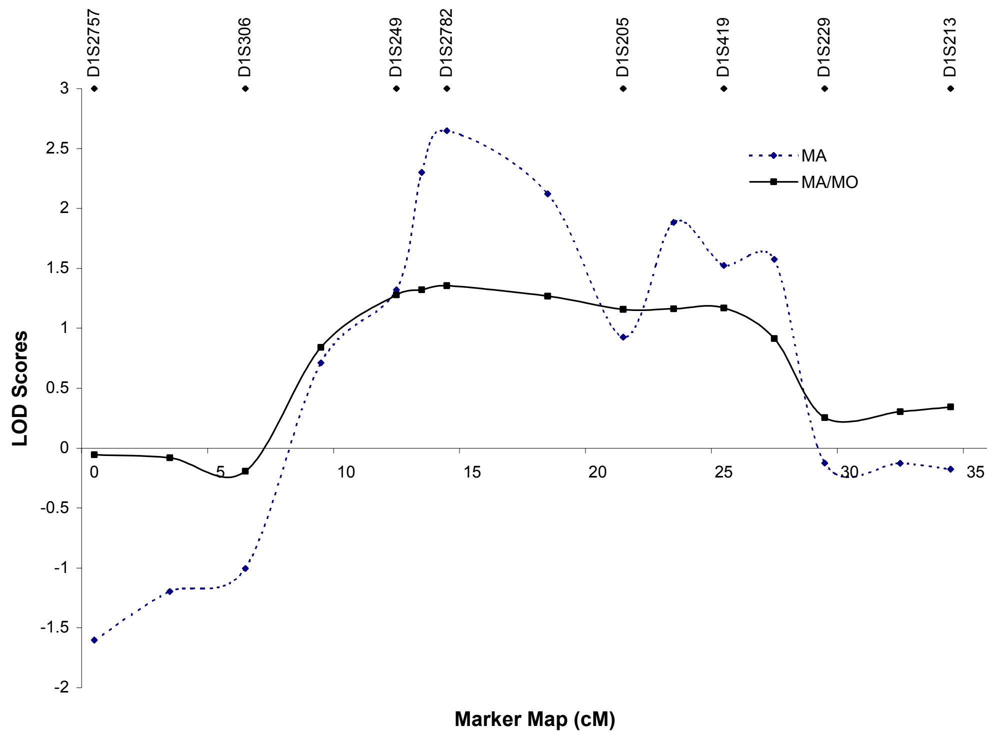

Figure 6.03. Results of multipoint *model-based* ALLEGRO linkage analysis utilising 8 markers on chromosome 1q31, for MF14 only. The Y axis shows the parametric LOD scores resulting from analyses of the typical migraine (MA/MO, unbroken line) and MA (dotted line) phenotypes. MA is migraine with aura and MO is migraine without aura.. Both disease phenotypes were assumed to exhibit an autosomal dominant mode of inheritance. For the typical migraine (MA/MO) disease phenotype, a 70% penetrance with a phenocopy rate of 0.7% and disease gene frequency of 12% was specified. For the MA phenotype, 90% penetrance with a 1% phenocopy rate and 6% disease gene frequency was set. The X axis shows the genetic distance in Kosambi cM across these markers.

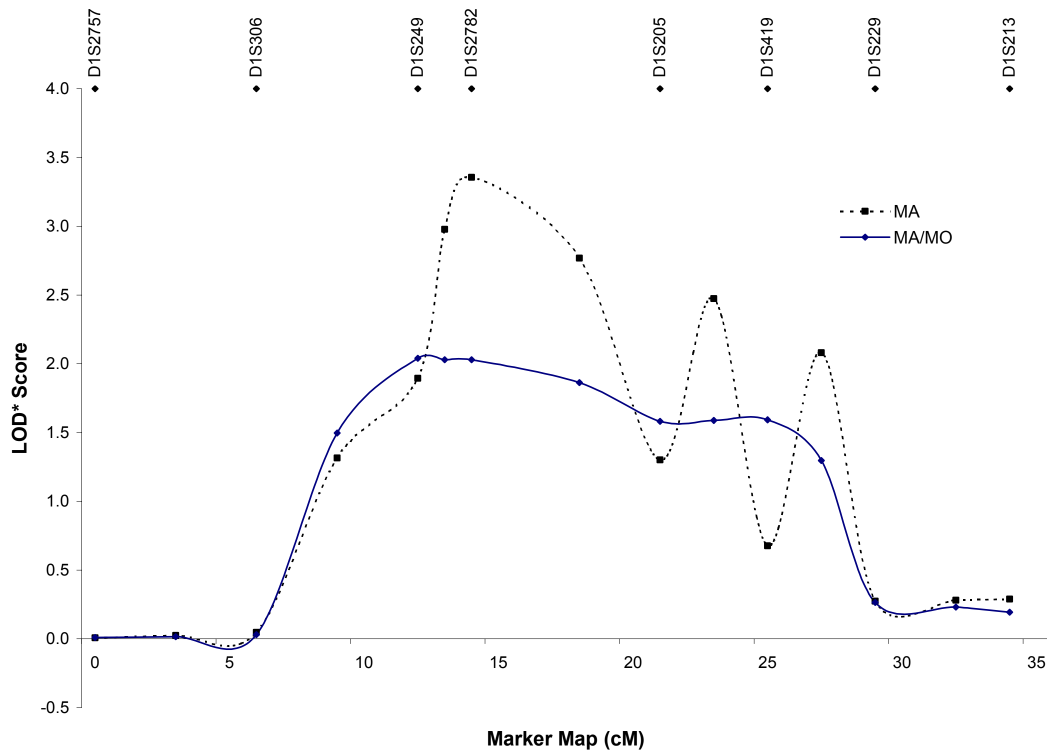

Figure 6.04 Results of multipoint *model-free* ALLEGRO allele sharing analysis for 8 markers on chromosome 1q31, for MF14 only. MA is migraine with aura and MO is migraine without aura. The Y axis shows the allele sharing LOD* scores resulting from analyses of the typical migraine (MA/MO, unbroken line) and MA (dotted line) phenotypes. The X axis shows the genetic distance in Kosambi cM across these markers.

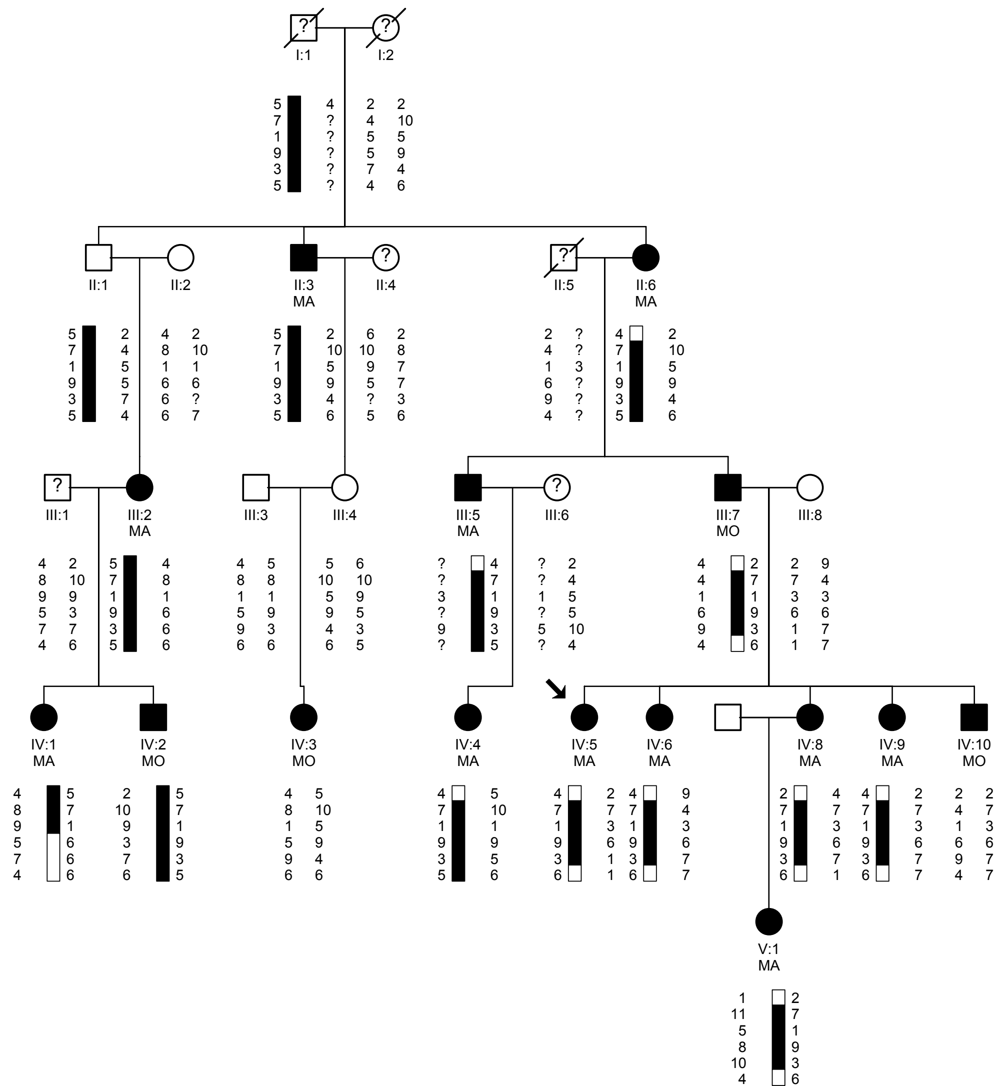

Figure 6.05. MF14 showing segregation of typical migraine susceptibility haplotypes estimated by ALLEGRO for chromosome 1q31 microsatellite markers (D1S306, D1S249, D1S2782, D1S205, D1S419 and D1S229 from top to bottom). Blackened symbols indicate affected individuals, with migraine diagnosis determined to be MA or MO. Key recombination events occur at individuals II:6 and IV:1 between markers (D1S306×D1S249) and (D1S2782×D1S205). This limits the disease locus to the 14.1cM region between D1S306 and D1S205.

In an attempt to substantiate the involvement of this region on Chr1q31 in typical migraine, we also tested 4 highly polymorphic and potentially implicated microsatellite markers in a large independent sample of 82 additional typical migraine families. These markers (D1S2757, D1S306, D1S249, D1S205) were from our initial map set. They were evenly spaced, and separated by a genetic distance of ~7cM.

Multipoint ALLEGRO results produced marginal allele sharing LOD* scores of 1.16 ($P = 0.01$) for typical migraine and 1.24 ($P = 0.008$) for MA peaking between markers D1S249 and D1S205 (Figure 6.06). The model-based LOD scores were all negative for this dataset. The genotype data from the 3 large pedigrees and 82 additional pedigrees was then pooled and re-analysed. This resulted in a maximum allele sharing LOD* score of 2.55 ($P = 0.0003$) for the MA phenotype and 1.89 ($P = 0.0015$) for typical migraine (MA/MO), between markers D1S249 and D1S205. The pairwise LOD* scores of this dataset also equated to marginally significant P-values (< 0.05) at markers D1S249 and D1S205 for ALLEGRO (Table 6.04).

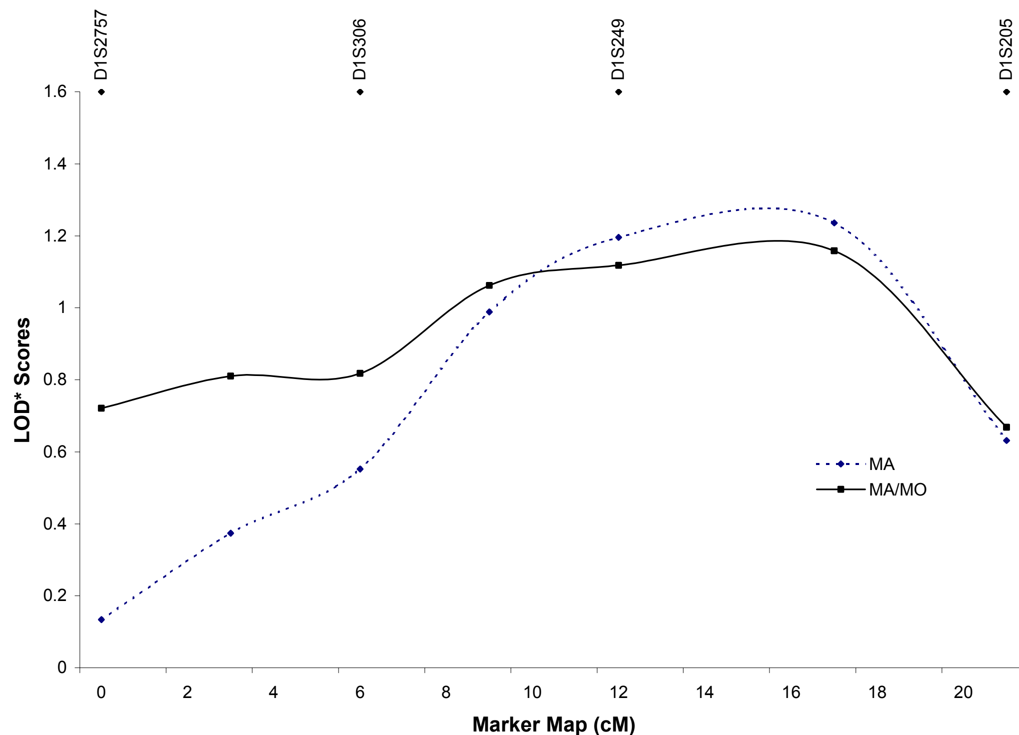

Figure 6.06. Results of multipoint *model-free* ALLEGRO allele sharing analysis for 4 markers on chromosome 1q31, for 82 typical migraine families. MA is migraine with aura and MO is migraine without aura. The Y axis shows the allele sharing LOD* scores resulting from analyses of the typical migraine (MA/MO, unbroken line) and MA (dotted line) phenotypes. The X axis shows the genetic distance in Kosambi cM across these markers.

6.3.2 Family-Based Association Analysis

The complementary mapping strategy of allelic association testing using the family-based approach was also carried out for the 82 additional families using the FBAT program. The test statistic for the FBAT analysis was marginally positive at marker D1S249 for this large sample of typical migraine families under both phenotypes. A global χ^2 of 15.00 for 5df (P = 0.010) for MA and χ^2 of 17.98 for 7df (P = 0.012) was obtained for MA/MO. As either linkage or association in the data may explain these small P-values we also tested for association using the empirical variance to account for correlation between transmissions in families when linkage is present. In this analysis, the evidence for association of alleles at the D1S249 locus decreased slightly for both phenotypes to P = 0.048 and P = 0.033 for MA and MA/MO, respectively. Specifically, alleles 9 -12 contributed largely to the global allele transmission distortion by showing slight overtransmission than what was expected ($P < 0.05$).

The TRANSMIT TDT analysis was also positive at marker D1S205 for this large sample of typical migraine families, producing a global χ^2 of 22.47 for 11df (P = 0.021) for 140 affected offspring. Specifically, alleles 4 and 5 were shown to contribute largely to the overall allele transmission distortion seen at this marker ($\chi^2_{(1)}$ = 8.56, P = 0.003 and $\chi^2_{(1)}$ = 7.11, P = 0.008 for alleles 4 and 5, respectively) (Table 6.04).

Table 6.04. Pairwise linkage and association results for 82 typical migraine pedigrees

Microsatellite Marker	Heterozygosity (no. of alleles)	Allegro LOD* (P-value)	FBAT (Global χ^2) MA/MO	TRANSMIT (Global χ^2) MA/MO
D1S2757	0.85 (11)	0.640 (**0.043**)	P = 0.914	7.36, 10 df, (P = 0.691)
D1S306	0.62 (9)	0.479 (0.069)	P = 0.349	8.07, 8 df, (P = 0.427)
D1S249	0.88 (14)	0.609 (**0.047**)	***P* = 0.012**	16.6, 12 df, (P = 0.165)
D1S205	0.80 (13)	0.010 (0.415)	P = 0.096	22.47, 11 df,(***P* = 0.021)**

6.4 Discussion

Typical Migraine is a multifactorial condition influenced by genetic and lifestyle characteristics. At present the mode(s) of inheritance is unclear and the type and number of genes involved in the disease is not known.. The *CACNA1A* gene, which causes some cases of the rare migraine subtype FHM, encodes the α1A subunit of the P/Q-type voltage gated Ca^{2+} channel that is predominantly expressed in the brain (Ophoff et al. 1996). These calcium channels control a number of fundamental neuronal processes including the mediation and release of neurotransmitters such as serotonin (Codignola et al. 1993). Since both FHM and typical migraine display some clinical overlap, it has been postulated that the more prevalent typical migraine (with and without aura) may also be a channelopathy. We have recently reported evidence for linkage to this same *CACNA1A* region on chromosome 19 in one of our large Australian typical migraine pedigrees, although direct sequencing of this gene in affected family members from this pedigree revealed no disease causing mutation (Nyholt et al. 1998a, Lea et al, 2001).

Linkage to *CACNA1A* occurs in approximately 50% of families affected with FHM indicating at least one other gene is involved in this disease (Ophoff et al. 1996). In 1997 Gardner et al, reported results of a linkage study investigating a large pedigree clearly affected with autosomal dominant FHM. These researchers excluded the 19p13.1 *CACNA1A* gene region for involvement in this pedigree and therefore tested 12 microsatellite markers spanning 44cM on chromosome 1q31 (Gardner et al. 1997). This region reportedly contains another voltage-gated neuronal calcium channel subunit gene, *CACNA1E* (Diriong et al. 1995). Model-based analyses of the markers utilised by Gardner et al, indicated significant linkage to FHM with a maximum LOD score peaking slightly between markers D1S249 and D1S2782 (Z_{max} = 3.328) (Gardner et al. 1997). Therefore, *CACNA1E* is an excellent candidate gene for FHM in this linked pedigree. To investigate the 1q31, *CACNA1E* region for involvement in the more prevalent forms of typical migraine (with and without aura), we conducted both linkage and association studies in Australian Caucasian families affected with this disease.

Utilising 3 large multigenerational pedigrees we initially tested 8 chromosome 1q31 markers for linkage to typical migraine. The results of both the model-based and model-free analyses showed linkage in one of the pedigrees tested. Haplotype analysis of this pedigree showed key recombination events limiting this region to 14.1cM. Interpretation of this haplotype information combined with the linkage results suggests that the most likely location of a disease susceptibility gene is within an 8.2cM region between markers D1S205 and D1S249. Follow up linkage analysis of an independent population of 82 typical migraine pedigrees was then performed. Four markers spanning the FHM region, and the *CACNA1E* gene, were tested and both multipoint and singlepoint analyses also provided nominal evidence for linkage in this region. These additional results offer good support to our initial findings in MF14.

An overall maximum allele sharing LOD* score of 2.55 (P = 0.0003) obtained for all pedigrees combined, whilst not significant at the genomewide level, is strongly suggestive of linkage in this entire population and thus warrants further investigation, especially considering the significant LOD* score (3.36) obtained for MF14 when the MA only phenotype was tested. It is also interesting to note that all our linkage peaks were maximised near the very same locus (D1S249) as the FHM results reported by Gardner et al (Gardner et al. 1997). The results of the present study interpreted together with those reported by Gardner et al. strongly support the idea that a common defective gene may be influencing both FHM and typical migraine.

Linkage analysis employing allele (or haplotype) sharing methods, whilst being a useful first step strategy for implicating regions of interest in multifactorial traits, may not be useful for pinpointing disease genes since true peaks resulting from these tests often span genetically broad regions (Terwilliger et al. 1997). Fortunately, by measuring allelic association of marker and disease alleles using methods such as the TDT, there is the potential to localise a susceptibility gene to a more narrowed region (Herr et al. 2000). Using the FBAT program, we incorporated allelic association into our analysis and found distortion of allele transmission at marker D1S249, thus providing evidence for linkage disequilibrium at this marker locus. The positive association results reported here for D1S249 will also require further independent study for confirmation, given the marginal P-value for this marker. If corroboration is

established then a gene affecting typical migraine at the population level may localise to the immediate vicinity of the D1S249 marker.

We also incorporated the TDT using TRANSMIT into our analysis and found distortion of allele transmission from heterozygous parents to affected offspring at marker D1S205, thus providing evidence for allelic association at this marker locus. These positive TDT results reported here for D1S205 will also require further independent study for confirmation, given the marginal P-value for this marker. Whilst it would have been more reassuring to detect allelic association at exactly the same position as the linkage peak and FBAT signal (ie. D1S249), the association and linkage results are not inconsistent with indicating a single typical migraine risk locus, since the marker showing positive association (D1S205) is within the 1-LOD* unit confidence interval for suggestive linkage.

This is the first study to provide evidence for the localisation of a typical migraine susceptibility region on chromosome 1. Overall, our linkage results indicate that the most plausible location of a disease gene is within the 8.2cM region between markers D1S249 and D1S205. If the allelic association indicated by TDT analysis is a true positive then it is plausible that a gene involved in typical migraine resides within a region proximal to D1S205 depending on the distance of linkage disequilibrium extended by alleles at this marker. We are now investigating the *CACNA1E* gene as a potential candidate for typical migraine. We are also testing marker loci around chromosome 1q21 specifically to determine whether the FHM susceptibility region implicated by Ducros et al. is also involved in typical migraine in our populations.

6.5 Conclusion

In conclusion, it is interesting and important to note that we have previously reported linkage of chromosome Xq markers to migraine in affected pedigree MF14 (Nyholt et al. 1998b; Nyholt et al. 2000). A case of male-to-male transmission of migraine exists within this pedigree indicating genetic factors other than X-linked may explain some of the disease in this pedigree. The significant excess allele sharing demonstrated in this pedigree with both Xq and 1q31 markers raises the possibility that "within family" locus heterogeneity exists, with genes on both chromosome

Xq24-28 and 1q31 potentially contributing to the disease either independently or interacting epistatically. The identification of the specific genes involved in this pedigree could greatly aid in the understanding of this polygenic neurovascular disorder.

CHAPTER 7
MIGRAINE CANDIDATE GENE STUDIES

7.1 Investiagtion of Serotonin- and Dopamine-Related Genes as Candidates for Migraine Susceptibility

7.1.1 Introduction

The serotonergic system has long been implicated in the pathophysiology of migraine. Researchers have therefore focused on the serotonin receptors and the genes that code for them when investigating this disease. Although serotonin receptor agonists have proven to be effective in the treatment of migraine, there has been no evidence of a serotonin receptor gene being associated with the disorder. However, in 1998, Ogilvie_et al reported that a VNTR in the serotonin transporter gene (*SERT*) showed altered allelic distributions in a Danish migraine population (Ogilvie, 1998).

In addition to serotonin, there has been ongoing interest in the involvement of the dopaminergic pathways in migraine. This interest has gained impetus since the recent findings of Peroutka et al who reported an allelic association between the dopamine receptor gene *DRD2* and migraine with aura (MA) (Peroutka et al, 1997). Another dopamine related gene, the dopamine beta-hydroxylase gene (*DBH*), has been localised to Chr 9q34 and codes for the enzyme that catalyses the conversion of dopamine to norepinephrine (Wilson et al, 1998). It therefore plays an important role in dopaminergic and noradrenergic neurotransmission. Serum levels of *DβH* enzyme have been reported to be elevated in migrainous patients during the headache phase of an attack (Anthony, 1981). Also, significantly increased *DβH* enzyme activity has been observed in migraine patients during the headache-free interval (Gotoh, 1976). Thus, the *DBH* gene is another good candidate for involvement in migraine pathophysiology and, to our knowledge, has not been previously implicated in this disease.

Here we report the results of affected relative pair linkage analyses and transmission/disequilibrium tests (TDT) in Australian Caucasian families affected

with migraine as well as results of a cross-sectional association test in unrelated migraineurs and controls. The focus of this study was on the serotonin transporter (*SERT*) gene, the dopamine receptor D2 (*DRD2*) gene and the dopamine beta-hydroxylase (*DBH*) gene.

7.1.2 Methods

7.1.2.1 Study Populations

The 82 migraine pedigrees used in this study have been described in previous chapters. Unrelated case control groups were also studied. This age and sex matched Caucasian population, comprised 177 unrelated migraineurs (including 98 MA and 79 MO clearly defined subjects) and 182 non-migraine controls. It was estimated that these groups would have >80% power to detect an allelic association conferring a 2-fold increase in risk to the disease.

7.1.2.2 Markers/Genotyping

This study employed a candidate gene approach to investigating the genetics of typical migraine. In particular, three migraine candidate genes *DRD2*, *DBH* and *SERT* were examined. An intragenic polymorphism within each candidate gene was used for linkage and association testing. The *DRD2* gene contains a NcoI RFLP, *SERT* contains a tri-allelic VNTR in intron 2. The primers for the *DBH* gene simultaneously amplify alleles of an $(AC)_n$ dinucleotide repeat and a 19bp insertion/deletion polymorphism located 118bp downstream. These markers will hereafter be called *DBH-1* and *DBH-2* respectively, where *DBH-1* alleles 1 - 4 combined are equivalent to the *DBH-2* deletion allele, and *DBH-1* alleles 5 - 8 combined are equivalent to the *DBH-2* insertion allele [20]. PCR amplification of *DRD2*, *DBH* and *SERT* markers was performed using previously published conditions (Peroutka et al, 1997; Nahmias et al, 1992; Ogilvie et al, 1998, respectively). The *DBH* and *SERT* markers underwent PCR with fluorescently labelled primers and alleles detected using an ABI 310 Genetic Analyser (Figures 7.1 and 7.2). *DRD2* alleles were discriminated using the NcoI restriction enzyme and agarose gel electrophoresis (Figure 7.3).

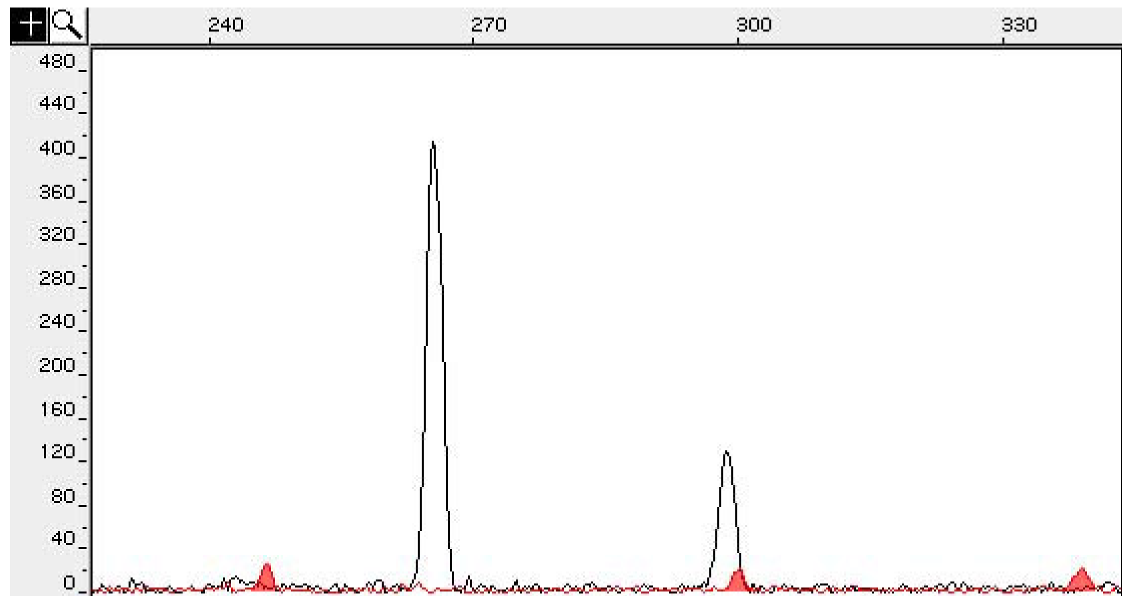

Figure 7.1. An Electrophoretogram showing a heterozygote for the *SERT* VNTR. The marker is triallelic with fragments (alleles) differing in size by 17bp

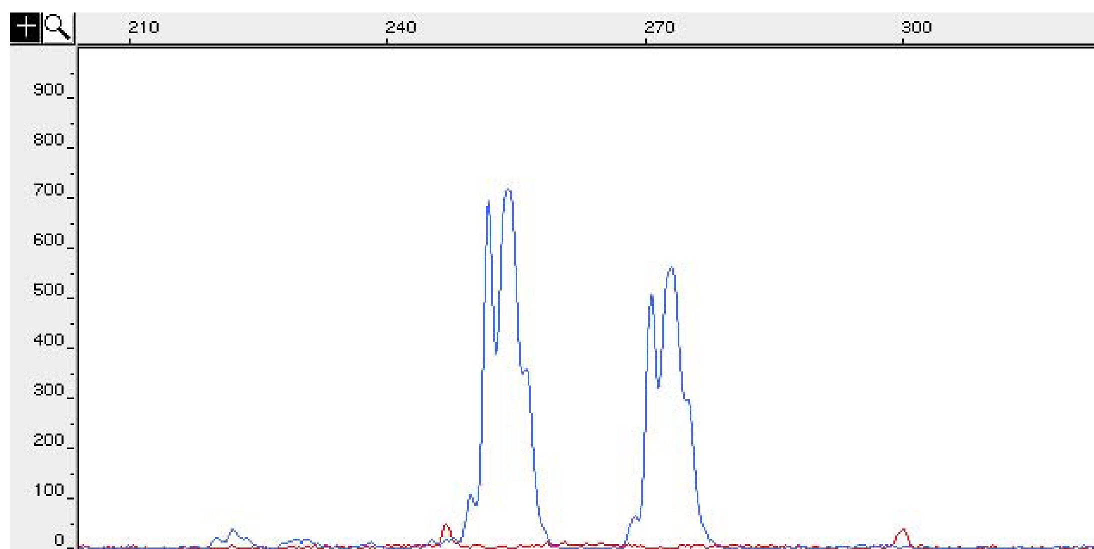

Figure 7.2. An Electrophoretogram showing a heterozygote for the *DBH* marker. PCR fragments of 255bp and 272bp are shown and represent alleles 1 and 2.

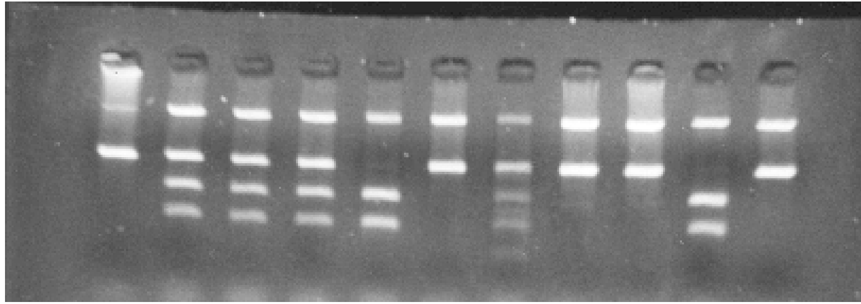

Figure 7.3. A slab gel photo illustrating allelic discrimination of the *DRD2* polymorphism. The bottom two bands indicate the rarer allele containing the restriction site. Heterozygotes for this marker show all four bands.

7.1.2.3 Statistical Analysis

This study employed the complementary strategies of family-based linkage and association analyses as well as cross-sectional case/control approach as described in detail in Section 5.2.3 in Chapter 5. In summary, linkage analysis was carried out using the GENEHUNTER-PLUS (modification of the GENEHUNTER) and SimIBD programs. The model-based analysis was performed using conservative disease model parameters previously described. In addition, the TRANSMIT program was used to perform the TDT on the data from nuclear families.

A population-based cross-sectional association analysis was also implemented on an unrelated group of migraineurs and unaffected controls matched for age, sex and ethnicity. The allele frequency distributions for each of the three markers in case and control groups were compared using the standard χ^2 test. In addition, the CLUMP program, which is useful for association testing when markers produce sparse contingency tables, was used for the multiallelic *DBH-1* polymorphism. Since it is difficult to determine an accurate correction for multiple comparisons, we chose to adjust the conventional α = 0.05 significance level according to the number of markers tested. In total, we examined 4 markers and therefore reduced the significance level by a factor of 4 (0.05/4) to α = 0.01.

7.1.3 Results

7.1.3.1 Family-Based Analysis

To determine whether variations at three migraine candidate gene loci were associated with typical migraine we tested an RFLP and a VNTR in the *DRD2* and *SERT* genes, respectively, as well as two adjacent markers in the *DBH* gene (*DBH-1* and *DBH-2*. Genotype data from 82 unrelated families affected with migraine were analysed for both linkage and association. Allele sharing statistics obtained from both GENEHUNTER-PLUS and SimIBD single-point analyses provided no evidence for linkage of any of the tested markers to the disease (Table 7.1). However, TDT analysis indicated distortion of allele transmission at the *DBH-1* marker for nuclear families affected with migraine. In particular, transmission of allele 3 of this polymorphism occurred less than expected (observed 30, expected 36.45; χ^2 = 4.44, 1df, P = 0.035). The biallelic *DBH-2* polymorphism however showed no distortion of allele transmission in the dataset.

Table 7.01. Linkage Analysis results using GENEHUNTER-PLUS (modification of GENEHUNTER) and SimIBD programs

	Genehunter-Plus		SimIBD
	Parametric Lod	GHP LOD*	*P*-value
DBH(1)	-2.116	0.017	0.503
DBH(2)	-1.470	0.000	0.601
DRD2	-1.068	0.000	0.679
SERT	-2.012	0.024	0.461

*Genehunter Plus LOD score

7.1.3.2 Case-Control Analysis

Independent cross-sectional association analysis was also performed using each marker in a sex and age-matched sample of 177 migraine sufferers and 182 unrelated non-migraine controls. The total combined typical migraine population was sub-categorised into individuals clearly affected with MO and MA. Allele frequency distributions for each migraine category were compared using the chi-squared (χ^2) test to determine whether an association exists between these gene markers and the disease.

Chi-squared analysis of the *DBH* gene using Monte Carlo simulations implemented in the CLUMP program, indicated that the allele frequency distributions of the *DBH-1* marker were altered between the total migraine combined group and controls (Table 7.2). Specifically, the normal T1 analysis produced a χ^2 of 16.53 (P = 0.019). This unrelated case-control P-value together with the P-value of the independent TDT analysis for *DBH-1* provide overall, significant evidence at α= 0.01 for association of the *DBH-1* polymorphism to migraine susceptibility - Fisher's combined χ^2 of 14.6 (P = 0.006, 4 d.f) {-2*ln*(0.035) + -2*ln*(0.019)}. Examination of Table 7.2 shows that there was no individual allele which was independently responsible for the altered allele frequency distributions between the typical migraine combined group and controls. However, increased frequencies of the rarer alleles 4, 5 and 8 in the affected group were seen to contribute largely to the overall effect. Alleles of the *DBH-2* marker, which were found to be in strong linkage disequilibrium with *DBH-1* alleles, did not differ significantly between the control and migraine groups, with alleles being almost equifrequent in each group (Table 7.3). There was also no evidence for association between the *DRD2* and *SERT* gene polymorphisms and any migraine group (Tables 7.4 and 7.5).

Table 7.02. Allele frequency distribution for the *DBH-1* polymorphism in the DBH gene in migraine groups and controls

Group	*n* (alleles)	*DBH-1* Allele Frequency Distribution							
		1	2	3	4	5	6	7	8
Migraine* (Combined)	284	11(4%)	65(23%)	30(10%)	28(10%)	5(2%)	100(35%)	39(14%)	6(2%)
MA	156	7(4%)	33(21%)	17(11%)	16(10%)	3(2%)	56(36%)	21(14%)	3(2%)
MO	128	4(3%)	32(25%)	13(10%)	12(9%)	2(2%)	44(34%)	18(14%)	3(3%)
Control	272	19(7%)	52(19%)	39(14%)	16(6%)	1(0.5%)	115(42%)	29(11%)	1(0.5%)

* Chi-squared analysis showed a significant difference in allele frequency distribution between the migraine (combined) group and control (CLUMP T1 χ^2 = 16.53, P = 0.019).

Table 7.03. Allele and genotype frequency distributions for the 19bp insertion/deletion polymorphism (*DBH-2*) in the *DBH* gene

Group	n (alleles)	Allele Frequencies		Genotype Frequencies		
		1	2	1/2	2/2	1/1
Migraine (combined)	284	134 (47%)	150 (53%)	70 (49%)	40 (28%)	32 (23%)
MA	156	68 (51%)	88 (49%)	38 (49%)	25 (32%)	15 (19%)
MO	128	61 (48%)	67 (52%)	27 (42%)	20 (31%)	17 (27%)
Control	272	126 (46%)	146 (54%)	78 (57%)	34 (25%)	24 (18%)

* Allele 1 represents the 19bp deletion and allele 2 represents the 19bp insertion allele of the *DBH* gene. There was no significant difference between allele frequencies for any migraine groups and controls. Migraine (combined) against control alleles (χ^2 = 0.04, P = 0.84).

Table 7.04. Allele and genotype frequencies for *DRD2 NcoI* Polymorphism in Migraine and Control Groups

Group	Allele Frequencies			Genotype Frequencies		
	n (alleles)	1	2	A1/A1	A1/A2	A2/A2
Migraine (combined)	354	241 (68%)	113 (32%)	80 (45%)	81 (46%)	16 (9%)
MA	196	130 (66%)	66 (34%)	41 (42%)	48 (49%)	9 (9%)
MO	158	111 (70%)	47 (30%)	39 (49%)	33 (42%)	7 (9%)
Control	364	262 (72%)	102 (28%)	92 (51%)	78 (43%)	12 (7%)

There was no significant difference between allele frequencies for any migraine groups and controls. Migraine (Combined) allele group against control (χ^2 = 1.3, P = 0.25).

Table 7.05. Distribution of Allele and Genotype frequencies for *SERT* polymorphism in Migraine and Control Groups

Group	Allele Frequencies				Genotype Frequencies				
	n (alleles)	1	2	3	1/2	1/3	2/2	2/3	3/3
Migraine (combined)	296	5 (2%)	116 (39%)	175 (59%)	2 (1%)	3 (2%)	34 (23%)	46 (31%)	63 (43%)
MA	162	3 (1%)	64 (40%)	95 (59%)	1 (1%)	2 (3%)	19 (23%)	25 (31%)	34 (42%)
MO	134	2 (1%)	52 (39%)	80 (60%)	1 (2%)	1(2%)	15 (22%)	21 (31%)	29 (43%)
Control	282	4 (1%)	112 (40%)	166 (59%)	1 (1%)	3 (2%)	33 (23%)	45 (32%)	59 (42%)

There was no significant difference between any migraine group and controls. Migraine (combined) group against controls (χ^2 = 0.08, 2df, P = 0.96).

7.1.4 Discussion

Migraine is a complex condition influenced by genetic and lifestyle characteristics. At present the mode(s) of inheritance is unclear and the type and number of genes involved in the disease is not known. Serotonin and dopamine have been implicated in the pathogenesis of migraine and recent genetic studies suggest that genes encoding proteins related to these neurotransmitters may play a role in the disease. In this study we investigated three neurotransmitter related genes *DRD2*, *DBH* and *SERT*. To increase the chance of detecting any involvement of these genes in typical migraine, we employed the complementary strategies of family-based linkage and case-control association testing.

7.1.4.1 *DBH* Allelic Association

Overall, the results of this study provide good evidence for an allelic association of the *DBH* gene with typical migraine. Association analyses of the *DBH* gene indicated both allelic transmission distortion in families and altered allele frequency distribution between unrelated cases and controls at the *DBH-1* microsatellite marker. The overall association seen at this marker is apparently the result of several alleles each contributing only a minor effect to disease risk. The findings of this study are particularly interesting since the likelihood of a false positive allelic association due to population stratification in the case-controls is reduced due to prior matching of test groups for sex, age and ethnicity. In addition, TDT analysis performed on an independent family dataset confirmed a transmission disturbance of alleles at the *DBH-1* marker in migraineurs. The TDT can be used to overcome the problem of hidden genetic heterogeneity due to sub-populations reducing the chance of a spurious association (Risch, 1997).

Alleles for the adjacent *DBH-2* marker are in strong linkage disequilibrium with alleles at *DBH-1*. However, *DBH-2* alleles were not shown to be associated with migraine in these populations. This lack of co-association of *DBH-1* and *DBH-2* alleles with the disease may be due to the minor association of multiple rarer alleles at *DBH-1* being swamped by common alleles conferring no effect. We are currently examining nearby *DBH* gene markers, including adjacent single nucleotide polymorphisms (SNPs), to elucidate our current findings of allelic association of the

DBH-1 marker with migraine susceptibility. In addition, a replication association study should be performed, preferably in larger populations, to confirm the involvement of the *DBH* gene to typical migraine.

7.1.4.2 *DBH* Linkage and Allele Sharing

The linkage results obtained from family data in this study do not support involvement of the *DBH* gene in migraine aetiology. It should be noted that failure to detect linkage to the *DBH* gene might be due to lack of statistical power using this migraine family data. The recurrence risk to a relative of an affected individual can be used directly to estimate power to detect linkage. Risch has reported that by using 100, 3rd or 2nd degree relatives, the recurrence relative risk (λ_o) to achieve >95% power to detect linkage should be >3.5. The overall λ_o for migraine is estimated to be 2.5 based on a migraine relative risk study by Russell et al (1996). Therefore, using 151 relative pairs of this type, this study should have ~80% power to detect linkage assuming a recombination fraction of zero between disease and marker loci (Risch, 1990a). However, Risch also suggests that the information content of the marker be >0.85 when using second and third degree relatives (Risch, 1990b). The information content of the *DBH-1* polymorphism was calculated to be 0.73. Consequently, the power of this study may have actually been <80%. If *DBH* is involved in migraine predisposition this reduction in power, in combination with a possible weak gene effect, may allow linkage of the locus in this population to go undetected.

7.1.4.3 The *DRD2* Gene

Although our case-control populations had sufficient power, the *DRD2* results of this study did not concur with the previous findings of Peroutka et al, who reported that the *DRD2* NcoI C allele was over-represented in a group of unrelated MA patients. Similarly, we could not support results of Del Zompo et al who employed a TDT analysis in a population of 50 Sardinian triad families and provided evidence that an allele of a *DRD2* dinucleotide was in linkage disequilibrium with MO in individuals enriched with dopaminergic activity. The fact that both Peroutka et al and Del Zompo et al tested different intragenic *DRD2* polymorphisms and employed different population sampling strategies provides some evidence that variations in the *DRD2* gene may impact on migraine. It should be noted, however, that both studies found

association in different migraine subtypes (MA and MO, respectively) using groups of relatively small size. Since there are clinical differences between IHS defined MA and MO, it is worthwhile distinguishing between them when performing allelic association studies. However, the different disease subtype associations reported by Peroutka et al and Del Zompo et al, for MA and MO, respectively, may indicate the presence of a single *DRD2* gene mutation influencing typical migraine. For this reason it may be beneficial, in future studies, to consider MA and MO subtypes as one disease group. A larger overall sample size may subsequently increase the power to detect an association, and reduce type I (false positive) error rate when investigating the DRD2 gene in migraine populations.

7.1.4.4 The *SERT* gene

The *SERT* gene is an excellent candidate gene for involvement in neurological diseases and has previously been shown to be associated with susceptibility to bipolar disorder (Ogilvie et al, 1996). Recently, Olgivie et al (1998) also reported altered allelic distributions of the *SERT* gene in both MA and MO in a Danish population. The results of our *SERT* association analysis gave no evidence of any genotypic or allelic alteration in our migraine groups. One reason for differing conclusions between Ogilvie et al and ourselves is that there may be some different genetic features between the Danish population and our Australian groups. Migraine is genetically heterogeneous and it is possible that different mutations are responsible for causing migraine in different populations. This idea may be substantiated by an Italian family study performed by Monari et al (1997). These researchers provided no evidence for linkage of microsatellite markers, surrounding the *SERT* gene on chromosome 17, in 14 families affected with either MA or MO (LOD < 0)[33]. These results excluded involvement of the *SERT* gene in these Italian families affected with migraine and offer support to our findings of no linkage to *SERT* using the Australian families in this study.

In conclusion, our results do not confirm previous association of migraine subtypes to the *DRD2* and *SERT* genes, however they do suggest that allelic variation at the *DBH* locus may predispose some individuals to the common forms of migraine. Hence, this study suggests that the dopaminergic system may play a role in migraine aetiology. However, further investigation is needed since these results alone may not

be used to infer any biological changes in migraineurs as a consequence of *DBH* gene variation.

7.2 Inducible Nitric Oxide Synthase Gene as a Candidate for Typical Migraine

7.2.1 Introduction

At present, the pathophysiological mechanisms underlying migraine are largely speculative. Whilst the strong therapeutic effect of serotonin (5-HT) receptor agonists suggests that serotonin is implicated in the disease, injection of serotonin, intravenously or locally into superficial temporal muscle does not cause pain (Jensen *et al.*, 1990; Roberts, 1992). In addition, calcitonin gene related peptide (CGRP), which is known to be released during a migraine attack (Goadsby *et al.*, 1990), does not result in pain after injection (Sheker *et al.*, 1991). Therefore, the nociception responsible for migraine pain is not likely to be caused by either serotonin or CGRP.

It has been hypothesised that nitric oxide (NO) may be a more likely candidate molecule involved in migraine headache (Thomsen and Olesen, 1998). NO is a small messenger molecule that exhibits various biological functions in different tissues including mediation of neurotransmission and vasodilation. Synthesis of NO is catalysed by a family of nitric oxide synthase (NOS) enzymes. These proteins are directly responsible for catalysing NO synthesis, and have distinct tissue localisations, although all isoforms can be found in neuronal tissue. They include endothelial NOS (*e*NOS), neuronal NOS (*n*NOS) and inducible NOS (*i*NOS). A recent study reported that the non-specific NOS-inhibitor L-NG methylarginine hydrochloride may be effective in the acute treatment of migraine headache (Lassen *et al.*, 1998). Therefore, alteration of NOS itself may be involved in disease causation, making NOS genes good candidates for investigating the genetic basis of migraine. In particular, the *i*NOS enzyme is involved in synthesis of NO during inflammatory states and NO released from *i*NOS also plays a role in vasodilation (Gross *et al.*, 1996). It has been suggested that neurogenic inflammation within blood vessels of the dura mater may account for vascular head pains, including migraine (Moskowitz 1984). Thus, alterations in *i*NOS function specifically, may contribute to the disease.

The aim of this study was to investigate the possible involvement of *i*NOS in migraine susceptibility. We examined a bi-allelic tetranucleotide polymorphism in the

promoter region of the human *i*NOS gene by employing an association study approach incorporating a large group of unrelated migraine sufferers and carefully matched controls.

7.2.2 Methods

7.2.2.1 Subjects

In total, 513 DNA samples from unrelated individuals were analysed in this study (252 controls and 261 migraineurs). Of the migraine affected group, 90% of patients had a known family history of the disease, or at least one first degree relative suffering from migraine. This group was comprised of 156 individuals diagnosed as clearly having MA, whilst 105 were diagnosed as having MO.

7.2.2.2 Genotyping

Genomic DNA was extracted from whole venous blood samples by a standard SDS-Proteinase K (Blin and Stafford, 1976) incorporating a salting out procedure (Miller *et al.*, 1988) and used as a template to generate PCR products for genotyping. Within the 5' flanking DNA of the *i*NOS gene there is a AAAT/AAAAT repeat sequence extending from –756 to –716bp 5' to the main TATA-directed transcription initiation site [Nunokawa *et al.*, 1994). Primers specific for this bi-allelic tetranucleotide repeat polymorphism located between –891 to –575bp of the *i*NOS gene were employed to perform PCR (5'-TGG TGC ATG CCT GTA GTC C-3' and Reverse-TGG GGC CTG GGT ACC TCC GA) (Bellamy *et al*., 1997). The forward primer was fluorescently labelled with FAM dye and PCR amplification according to the following procedure was performed. A total volume of 15μl was prepared containing 30ng of DNA, 1 unit of *Taq* polymerase, 1.75mM $MgCl_2$, 5mM dNTPs and buffer. Samples were then subjected to thermal cycling conditions of 1 cycle at 94^oC for 4min, 35 cycles of 94^oC for 1min, 60^oC for 1min, and 1 cycle of 72^oC for 2min. All PCR products were then electrophoresed and genotyped using an ABI 310 Genetic Analyser and GENOTYPER software.

Allele and genotype frequencies were compared using standard chi-square (independence) analysis implemented in the SPSS program. Power analysis indicated that if the *i*NOS tetranucleotide polymorphism were to confer a two-fold increase in

relative risk of migraine, the case and control groups used in this study were of sufficient size to have 99% power to detect an allelic association and 89% power to detect a genotypic association at the 0.05 level.

7.2.3 Results

Genotypes for the *i*NOS bi-allelic tetranucleotide marker were determined for 261 unrelated migraineurs and 252 unaffected controls. Genotype and allele distributions are presented in Table 7.6. DNA fragments 313bp and 317bp in length represent the two alleles detected for the *i*NOS polymorphism. The normal frequencies for these alleles are similar to those found in other populations (Bellamy *et al.*, 1997). Genotype frequencies for the control group display Hardy-Weinberg equilibrium reducing the chance of false results due to genotyping errors.

Results of the chi-squared analysis for the *i*NOS polymorphism indicate the allele frequency distributions for the migraine affected and unaffected groups were not significantly different ($\chi^2 = 1.93$, $P = 0.16$). The migraine test group was divided into subgroups affected with either MO (n = 105) or MA (n = 156). There was no significant difference seen from comparison of allele frequencies for the MA and MO subgroups and the control population ($\chi^2 = 0.69$, $P = 0.41$ and $\chi^2 = 2.27$, $P = 0.13$, respectively). Similarly, comparison of genotypes indicated no significant alteration in the distribution between any migraine groups and controls.

Table 7.06. Distribution of Genotype and Allele Frequencies for *i*NOS Tetranucleotide Polymorphism in Migraine and Control Groups*

Group	i*NOS Genotypes*			N *(Genotypes)*	*i*NOS Alleles	
	313/313	*313/317*	*317/317*		313	317
Migraine (combined)	216 (83%)	39 (15%)	6 (2%)	261	471 (90%)	51 (10%)
MA	126 (81%)	27 (17%)	3 (2%)	156	279 (89%)	33 (11%)
MO	90 (86%)	12 (11%)	3 (3%)	105	192 (91%)	18 (9%)
Control	193 (77%)	55 (21%)	4 (2%)	252	441 (88%)	63 (12%)

*$\chi^2 = 1.93$, $P = 0.16$ for migraine (combined) allele frequency compared to controls

7.2.4 Discussion

The complexity of typical migraine has made the identification of causative genes difficult. Investigation of heterogeneous polygenic disorders using a candidate gene association approach may be an important strategy in the detection of genes contributing a small effect to the disease (Risch and Merikangas, 1996). To date, migraine association studies investigating candidate genes have revealed some interesting results. Polymorphisms in the dopamine receptor gene, *DRD2*, have recently been shown to be associated with migraine in two independent studies (Peroutka *et al.*, 1997; Del Zompo *et al.*, 1998). In addition, we have recently found evidence for association of the dopamine beta-hydroxylase gene (*DBH*) gene and migraine using both families and unrelated cases and controls (Lea *et al.*, 2000). Finally, a Danish association study investigating the serotonin transporter gene (*SERT*) reported significant alterations in allelic distributions of a *SERT* repeat polymorphism (VNTR) between migraine groups and controls (Ogilvie *et al.*, 1998).

Nitric Oxide is believed to play a key role in the pain associated with a migraine attack. At present the underlying mechanisms by which NO may trigger migraine headache are theoretical. However, a recent study investigating the effect of L-NG methylarginine hydrochloride (546C88), a non-specific NOS inhibitor, found that 67% of migraine patients treated with 546C88 experienced headache relief two hours after infusion compared to 14% of patients given a placebo ($P = 0.01$) (Lassen *et al.*, 1998). These results provide evidence that NOS is involved in migraine and suggests that NOS genes are good candidates for migraine molecular genetic studies. We have previously reported results of a linkage and association study investigating an *e*NOS dinucleotide polymorphism in four large migraine families and unrelated case-control groups. The results of these analyses did not support a role for the *e*NOS gene in migraine aetiology (Griffiths *et al.*, 1997). Unlike *e*NOS, the *i*NOS enzyme is responsible for the release of relatively large quantities of NO from macrophages, astrocytes and microglia (Moncada *et al.*, 1991). This may be important since severe migraine headaches are commonly associated with intracranial infection and neurogenic inflammation. For this reason, alterations in the *i*NOS gene may be specifically involved in migraine predisposition. The *i*NOS gene is localised to chromosome 17q11.2-q12 (Marsden *et al.*, 1994) and comprises 26 exons (Chartrain

et al., 1994). The complex promoter of the human *i*NOS gene contains multiple transcription initiation sites and eight partially overlapping open reading frames. It has been suggested that this region may have an important role in translational regulation of the human *i*NOS mRNA (Chu *et al.*, 1995). In this association study we tested a bi-allelic tetranucleotide polymorphism in the promoter region of the *i*NOS gene that could potentially affect expression of the *i*NOS enzyme.

7.2.5 Summary

The results of this study provide no evidence for an allelic association of this particular *i*NOS variant with the common forms of migraine. However, it is important to consider the possibility that segments elsewhere in the gene, especially in the 3' region, which are not in linkage disequilibrium with this polymorphism may contain a mutation(s) responsible for modifying the expression of the *i*NOS gene. Therefore, whilst we have established that this functional polymorphism is not associated with susceptibility to migraine in our tested populations, we cannot conclusively exclude the entire gene for involvement in the disease pathogenesis.

CHAPTER 8
RESEARCH SUMMARY AND FUTURE DIRECTIONS

8.1 Summary of Results

The research presented in this thesis describes genetic studies into the molecular basis of typical migraine. In particular, genomic susceptibility regions on chromosome 19p and chromosome 1q as well as the migraine candidate genes *DRD2, SERT, DBH, INSR, CACNA1A* and *iNOS* were investigated in an attempt to localise and identify some of the variants that predispose individuals to the disease. The research design involved the use of a large cohort of migraine affected pedigrees as well as unrelated case and control groups. DNA markers including microsatellites and SNPs were utilised to obtain genetic data from the study populations. This data was analysed employing the complementary strategies of linkage, allele-sharing and association (LD) methods incorporating computer programs such as GENEHUNTER and CLUMP.

Results of the chromosome 19p13 - *CACNA1A* gene study, although indicating initial linkage of MF1 to this genomic region, did not provide further linkage or association evidence implicating this gene in typical migraine. Furthermore, direct sequencing of all exonic fragments in this gene revealed no disease causing mutations. It was concluded that if the *CACNA1A* gene played a role in the common forms of migraine its contribution to the disease was minor. A follow up case-control association study of the *INSR* gene, which resides ~15cM telomeric to *CACNA1A*, indicated evidence for association of an intragenic SNP for this gene. These results taken together with the previous findings of McCarthy et al strongly implicate the *INSR* gene as a migraine susceptibility gene.

An additional family-based linkage and association study was also performed using markers spanning the existing FHM region on chromosome 1q. The results of this analysis provided highly suggestive evidence for the localisation of a new typical

migraine susceptibility locus at this genomic region and reinforced the notion that FHM and typical migraine may in part be due to a common aetiology.

Candidate gene studies may be useful strategies for identifying genes involved in complex diseases such as migraine, especially if the gene being examined contributes only a minor effect to the overall phenotype. Therefore, this research also involved a linkage and association approach to investigating neurotransmitter related migraine candidate genes. Specifically, polymorphisms within the serotonin transporter gene (*SERT*), the dopamine receptor gene (*DRD2*), the dopamine beta-hydroxylase (*DBH*) gene and the inducible nitric oxide synthase (*iNOS*) gene were tested in unrelated Caucasian migraineurs and non-migraine control individuals. Although these studies provided no evidence for involvement of the *SERT*, *DRD2* and *iNOS* genes in migraine, unrelated case-control association analysis of a *DBH* intragenic dinucleotide polymorphism indicated significant altered allelic distribution between migraine and control groups. Furthermore, the transmission/disequilibrium test (TDT), which was implemented on the family data, also indicated distortion of allele transmission for the same *DBH* marker. Together, these results provided evidence for allelic association of the *DBH* gene with typical migraine susceptibility and indicate that further research into the role of the *DBH* gene in migraine aetiology is warranted.

8.2 Future Directions

8.2.1 Pharmacogenomics and Migraine

Recently, there has been considerable medical interest in developing tailored individual-specific treatments for patients suffering from common diseases such as migraine. This research focus combines the disciplines of pharmacology and genomics to form the new field of pharmacogenomics and is aimed at diagnosing and treating patients according to their drug-specific genetic profile. Migraine is a common and often extremely debilitating neurovascular disorder. It therefore incurs a huge economic burden on the population. The broad range of clinical features that manifests in migraineurs has made it difficult to diagnose and the current treatment strategies exhibit variable efficacy among sufferers. This is most likely due to treatments failing to selectively target molecular mechanisms causing susceptibility to

the disease. For this reason there is enormous potential for the development of individual genetic profiling of patients to enable more accurate diagnosis and customised treatments to be prescribed.

Pharmacogenomics involves, through an understanding of the genome, the application of modern genomic techniques, including SNP and transcript (by microarray) profiling as they relate to drug response. The ultimate objective of pharmacogenomics is to develop and prescribe drugs targeted to patients with a specific genetic profile determined at the genome-wide level. This will allow optimisation of disease treatment by maximising drug efficacy whilst minimising adverse events (side effects). Pharmacogenetics is a subtype of pharmacogenomics encompassing the study of specific gene variation that underlies differential response to drugs, particularly genes involved in drug metabolism. With the implementation of pharmacogenetics, diseases such as migraine will be assessed by the genetic mechanisms that govern them, rather than the symptomatic manifestation. This will allow for early individual specific treatment to be applied based on genetic susceptibility rather than just diagnosis. New drugs will target receptors and drug metabolising enzymes, making genes that code for these, good candidates for molecular pharmacogenetic studies.

Many studies have examined pharmacological treatment variation in relation to genetically different populations. However, the pharmacogenetics of migraine is an emerging field and there have been only a few studies to date investigating the association of specific gene polymorphisms and response to medication. Sumatriptan, a 5-HT_1 receptor agonist is a popular migraine analgesic but is known to be ineffective, or short lasting, in some suffers. A study by MaassenVanDenBrink et al, tested the hypothesis that polymorphisms within the 5-HT_1B receptor gene may explain the differences in response to sumatriptan. Allele frequencies for two SNPs were compared in groups that responded, did not respond at all, or where migraine recurred after treatment. No significant alteration in allele frequency was detected in this study (MaassenVanDenBrink et al, 1998).

A more recent study of a Phe-124-Cys amino acid substitution in the 5-HT_1B receptor was designed to assess the affect of this mutation on the pharmacological properties of

the receptor, and whether the mutation was associated with schizophrenia and bipolar disorder. Whilst there was no indication for involvement in disease, the variant was shown to significantly affect the pharmacological properties of the 5-HT1B receptor (Bruss et al, 1999). Additional pharmacogenetics work carried out by the same researchers indicated that the Phe-124-Cys mutation modifies the pharmacological properties of the 5-HT1B receptor and suggested that the 124-Cys variant may explain certain adverse events in some migraineurs being treated with sumitriptan (Kiel et al, 2000).

There is a considerably strong co-morbidity of migraine with depression (Breslau et al. 1991; Merikangas et al. 1993). By inference, the successful treatment of both conditions with selective serotonin re-uptake inhibitors supports the idea that an overlap of mechanisms involving serotonergic function may be involved. The human serotonin transporter protein actively mediates the reuptake of serotonin into the presynaptic terminal in the brain and thus plays an important role in the regulation of synaptic serotonin levels (Kelsoe et al. 1996). The selective serotonin re-uptake inhibitors (SSRIs) modulate serotonergic activity and are effective in the treatment of serotonin-related mental disorders, and also migraine. The serotonin transporters capacity to transport serotonin is known to be reduced after SSRI binding. A recent review examined the relationship between a functional polymorphism in the promoter region of the serotonin transporter gene (*SERT*), mental disorders and response to serotonergic agents (Weizman and Weizman, 2000). It was suggested that a *SERT* gene polymorphism is associated with level of expression of the *SERT* in the brain. Also, *SERT* polymorphisms were found to be related to a better and faster response to SSRIs in depressed patients. This may also have implications for migraine therapeutics (Weizman and Weizman, 2000). Although the results of this thesis do not confirm a relationship of this *SERT* gene variant and migraine, the polymorphism has been associated with typical migraine in a Danish population (Ogilvie, 1997). These findings interpreted with those of Weizman and Weizman (2000) make the *SERT* polymorphism a worthy candidate for future migraine pharmacogenetics studies.

Polymorphisms in the *DRD2* gene have been repeatedly associated with neuropsychiatric disorders thus providing an attractive opportunity for

pharmacogenetics studies testing this gene (Noble, 2000; Peroutka, 1997). A study investigating the association of response to short-term treatment of the antipsychotic drug haloperidol (a dopamine antagonist) treatment with the *Taq*I polymorphism in the *DRD2* gene found that patients that were heterozygous for the marker exhibited a significant improvement in positive symptoms after treatment. Thus, these results indicated that genetic variations in the *DRD2* gene might influence individual response to antipsychotics (Schafer et al, 2001). Migraine is known to coexist with several psychiatric disorders (Merikangas and Stevens, 1997) and antidepressants are often used as a prevention for chronic headache. A meta-analysis of clinical trials of antidepressants as prophylaxis for chronic headache was performed to determine the effectiveness of these drugs in the prevention of chronic headache. It was found that antidepressants are effective, but whether this is independent of depression was not determined (Tomkins et al, 2001). Interestingly, Peroutka et al., has reported a significant association between comorbid migraine with aura, anxiety and depression with *DRD2* NcoI C/C genotypes (Peroutka et al, 1998). These findings suggest that migraine could be considered together with certain mental disorders in candidate gene studies and pharmacogenetic studies of disorders such as depression may be useful models for similar studies in migraine.

Finally, the GRC now has several large migraine pedigrees showing linkage to different genomic regions. Thus, future pharmacogenetics research may involve re-interview of all affected patients from these linked migraine families to ascertain detailed information about treatments prescribed, time taken, dosages, responses to medication and adverse events. This new patient information could potentially be used to perform statistical correlation analysis of genomic susceptibility regions and family-specific clinical (specifically drug response) characteristics. It is hypothesised that differences at the genomic level between our pedigrees may in part explain the variation in symptoms and treatment responses apparent at the clinical level. If different genomic regions are involved in explaining family-specific treatment response differences, this may lead to the targeting of different genes for more effective medication development.

8.2.2 Migraine Genome Scan and Fine Mapping of Regions

Localisation of migraine susceptibility regions has been based on linkage studies using large multigenerational pedigrees, which have indicated that migraine is a genetically heterogeneous disorder involving at least three susceptibility regions. The GRC, in collaboration with GlaxoSmithKline, is currently completing a full genome scan using many migraine pedigrees to determine whether there are other gene loci involved in the disorder. Following this study, future research will be focused on the next step of the gene mapping process - fine mapping and identification of relevant sequence variants within migraine susceptibility genes, such as that already done with the chromosome 19p region and the *INSR* gene (McCarthy et al, 2001). More specifically, future studies will employ fine mapping approaches to narrow already implicated migraine susceptibility regions on chromosome Xq and 1q, as well as any new loci identified by the migraine genome scan. Closely spaced polymorphic markers, including intragenic candidate gene markers, spanning the previously implicated regions will be tested for linkage in families and undergoe haplotype analysis to refine the first pass map. Furthermore, intragenic markers (including triplet repeats, RFLPs and newly identified SNPs) will also be employed for high resolution LD mapping in large cross-sectional affected and unaffected populations. In addition, high throughput mutation screening of potential migraine candidate genes will be undertaken using new DHPLC methods in conjunction with standard direct sequencing analysis. Information gained from these future linkage and association tests as well as mutation screening will aid in the identification of new underlying genetic risk variants responsible for the disorder and make it easier for future researchers to generate new hypotheses for studies such as those investigating migraine pharmacogenetics.

8.2.3 Genotype/Symptom Correlation

The genetics of migraine is complex, involving multiple and possibly interacting loci. The current IHS criteria, which lead to the ultimate diagnosis of MA and MO may, in part, be hindering molecular genetic studies because it is likely that genetic variants are involved in influencing the risk of specific characteristics and symptoms rather than the disease as a whole. To identify the relationship of such genotypic variation on pheno-subtypes, future migraine research may involve an in-depth investigation of

the types of migraine symptoms and characteristics such as headache severity and duration, age of onset, triggers and certainly responses to specific types of medication. Such correlation studies may be undertaken in families showing linkage to specific genomic regions and also in cross-sectional populations genotyped for specific associated genes. As an example, it may be possible that individuals with a specific *DBH* genotype are more likely to have severe nausea/vomiting symptoms and individuals with a specific *INSR* genotype may be more prone to specific environmental triggers than those with other genotypes. Indeed, it is not unreasonable to think that a candidate gene polymorphism is associated with some particular migraine symptom even though that polymorphism failed to show previous involvement with typical migraine or one of the subtypes, MA or MO. The detection of genotypes associated with specific migraine characteristics and symptoms may have implications for both diagnosis and also the design of more appropriate treatments

8.2.4 New Analysis Strategies for Migraine Genetic Studies

Traditionally, researchers have treated migraine as a categorical (dichotomous) variable when investigating the molecular genetics of the disease. Future work might involve transformation of the current migraine phenotype (MA/MO) into a quantitative trait based on severity. In particular, the latest diagnostic variables including migraine subtype, headache frequency and duration, age of onset, associated symptoms, response to medication and gender, could be analysed to create a "migraine severity" variable. This new quantitative measure of severity could be constructed using a procedure known as latent class analysis to assign probabilities of a migraineur having a certain combination of specific clinical characteristics. Treating migraine as a type of quantitative trait should allow more powerful regression interaction modelling to be performed. These types of analyses will incorporate both genetic and clinical data to identify combined risk factors that could explain variation in the disease.

8.3 Conclusion

The complementary strategies of family-based linkage analysis and the candidate gene/association approach used in this research have proven useful in the

identification two novel migraine susceptibility genes DBH and INSR and a new susceptibility region on chromosome 1q31. Despite this, a specific risk variant conferring a functional effect on the disease has yet to be uncovered. Migraine is known as a complex disorder with the heterogeneity apparent at the clinical level likely to be explained by a combination of many "small-modest effect" genes as well as environmental influences. Moreover, it is likely that these genetic and environmental risk factors interact, further confounding the dissection of the disease aetiology. Hence, there is now great potential for new drug research targeting genes such as *INSR* in migraineurs, factoring in individual variations at the molecular genetic level. Certainly, advancement in pharmocogenomics techniques will greatly aid in the understanding and development of individual-specific diagnosis and treatment of this complex disease.

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Electronic Database Information

Accession numbers and URLs for data in this article are as follows;
www.genethon.fr/genethon_en.html
gdbwww.gdb.org
www.ncbi.nlm.nih.gov/genemap99
www-genome.wi.mit.edu/ftp/distribution/software/pedmanager/
www.mrc-bsu.cam.ac.uk/pub/methodology/genetics/ (ClaytonD (1997) TRANSMIT)

APPENDIX I. Migraine Questionnaire

Migraine Questionnaire

Is this a ☐ NEW Record or a ☐ CORRECTION to a completed Record?

Center: ☐☐☐ Family#: ☐☐☐☐☐ Individual#: ☐☐☐☐ Local ID: ☐☐☐☐☐☐☐☐☐☐

Exam Date: ☐☐ / ☐☐☐ / ☐☐☐☐ (Day / Month* / Year) Examiner: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

We would be most grateful if you could answer the following questions:

Morphometry

Height: ☐☐☐ cm Weight: ☐☐☐ kg

Migraine Information

1. Do you or have you ever suffered from headache attacks? ☐ Yes ☐ No

   If **NO**, skip to Medical History.

2. Do/or did you have different types of headaches? ☐ Yes ☐ No

   If **YES**, consider your most severe headache (migraine) type to answer the following questions:

3. What is the duration of your longest migraine attack? ☐ <1 hr ☐ 1-<3 hrs ☐ 3-<4 hrs ☐ 4-<12 hrs ☐ 12-<24 hrs ☐ 1-<3 days ☐ 3-<4 days ☐ 4-6 days ☐ >6 days

4. How long do your headache attacks usually last? ☐ <1 hr ☐ 1-<3 hrs ☐ 3-<4 hrs ☐ 4-<12 hrs ☐ 12-<24 hrs ☐ 1-<3 days ☐ 3-<4 days ☐ 4-6 days ☐ >6 days

5. How often do you have a migraine attack? ☐ <1/month ☐ 1-2/month ☐ 3-4/month ☐ >4/month

6. How many migraine attacks have you experienced? ☐ 1-4 ☐ 5-<10 ☐ 10-20 ☐ >20

7. How old were you when you started to get migraines? ☐☐☐

8. Do you still have migraines? ☐ Yes ☐ No

9. Do you have any of the following symptoms before a migraine:

   a. Visual disturbances ☐ Yes ☐ No

   b. Numbness or tingling ☐ Yes ☐ No

   c. Speech problems ☐ Yes ☐ No

*Valid month entries are: JAN, FEB, MAR, APR, MAY, JUN, JUL, AUG, SEP, OCT, NOV, DEC

Form Name: migraine_quest_37202
Filename: x_migraine_quest_37202.del
Revised: 10/7/1999

1 of 4

37202

Center: | | | **Family #:** | | | | | **Individual #:** | | | |

10. When you have a migraine, or just prior to the migraine, do you suffer from:

	Yes	No		Yes	No
a. Nausea	☐	☐	j. Numbness or tingling	☐	☐
b. Vomiting	☐	☐	k. Weakness	☐	☐
c. Diarrhoea	☐	☐	l. Throbbing pain	☐	☐
d. Increased sound sensitivity	☐	☐	m. Pain on one side of the head	☐	☐
e. Increased light sensitivity	☐	☐	n. Pain on both sides of the head	☐	☐
f. Increased sensitivity to smells	☐	☐	o. Discomfort on moving your head	☐	☐
g. Alteration of vision	☐	☐	p. Discomfort on moving your eyes	☐	☐
h. Dizziness or double vision	☐	☐	q. Prevention of normal physical activity	☐	☐
i. Speech problems	☐	☐			

11. Are your migraine attacks associated with or triggered by any of the following?

	Yes	No
a. FOR WOMEN ONLY: Menses	☐	☐

FOR ALL RESPONDERS:	Yes	No
b. Weather changes	☐	☐
c. Stress	☐	☐
d. Holiday/Relaxation	☐	☐
e. Other	☐	☐

If other, please specify: ____________________

f. Food and Beverages

	Yes	No
1. red wine	☐	☐
2. other alcohol	☐	☐
3. chocolate	☐	☐
4. oranges	☐	☐
5. ripe cheese	☐	☐
6. other dairy products	☐	☐
7. other foods and beverages	☐	☐

If other, please specify: ____________________

37202

Center: ☐☐☐ Family #: ☐☐☐☐☐ Individual #: ☐☐☐☐

12. Do you take any prescribed treatment or medication for migraine? ☐ Yes ☐ No

If **YES:**

13. Have you ever been treated with 5-HT_1 type drugs (e.g. 'triptans': sumatriptan, naratriptan, or zolmitriptan)? ☐ Yes ☐ No

If **YES:**

14. Do you find this medication effective? ☐ Yes ☐ No

15. Do you take any **other** treatments/medication?

a. Pain killers . . . . ☐ Yes ☐ No

b. Natural remedy . . . . ☐ Yes ☐ No

c. Medication for nausea . . . . ☐ Yes ☐ No

d. Other . . . . ☐ Yes ☐ No

please specify: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

If **YES** to any of question 15:

16. Do you find this treatment/medication effective? ☐ Yes ☐ No

Medical History:

17. Have you ever been diagnosed with or treated for any of the following:

	Yes	No
a. Severe pain problems (other than headache)	☐	☐
b. Chronic neck pain	☐	☐
c. High blood pressure	☐	☐
d. Stroke	☐	☐
e. Heart disease	☐	☐
f. Depression	☐	☐
g. Anxiety disorder	☐	☐
h. Panic disorder	☐	☐
i. Chronic fatigue syndrome	☐	☐
j. Irritable bowel syndrome	☐	☐
k. FOR WOMEN ONLY: Severe menstrual problems	☐	☐
l. FOR ALL SUBJECTS: Chronic back pain	☐	☐

37202

Center: ☐☐☐ **Family #:** ☐☐☐☐☐ **Individual #:** ☐☐☐☐

18. FOR WOMEN ONLY: Are you or have you in the past taken the contraceptive pill? ☐ Yes ☐ No

19. Do you take any prescribed treatment or medication for disorders other than migraine? ☐ Yes ☐ No

    If **YES**, please specify:

20. Do you drink alcohol? . . . . ☐ Yes ☐ No

    If **YES**, indicate average amount: ☐ <3 glasses/day ☐ 3-6 glasses/day ☐ >6 glasses/day

21. Do you smoke cigarettes? . . . . ☐ Yes ☐ No

    If **YES**, indicate amount: ☐ <5 cigarettes/day ☐ 5-<10/day ☐ 10-20/day ☐ >20/day

22. Are you regularly exposed to someone else's smoke at work, school or home? ☐ Yes ☐ No

23. Have any of your **immediate** family been diagnosed or treated for the following disorders? If so, indicate the **number** of affected relatives. If not, please leave boxes blank.

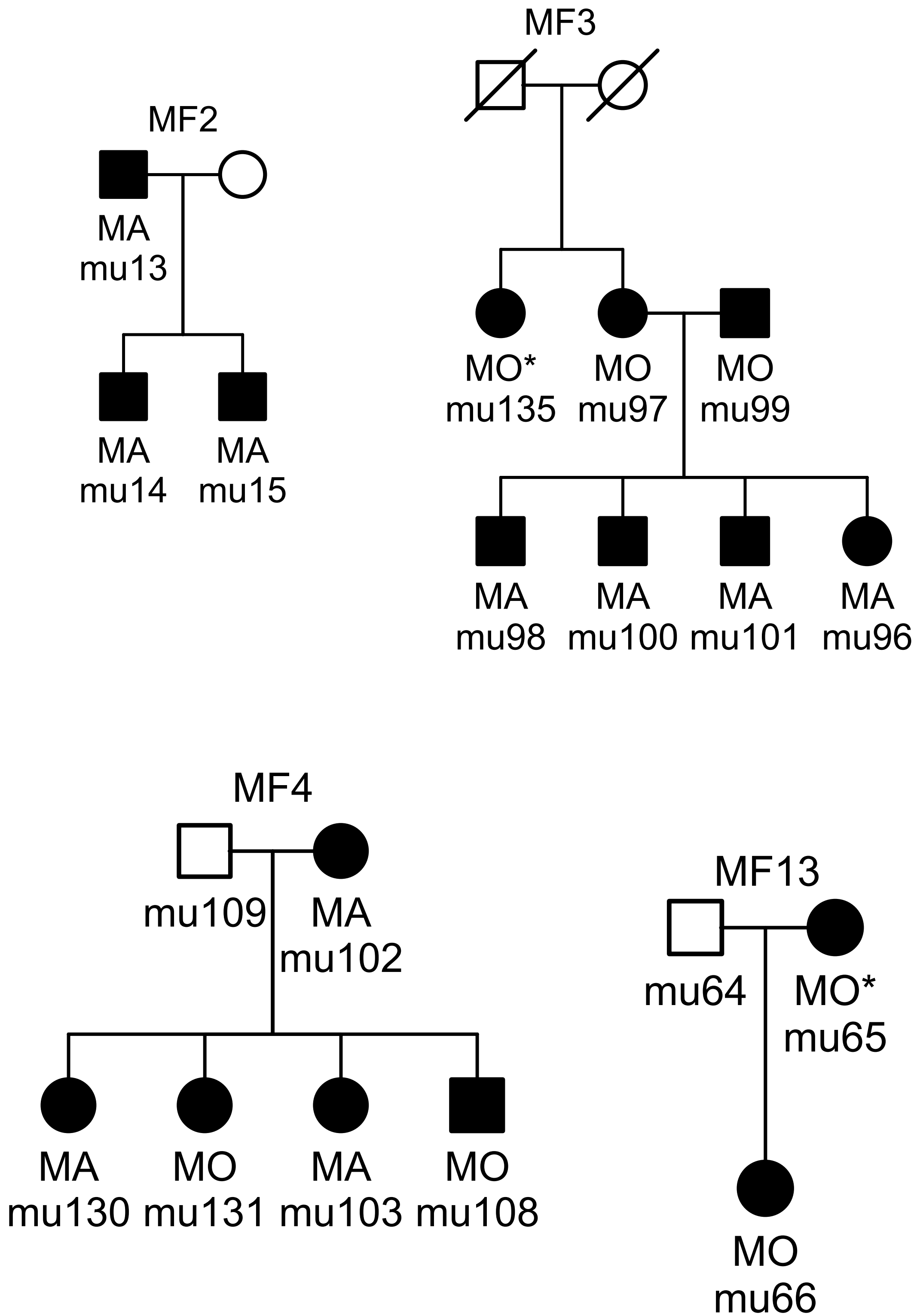
	Father	Mother	Brother(s)	Sister(s)	Children	Grandparent(s)
a. Migraine	☐	☐	☐☐	☐☐	☐☐	☐
b. Heart disease	☐	☐	☐☐	☐☐	☐☐	☐
c. Stroke	☐	☐	☐☐	☐☐	☐☐	☐
d. High blood pressure	☐	☐	☐☐	☐☐	☐☐	☐
e. Depression	☐	☐	☐☐	☐☐	☐☐	☐
f. Anxiety disorder	☐	☐	☐☐	☐☐	☐☐	☐

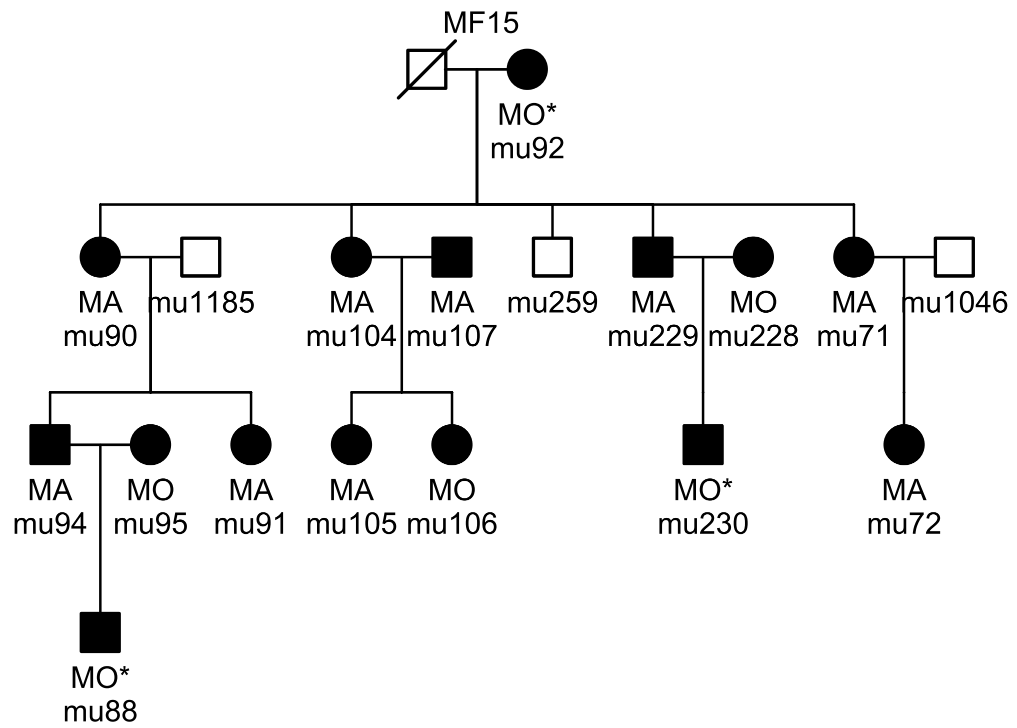
24. Impression
    - ☐ MA migraine - meets IHS criteria
    - ☐ MO migraine - meets IHS criteria
    - ☐ MA migraine - does not meet strict IHS criteria
    - ☐ MO migraine - does not meet strict IHS criteria
    - ☐ Unaffected

Thank you very much for your time and help with our research.

37202

Appendix II. 82 migraine affected pedigrees examined in this research. Blackened symbols indicate subjects affected with MO or MA. MU* indicates individuals from which blood (and DNA) was available.

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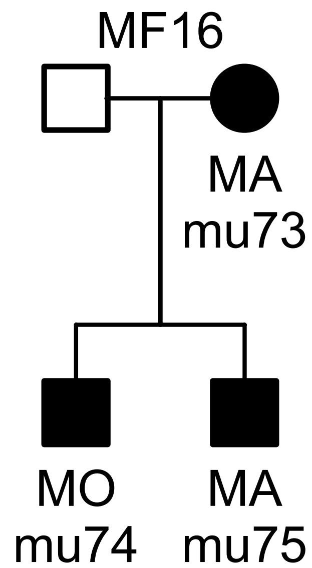
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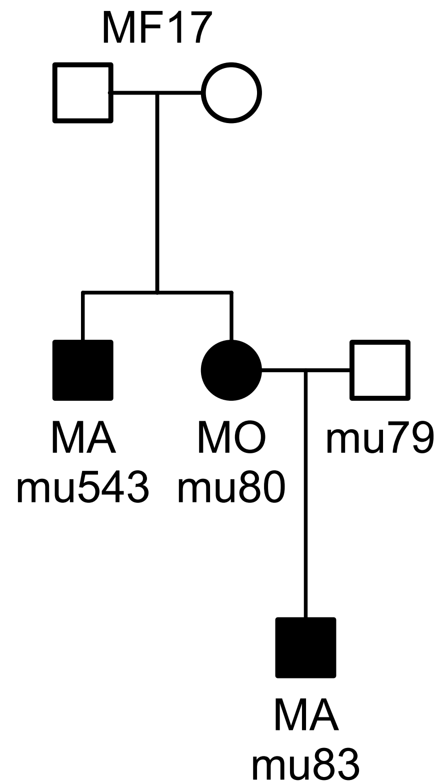
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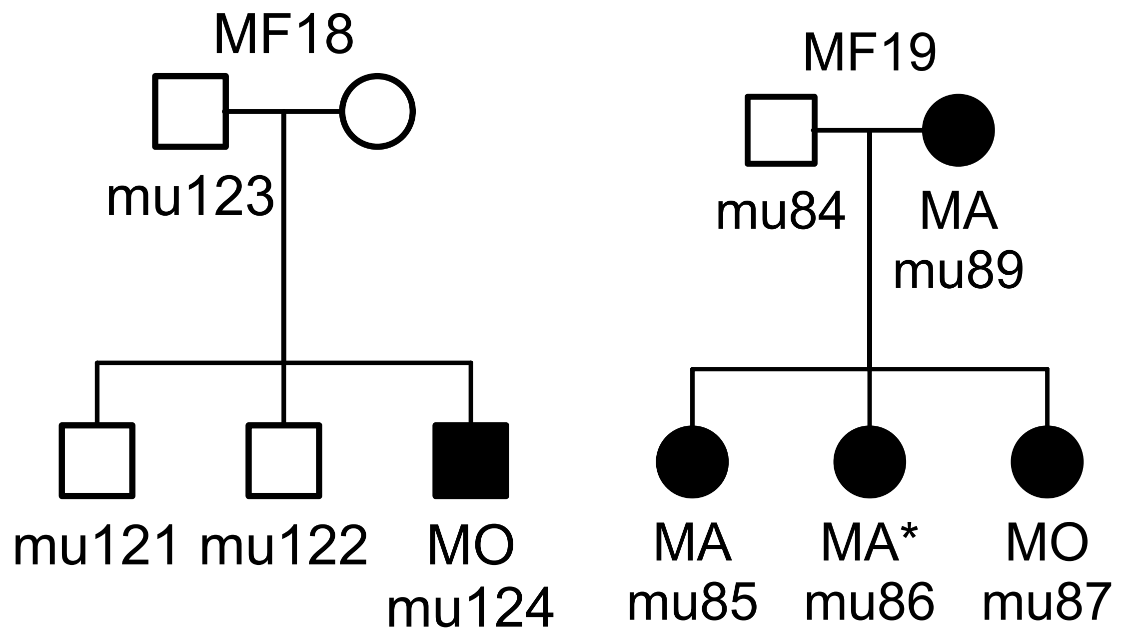
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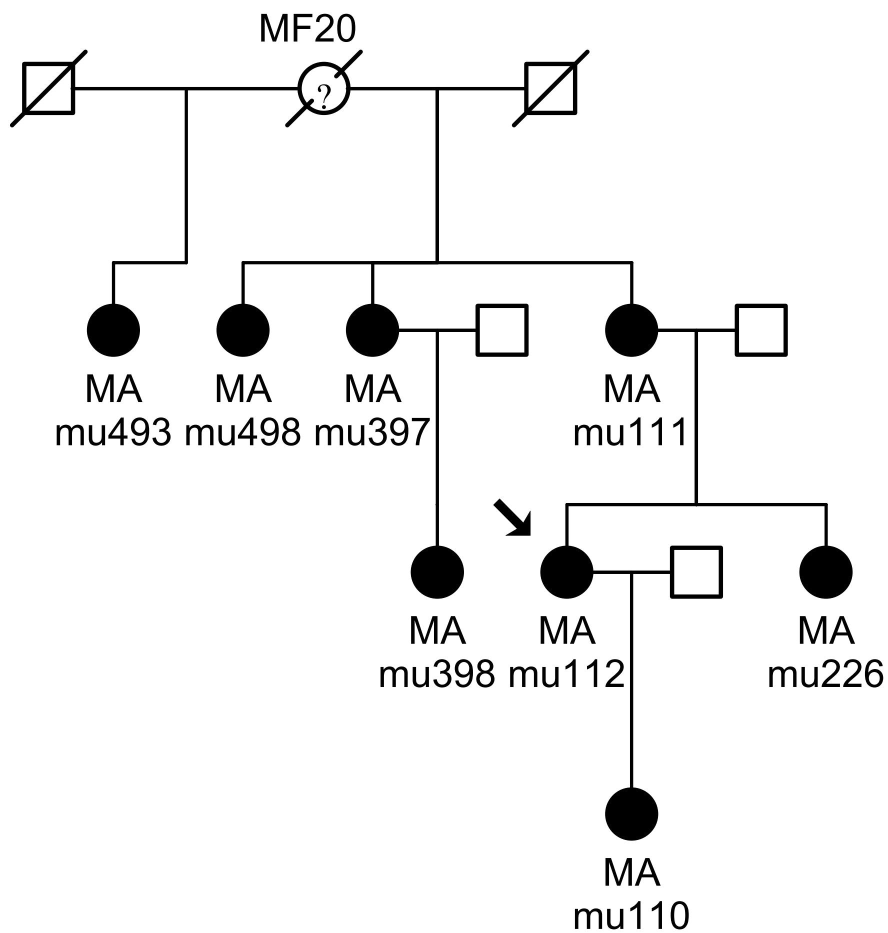
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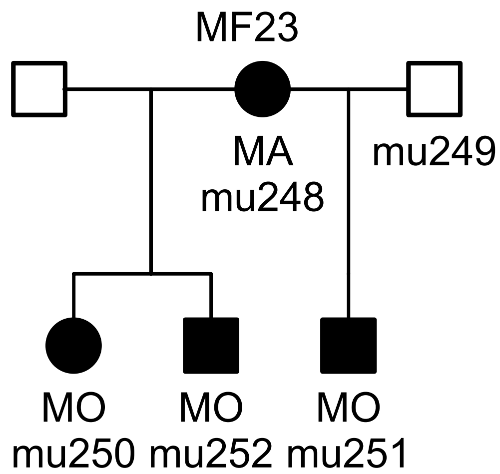
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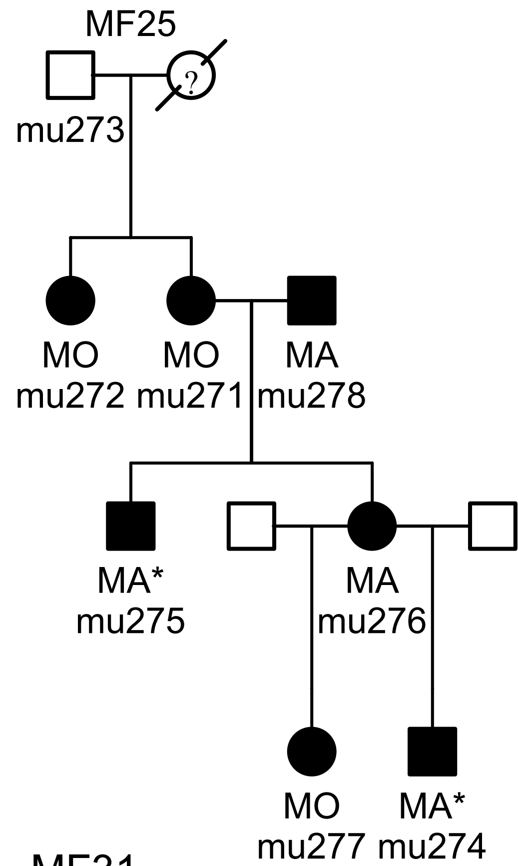
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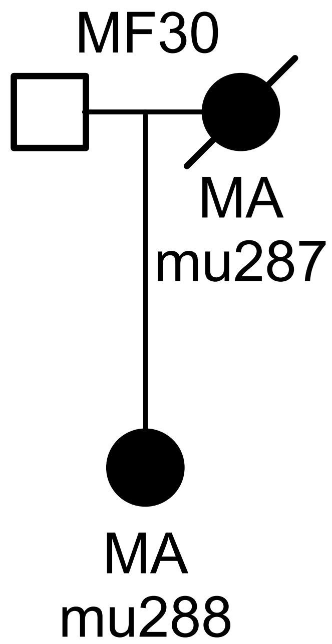
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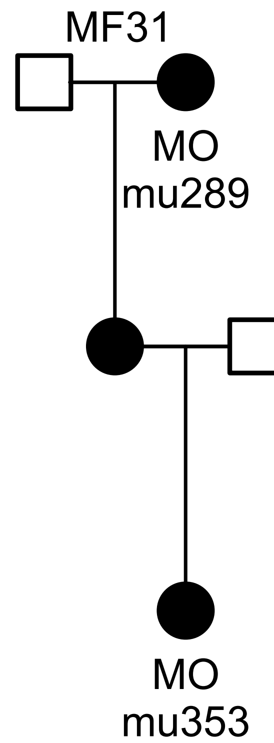
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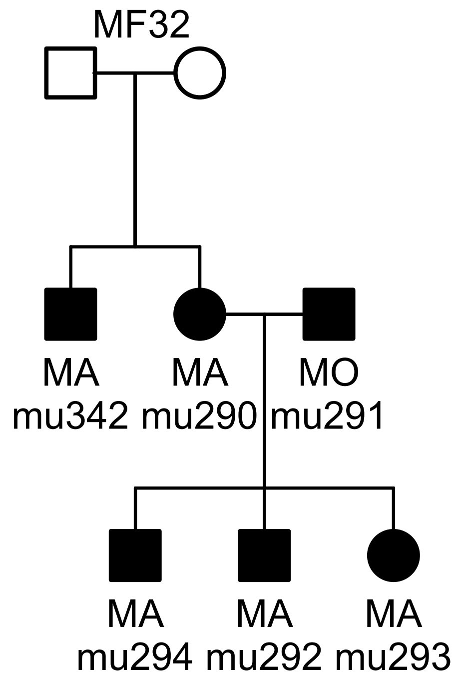
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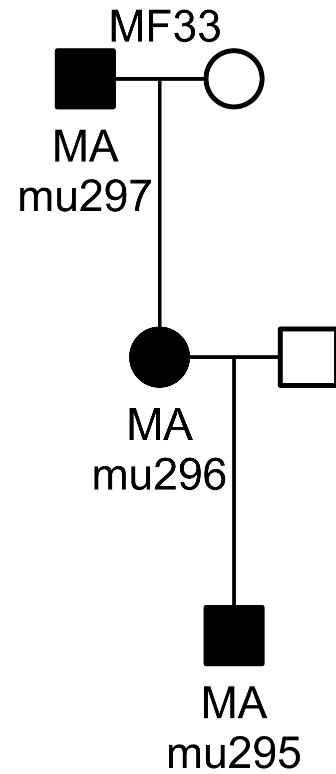
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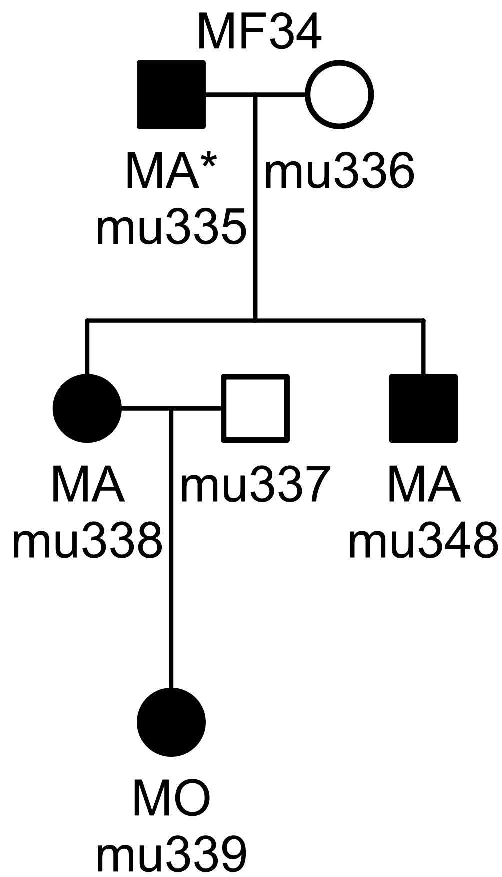
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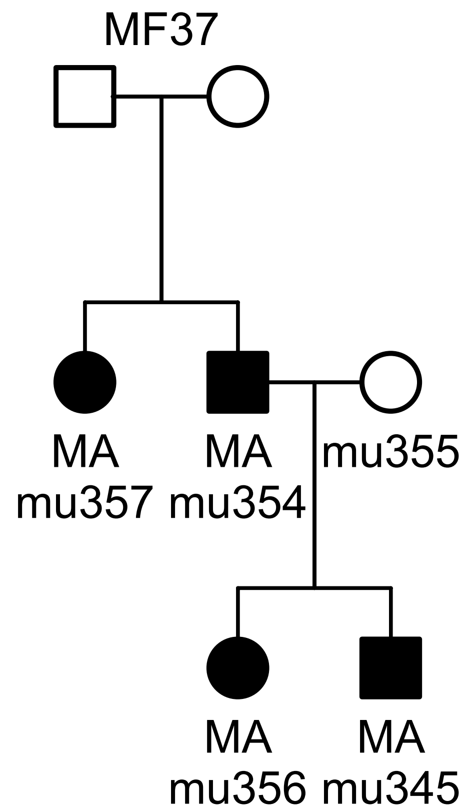
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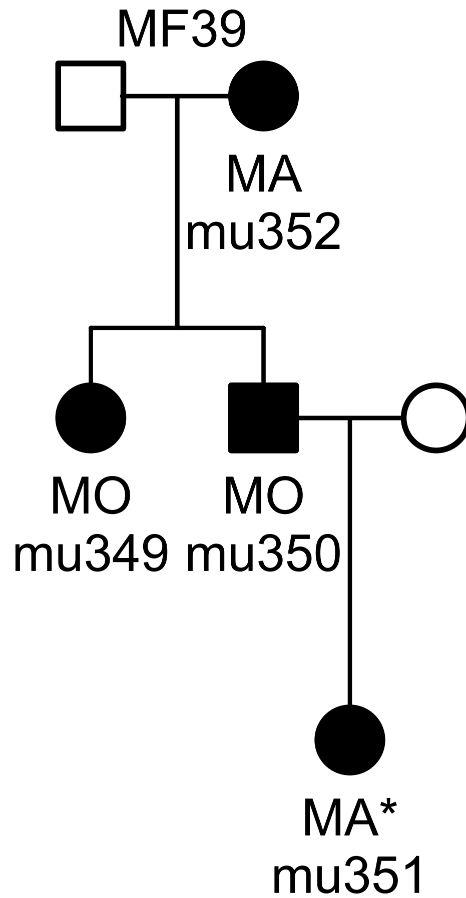
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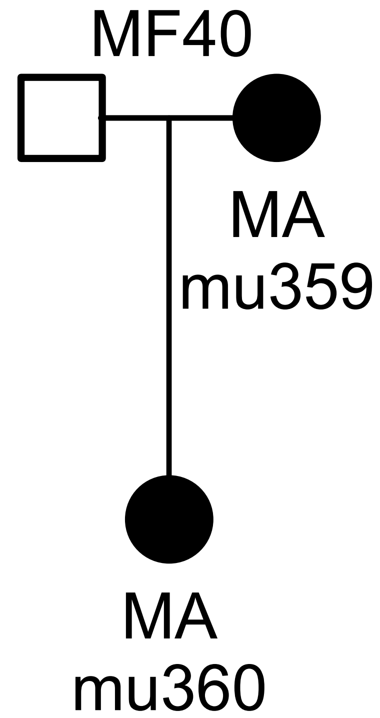
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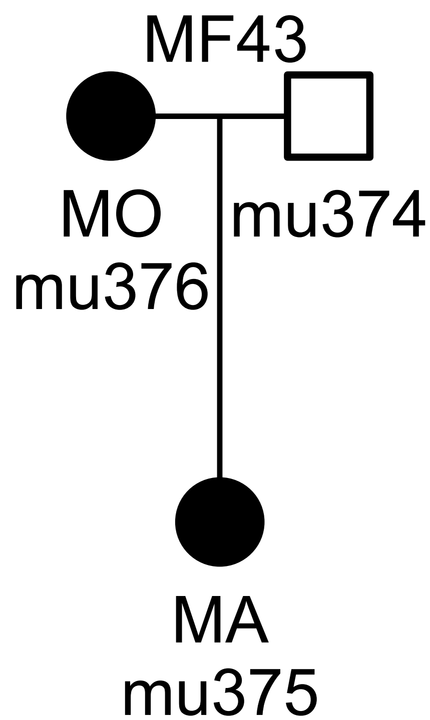
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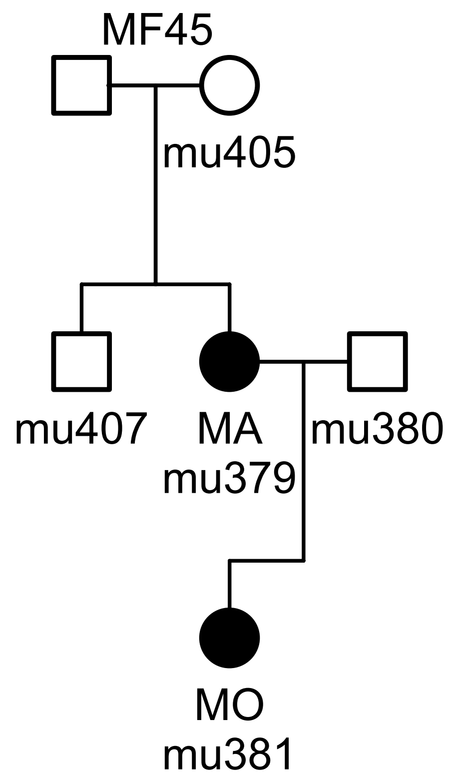
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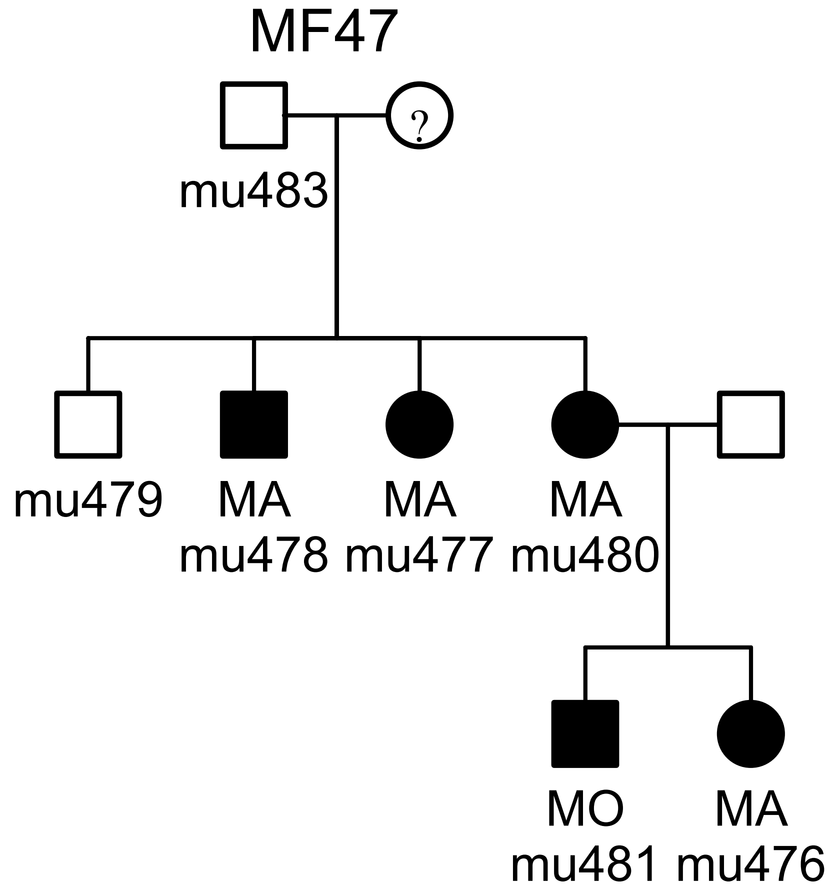
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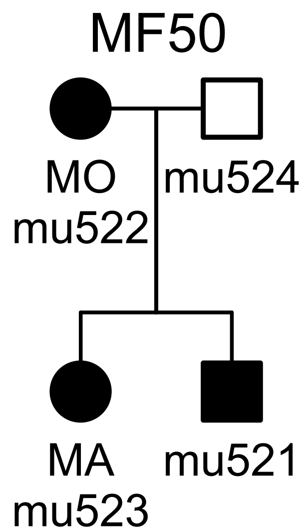
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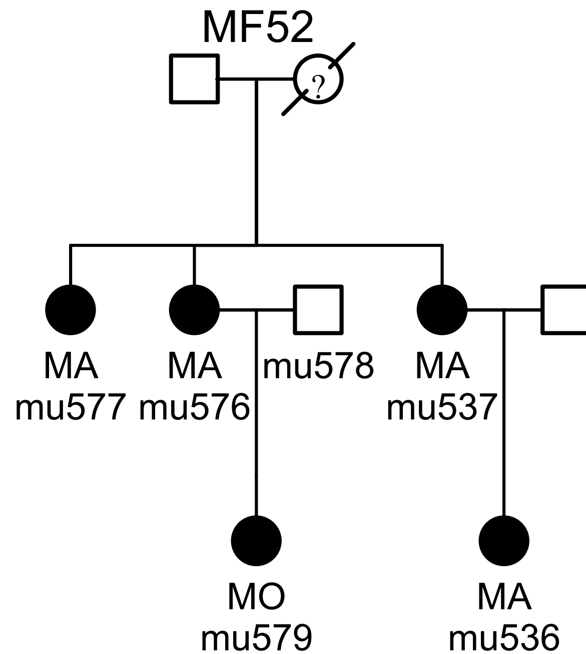
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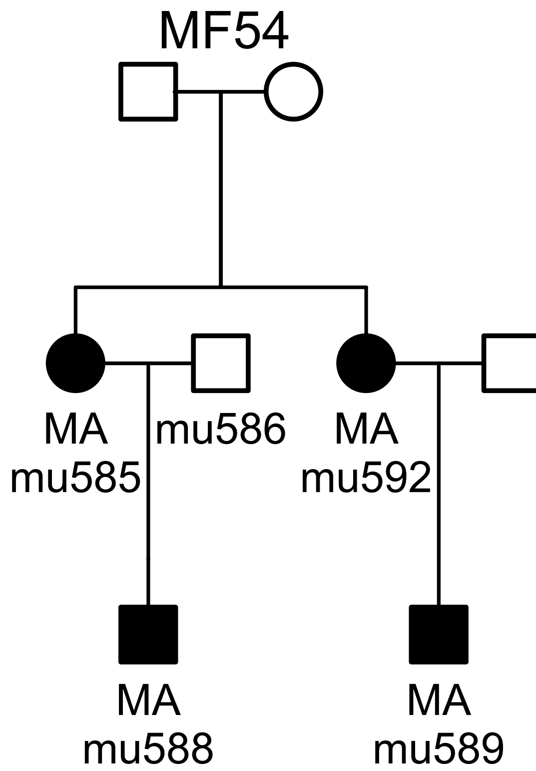
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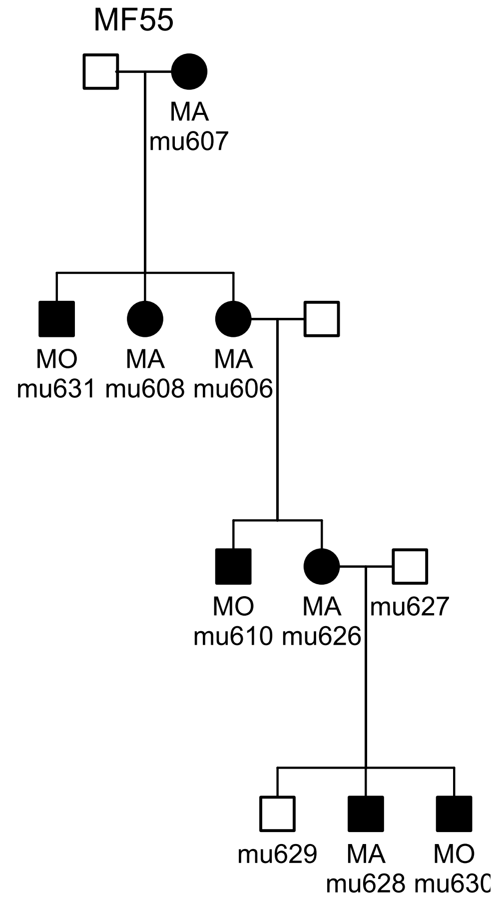
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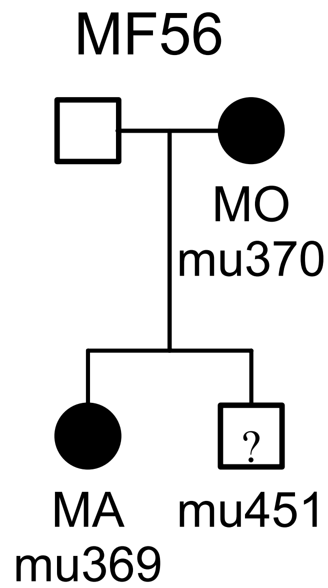
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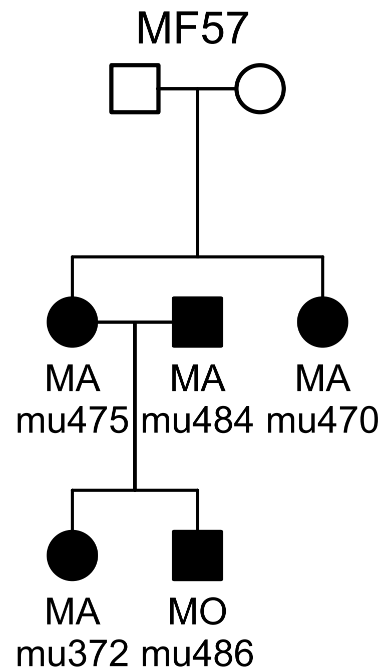
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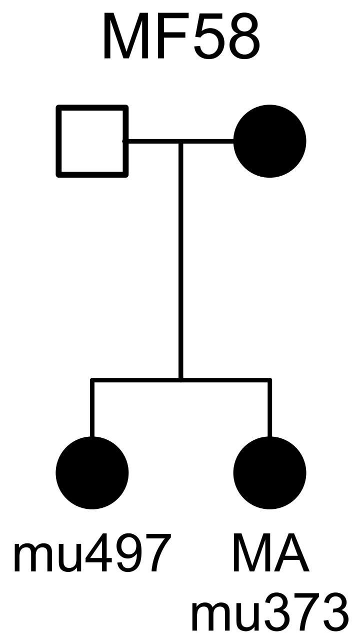
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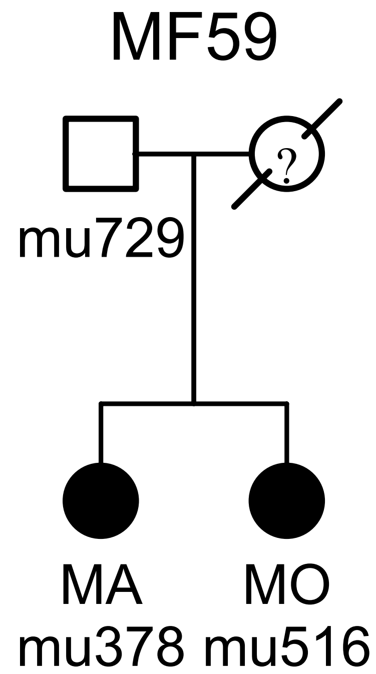
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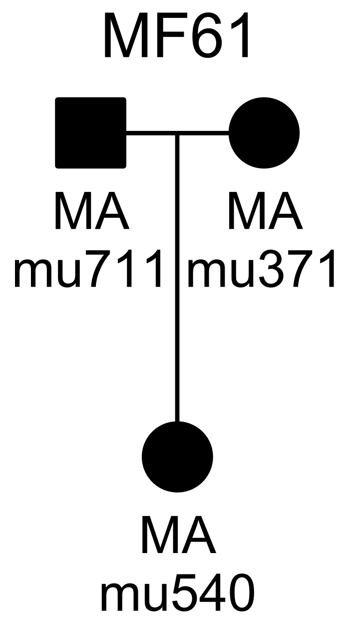
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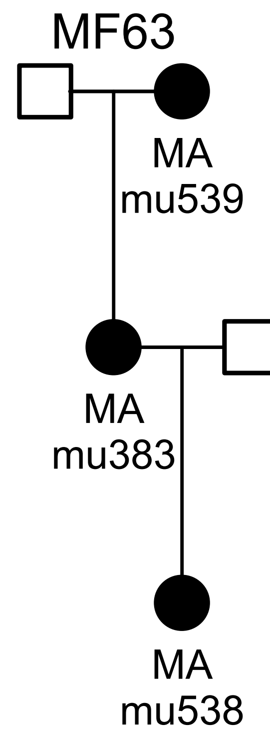
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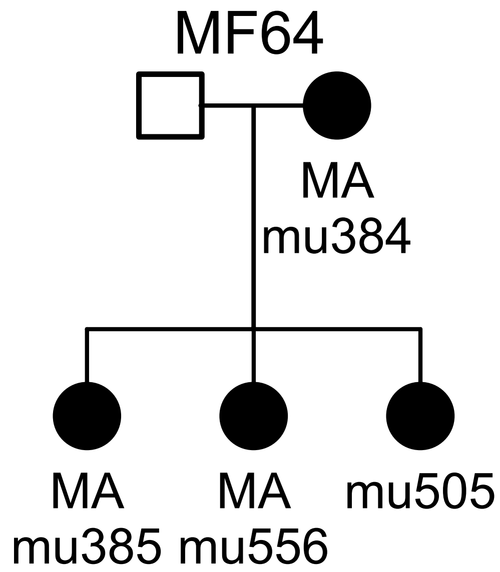
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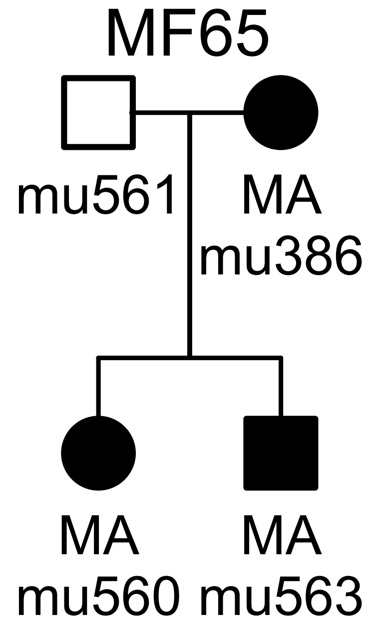
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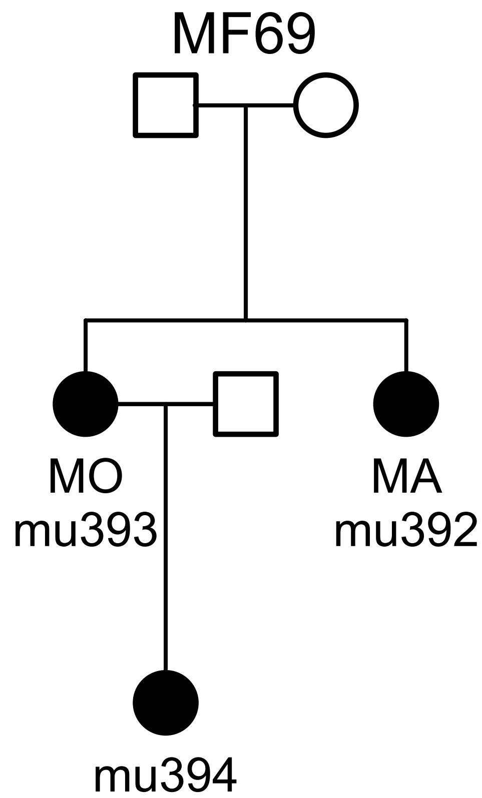
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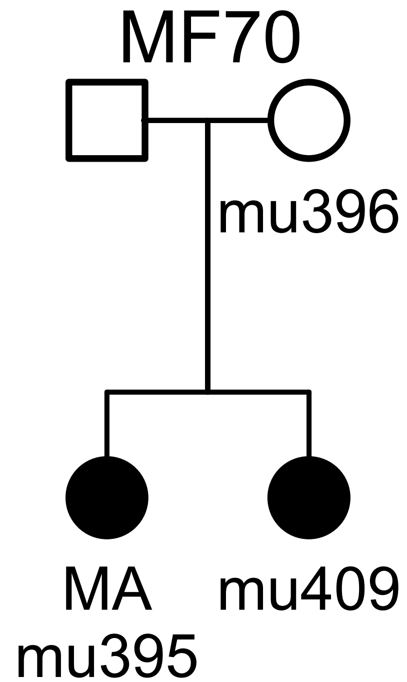
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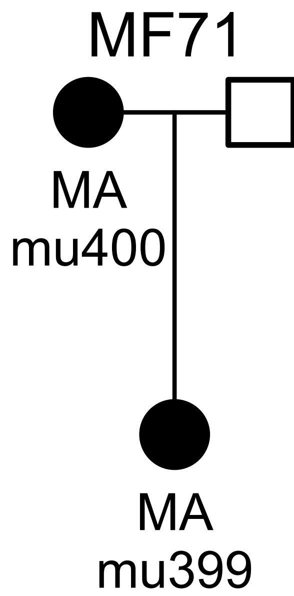
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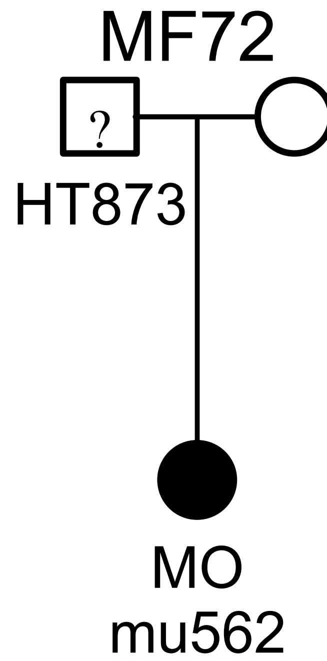
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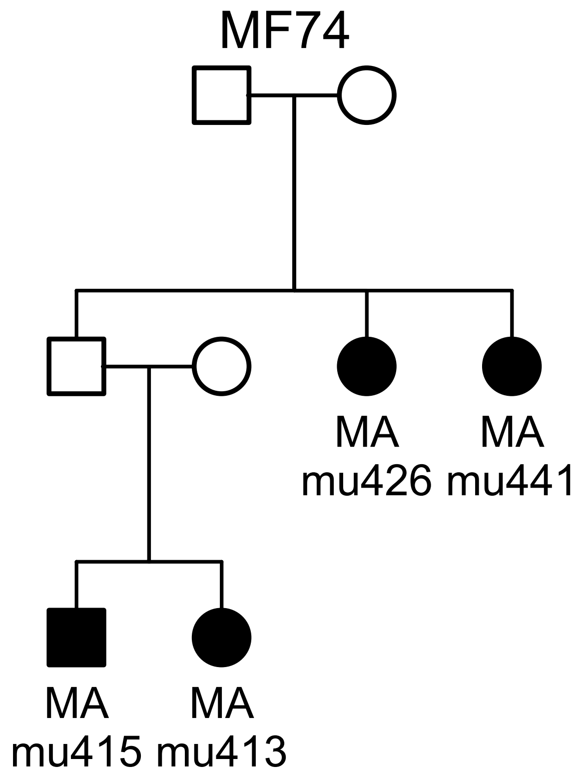
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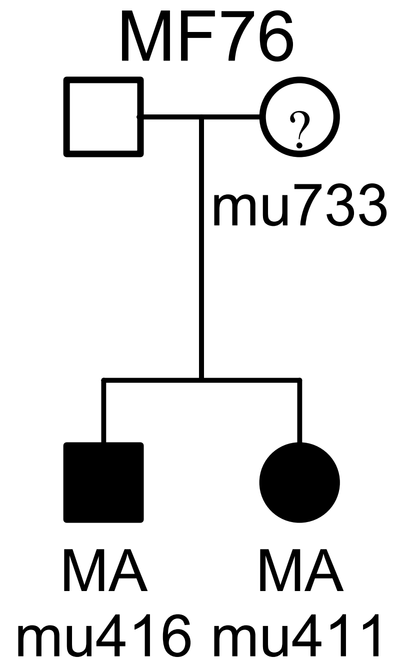
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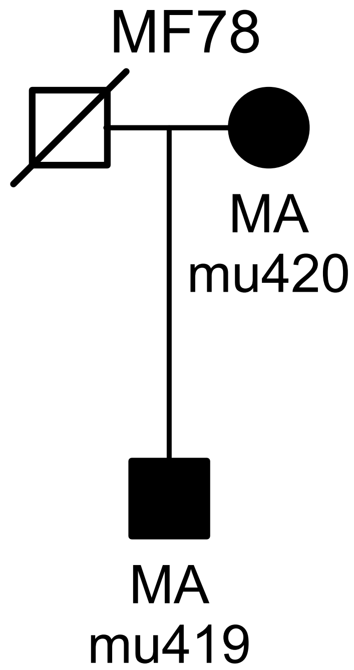
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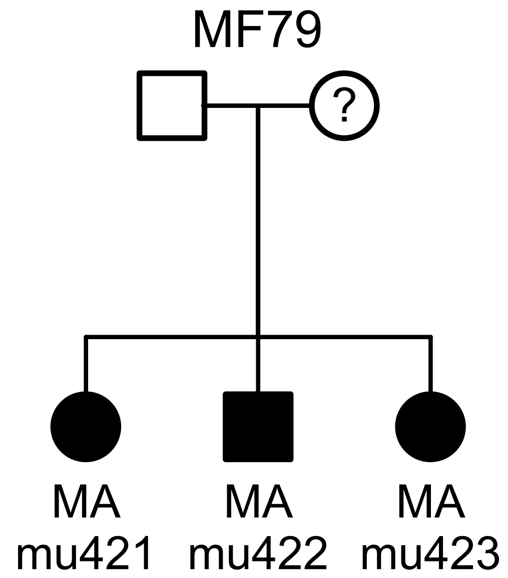
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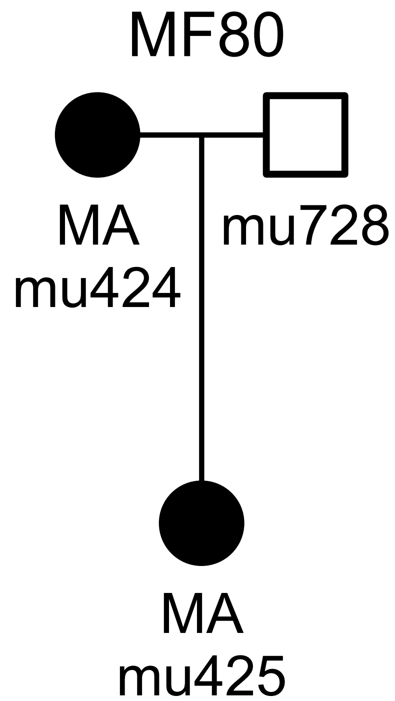
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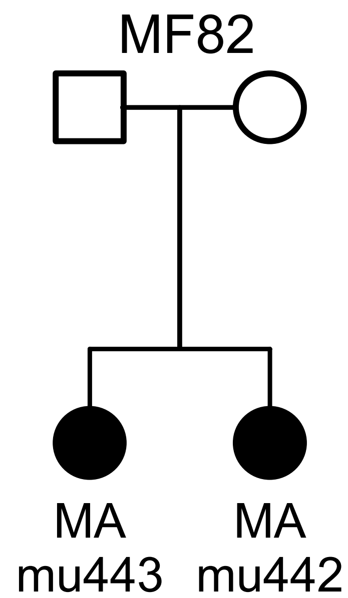
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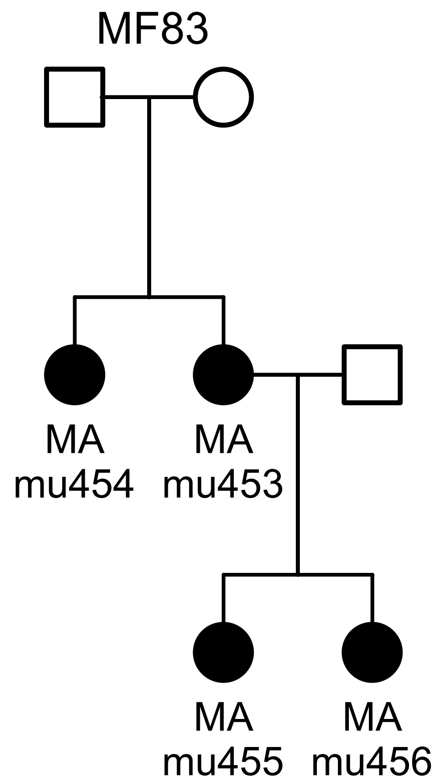
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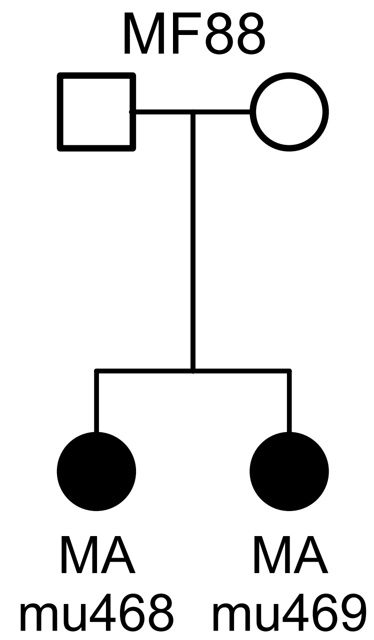
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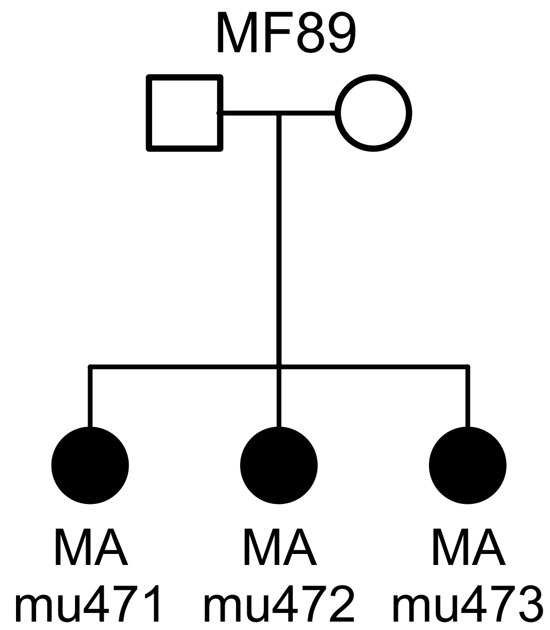
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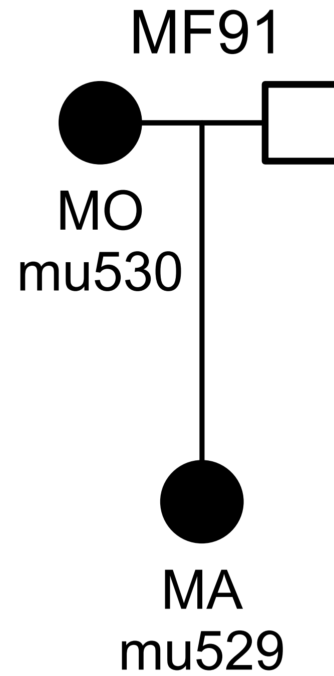
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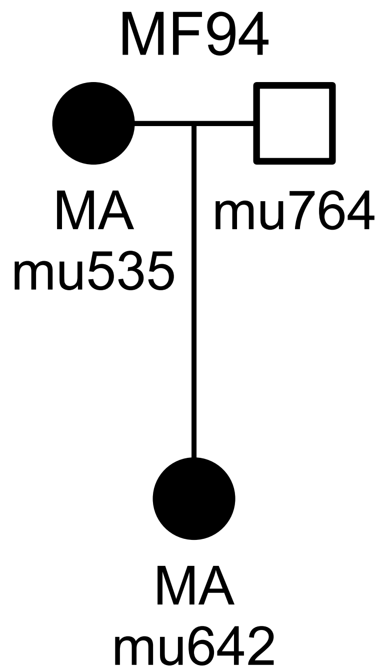
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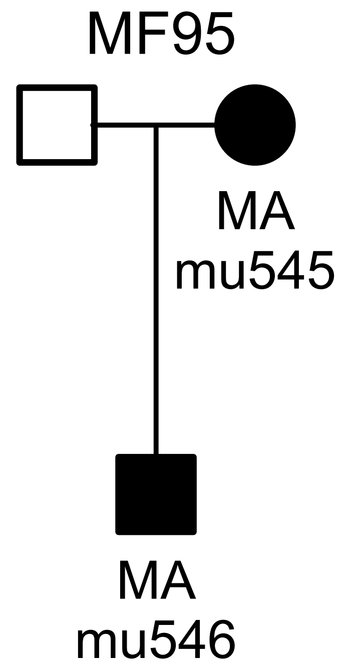
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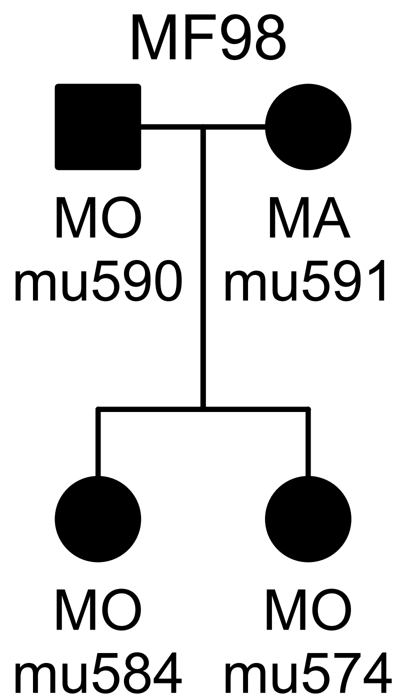
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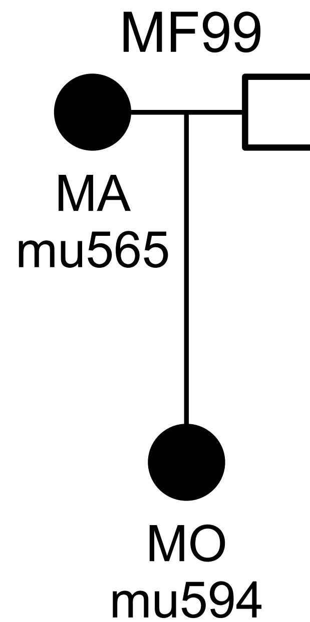
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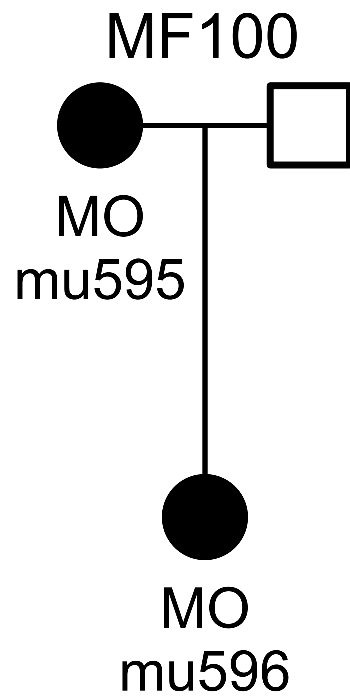
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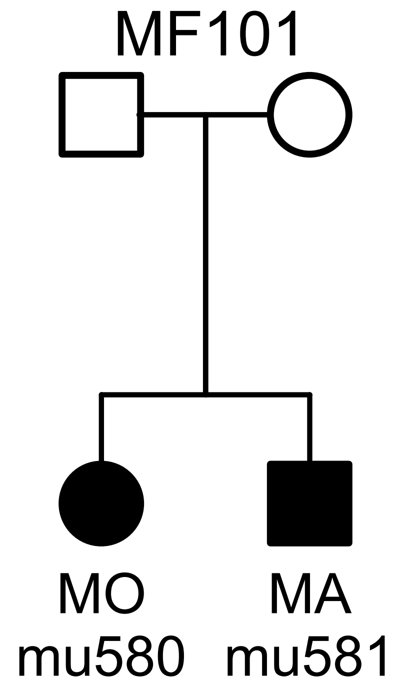
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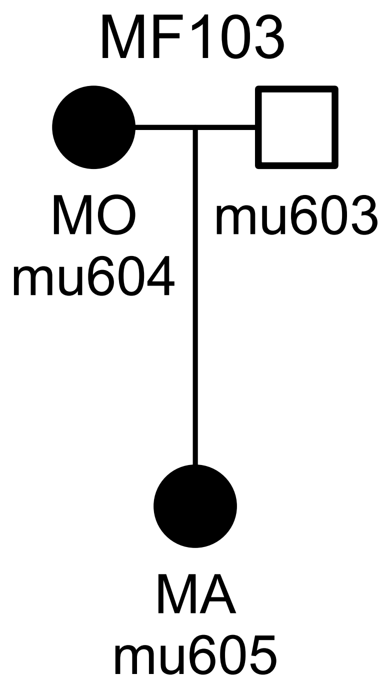
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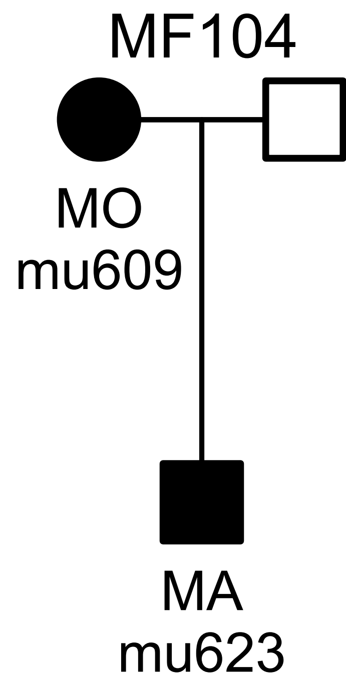
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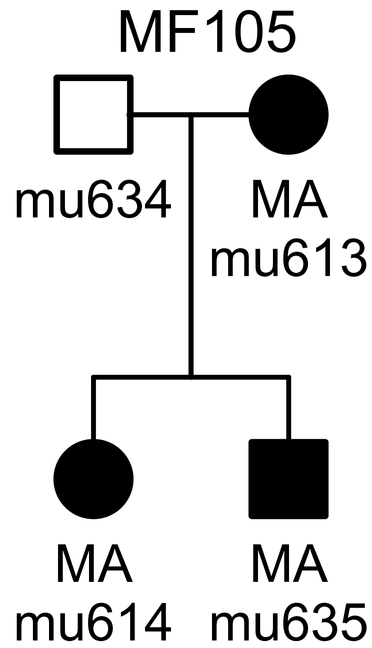
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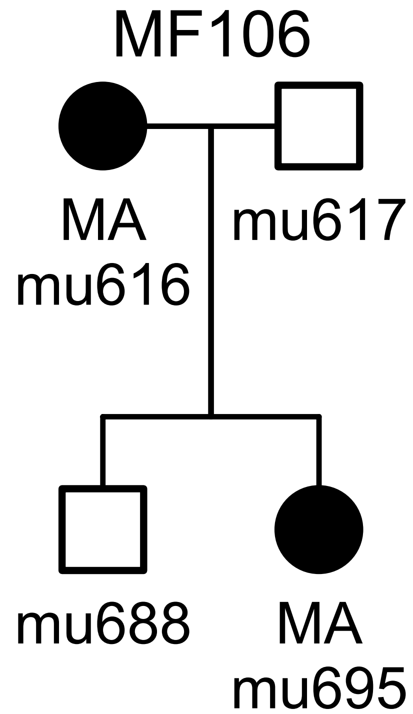
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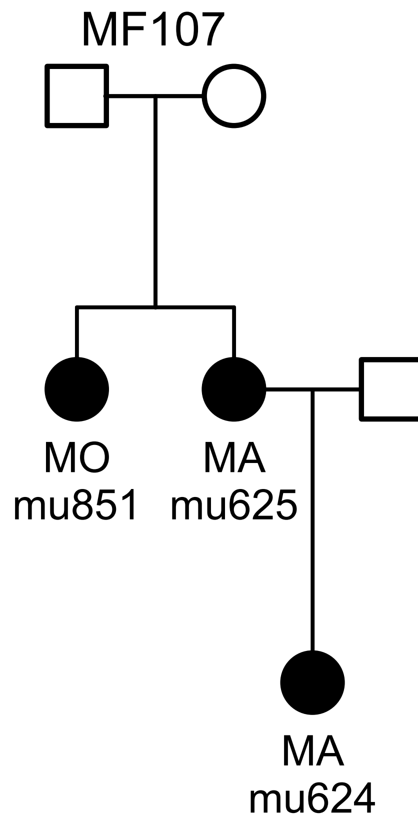
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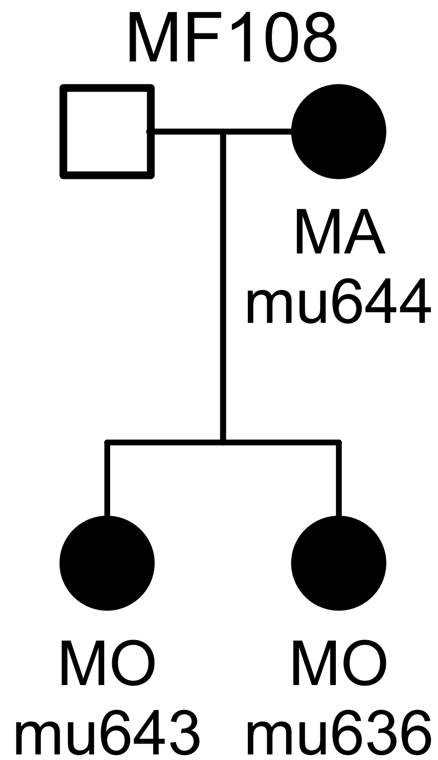
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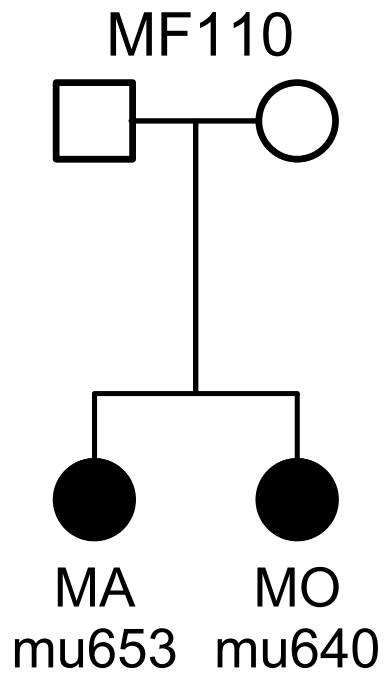
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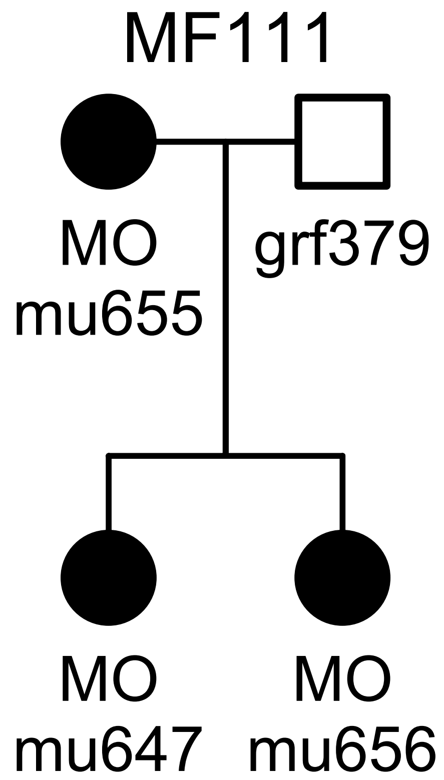
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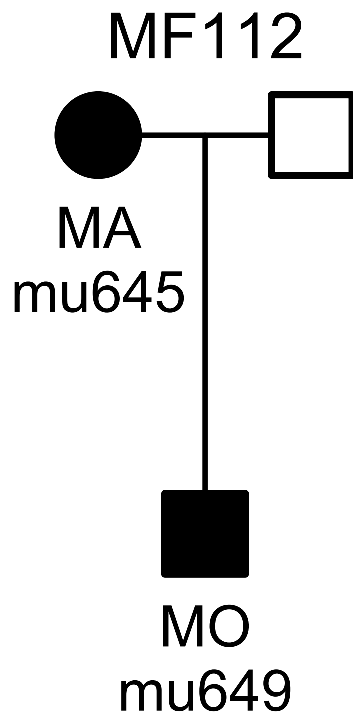
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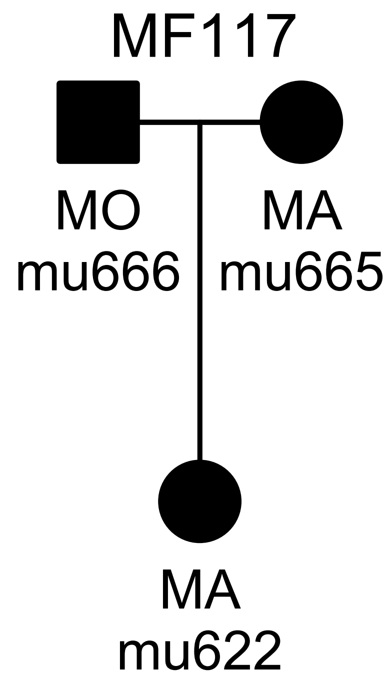
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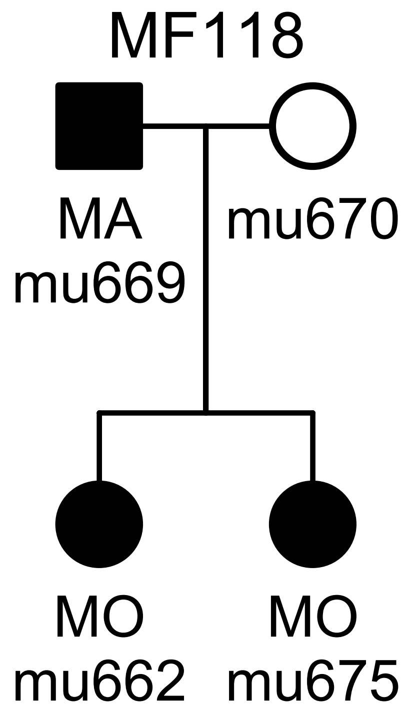
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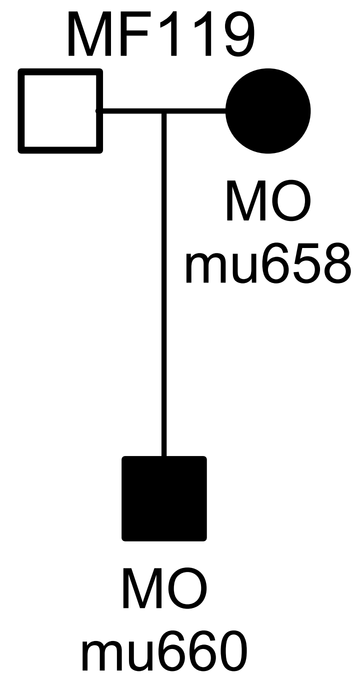
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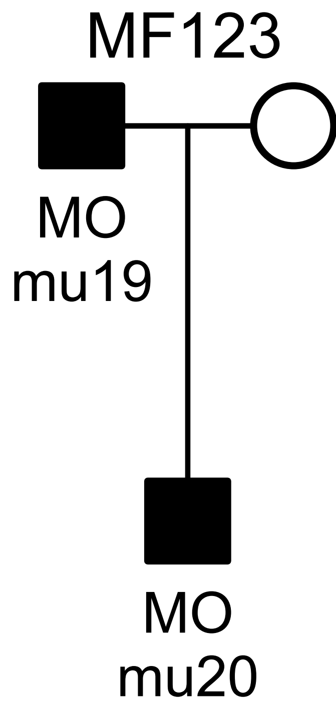
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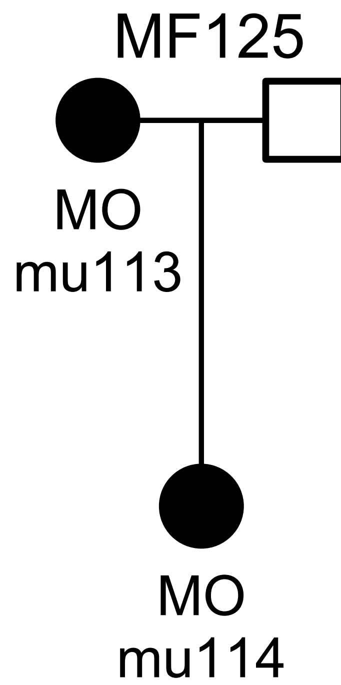
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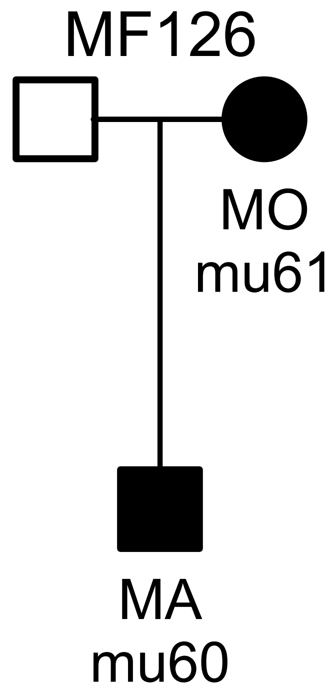
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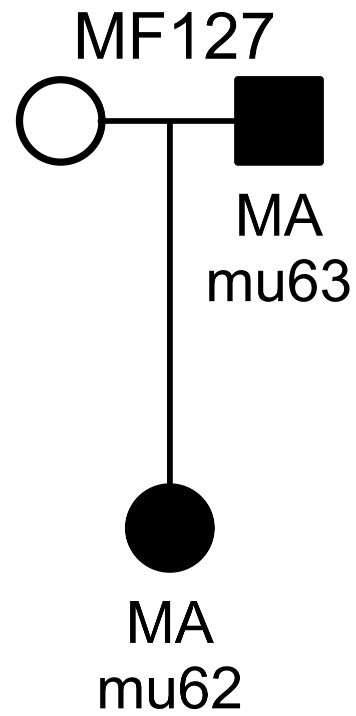
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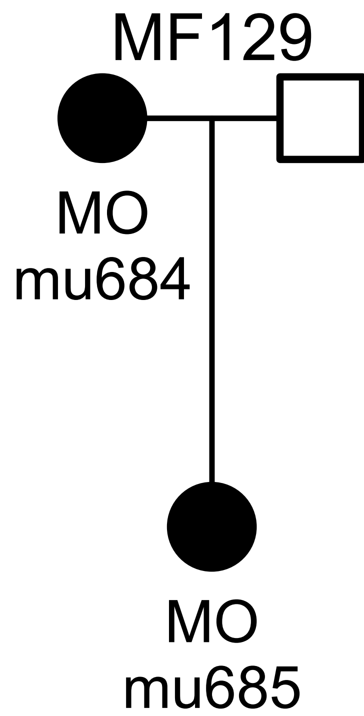
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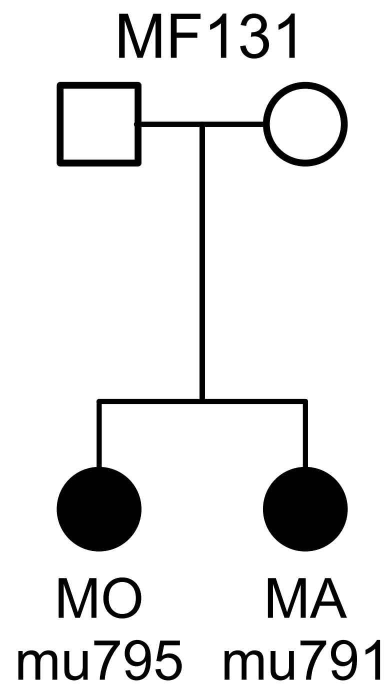
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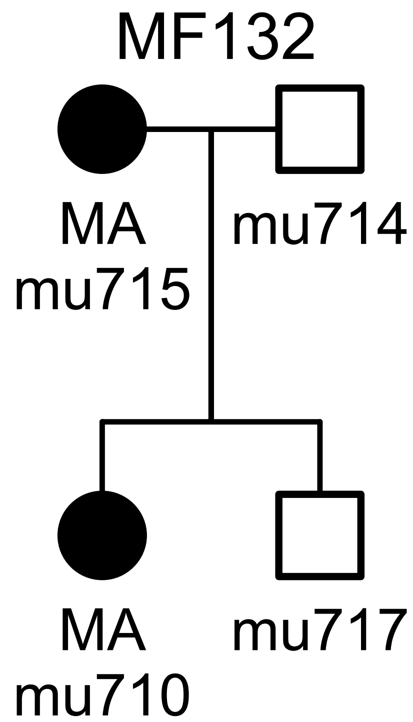
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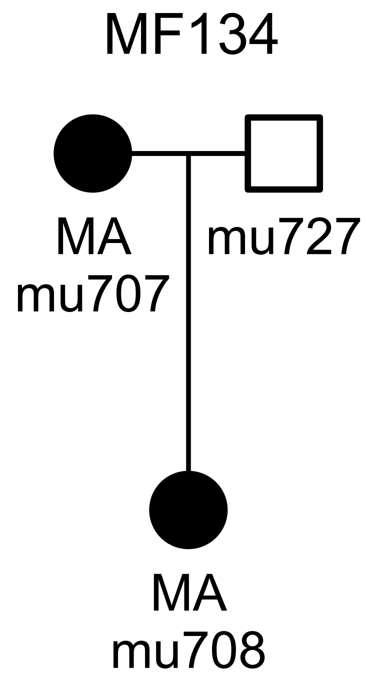
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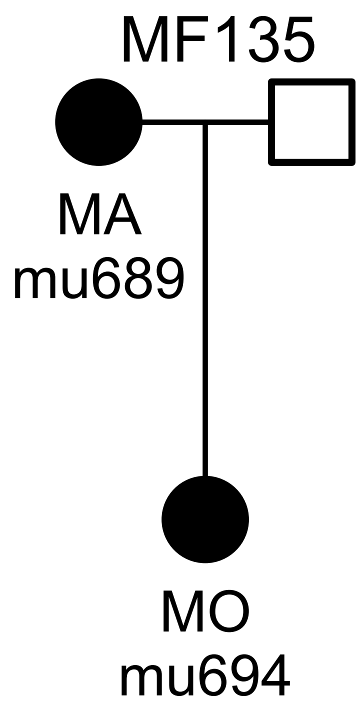
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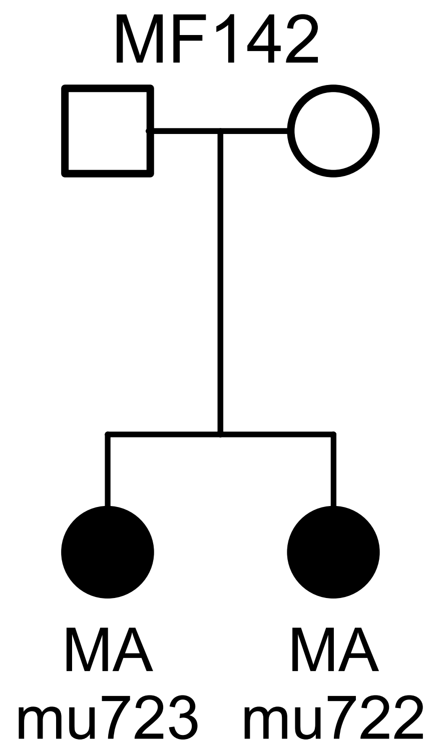
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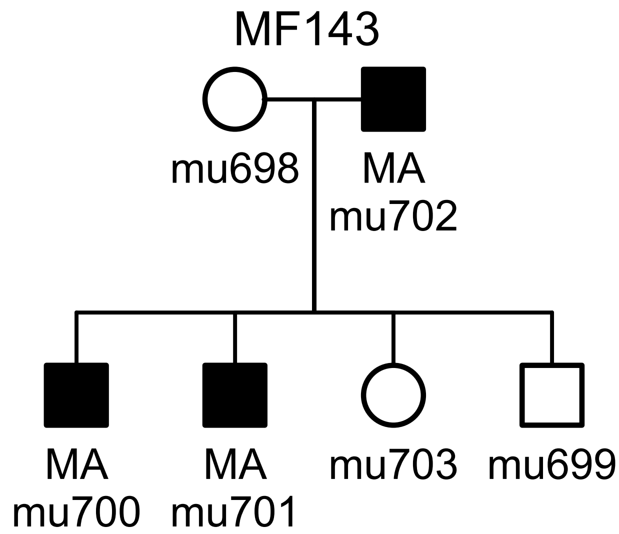
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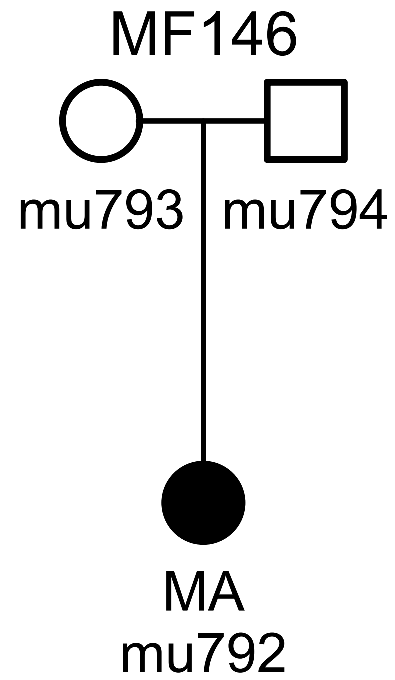
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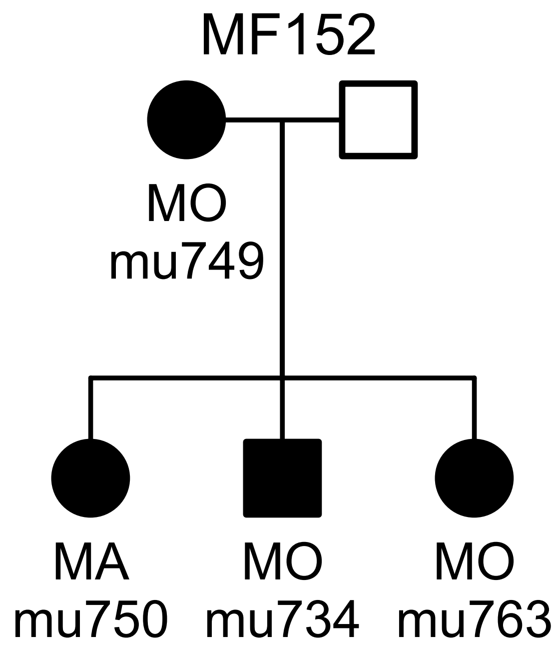
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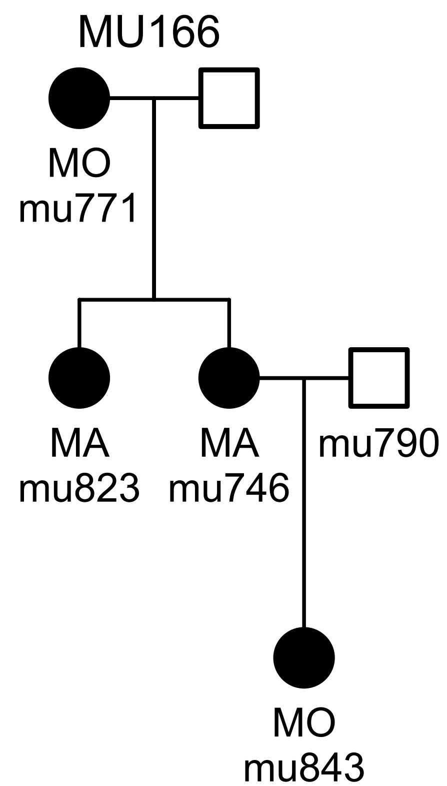
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APPENDIX III. LINKAGE INPUT FILES

Chromosome 19p13 (D19S221, CACNA1A (CAG)n, D19179)

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111	2	0	0	1	1	1	8	7	7	0	0	3	3
111	3	2	1	2	2	1	8	7	8	0	0	3	5
111	4	2	1	2	2	1	8	0	0	8	8	3	5
112	1	0	0	2	2	5	7	8	8	5	8	0	0
112	2	0	0	1	0	0	0	0	0	0	0	0	0
112	3	2	1	1	2	4	5	8	9	4	5	3	3
117	1	0	0	2	1	8	10	7	7	4	8	3	3
117	2	0	0	1	1	5	9	8	9	0	0	3	5
117	3	2	1	2	2	9	10	7	9	4	9	3	5
118	1	0	0	2	1	0	0	3	9	0	0	3	3
118	2	0	0	1	2	7	8	3	9	0	0	3	3
118	3	2	1	2	2	3	8	9	9	5	7	3	3
118	4	2	1	2	2	0	0	9	9	0	0	3	3
119	1	0	0	2	2	0	0	0	0	8	8	0	0
119	2	0	0	1	0	0	0	0	0	7	8	0	0
119	3	2	1	1	2	8	8	7	7	0	0	5	5
123	1	0	0	1	2	5	8	0	0	0	0	3	7
123	2	0	0	2	0	0	0	0	0	0	0	0	0
123	3	1	2	1	2	4	5	0	0	0	0	5	7
125	1	0	0	2	2	4	8	3	9	0	0	5	5
125	2	0	0	1	0	0	0	0	0	8	8	0	0
125	3	2	1	2	2	4	9	9	9	6	8	3	5
126	1	0	0	2	2	0	0	8	8	5	7	0	0
126	2	0	0	1	0	0	0	0	0	8	9	0	0
126	3	2	1	1	2	5	7	8	10	5	9	3	5
127	1	0	0	2	0	0	0	0	0	5	8	0	0

127	2	0	0	1	2	8	8	8	9	0	0	0	0
127	3	2	1	2	2	7	8	9	12	0	0	3	3
129	1	0	0	2	2	0	0	8	9	0	0	3	3
129	2	0	0	1	0	0	0	0	0	0	0	0	0
129	3	2	1	2	2	0	0	7	9	4	6	0	0
131	1	0	0	2	0	0	0	0	0	0	0	0	0
131	2	0	0	1	0	0	0	0	0	7	8	0	0
131	3	2	1	2	2	8	8	7	8	4	6	5	5
131	4	2	1	2	2	6	8	7	9	0	0	3	7
132	1	0	0	2	2	5	7	7	7	0	0	0	0
132	2	0	0	1	1	8	9	7	9	0	0	0	0
132	3	2	1	2	2	5	9	7	9	0	0	0	0
132	4	2	1	1	1	5	8	7	7	0	0	0	0
134	1	0	0	2	0	0	0	0	0	1	9	0	0
134	2	0	0	1	0	0	0	0	0	5	9	0	0
134	3	0	0	2	0	0	0	0	0	0	0	0	0
134	4	0	0	1	0	0	0	0	0	0	0	0	0
134	5	2	1	2	2	4	6	7	9	8	9	3	3
134	6	0	0	1	1	0	0	3	9	1	8	0	0
134	7	4	3	2	2	7	8	8	8	0	0	3	3
134	8	6	5	2	2	4	6	3	7	1	8	3	3
135	1	0	0	2	2	0	0	7	7	4	8	3	5
135	2	0	0	1	0	0	0	0	0	7	10	0	0
135	3	2	1	2	2	0	0	7	7	8	8	0	0
142	1	0	0	2	0	0	0	0	0	8	10	0	0
142	2	0	0	1	0	0	0	0	0	3	7	0	0
142	3	2	1	1	1	1	9	3	8	0	0	3	7
142	4	2	1	2	2	5	9	8	9	3	7	5	7
142	5	2	1	2	2	0	0	3	7	3	5	3	3
143	1	0	0	2	1	0	0	0	0	3	7	0	0
143	2	0	0	1	1	8	9	0	0	3	10	0	0
143	3	2	1	1	2	1	8	7	9	0	0	3	5
143	4	2	1	1	1	0	0	0	0	3	10	5	5
143	5	2	1	2	1	1	8	7	9	3	10	3	3
143	6	2	1	1	1	4	8	9	9	3	9	3	5
146	1	0	0	2	1	7	10	7	7	0	0	3	7
146	2	0	0	1	1	8	8	8	8	4	6	3	5
146	3	2	1	2	2	8	10	7	8	0	0	3	7
152	1	0	0	2	2	3	7	7	9	7	8	3	3
152	2	0	0	1	0	0	0	0	0	4	6	0	0
152	3	2	1	1	2	3	7	7	9	0	0	3	3
152	4	2	1	2	2	3	5	7	9	0	0	3	3
152	5	2	1	2	2	3	7	0	0	0	0	3	3
166	1	0	0	2	2	3	10	0	0	0	0	3	7
166	2	0	0	1	0	0	0	0	0	0	0	0	0
166	3	2	1	2	2	3	10	3	7	1	9	3	3
166	4	2	1	2	2	3	10	0	0	5	9	3	3
166	5	0	0	1	1	3	9	0	0	0	0	3	8
166	6	5	4	2	2	0	0	7	10	0	0	0	0

Chromosome 1q31 (D1S2757, d1s306, d1s249, D1s205)

2	1	0	0	1	2	7	8	2	9	11	14	1	5
2	2	0	0	2	1	0	0	0	0	0	0	0	0
2	3	1	2	1	2	6	8	1	2	11	12	1	7
2	4	1	2	1	2	6	7	1	9	4	14	5	7
3	1	0	0	2	0	0	0	0	0	0	0	0	0
3	2	0	0	1	1	0	0	0	0	0	0	0	0
3	3	2	1	2	2	7	7	1	1	7	12	8	11
3	4	2	1	2	2	1	7	1	6	10	12	8	11
3	5	0	0	1	2	4	6	1	6	4	9	7	9
3	6	5	4	1	2	1	4	6	6	4	10	7	8
3	7	5	4	1	2	1	4	6	6	4	10	7	8
3	8	5	4	1	2	4	7	1	6	4	12	7	11
3	9	5	4	2	2	6	7	1	1	9	12	9	11
4	1	0	0	1	1	3	8	1	3	11	12	8	9
4	2	0	0	2	2	6	9	1	1	6	12	1	11
4	3	1	2	2	2	8	9	1	1	6	12	1	8
4	4	1	2	2	2	3	6	1	1	11	12	8	11
4	5	1	2	2	2	8	9	1	1	11	12	8	11
4	6	1	2	1	2	8	9	1	3	11	12	8	11
13	1	0	0	2	2	4	5	6	8	4	5	5	6

13	2	0	0	1	1	3	9	1	4	10	12	8	8
13	3	2	1	2	2	3	5	4	6	4	10	5	8
16	1	0	0	2	2	6	7	1	7	12	12	5	7
16	2	0	0	1	1	0	0	0	0	0	0	0	0
16	3	2	1	1	2	6	9	6	7	12	13	7	9
16	4	2	1	1	2	2	6	1	7	10	12	5	9
17	1	0	0	1	0	0	0	0	0	0	0	0	0
17	2	0	0	2	0	0	0	0	0	0	0	0	0
17	3	1	2	1	2	6	8	1	1	5	11	8	9
17	4	1	2	2	2	3	6	1	1	3	5	8	9
17	5	0	0	1	1	3	10	2	7	7	14	5	8
17	6	5	4	1	2	6	10	1	7	5	14	8	9
18	1	0	0	2	2	0	0	0	0	0	0	0	0
18	2	0	0	1	1	7	8	1	1	4	10	8	11
18	3	2	1	1	2	3	8	1	1	9	10	5	8
18	4	2	1	1	2	3	8	1	1	3	10	5	8
18	5	2	1	1	2	6	8	1	1	9	10	5	8
19	1	0	0	1	1	0	0	0	0	0	0	0	0
19	2	0	0	2	2	3	4	1	1	0	0	5	8
19	3	1	2	2	2	4	8	1	1	0	0	8	11
19	4	1	2	2	2	3	8	1	7	0	0	8	8
19	5	1	2	2	2	3	7	1	7	0	0	5	8
20	1	0	0	1	0	0	0	0	0	0	0	0	0
20	2	0	0	2	2	0	0	0	0	0	0	0	0
20	3	0	0	1	0	0	0	0	0	0	0	0	0
20	4	1	2	2	2	4	9	0	0	6	13	5	6
20	5	3	2	2	2	7	8	1	5	10	10	5	8
20	6	3	2	2	2	8	9	1	3	9	10	7	8
20	7	0	0	1	1	0	0	0	0	0	0	0	0
20	8	3	2	2	2	3	7	1	1	6	10	5	6
20	9	0	0	1	0	0	0	0	0	0	0	0	0
20	10	7	6	2	2	3	9	1	1	10	12	7	9
20	11	9	8	1	0	0	0	0	0	0	0	0	0
20	12	9	8	2	2	3	8	1	1	6	10	5	7
20	13	0	0	1	0	0	0	0	0	0	0	0	0
20	14	9	8	2	2	7	8	1	1	5	10	5	8
20	15	13	12	2	2	8	8	1	1	10	10	7	7
23	1	0	0	1	0	0	0	0	0	0	0	0	0
23	2	0	0	2	2	2	10	1	6	10	14	5	5
23	3	0	0	1	1	5	7	1	3	10	11	7	8
23	4	1	2	2	2	2	10	1	1	0	0	5	6
23	5	1	2	1	2	2	10	1	6	10	14	5	8
23	6	3	2	1	2	2	5	3	6	11	14	5	8
25	1	0	0	1	1	4	7	1	6	1	9	8	11
25	2	0	0	2	2	0	0	0	0	0	0	0	0
25	3	1	2	2	2	4	4	1	3	1	12	5	11
25	4	1	2	2	2	3	4	1	1	1	10	7	11
25	5	0	0	1	1	3	6	3	6	10	11	5	8
25	6	5	4	1	2	3	6	1	3	10	11	7	8
25	7	0	0	1	0	0	0	0	0	0	0	0	0
25	8	5	4	2	2	4	6	1	3	1	10	5	11
25	9	0	0	1	0	0	0	0	0	0	0	0	0
25	10	7	8	2	2	4	9	1	1	1	12	7	11
25	11	9	8	1	2	2	4	1	1	1	4	8	11
30	1	0	0	1	0	0	0	0	0	0	0	0	0
30	2	0	0	2	2	3	4	1	1	0	0	0	0
30	3	1	2	2	2	4	4	1	1	0	0	0	0
31	1	0	0	2	2	3	7	1	1	0	0	0	0
31	2	0	0	1	0	0	0	0	0	0	0	0	0
31	3	2	1	2	2	0	0	0	0	0	0	0	0
31	4	0	0	1	0	0	0	0	0	0	0	0	0
31	5	4	3	2	2	8	8	1	1	0	0	0	0
32	1	0	0	2	0	0	0	0	0	0	0	0	0
32	2	0	0	1	0	0	0	0	0	0	0	0	0
32	3	2	1	1	2	3	8	3	3	10	12	7	8
32	4	2	1	2	2	5	7	1	1	5	11	7	8
32	5	0	0	1	2	3	8	3	3	12	12	5	8
32	6	5	4	1	2	5	8	1	3	5	12	7	8
32	7	5	4	1	2	7	8	1	3	11	12	5	7
32	8	5	4	2	2	5	8	1	3	5	12	7	8
33	1	0	0	2	0	0	0	0	0	0	0	0	0
33	2	0	0	1	2	3	7	1	6	5	9	5	5
33	3	2	1	2	2	3	4	1	1	5	12	5	7
33	4	0	0	1	0	0	0	0	0	0	0	0	0
33	5	4	3	1	2	2	4	1	8	10	12	4	7
34	1	0	0	2	1	5	8	1	6	10	10	5	8

34	2	0	0	1	2	6	10	1	8	8	14	7	11
34	3	2	1	2	2	5	6	1	6	8	10	7	8
34	4	0	0	1	1	3	7	1	1	10	11	8	9
34	5	2	1	1	2	5	10	6	8	10	14	8	11
34	6	4	3	2	2	3	6	1	1	10	11	8	9
35	1	0	0	2	2	3	8	1	5	9	13	0	0
35	2	0	0	1	0	0	0	0	0	0	0	0	0
35	3	2	1	2	2	3	8	1	1	12	13	0	0
35	4	2	1	2	0	0	0	0	0	0	0	0	0
37	1	0	0	2	0	0	0	0	0	0	0	0	0
37	2	0	0	1	0	0	0	0	0	0	0	0	0
37	3	2	1	2	2	5	7	1	1	10	14	8	9
37	4	2	1	1	2	3	7	0	0	10	13	8	11
37	5	0	0	2	1	7	8	1	1	5	12	5	7
37	6	4	5	2	2	3	7	1	1	10	12	7	11
37	7	4	5	1	2	3	7	1	1	5	10	5	11
39	1	0	0	2	2	0	0	1	2	0	0	5	13
39	2	0	0	1	0	0	0	0	0	0	0	0	0
39	3	2	1	2	2	0	0	1	1	9	9	5	7
39	4	2	1	1	2	5	8	1	3	5	9	7	13
39	5	0	0	2	0	0	0	0	0	0	0	0	0
39	6	4	5	2	2	5	5	1	3	5	9	5	13
40	1	0	0	2	2	3	8	1	9	0	0	0	0
40	2	0	0	1	0	0	0	0	0	0	0	0	0
40	3	2	1	2	2	3	8	1	1	0	0	0	0
43	1	0	0	2	2	3	4	0	0	0	0	0	0
43	2	0	0	1	1	8	8	0	0	0	0	0	0
43	3	2	1	2	2	3	8	0	0	0	0	0	0
45	1	0	0	2	1	3	7	1	1	10	11	5	8
45	2	0	0	1	0	0	0	0	0	0	0	0	0
45	3	2	1	1	2	3	9	1	4	10	12	8	9
45	4	2	1	2	2	7	8	1	8	8	10	5	8
45	5	0	0	1	1	6	8	1	3	5	6	5	8
45	6	5	4	2	2	6	8	3	8	6	8	8	8
47	1	0	0	1	1	3	4	1	1	5	12	5	6
47	2	0	0	2	0	0	0	0	0	0	0	0	0
47	3	1	2	1	1	0	0	1	1	0	0	0	0
47	4	1	2	1	2	3	6	1	1	11	12	6	7
47	5	1	2	2	2	3	6	1	1	5	11	5	7
47	6	1	2	2	2	3	9	1	1	4	5	5	6
47	7	0	0	1	0	0	0	0	0	0	0	0	0
47	8	7	6	1	2	3	9	1	1	5	13	6	7
47	9	7	6	2	2	3	9	1	1	5	13	6	7
50	1	0	0	2	2	6	6	1	3	9	12	5	8
50	2	0	0	1	1	2	7	1	1	10	13	8	9
50	3	2	1	2	2	2	6	1	1	10	12	8	8
50	4	2	1	1	2	2	6	1	3	10	12	8	9
51	1	0	0	2	0	0	0	0	0	0	0	0	0
51	2	0	0	1	0	0	0	0	0	0	0	0	0
51	3	2	1	2	2	3	4	1	1	7	10	8	11
51	4	2	1	2	2	3	7	1	3	6	7	8	12
51	5	0	0	1	1	0	0	1	8	11	12	5	7
51	6	2	1	2	2	3	4	1	1	7	10	8	11
51	7	0	0	1	0	0	0	0	0	0	0	0	0
51	8	5	4	2	2	0	0	3	8	6	12	5	12
51	9	7	6	2	2	4	8	1	1	7	13	8	9
52	1	0	0	2	0	0	0	0	0	0	0	0	0
52	2	0	0	1	0	0	0	0	0	0	0	0	0
52	3	0	0	1	0	0	0	0	0	0	0	0	0
52	4	2	1	2	2	3	10	1	8	9	12	5	8
52	5	2	1	2	2	8	8	3	8	6	9	5	7
52	6	0	0	1	0	0	0	0	0	0	0	0	0
52	7	0	0	1	0	0	0	0	0	0	0	0	0
52	8	3	4	2	2	3	5	1	1	12	13	8	9
52	9	3	4	2	2	7	10	1	8	7	9	5	7
52	10	6	5	1	0	0	0	0	0	0	0	0	0
52	11	0	0	2	2	4	11	1	6	6	12	9	10
52	12	7	8	1	2	2	3	1	1	12	13	8	9
52	13	10	11	2	2	2	4	1	6	12	13	9	10
54	1	0	0	2	0	0	0	0	0	0	0	0	0
54	2	0	0	1	0	0	0	0	0	0	0	0	0
54	3	0	0	1	1	5	9	1	3	6	8	0	0
54	4	2	1	2	2	3	8	1	1	5	8	7	7
54	5	2	1	2	2	3	8	1	1	5	8	7	8
54	6	0	0	1	0	0	0	0	0	0	0	0	0
54	7	3	4	2	1	5	8	1	1	0	0	0	0

54	8	3	4	1	2	8	9	1	3	5	8	0	0
54	9	6	5	1	2	3	5	1	3	8	10	0	0
55	1	0	0	2	2	3	3	1	5	10	12	5	11
55	2	0	0	1	0	0	0	0	0	0	0	0	0
55	3	2	1	1	2	3	4	3	5	10	10	5	7
55	4	2	1	2	2	3	3	1	3	10	12	7	11
55	5	2	1	2	2	3	4	3	5	10	10	5	7
55	6	0	0	1	0	0	0	0	0	0	0	0	0
55	7	6	5	1	2	3	4	1	3	10	12	7	9
55	8	6	5	2	2	3	8	1	5	10	13	5	6
55	9	0	0	1	0	7	9	3	6	12	13	5	13
55	10	9	8	1	0	3	9	5	6	10	12	5	13
55	11	9	8	1	2	0	0	1	3	12	13	5	5
55	12	9	8	1	2	7	8	1	3	12	13	5	6
56	1	0	0	1	0	0	0	0	0	0	0	0	0
56	2	0	0	2	2	3	5	0	0	6	12	5	8
56	3	1	2	2	2	3	5	0	0	6	12	4	8
56	4	1	2	1	2	2	5	0	0	11	12	5	6
57	1	0	0	1	0	0	0	0	0	0	0	0	0
57	2	0	0	2	0	0	0	0	0	0	0	0	0
57	3	0	0	1	2	3	8	1	5	9	10	10	12
57	4	1	2	2	2	3	7	1	4	9	13	7	8
57	5	1	2	2	2	3	5	1	4	9	12	8	11
57	6	3	4	2	2	3	8	4	5	9	10	8	10
57	7	3	4	1	2	3	7	1	1	9	13	8	12
58	1	0	0	2	0	0	0	0	0	0	0	0	0
58	2	0	0	1	0	0	0	0	0	0	0	0	0
58	3	2	1	2	2	3	3	3	4	9	12	8	9
58	4	2	1	2	2	3	3	3	3	12	14	8	9
59	1	0	0	2	0	0	0	0	0	0	0	0	0
59	2	0	0	1	0	0	0	0	0	0	0	0	0
59	3	2	1	2	2	4	6	1	4	0	0	1	8
59	4	2	1	2	2	5	9	1	6	0	0	7	11
61	1	0	0	2	2	0	0	1	8	0	0	0	0
61	2	0	0	1	0	0	0	0	0	0	0	0	0
61	3	2	1	1	0	0	0	0	0	0	0	0	0
61	4	2	1	2	2	0	0	1	1	0	0	0	0
63	1	0	0	2	2	0	0	1	1	0	0	0	0
63	2	0	0	1	0	0	0	0	0	0	0	0	0
63	3	2	1	2	2	0	0	1	1	0	0	0	0
63	4	0	0	1	0	0	0	0	0	0	0	0	0
63	5	4	3	2	2	0	0	1	8	0	0	0	0
64	1	0	0	2	2	7	8	1	1	12	14	8	9
64	2	0	0	1	0	0	0	0	0	0	0	0	0
64	3	2	1	2	2	5	7	1	8	10	12	8	13
64	4	2	1	2	2	2	7	1	1	6	14	8	9
64	5	2	1	2	2	5	8	1	8	10	14	9	13
65	1	0	0	2	2	3	8	1	1	10	12	10	11
65	2	0	0	1	1	3	8	1	3	9	10	5	13
65	3	2	1	2	2	3	8	1	3	10	12	11	13
65	4	2	1	1	2	3	3	1	1	10	12	5	10
69	1	0	0	2	0	0	0	0	0	0	0	0	0
69	2	0	0	1	0	0	0	0	0	0	0	0	0
69	3	0	0	1	0	0	0	0	0	0	0	0	0
69	4	2	1	2	2	3	8	1	1	5	10	8	11
69	5	2	1	2	2	5	9	1	9	10	12	7	8
69	6	3	4	2	2	3	8	1	1	5	8	0	0
70	1	0	0	2	1	8	9	3	4	10	12	7	7
70	2	0	0	1	0	0	0	0	0	0	0	0	0
70	3	2	1	2	2	6	9	1	3	12	12	5	7
70	4	2	1	2	2	6	9	1	3	12	12	5	7
71	1	0	0	2	2	3	8	0	0	0	0	0	0
71	2	0	0	1	0	0	0	0	0	0	0	0	0
71	3	2	1	2	2	3	3	0	0	0	0	0	0
72	1	0	0	1	2	4	8	1	3	0	0	0	0
72	2	0	0	2	0	0	0	0	0	0	0	0	0
72	3	1	2	2	2	8	10	3	3	0	0	0	0
74	1	0	0	2	0	0	0	0	0	0	0	0	0
74	2	0	0	1	0	0	0	0	0	0	0	0	0
74	3	0	0	2	0	0	0	0	0	0	0	0	0
74	4	2	1	1	0	0	0	0	0	0	0	0	0
74	5	2	1	2	2	3	4	1	1	8	9	11	12
74	6	2	1	2	2	4	5	1	1	6	8	10	11
74	7	4	3	1	2	7	8	3	4	10	11	7	8
74	8	4	3	2	2	7	9	3	4	10	11	8	10
76	1	0	0	1	0	0	0	0	0	0	0	0	0

76	2	0	0	2	0	0	0	0	0	0	0	0	0
76	3	1	2	1	2	6	6	1	1	12	13	10	11
76	4	1	2	2	2	2	6	1	3	9	10	5	8
78	1	0	0	2	2	0	0	0	0	0	0	0	0
78	2	0	0	1	0	0	0	0	0	0	0	0	0
78	3	2	1	1	2	0	0	0	0	0	0	0	0
79	1	0	0	1	0	0	0	0	0	0	0	0	0
79	2	0	0	2	0	0	0	0	0	0	0	0	0
79	3	1	2	2	2	8	8	1	1	9	11	8	8
79	4	1	2	1	2	8	8	1	1	9	10	8	9
79	5	1	2	2	2	8	9	1	1	10	11	8	8
80	1	0	0	2	2	0	0	0	0	0	0	0	0
80	2	0	0	1	0	0	0	0	0	0	0	0	0
80	3	2	1	2	2	0	0	0	0	0	0	0	0
82	1	0	0	2	0	0	0	0	0	0	0	0	0
82	2	0	0	1	0	0	0	0	0	0	0	0	0
82	3	2	1	2	2	5	8	1	1	14	14	8	9
82	4	2	1	2	2	3	8	1	1	9	12	7	8
83	1	0	0	2	0	0	0	0	0	0	0	0	0
83	2	0	0	1	0	0	0	0	0	0	0	0	0
83	3	2	1	2	2	7	8	1	1	5	12	5	8
83	4	2	1	2	2	5	7	1	1	5	10	5	8
83	5	0	0	1	0	0	0	0	0	0	0	0	0
83	6	5	4	2	2	5	6	1	1	10	10	5	8
83	7	5	4	2	2	7	7	1	1	6	10	5	8
88	1	0	0	1	0	0	0	0	0	0	0	0	0
88	2	0	0	2	0	0	0	0	0	0	0	0	0
88	3	1	2	2	2	2	8	2	7	5	5	8	9
88	4	1	2	2	2	2	8	2	7	5	5	8	9
89	1	0	0	1	0	0	0	0	0	0	0	0	0
89	2	0	0	2	0	0	0	0	0	0	0	0	0
89	3	1	2	2	2	6	8	3	3	5	14	4	8
89	4	1	2	2	2	3	6	1	4	5	14	4	5
89	5	1	2	2	2	3	6	1	4	5	14	4	8
91	1	0	0	2	2	0	0	0	0	0	0	0	0
91	2	0	0	1	0	0	0	0	0	0	0	0	0
91	3	2	1	2	2	0	0	0	0	0	0	0	0
95	1	0	0	1	0	0	0	0	0	0	0	0	0
95	2	0	0	2	2	0	0	1	6	0	0	0	0
95	3	1	2	1	2	0	0	1	1	0	0	0	0
98	1	0	0	2	2	3	8	3	6	12	12	4	10
98	2	0	0	1	1	3	3	1	6	10	13	7	8
98	3	2	1	2	2	3	8	6	6	10	12	4	7
98	4	2	1	2	2	3	8	6	6	10	12	4	7
99	1	0	0	2	2	0	0	0	0	0	0	0	0
99	2	0	0	1	0	0	0	0	0	0	0	0	0
99	3	2	1	2	2	0	0	0	0	0	0	0	0
100	1	0	0	2	2	9	9	0	0	0	0	0	0
100	2	0	0	1	0	0	0	0	0	0	0	0	0
100	3	2	1	2	2	5	9	0	0	0	0	0	0
101	1	0	0	1	0	0	0	0	0	0	0	0	0
101	2	0	0	2	0	0	0	0	0	0	0	0	0
101	3	1	2	2	2	5	8	1	3	12	14	7	11
101	4	1	2	1	2	8	8	1	1	9	12	7	12
103	1	0	0	2	2	8	9	1	4	0	0	0	0
103	2	0	0	1	1	5	9	1	1	0	0	0	0
103	3	2	1	2	2	9	9	1	4	0	0	0	0
104	1	0	0	2	2	5	7	1	1	0	0	0	0
104	2	0	0	1	0	0	0	0	0	0	0	0	0
104	3	2	1	1	2	7	8	1	1	0	0	0	0
105	1	0	0	2	2	7	8	1	3	12	13	8	12
105	2	0	0	1	1	3	3	1	6	10	11	5	10
105	3	2	1	2	2	3	7	3	6	10	12	8	10
105	4	2	1	1	2	3	7	3	6	11	12	8	10
106	1	0	0	2	2	3	7	1	2	5	13	7	11
106	2	0	0	1	1	3	7	1	5	5	9	5	5
106	3	2	1	1	1	0	0	0	0	0	0	5	7
106	4	2	1	1	2	7	7	1	1	5	9	5	7
107	1	0	0	2	2	0	0	1	6	0	0	0	0
107	2	0	0	1	0	0	0	0	0	0	0	0	0
107	3	2	1	2	2	0	0	1	6	0	0	0	0
108	1	0	0	2	2	5	6	1	2	5	13	0	0
108	2	0	0	1	0	0	0	0	0	0	0	0	0
108	3	2	1	2	2	3	5	1	2	4	5	0	0
108	4	2	1	2	2	6	6	1	1	12	13	0	0
110	1	0	0	2	0	0	0	0	0	0	0	0	0

110	2	0	0	1	0	0	0	0	0	0	0	0	0
110	3	2	1	2	2	2	9	1	1	12	12	7	9
110	4	2	1	2	2	6	9	1	2	5	12	9	13
111	1	0	0	2	2	2	4	1	6	9	12	7	7
111	2	0	0	1	1	5	5	1	2	9	12	4	10
111	3	2	1	2	2	2	5	1	1	9	12	4	7
111	4	2	1	2	2	4	5	2	6	9	12	4	7
112	1	0	0	2	2	3	10	1	6	0	0	0	0
112	2	0	0	1	0	0	0	0	0	0	0	0	0
112	3	2	1	1	2	2	10	6	8	0	0	0	0
117	1	0	0	2	2	3	5	1	6	0	0	0	0
117	2	0	0	1	1	3	7	1	3	0	0	0	0
117	3	2	1	2	2	3	3	1	6	0	0	0	0
118	1	0	0	2	1	2	5	1	1	11	12	8	11
118	2	0	0	1	2	5	7	1	1	5	9	2	7
118	3	2	1	2	2	2	7	1	1	5	12	2	8
118	4	2	1	2	2	5	7	1	1	0	0	7	11
119	1	0	0	2	2	0	0	0	0	0	0	0	0
119	2	0	0	1	0	0	0	0	0	0	0	0	0
119	3	2	1	1	2	0	0	0	0	0	0	0	0
123	1	0	0	1	2	5	5	1	8	0	0	0	0
123	2	0	0	2	0	0	0	0	0	0	0	0	0
123	3	1	2	1	2	3	5	1	1	0	0	0	0
125	1	0	0	2	2	1	8	0	0	0	0	0	0
125	2	0	0	1	0	0	0	0	0	0	0	0	0
125	3	2	1	2	2	1	6	0	0	0	0	0	0
126	1	0	0	2	2	4	5	0	0	0	0	0	0
126	2	0	0	1	0	0	0	0	0	0	0	0	0
126	3	2	1	1	2	3	4	0	0	0	0	0	0
127	1	0	0	2	0	0	0	0	0	0	0	0	0
127	2	0	0	1	2	5	7	0	0	0	0	0	0
127	3	2	1	2	2	3	7	0	0	0	0	0	0
129	1	0	0	2	2	3	4	0	0	0	0	0	0
129	2	0	0	1	0	0	0	0	0	0	0	0	0
129	3	2	1	2	2	3	8	0	0	0	0	0	0
139	1	0	0	2	2	2	8	0	0	0	0	0	0
139	2	0	0	1	0	0	0	0	0	0	0	0	0
139	3	2	1	2	2	2	7	0	0	0	0	0	0
143	1	0	0	2	1	6	8	1	7	5	12	5	7
143	2	0	0	1	1	0	0	1	4	9	14	7	8
143	3	2	1	1	2	3	6	1	1	12	14	7	8
143	4	2	1	1	1	3	6	1	1	12	14	5	8
143	5	2	1	2	2	3	8	1	7	5	14	5	7
143	6	2	1	1	1	0	0	1	1	5	9	5	8

Candidate Genes (DBH-1,DBH-2,SERT,DRD2)

2	1	0	0	1	2	1	4	1	2	2	3	1	1
2	2	0	0	2	1	0	0	0	0	0	0	0	0
2	3	1	2	1	2	1	4	1	2	3	4	1	2
2	4	1	2	1	2	1	4	1	2	0	0	1	1
3	1	0	0	2	0	0	0	0	0	4	4	0	0
3	2	0	0	1	1	0	0	0	0	4	4	0	0
3	3	2	1	2	2	1	5	1	2	0	0	1	1
3	4	2	1	2	2	2	5	1	2	4	5	1	1
3	5	0	0	1	2	3	3	1	1	4	4	1	2
3	6	5	4	1	2	3	5	1	2	4	4	1	1
3	7	5	4	1	2	2	3	1	1	0	0	1	1
3	8	5	4	1	2	2	3	1	1	0	0	1	2
3	9	5	4	2	2	2	3	1	1	0	0	1	1
4	1	0	0	1	1	1	4	1	2	1	4	1	1
4	2	0	0	2	2	4	5	2	2	0	0	1	1
4	3	1	2	2	2	1	4	1	2	0	0	1	1
4	4	1	2	2	2	1	4	1	2	2	4	1	1
4	5	1	2	2	2	4	4	2	2	2	4	1	1
4	6	1	2	1	2	4	4	2	2	2	4	1	1
13	1	0	0	2	2	0	0	0	0	2	2	1	1
13	2	0	0	1	1	0	0	0	0	4	4	1	1
13	3	2	1	2	2	0	0	0	0	4	4	1	1
15	1	0	0	1	0	0	0	0	0	0	0	0	0
15	2	0	0	2	2	2	5	1	2	4	4	1	2
15	3	0	0	1	0	0	0	0	0	4	4	0	0
15	4	1	2	2	2	2	5	1	2	0	0	2	2
15	5	1	2	2	2	0	0	0	0	4	4	1	2

15	6	0	0	1	2	1	2	1	1	1	2	1	2
15	7	1	2	1	1	0	0	0	0	3	4	0	0
15	8	1	2	1	2	2	5	1	2	1	4	2	2
15	9	0	0	2	2	4	4	2	2	2	5	1	2
15	10	1	2	2	2	4	5	2	2	1	4	1	2
15	11	0	0	1	0	0	0	0	0	4	5	0	0
15	12	0	0	2	2	2	4	1	2	2	4	2	2
15	13	3	4	1	2	2	2	1	1	0	0	1	2
15	14	3	4	2	2	2	5	1	2	2	3	1	2
15	15	6	5	2	2	2	5	1	2	0	0	1	2
15	16	6	5	2	2	2	5	1	2	0	0	2	2
15	17	8	9	1	2	4	5	2	2	0	0	2	2
15	18	11	10	2	2	3	5	1	2	1	1	1	1
15	19	13	12	1	2	0	0	0	0	1	4	0	0
16	1	0	0	2	2	2	3	1	1	4	4	1	1
16	2	0	0	1	1	0	0	0	0	4	4	0	0
16	3	2	1	1	2	3	3	1	1	0	0	1	1
16	4	2	1	1	2	3	4	1	2	0	0	1	1
17	1	0	0	1	0	0	0	0	0	0	0	0	0
17	2	0	0	2	0	0	0	0	0	4	5	0	0
17	3	1	2	1	2	4	5	2	2	4	4	1	2
17	4	1	2	2	2	4	5	2	2	0	0	1	2
17	5	0	0	1	1	4	4	2	2	3	4	1	1
17	6	5	4	1	2	4	5	2	2	4	4	1	1
18	1	0	0	2	2	0	0	0	0	0	0	0	0
18	2	0	0	1	1	2	4	1	2	1	4	1	2
18	3	2	1	1	2	4	4	2	2	2	3	1	2
18	4	2	1	1	2	4	4	2	2	4	5	1	1
18	5	2	1	1	2	4	4	2	2	3	4	1	1
19	1	0	0	1	1	0	0	0	0	1	4	0	0
19	2	0	0	2	2	2	3	1	1	0	0	1	2
19	3	1	2	2	2	2	4	1	2	2	4	1	1
19	4	1	2	2	2	2	4	1	2	2	4	1	2
19	5	1	2	2	2	3	4	1	2	2	4	2	2
20	1	0	0	1	0	0	0	0	0	2	2	0	0
20	2	0	0	2	2	0	0	0	0	2	2	0	0
20	3	0	0	1	0	0	0	0	0	0	0	0	0
20	4	1	2	2	2	2	3	1	1	0	0	2	2
20	5	3	2	2	2	2	2	1	1	2	4	1	2
20	6	3	2	2	2	2	3	1	1	1	2	1	2
20	7	0	0	1	1	0	0	0	0	2	4	0	0
20	8	3	2	2	2	3	5	1	2	0	0	1	2
20	9	0	0	1	0	0	0	0	0	4	5	0	0
20	10	7	6	2	2	2	2	1	1	4	5	1	1
20	11	9	8	1	0	0	0	0	0	4	4	0	0
20	12	9	8	2	2	2	5	1	2	4	5	1	1
20	13	0	0	1	0	0	0	0	0	4	5	0	0
20	14	9	8	2	2	4	5	2	2	4	5	1	1
20	15	13	12	2	2	0	0	0	0	0	0	1	2
23	1	0	0	1	0	0	0	0	0	0	0	0	0
23	2	0	0	2	2	2	4	1	2	2	3	2	2
23	3	0	0	1	1	2	5	1	2	2	4	1	1
23	4	1	2	2	2	1	4	1	2	1	4	1	2
23	5	1	2	1	2	2	4	1	2	4	4	2	2
23	6	3	2	1	2	4	5	2	2	0	0	1	2
25	1	0	0	1	1	2	4	1	2	1	4	2	2
25	2	0	0	2	2	0	0	0	0	4	4	0	0
25	3	1	2	2	2	2	2	1	1	0	0	2	2
25	4	1	2	2	2	3	4	1	2	0	0	2	2
25	5	0	0	1	1	2	3	1	1	0	0	1	2
25	6	5	4	1	2	2	3	1	1	2	4	2	2
25	7	0	0	1	0	0	0	0	0	2	3	0	0
25	8	5	4	2	2	3	4	1	2	0	0	2	2
25	9	0	0	1	0	0	0	0	0	0	0	0	0
25	10	7	8	2	2	4	4	2	2	2	3	2	2
25	11	9	8	1	2	4	4	2	2	4	4	1	2
30	1	0	0	1	0	0	0	0	0	0	0	0	0
30	2	0	0	2	2	4	5	2	2	2	5	1	1
30	3	1	2	2	2	4	4	2	2	2	5	1	2
31	1	0	0	2	2	4	4	2	2	5	5	1	2
31	2	0	0	1	0	0	0	0	0	0	0	0	0
31	3	2	1	2	2	0	0	0	0	0	0	0	0
31	4	0	0	1	0	0	0	0	0	1	4	0	0
31	5	4	3	2	2	1	4	1	2	1	4	1	2
32	1	0	0	2	0	0	0	0	0	4	4	0	0
32	2	0	0	1	0	0	0	0	0	0	0	0	0

32	3	2	1	1	2	2	4	1	2	1	4	1	2
32	4	2	1	2	2	2	4	1	2	1	1	1	2
32	5	0	0	1	2	2	4	1	2	1	4	1	1
32	6	5	4	1	2	2	2	1	1	2	3	1	1
32	7	5	4	1	2	4	4	2	2	0	0	1	2
32	8	5	4	2	2	4	4	2	2	1	2	1	1
33	1	0	0	2	0	0	0	0	0	3	4	0	0
33	2	0	0	1	2	4	4	2	2	1	2	1	1
33	3	2	1	2	2	4	4	2	2	0	0	1	1
33	4	0	0	1	0	0	0	0	0	2	5	0	0
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