The Genetic Analysis and Clinical Features of Early Onset Familial Alzheimer's Disease

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ABSTRACT

This thesis details the genetic analysis and clinico-genetic correlates of early onset familial Alzheimer's disease (AD). Multiply affected families have been examined for linkage to markers on two candidate chromosomes, 21 and 14. The analysis demonstrates linkage between the ß amyloid precursor protein (ßAPP) gene and AD in five early onset (<65 years) families in which three different mutations were subsequently discovered. Simulation studies were used extensively and enabled the evaluation of lod values below the accepted criterion of 3.0 in single families. Clinical details of these families are presented. In general, these families show classical AD symptoms and signs but with additional prominent features previously recognised in early onset disease. Linkage to *ßAPP* is excluded in analyses of other early onset families which are shown to be linked to the long arm of chromosome 14. A comparison of clinical features is made between allelic variants of the *ßAPP* locus and chromosome 14 linked families. In particular, analysis of variance of age of onset in early onset families supports the notion of clinico-genetic heterogeneity by demonstrating family specific ages of onset and correlation between genetic aetiology and age of onset; the very early onset families show collective evidence of linkage to chromosome 14 and the families with mean onset in the 50s have *ßAPP* mutations.

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CHAPTER 1

Introduction

1.1 The Problems, Previous Work and the Purpose of the Present Study

Advancing our understanding the aetiology of Alzheimer's disease (AD) is a worthy goal for obvious reasons: in all modern societies the numbers of individuals at risk for this disorder increases each year with increased longevity. In the US, 25 million people are over age 65 years (over 10% of the population) and this group is increasing at 2.5 times the rate of the general population. The prevalence of Alzheimer's is age related and it has been estimated that 11% of the over 65 year olds will have mild to moderate dementia and over 4% are severely demented (Katzman, 1976). Other estimates are more conservative but only marginally less alarming; in the UK 2% of over 65 year olds have dementia and 20% of the over 80 year olds (Royal College of Physicians, 1981). Not all of these dementias are due to Alzheimer's disease but approximately 70% will be either pure AD or a mix of vessel disease and AD (Tomlinson *et al.*, 1970). There is no treatment for these cases and as the average life expectancy is 7 years post onset, the medical and sociological burdens are great.

In looking for causes of AD an examination of the risk factors is helpful; although many cases of AD are sporadic, the most consistent risk factor detected in several epidemiological studies has been a positive family history of the disease (Heyman *et al.*, 1984; Fitch *et al.*, 1988; Amaducci *et al.*, 1986; Hofman *et al.*, 1989; van Duijn *et al.*, 1991). In addition, many large early onset pedigrees have been described in which AD appears to be inherited as an autosomal dominant disorder (Lowenberg, Waggoner, 1934; Feldman *et al.*, 1963; Heston *et al.*, 1966; Cook *et al.*, 1979; Nee *et al.*, 1983; Bird *et al.*, 1988; Sadovnick *et al.*, 1988; Fukutani *et al.*, 1989; Martin *et al.*, 1991; Karlinsky *et al.*, 1992; Karlinsky *et al.*, 1991; Frommelt *et al.*, 1991). The existence of such families prompted the enthusiastic application of the lod score method of linkage analysis to localise disease causing genes. The association of AD pathology with Down's syndrome (Mann *et al.*, 1984; Mann, 1988; Mann *et al.*, 1986) forwarded chromosome 21 as a candidate region and the general pathology of AD, namely amyloid deposition, promoted the ß-amyloid precursor protein (ßAPP) gene as a candidate locus.

However, there are problems in the genetic analysis of Alzheimer's disease which are common to the analysis of complex disorders; heterogeneity, unquantified genetic parameters and diagnostic validity. The errors which arise as a result of undetected heterogeneity reflect the

way in which linkage is mathematically related to genetic distance in the lod score method (discussed below). The problems of diagnosis occur because there is no laboratory test, clinical feature, scan or EEG measure that yields a definitive diagnosis (excepting cerebral biopsy or autopsy). Rather, the accurate diagnosis of AD is one of exclusion of competing diagnosis, requiring a careful history and examination covering several broad areas; neuropsychometry, psychiatry, general medicine and neurology. An integrative and often multi-disciplinary approach to the diagnosis is therefore required. However, the gold standard of diagnosis is neuropathology. Even then, the distinction between AD and normal aging is not always easy. Typical AD neuropathology depends on the observation of intracellular neurofibrillary tangles and extracellular deposits of ß-amyloid (Aß) in the form of spherical plaque. These structures, up to 1mm across in the cortex, have an Aß core and in the mature form are surrounded by neuritic outgrowths from nearby neurons. These features allow a high degree of diagnostic certainty at autopsy. By contrast at the clinical level, although there is a core syndrome with characteristic onset and progression, there are also many atypical cases. In addition, non-standardisation of diagnostic practice has been a problem until relatively recently when some broad criteria for the diagnosis of AD were established (McKhann *et al.*, 1984). The consequence of diagnostic uncertainty for linkage studies, particularly the lod score method, have been stressed many times in the literature. The sensitivity of the lod score method to apparent recombinants in highly penetrant disorders requires strict diagnostic certainty. If this requirement is not met, inaccurate estimation of the recombination fraction results. This requirement is less important if other methods of analysis are used, for instance the sib pair methods (Dawson *et al.*, 1990) or the affected pedigree member method (Weeks, Lange, 1988). However, in most analyses of early onset disease most groups have felt confident enough to apply the lod score method despite the limitations discussed below and in consequence there have been several misleading results.

1.2 The Lod Score Method of Linkage Analysis

1.2.1 Two Point Analysis

The general aim of the lod score method of linkage analysis is to estimate the actual recombination fraction, theta (θ), between two or more loci, one of which may be a disease associated or disease causing locus. This method compares the probabilities that the data reflect either linkage or non-linkage. The alternative values associated with these different hypotheses (likelihoods) are probabilities calculated in terms of θ (varied) and $\theta = 0.5$, respectively. A measure of the ratio of the two hypotheses (linked/unlinked) is obtained by comparing the ratio of these likelihoods ($L(\theta)/L(0.5)$) and the maximum value of this ratio

corresponds to the most likely value of θ. The lod score is the logarithm of this likelihood ratio. The calculation of L(θ) is given by the probabilities of the occurrence of the observed recombinants and non-recombinants through a pedigree, i.e., $\theta^x(1-\theta)^y$ where x is the number of observed recombinants and y is the number of non-recombinants (the probability of which is 1-θ as in each meiosis a recombination event can either occur, with probability θ, or not occur). X + y is the number of fully informative meosis, (Morton, 1955).
The value of θ is unknown of course and L(θ) varies with θ. L(0.5) simply reflects the probability of observing the number of fully informative meioses under non-linkage; $0.5^{(x+y)}$. The lod score between any two loci is therefore given by:

$$Z(\theta) = \log_{10}[\theta^x(1-\theta)^y/0.5^{(x+y)}]$$

As above, the maximum value of L(θ) defines the value of θ_e and the corresponding maximum value of lod, Z_{max};

$$Z_{max}(\theta_e) = \log_{10}[\theta_e{}^x(1-\theta_e)^y/0.5^{(x+y)}]$$

From this definition, at θ = 0.0 between two loci, if there is a complete recombinant, Z(θ) becomes $\log_{10}(0.0) = -\infty$. Also, it is possible to see that if there is complete linkage between two loci, each additional fully informative meioses contributes

$$\log_{10}(1/0.5) = 0.3 \text{ lod}$$

The **assumptions of the lod score linkage** method are to be borne in mind when dealing with an aetiologically heterogeneous disorder. Unless special precautions are taken to mimic more complex inheritance, the most important implicit assumption in the lod score method of analysis is that families pooled for a single estimate of θ_e have lesions at the same locus. They may be allelic variants, but in this circumstance the mode of inheritance, penetrance and other parameters are assumed constant between allelic subgroups. For instance, a combined estimation of θ_e between AD and *βAPP* in codon 717 mutated families (Val→Ile and Val→ Gly) and *βAPP* codon 670/671 families would successfully yield $\theta_e = 0.0$. However, the combination of any of these families with non-allelic variants (the chromosome 14 linked families) would yield misleading values of θ_e. If there are no distinguishing phenotypic characteristics between the non-allelic variants, then such analyses cannot be declared invalid on an *a priori* basis. Any phenotype that is scored as recombinant at a disease or marker locus will generate negative scores. In the case of unaffected individuals in AD families the use of liability classes avoids recombinant scores in individuals yet to be affected. The problem of individuals affected due to causes other then the locus under consideration is

potentially dealt with by the phenocopy rate. The use of high phenocopy rates however, will tend to reduce all lod scores, positive or negative.

1.2.2 Multi-Point Analysis (Lathrop *et al*, 1984)

This extension of two point analysis to n loci introduces n(n-1)/2 recombination fractions to be estimated and n!/2 possible orders of loci. The estimate of marker order and of the maximum thetas between loci is not usually done simultaneously, nor usually on the same data sets. Analysis of data in large multi-generation reference pedigrees provides the most likely order of loci which is subsequently used in the localisation of disease pedigrees. Likelihoods can be calculated as the disease locus is positioned along a fixed map of markers (using either the MLINK or LINKMAP programs). The value of the different estimated thetas between each of the loci and the disease locus corresponds to the maximum likelihood calculated for the data set. Where the order of markers is not well defined both this and the most likely position of the disease locus can be estimated by comparing the maximum likelihoods (with corresponding loci order and distance) between all possible orders using the ILINK program. Due to the large number of parameters involved in these iterative analyses, it is preferable to estimate the marker map and disease position in separate pedigrees.

1.3 The Problem of Heterogeneity in Genetic Linkage Studies

There are several types of heterogeneity. Allelic heterogeneity refers to the situation where several mutations at the same locus are causing disease. Gerstmann-Straussler syndrome, Jakob-Creutzfeldt disease (Hsiao *et al.*, 1989; Hsiao *et al.*, 1991) and fatal familial insomnia (Medori, et al., 1992) are clinically distinct allelic variants of the prion protein gene. However, clinically identical allelic variants can also occur; the families described in Chapters 3, 5 and 6 are good examples of this phenomenon. In this latter case, allelic heterogeneity does not interfere with the identification of a disease locus by linkage and may only be detected once the locus is identified. Non-allelic heterogeneity refers to the situation when mutations at different loci cause the same disease phenotype. Non-genetic heterogeneity occurs when non-genetic factors, such as environmental ones, predispose to the disease phenotype. Intra-familial heterogeneity can also occur where two or more genes act independently to cause the disease within families. A more complex situation still is where several combinations of two or more of genes act dependently within families. Such combinations may be important in different families. The lod score method of linkage can easily accommodate heterogeneity limited to the Mendelian transmission (for both the

detected and undetected loci). More complex analyses under heterogeneity are feasible but are beyond the requirements of the analysis of early onset familial AD.

Linkage analysis may be applied in a situation where non-allelic and non-genetic heterogeneity are suspected. Non allelic heterogeneity has no effect on localisation of genes by linkage analysis and the presence of non-genetic heterogeneity can be detected by statistical analysis of the linkage data in the family data set. Statistical methods compare the hypothesis of linkage with heterogeneity, linkage with homogeneity and none linkage using likelihood ratio tests (approximating a chi squared statistic, Ott, 1991). However, there are practical considerations which limit the applicability of such tests. An individual pedigree may not contain enough information to define recombinants. When this problem is compounded by large genetic distances between marker and disease locus the test results are inconclusive. Thus, true reverse genetic approaches are unlikely to detect heterogeneity whether or not it exits, by such tests. This is essentially because with small lod scores at large genetic distance it is not possible to discriminate between linkage with recombination and non-linkage. Studies of small families with 10cM marker-disease distance demonstrate very poor heterogeneity detection rates (Cavalli-Sforza, King, 1986).

The consequences of not detecting heterogeneity are severe and are discussed in the final chapter but always result in a false estimation of the recombination fraction between marker and disease.

From the above discussion, large families with multiple meioses are the ideal for linkage analysis and to some extent avoid the need for heterogeneity testing. The families in this data set were intermediate in size, few of them able to individually generate a lod score above 3.0. The use of simulation studies to assess the significance of lod scores with such families is a useful adjunct and is detailed in the next chapter. Such studies are as versatile as lod score linkage itself and the effects of heterogeneity can be assessed by simulation with close or distantly linked marker loci.

1.3.1 Heterogeneity at the Phenotypic Level in Alzheimer's Disease

Alzheimer's first description of a case of cerebral plaque and neurofibrillary tangles (NFTs) was in a 51 year old and the initial clinico-neuropathological concepts were of a presenile dementia. It was later discovered that the senile plaques and NFTs seen in senile dementia (and to a lesser extent in the normal elderly) were essentially the same as those seen in the rare pre-senile cases. From the 1960s onward the concept of a single neuropathological entity emerged (Lauter, Meyer, 1968; Corsellis, 1969; Terry, Katzman, 1983), although there were many attempts to divide early and late onset disease, by pathology (Sourander, Sjorgren,

1970), neurochemistry (Rossor *et al.*, 1984) and clinical features, particularly aphasia. Aphasia has been proposed as a discriminating feature between early and late onset cases. In particular, aphasia has been associated with early onset disease (Chui *et al.*, 1985; Seltzer, Sherwin, 1983) and with increased rate of progression of disease (reduced life expectancy, Berg *et al.*, 1984). Aphasia has been seen as a part of a broader syndrome of amnesia, apraxia and agnosia (AAAA, Folstein *et al.*, 1988). AAAA has also been associated with a positive family history (Powell, Folstein, 1984; Breitner, Folstein, 1984). Other clinical features have been examined in an attempt to subdivide the disorder. Extra pyramidal signs, particularly bradykinesia and rigidity, were found in excess in those cases with a rapid progression (Chui *et al.*, 1985; Mayeux *et al.*, 1985). Myoclonus has been highlighted as a sign of early onset disease (Chui *et al.*, 1985; Mayeux *et al.*, 1985), again associated with a more rapid progression. However, in another study with a prevalence of myoclonus of 10% cases did not develop the disorder in the early stages of the disease and it did not predict a more rapid course (Hauser *et al.*, 1986).

One problem with these many of these studies is that it has not been possible to examine clinical features in terms of time post-onset. All the studies described above were essentially cross-sectional in nature. It remains quite possible then that the subgroups defined by these clinical features are actually representative of different stages of the same process. It is also possible that the rate of progression of disease is related to emphasis of particular features. Two studies of myoclonus shed some light on this (Clark *et al.*, 1991). A cross sectional study of 285 cases of AD identified 22 with myoclonic jerks. These were matched by age and duration of symptoms. When matched in this way, there were no differences in overall disability or mini-mental state examination, although there was a trend towards the myoclonic group scoring lower (more impaired) in both these areas. In the accompanying longitudinal study, 5 of 93 patients had myoclonus at entry (and all had an onset before age 59) and 6 more developed it over eight years. Myoclonus did not predict a more severe prognosis in this study. 16 of the 93 developed major convulsions during the late stage of the disease, including 8 of 11 of the myoclonic patients. The authors felt that the multifocal nature of the myoclonus was related to the subsequent seizures. The seizure group too had a generally earlier onset. In summary, then early stage myoclonus does seem to be associated with both earlier onset of disease and perhaps with late stage seizures. The occurrence of myoclonus in early onset familial disease is also well documented, but it does not appear to be particularly associated with familial disease (Clark *et al.*, 1991).

From the point of view of linkage studies, there is no particular feature which defines an homogeneous subgroup and therefore no *a priori* reason not to pool all AD families for linkage analysis. These considerations have a particular importance for the analysis of late

onset familial disease which is not explored here but also for the problem of phenocopies in early onset analysis which is discussed below. In addition, mean age of onset has emerged as a very useful partition of aetiologic subgroups and is explored throughout this thesis.

1.4 Other Genetic Parameters

A number of additional genetic parameters are important in the analysis of AD: delayed onset (age dependent penetrance), phenocopy rates and mutation and marker allele frequencies.

1.4.1 Penetrance

Alzheimer's disease accruing as it does in later life, requires the modification of linkage calculations to incorporate age dependent penetrance. This is easily accommodated in the likelihood method, by the attribution of a conditional probability to the observed phenotype. This is achieved by the construction of a liability table which represent the conditional probabilities of having the disease phenotype given a particular genotype. It is probably reasonable to assume a heterozygote penetrance of about 50% in the middle liability class and to consider age of onset as normally distributed.

Two putative examples of non-penetrance have been reported in the literature (Nee *et al.*, 1983; Bird *et al.*, 1988) - however, neither are convincing. For instance, a case of non-penetrance in an individual dying at age 36 with a mean family age of onset of 58. All the early onset families recorded in the literature are consistent with the transmission of an autosomal dominant gene with age dependent penetrance reaching 100%. The penetrance of any genes which operate in late onset disease is undoubtedly more complex than this but is beyond the scope of this thesis.

1.4.2 Phenocopy Rates

Although the mean ages of onset of the families studied in this thesis are below 60 years there are individuals in the families who develop the disease in their 60s. Such individuals may represent examples of autosomal dominant AD or may be examples of AD caused by other factors. The analysis of F372 (Chapter 4) contains an individual who had a disease onset well above the mean for the family and did not share the mutation that the other affected family members had. This family was discovered by direct screening (Chapter 3) and by linkage analysis this individual would have resulted in a false estimation of recombination between the disease and the disease locus. The use of the term phenocopy in this context is partly

inappropriate as the AD in this and similar cases may be due to other genes rather than purely environmental effects. In this instance, the cases arise as a result of intra-familial locus heterogeneity. In general though, for rare autosomal dominant disorders there are few phenocopies in the high density families selected for linkage (MacLean *et al.*, 1993).

1.4.3 Mutation and Marker Allele Frequencies

For the purposes of linkage analysis, the estimate of mutation frequency for genes for early onset disease are usually in the range 0.001 - 0.0001, based on the occurrence of the disease in the general population. A ten fold increase in frequency has little effect on lod score for most calculations, but higher frequencies does significantly reduce linked lod scores (i.e., .01). Marker allele frequencies can have a more profound effect on lod scores and in cases where there are unknown genotypes can effect the estimate of theta between the marker and any other locus (discussed further in Chapter 6).

1.5 Diagnostic Validity

Another source of error leading to inaccurate estimation of the recombination fraction is misdiagnosis. At least one analysis of early onset familial AD contained families that were not AD (Goate *et al.*, 1989). This analysis mis-localised the gene for familial AD to the centromere of chromosome 21 (for this and other reasons).

1.5.1 Differential Diagnosis of Early Onset Alzheimer's Disease

The clinical features of AD are those of any dementia - the triad of memory loss, other intellectual decline and personality deterioration. The first signs in biopsy confirmed cases was memory loss (Sim *et al.*, 1966). This study allowed the comparison of 35 cases of AD with 21 patients suffering from other dementias. The former group showed early signs of apraxia which tended to occur late in other dementias. Occurring late in AD but earlier in other dementias were fits, personality change, incontinence, confabulation, delusions, hallucinations and focal or gross neurological signs. In general though, the main competing diagnosis is multi-infarct dementia although a number of other early onset dementias do occur and were seen during the course of this thesis, for instance Gerstmann-Straussler syndrome and Jakob-Creutzfeldt disease. Also important differential diagnosis of memory impairment are anxiety and depressive disorders which sometimes occurred in the well siblings in these multiply affected families.

1.5.2 Clinical Features of Early Onset Familial Disease

Clinical details of large early onset families have been recorded in an attempt to identify a profile characteristic of this group. In general, these data are not collected prospectively, making comparisons difficult. Nevertheless, they do provide a general picture of the clinical features of early onset disease. Table 1 shows a summary of published family reports and the occurrence of specific clinical features.

Table 1. Summary of Published Family Reports and Occurrence of Specific Clinical Features

	Mean Age of Onset	Number of Detailed Case Reports	Apraxia	Depression	Myoclonus	Seizures	Extrapyramidal Signs
(Lowenberg, Waggoner, 1934)	34	5	0	1	3	4	0
(Feldman *et al.*, 1963)	38	10	2	2	4	4	4
(Armitage, Berry, 1987)	36	4	0	0	1	0	2
(Cook *et al.*, 1979)	47, 49	7	2	0	4	2	0
(Goudsmit *et al.*, 1981)	46, 48	18	0	0	0	0	4
(Nee *et al.*, 1983)	46	3	0	0	0	0	4
(Bird *et al.*, 1988)	52, 60, 67	7	0	0	0	0	2
(Bird *et al.*, 1989)	38, 42, 50, 62, 68	0	0	0	8	6	21
(Sadovnick *et al.*, 1988)	38	1	0	2	1	0	0
(Fukutani *et al.*, 1989)	33	1	0	0	1	1	1
(Martin *et al.*, 1991)	34, 35	0	0	0	common	common	common
(Frommelt *et al.*, 1991)	47	12	0	0	5	6	7
(Karlinsky *et al.*, 1991)	47	5	0	0	0	1	0

The families detailed in Table 1 may have different actiologies despite their (generally) early age of onset. The advent of molecular genetic analysis of similar families allows examination of the phenotype once the genetic lesion has been identified. One of the aims of the work of

this thesis is to detail the clinical features of families with known genetic loci. This allows comparison of families with different aetiologies and may provide clues to the underlying mechanisms accompanying each mutation.

Amnesia is common to all cases and in all but a few, is the first clinical feature. Aphasia and apraxia are infrequently detailed but are almost invariant accompaniments as the disease continues. Neither have been noted as a particular feature of many of these families (Table 1). Myoclonus and seizures have been frequently noted as have extra pyramidal features. Clinical and pathologically confirmed stroke have been infrequently noted (Bird *et al.*, 1988). Psychopathology, even depression, has been infrequently reported in these families.

1.6 The Aims of The Studies in this Thesis

The background to this thesis was the description of the families in Table 1 and the early linkage analysis by two groups finding linkage to chromosome 21 but not at the *βAPP* locus. The aim of the studies presented here was to identify families linked to chromosome 21 loci (specifically *βAPP*), and to describe their clinical features. In families not linked to chromosome 21 the aim was to detect linkage elsewhere and compare the clinical features between *βAPP*-linked and *βAPP*-unlinked families. These aims were achieved and resulted in the identification of six *βAPP* mutated families, nine families collectively showing linkage to chromosome 14 and the identification of age of onset as a clinico-genetic correlate.

CHAPTER 2

Materials And Methods

2.1 Clinical

2.1.1 Family Ascertainment

Families for the linkage studies were usually self-referred after advertisement in the Alzheimer Association newsletter, or by word of mouth via clinical colleagues. After initial (usually telephoned) assessment of the pattern of transmission in the family and the number of living relatives, the decision to collect or shelve was made. As such the families were highly selected for those that had a pattern of transmission consistent with autosomal dominance. Inclusion in the study was dependent upon the availability of enough samples to generate a significant lod score. Simulation studies (see below) to assess the power of individual families were conducted where necessary.

2.1.2 Clinical Assessment

Clinical assessment of family members included an initial screen with the mini-mental state examination (Folstein *et al.*, 1975) for all members, followed by a clinical, neuropsychological and physical examination for all living affected and selected unaffected members. The clinical assessment included a consensus history derived from the subject, the nearest relative, hospital medical and nursing records and general practitioner records. For affected individuals the National Institute for Neurological and Communicative Diseases (NINCDS) criteria (McKhann *et al.*, 1984) were used to diagnose possible, probable or definite Alzheimer's disease (AD). If cognitive function was well preserved in affected individuals (and in the case of well siblings) the following tests were administered: the cognitive examination of Camdex CAMCOG (Roth *et al.*, 1986), the Wechsler Adult Intelligence Test (WAIS) (Wechsler, 1981), the New Adult Reading Test (NART) (Nelson, O'Connell, 1978) and the Kendrick object learning subtest (Kendrick, 1987). The CAMCOG assessment was slightly expanded by the incorporation of four extra questions derived the Medical Research Council's criteria for assessment of dementia (Medical Research Council (UK), 1987). These questions added one point each to orientation for time, retrieval of remote information, reading comprehension and two points to the abstract thinking subsections of the CAMCOG. In all other ways, the scorings given are for the standard CAMCOG assessment. Where dementia was well

established an overall score of deterioration was obtained with the Blessed dementia scale (Blessed *et al.*, 1968).

2.1.3 Family Assessment for Linkage Studies, Power and Simulation Studies

As families were referred they were reviewed by clinicians and laboratory based staff for suitability for linkage studies. In general, families were collected if they were likely to provide sufficient power for linkage studies. Given the availability of simulation studies, the most likely lod scores obtainable were calculated in critical families. As a general rule though, families were opted into the study if they were able to generate a lod of 2.0. This was crudely assessed by the formula (informative meiosis)*0.3 (see Chapter 1).

2.2 Laboratory Methods (Maniatis *et al.*, 1982)

2.2.1 DNA Extraction from Whole Blood

DNA for RFLP, short tandem repeat and mutation analysis was extracted using following protocol.

Requires: Washing solution: (autoclaved): 0.1% Nonidet N-P40, 0.9% NaCl
Lysis buffer: 7M urea, 0.3M NaCl, 10mM EDTA, 10mM Tris/HCl pH:7.6

Blood samples (stored at -70°C or fresh) are transferred into 50ml tubes where an equal volume of washing solution is added. They are mixed and centrifuged for 15 minutes at 3000rpm. The supernatant is discarded and the pellet is re-suspended in 10-15ml of washing solution. The samples are centrifuged at 3000rpm for 15 minutes, the supernatant is poured off and 1ml of lysis buffer is added. After a gentle mix 9ml of lysis buffer is added and 2ml of 10% SDS. The samples are left at 55°C overnight. 7.5ml of phenol and 2.5ml $CHCl_3$ are then added. The samples are centrifuged at 3000rpm for 15 minutes and this step is repeated 2-3 times. The supernatant is kept in fresh tubes and 4ml $CHCl_3$ added. After a final centrifugation the supernatant is transferred to new tubes and the DNA is ethanol precipitated.

2.2.2 The Southern Blotting Procedure (Southern, 1975)

Random-Hexamer Labeling
Requires: OLB (Oligolabeling buffer)
OLB is a mixture of solutions A, B and C in a ratio 100:250:150.

Solution A: lml solution of 1.25M Tris pH 8.0, 0.125M MgCl2
18µl 2-mercaptoethanol, 5µl dATP (100mM), 5µl dTTP (100mM), 5µl dGTP (100mM)
Solution B: 2M Hepes adjusted to pH 6.6 with NaOH
Solution C: Hexadeoxyribonucleotides each suspended in TE at 90 OD units/ml
Klenow fragment of DNA polymerase (BRL)
Single stranded DNA is used as a template then random primers, Klenow polymerase, labeled and unlabeled nucleotide precursors are added in suitable oligolabeling buffer and the reaction allowed to proceed. The reaction is carried out at 37°C for 4-12 hours. 20-30ng of DNA is used in a 50µl reaction of oligolabeling buffer using 2µl BSA and 5µl [τ^{32}P] dCTP and 2 units of Klenow.

Hybridisation

Requires: SSC mixture: 7.5ml 20XSSC, 1.25ml 100XDenhardt's, 1.25 ml 10% SDS, 15mls dH20, 2.5g Dextran sulphate. The mixture is heated to 65°C to dissolve.

Washing solutions (when SSC mixture is used): 2XSSC, 0.1% SDS 20 minutes at 65°C lXSSC, 0.1% SDS 10 minutes at 65°C 0.lXSSC, 0.1% SDS 10 minutes at 65°C

100X Denhardt's solution [2% BSA, 2% Ficoll, 2% PVP (polyvinylpyrollidone)]

Church buffer: 180mls Na_2PO_4, lM 70mls NaH_2PO_4 lM, 3.5% SDS, lml 0.5M EDTA pH8.0, made up to 500mls with H_2O

Washing solution when Church buffer is used: Sodium Phosphate buffer pH 7.2, 72 mls of Na_2PO_4, 1M 28mls of NaH_2PO_4 lM
100mM Sodium phosphate buffer for 30min at 65°C
40mM Sodium phosphate buffer for 10min at 65°C
Sonicated Salmon Sperm DNA (competitor)
Hybond membrane N (Amersham)
20XSSC [3M NaCl, 0.3M Na_3citrate]
Denaturing solution [1.5M NaCl, 0.5M NaOH]
Neutralising solution [1.5M NaCl, 0.5 Tris-HCl pH 7.0, 0.001M EDTA]
Stripping solutions : 0.5% SDS or 10mM NaOH

The Procedure

Double stranded DNA can be denatured by various procedures that break the hydrogen bonds linking the complementary base pairs. Denaturation is reversible and when cooled the complementary strands re-associate. This process is known as annealing and can occur

between DNA strands that were not originally base-paired, provided they are sufficiently complementary. Specific sequences in the DNA are detected by hybridisation to radiolabeled probes of DNA. The binding of the probe can then be detected by autoradiography. The precise conditions under which hybridisation is performed may affect the extent of hybridisation with similar but not identical sequences.

The gel is placed in denaturing solution for 30 minutes. This solution is replaced with neutralising solution for another 30 minutes. Transfer apparatus is set up: a large tray is filled with 20XSSC, 3MM filter paper is soaked in 20X SSC and covers a glass plate. The paper must be flat when the gel is placed on it. The apparatus is covered with cling film. A piece of Hybond N membrane soaked in 2XSSC is directly placed on top of the gel. On top of the membrane, 4 pieces of 3MM paper are cut to the same size as the gel. Air bubbles trapped between the membrane or the papers are removed by rolling with a glass rod. Finally a stack of paper towels, a glass plate and a 500g weight are placed on top of the blot which is left overnight. When dismantled, the gel loading slots are marked and the Hybond membrane is soaked in 2XSSC to remove any agarose. The filter is dried between two sheets of 3MM paper and then wrapped in cling film. To fix the DNA on the filter, the membrane UV transilluminated (DNA side down).

The filter is placed in a hybridisation tube with 200mls 2XSSC and is smoothed to the sides. SSC is poured out and 5-10mls of hybridisation mixture is added. 100µl of salmon sperm DNA are then added to the mixture which is incubated in hybridisation oven for at least one hour. This prehybridisation mixture is poured off and the probe is denatured (10 minutes), held on ice for 10 minutes and then added to the hybridisation mixture which is poured into the tube. Incubation takes place for at least 4 hours (usually overnight). The labeled probe may be poured off and saved for another filter hybridisation or disposed down a radioactive sink. The tube is then filled with a washing solution. Washing proceeds until the filter is sufficiently radioactively deplete. Finally, it is dried on Whatman paper, covered with cling film and exposed to X-Ray film at -70°C for as long as is required to sufficiently irradiate the film (one to several days).

<u>Spun Columns</u>

Requires: Sepharose G-50, G-25 (Pharmacia)

1ml syringe barrels are used. One end is plugged with polymer wool, and Sepharose G-50 equilibrated in 3XSSC is added to fill a syringe using a plastic pipette. Syringes are placed inside 1.5ml plastic centrifuge tubes and are centrifuged for 10 minutes a 1600 rpm. If necessary, more Sepharose G-50 is added and centrifugation is repeated. The probe is loaded

onto the column and centrifuged for 5 minutes at 1600rpm. The columns are discarded and the radioactivity measured by liquid scintillation counter. A labeling of >5X106 counts per minute is adequate.

2.2.3 Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988)

Requires: Amplitaq DNA polymerase (Cetus and Cambio), Nucleotides(dNTPs, Pharmacia), PCR buffer: 1.5mM $MgCl_2$, H_2O, 0.01% glycerol, lOmM Tris.Cl pH:8.0, 50mM KCl Glycerol (BDH)

The PCR synthesises millions of copies of a specific DNA segment. The reaction is based on the annealing and extension of two oligonucleotide primers that flank the target region in duplex DNA; after denaturation of the DNA each primer hybridises to one separate strand so that extension from each 3' hydroxyl end is directed towards the other. The annealed primers are then extended on the template strand with DNA polymerase. Those steps (denaturation, primer annealing and DNA synthesis) when repeated result in the exponential accumulation of a discrete fragment whose termini are defined by the 5' ends of the primers. After n cycles there will be 2^n copies of the region between the two primers. The primer design can be considered as a critical factor in the reaction. Primers should have a random base distribution with average GC content. Their secondary structure must also be checked in order to avoid complementarity especially in the 3' ends. Taq DNA polymerase which is used for the synthesis of DNA is sensitive to the concentration of magnesium ions and monovalent ions. Since dNTPs (nucleotides) can bind magnesium, the precise concentration that is required to activate the enzyme maximally is dependent on the dNTPs concentration. The temperatures applied for primer annealing and DNA synthesis vary with the enzymes used and depends also to some degree on the base composition of the primers and template DNA.

Taq DNA polymerase, by allowing synthesis at elevated temperature, reduces the chances of unintended oligonucleotide priming by destabilising mismatch-pairing with unwanted target sequences.

Typical PCR: 10 minutes initial denaturation at 94°C followed by 35 cycles of 1 minute at 55C, 1 minute at 94°C and 1 minute at 72°C. The reaction volume usually used is 25 ~l of PCR buffer with a final concentration of primers at 0.2~M. All nucleotides are in a final concentration of 200~M and 1 unit of Taq DNA polymerase is added last. One drop of oil on the top of the reaction mixture ensures that there is limited condensation.

2.2.4 PCR and Enzymatic Digestion for Codon 717 Val→Ile Detection

PCR is carried out using the following intronic primers in order to amplify exon 17 of the APP gene: (1) 5'-CCTCATCCAAATGTCCCCGTCATT-3', and

(2) 5'-GCCTAATTCTCTCATAGTCTTAATTCCCAC-3'. PCR conditions were 94°C for 10 min to denature, then 35 cycles of 60°C for 1 min, 72°C for 3 min, 94°C for 1.5 min; and a single cycle of 72°C for 10 min. The reaction volume of 25µl contained 50 pmol of primers, dNTPs at 200µM, and $MgCl_2$ at 1.5mM. The 319bp product is cleaved to a 199bp and a 120bp product by *BclI* if the mutation is present.

BclI digests is carried out at 50°C for 2-4 hours then electrophoresed in 3% agarose and stained with ethidium bromide.

DNA Agarose and Polyacrylamide Gel Electrophoresis

DNA size markers: Lambda HindIII, 1 Kb Ladder(GIBCO BRL)

Gel Loading Buffers

Xylene cyanol FF(BDH), Bromophenol blue(SIGMA), Orange G (SIGMA)

The size of DNA can be determined by measuring its electrophoretic mobility in gels of polyacrylamide or agarose. The mobility of each sample depends on its size and on the concentration of the gel. In an electric field, DNA moves at a rate inversely proportional to its size so that bigger fragments move slower than small ones. Nucleic acids are visualised by staining the gels with ethidium bromide (Sigma). Ethidium bromide has an intense fluorescence when excited by ultraviolet radiation and complexed with nucleic acids. Usually, agarose gels are used to separate fragments of 200bp up to 5kb and polyacrylamide gels are used to separate smaller fragments.

Agarose gels were made with agarose powder (BRL) (desired concentration) in TAE buffer (40mM Tris, 20mM Acetate, 2mM EDTA at pH 8.0). The mixture was heated in a microwave oven until the agarose was in solution, cooled and then poured into the appropriate former. Polyacrylamide gels are made using a 40% solution of acrylamide (BDH) (19:1 acrylamide: N,N,methenebis-acrylamide) to the required concentration in TBE buffer (89mM Tris, 89mM Borate, EDTA pH:8.0), and ammonium persulphate (BRL) (10%) (w/v) and TEMED (BDH) are added as polymerisation initiator and catalyst respectively. The mixture is then poured between glass plates and allowed to set before use for about one hour. Gels are run usually at 50 volts (agarose) and 6 volts (polyacrylamide).

2.3 Linkage Data Errors

There are potentially serious consequences of entering flawed data in genetic linkage analyses. There are several kinds of flawed data:

2.3.1 Mis-Typed Genetic Markers

Mis-typings are a frequent occurrence in all linkage data - estimated in the order of 3% for RFLP data in the CEPH pedigrees. Laboratory experience suggests that mis-typing errors are relatively more common for short tandem repeat (STR) markers. This is due, in part, to the generally large number of STR marker alleles, each separated from the next by only two base pairs. Mis-typings may be eliminated by several strategies:

- Double and triple blind scoring of RFLP and short tandem repeat (STR) marker alleles can be compared leading to a consensus typing;

- Running known reference alleles on each gel;

- Electrophorese all family samples together or one sample from each family on the same gel;

- The use of sizing markers is standard but is subject to misreading due to gel distortion. Mis-typing may lead to non-Mendelian segregation of alleles which is detected either by observation or by the checking modules of the programs in the LINKAGE package. Mis-typing can result in data that are consistent with Mendelian inheritance and may therefore pass undetected, for instance the consistent mis-typing, in one branch of a family. This might result in linkage with false recombination between the disease and the marker. A common consequence of mis-typing alleles is the attribution of an incorrect allele frequencies.

2.3.2 Mis-Specification of the Allele Frequencies

As stated above, this can be a consequence of mis-typing or of the incorrect attribution of allele frequencies from published data or from the sampling of inappropriate reference populations. One research practice is to set unknown allele frequencies as equal - this is very likely to inflate lod scores in most cases (Ott, 1992). In general, mis-specification of allele frequencies will alter the value of a lod score but in pedigrees with little inferred data will not result in incorrect estimation of recombination fractions. The opposite is also true, that is, will not

pedigrees with unknown genotypes varying the allele frequencies can strongly influence recombination estimates (Knowles *et al.*, 1992; Nechiporuk *et al.*, 1993). This problem is discussed further in the situation where recoding has occurred and dummy allele frequencies interact with recoded allele frequencies.

2.3.3 Safeguards

The detection of mis-typings occurs at three levels. Firstly, by inspection of the segregation of alleles as they appear in families. Secondly, by the checks built into the LINKAGE programs. Mendelian incompatibilities are picked up in this way. Thirdly, in a situation where several families are typed for several markers, ILINK estimation of recombination (and therefore order) between linked markers should not differ significantly from genetic maps produced in reference pedigrees. This analysis was used to check for incompatibilities in chromosome 14 data discussed in Chapter 7.

2.4 Linkage

A figure of the general analysis by the linkage programs is shown in Figure 1. Two files are constructed for each analysis, a data input file, **family.inf**, containing the genotypes or affection status at each locus and a **locus.dat** file containing descriptions of each locus. The **family.inf** file is transformed into a **family.ped** file suitable for direct access by the linkage programs. The program MAKEPED (makeped.exe) rearranges the data in the **family.inf** files and checks for basic Mendelian incompatibilities, producing the **family.ped** output. Many typing errors can be intercepted at this level. An incompatibility message necessitates a return to the pedigree for examination of the segregating alleles. Other frequent errors include the mis-specification of relationships in the pedigree. Consanguinity loops can be specified at this level. One of these, two sisters marrying two cousins, occurs in the family structure of F206. In order to analyse this complex situation, the loop has to be broken; a file specify the position of the break is produced by MAKEPED.

Next, the UNKNOWN program is run. This program infers possible genotypes in untyped parents and outputs two files containing this information, **ipedfile.dat** and **speedfile.dat**. These two files and the **locus.dat** file are the input files for each of the three main linkage analysis programs MLINK, ILINK and LINKMAP (mlink.exe, ilink.exe and linkmap.exe). A batch file, **pedin.bat**, is produced by the linkage control program, lcp.exe, and specifies which of mlink.exe, ilink.exe and linkmap.exe is to be used and specifies the input files, **family.ped** and **locus.dat**. In addition, the **pedin.bat** file specifies which loci are to be

analysed, the known recombination fractions between loci and the mode of inheritance, sex linked or autosomal dominant. In addition, other parameters are specified depending on the program. If mlink.exe is to be used then the genetic distances between any two markers can be varied. The starting recombination fraction, the increment value and the stop value must be specified for this analysis. If linkmap.exe is used then the distance between any two markers is automatically divided and the lod score calculated at each of these points. Ilink.exe is an iterative program which can evaluate the most likely order and distances between loci, one of which can be a disease locus.

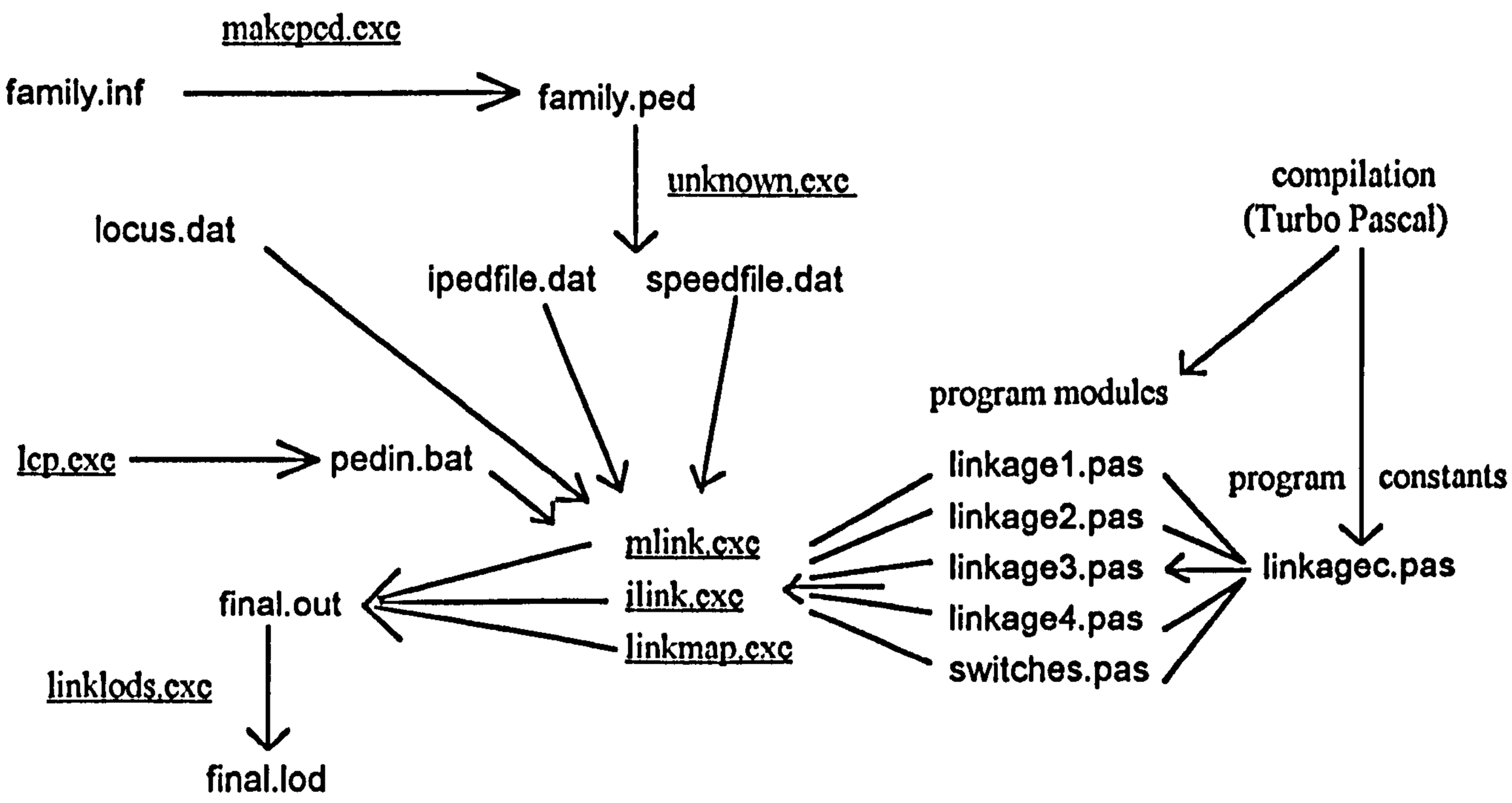


Figure 1.

The general relationship of files and programs in lod score linkage analysis with the LINKAGE suite of programs
(executable program files shown underlined -
correspond to the capitalised program name throughout text)

2.4.1 Compilation and Use of mlink.exe, ilink.exe and linkmap.exe

These three programs are related as they share Turbo Pascal modules and are compiled under direction of the same compilation control file, **linkagec.pas** (see Figure 1). There is overlap in the constants that need to be specified for each program during compilation. These constants (with typical values) are:

maxneed=800; {MAXIMUM NUMBER OF RECOMBINATION PROBABILITIES}
{THE FOLLOWING SHOULD BE LARGER THAN MININT}
maxcensor=10000; {MAXIMUM FOR CENSORING ARRAY}
maxlocus=3; {MAXIMUM NUMBER OF LOCI}
maxall=5; {MAX NUMBER OF ALLELES AT A SINGLE LOCUS}
maxhap=50; {MAXIMUM NUMBER OF HAPLOTYPES}
{ = n1 x n2 x ... ETC. WHERE ni = CURRENT NUMBER OF ALLELES AT LOCUS I}
maxind=80; {MAXIMUM NUMBER OF INDIVIDUALS in all pedigrees}
maxped=1; {MAXIMUM NUMBER OF PEDIGREES}
maxloop=1; {MAXIMUM NUMBER OF LOOPS}
maxchild=18; {MAXIMUM NUMBER OF FULLSIBS IN A SIBSHIP}
affall=2; {DISEASE ALLELE FOR QUANTITATIVE TRAITS IN X-LINKAGE}

{QUANTITATIVE TRAIT}
maxtrait=1; {MAXIMUM NUMBER OF QUANTITATIVE. FACTORS AT A SINGLE LOCUS}
missval=0.0; {MISSING VALUES FOR QUANTITATIVE TRAITS}

{AFFECTION STATUS}
missaff=0; {MISSING VALUE FOR AFFECTION STATUS}
affval=2; {CODE FOR AFFECTED INDIVIDUAL}
maxliab=7; {MAXIMUM NUMBER OF LIABILITY CLASSES}

{BINARY (FACTOR UNION) SYSTEM}
maxfact=7; {MAXIMUM NUMBER OF BINARY CODES AT A SINGLE LOCUS}
{An error in the programs require that for binary factor analysis, maxfact should be equal to maxall}

The size of the problem that can be analysed with the linkage programs is determined by the maximum array allowed by the particular operating system in which it is being run. Since, no array or other variable can exceed 64K in MS-DOS the upper limit on maxhap is 126. The maximum value of maxhap is the product of the maximum number of alleles at each locus. On a mainframe or Sparc 10 workstation or other VMS or unix based system, this constant can be increased. However, in either case this constant is a limiting factor in the multi-point analysis of highly polymorphic systems where the number of alleles occurring at each locus can be 10-20.

The product maxfem*maxped must not be larger than 65536, where maxfem = maxhap (maxhap+1)/2 = the maximum number of genotypes.

Maxneed and maxcensor are variables that are difficult to estimate prior to running a particular problem. Maxneed determines the dimensions of an array storing the probabilities of recombination classes. An error message is displayed when the program runs if maxneed is too small. Maxcensor determines an array which impacts on the efficiency of calculations. At the beginning of calculations, a message is displayed detailing how efficiency may be increased by varying this constant.

2.4.2 Penetrance

The following example is typical of a liability table for early onset AD (Table 1). The statistical effects of varying penetrance may be small (see Introduction). For instance, in the Swedish family, F139 (Figure 1), changing penetrance has little effect on lod score as the genotypes of the affected parent of the main sibship is not determined clearly. Changing penetrance, particularly to 100% has the greatest impact when recombinants are fully informative. In this situation the recombinant is capable of generating a lod of -∞. Under most other conditions, recombinants produce modest negative lod scores in families of the size examined in this thesis (see, for instance, analysis of unlinked and recombinant families, Chapter 7).

Table 1. Typical Liability Classes for Early Onset AD Lod Score Linkage Analysis

Liability Class	Genotype		
	A/A	A/a	a/a
1	0.001	0.01	0.01
2	0.001	0.05	0.05
3	0.002	0.3	0.3
4	0.002	0.5	0.5
5	0.002	0.8	0.8
6	0.003	0.95	0.95
7	0.003	0.99	0.99

Liability classes and corresponding conditional probabilities associated with the genotype above. 'A' represents the non-disease allele and the first column therefore represents the phenocopy frequency. The slight increase of phenocopy frequency with age (classes 6 and 7) reflects the risk of "sporadic" disease in the early 60s.

2.4.3 Heterogeneity of Two-Point and Multi-Point Data

If the assumptions of homogeneity noted in above cannot be readily made, it is useful to test for heterogeneity. Tests for heterogeneity with either two-point or multi-point data can be implemented with the program HOMOG. The principle behind the two-point analysis is covered in the introductory chapter. Multi-point data can be examine to determine whether the maximum likelihood position of the disease locus in the whole data set corresponds to the sum of the individual likelihoods for the same position. For the reasons discussed previously, such tests have limited usefulness in families containing only little information but nevertheless were applied to chromosome 14 data in Chapter 7.

2.4.4 Recoding Alleles of Highly Polymorphic Markers

One of the immediate consequences of employing multi-allelic systems in linkage analysis is the increased run-time required to perform the analysis. Five-point multi-point calculations in ten pedigrees by Schellenberg and colleagues with chromosome 14 markers, for instance, were not performed as they were estimated to take weeks to complete. In the multi-point analyses short tandem repeat markers run times were shortened by recoding multi-allele systems, employing only the number of alleles actually occurring in each data set. A recoding program was written in Turbo Pascal for Windows and is shown in expanded form in Addendum 1. This program could recode up to 10 loci with 14 alleles at each locus. In addition, a summary file of the recoded data is outputted if required (see Addendum 2). During recoding the allele frequencies were unchanged, so that the probability of a particular allele occurring at a "married-in" case was the same.

This strategy, although simplifying calculations and significantly reducing run time, necessarily entails a loss of data. For instance, the possibility of additional alleles occurring in individuals of genotype unknown is lost. The situation where recoding is most advantageous in this regard (many cases with unknown genotype) is also the one where most distortion of results could potentially occur. One precaution that can be taken is an additional dummy allele can be allowed to occur in the pedigree. The frequency of this allele can be the collective frequency of the alleles eliminated by the recoding. This problem was illustrated by the Swedish pedigree F139 discussed in Chapter 6 (Figure 2).

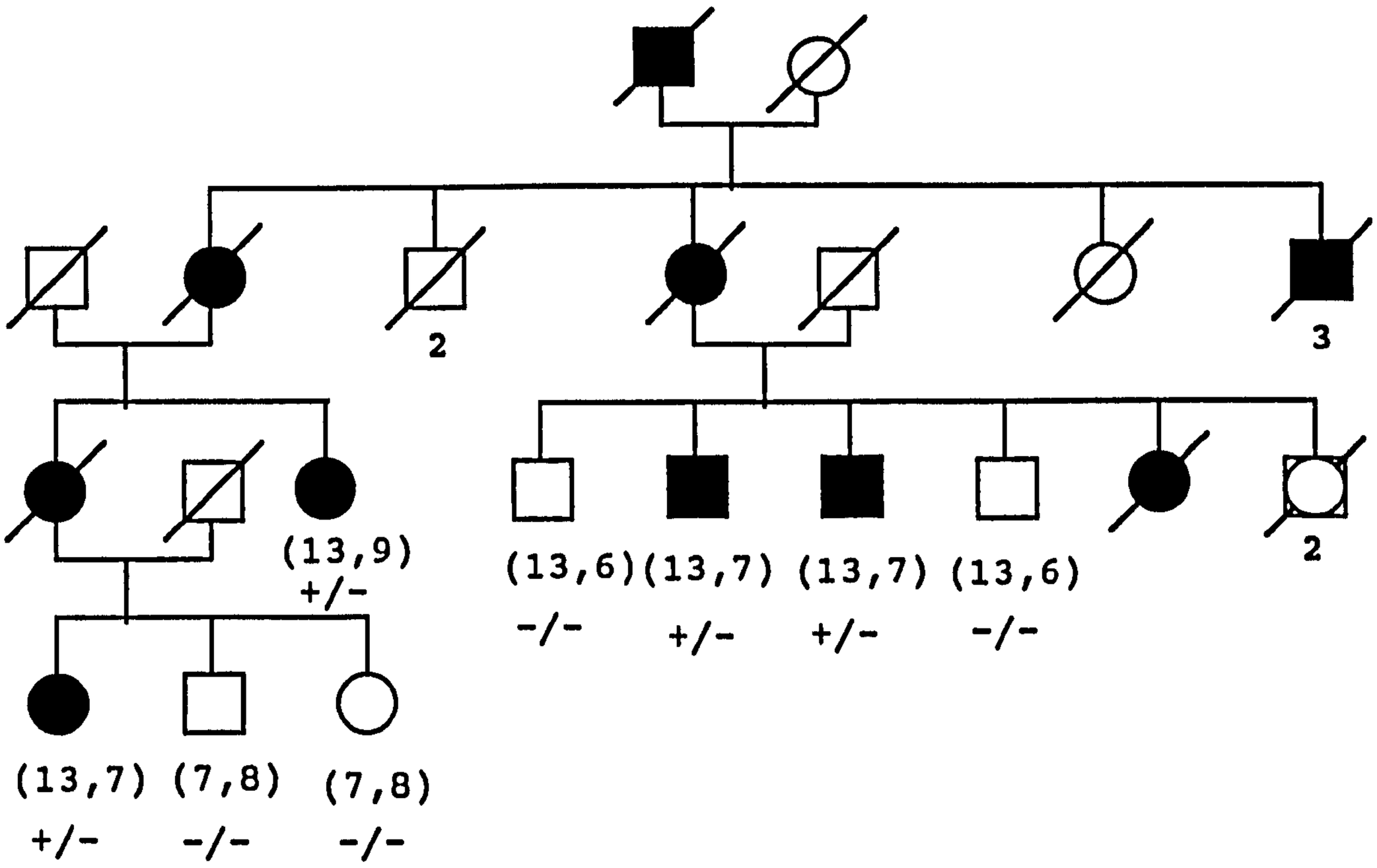


Figure 2.

Segregation of GT12 Alleles and Disease Causing Mutation in F139

The problem in this family is the lack of information from the parents of the main sibship. Although allele 13 occurs with the disease elsewhere in the pedigree it also occurs in two unaffecteds in this sibship. The use of a dummy allele here reflects the fact that other alleles other than the ones detected might occur. However, there is little difference in lod score when a dummy allele is used with high or low frequency. The most influential effect on the lod score in this family is the frequency of allele 13. This determines whether the configuration in the larger sibship is scored as a recombinant or not. There is an interplay between the frequency of the dummy allele and that of the segregating allele. The effects of the low frequency of the segregating allele implying a recombinant are compounded by the high frequency of a dummy allele as the occurrence of the latter becomes more probable implying a unilateral and recombinant source of allele 13.

In order to check the effects of recoding on multipoint analysis, all two-point data were checked before and after recoding. Usually, only second decimal place changes occurred. It was then a reasonable assumption that multipoint analysis would not be grossly distorted.

2.4.5 Assigning Liability Classes

A program, written in Turbo Pascal for Windows, was written to assign liability classes based on the existing ages of onset of members of a particular pedigree. The program is shown in Addendum 3. The program asks for the ages of onset (or date of birth) of affected individuals. The mean and standard error are calculated, and written to an output file. The program then asks for the present age of the individual requiring a liability class, and one is assigned under the assumption of equal distribution under the normal population of ages of onset. Although, there are good arguments for supposing that the age of onset as recorded in families selected for linkage may not represent a normal distribution, it is a reasonable approximation.

2.4.6 Polymorphism Information Content and Heterozygosity

The usefulness of a marker in genetic linkage studies depends on its degree of polymorphism i.e. the number and frequency of its alleles. In general, marker systems that depend on the presence or absence of an endonuclease restriction site have few alleles (usually two). Thus, the initial studies of Restriction Fragment Length Polymorphisms in F23 show poor informativeness through the family (see Table 1, Chapter 3). Many such markers are required in multi-point analyses to produce large enough lod scores to draw conclusive results. The highly polymorphic simple tandem repeat sequence markers (also called AC or TG repeats) enable more powerful assessment of linkage in single families. There are several ways to measure informativeness of a marker system. The polymorphism information content is the probability that the marker genotype of an offspring will allow deduction of which of the two alleles of the affected patient it had received. The heterozygosity value is a more popular measure with short tandem repeat markers, and values upwards of 75% providing excellent markers for linkage studies.

2.5 Simulation

2.5.1 General Methods

For the generation of simulated linkage data the structures of the families under consideration were ascertained and entered in the format for linkage analysis with the exception that an additional column was added in accordance with the following availability codes:

Code	Markers	Trait
0	Unavailable	Use original phenotype
1	Available	Use simulated phenotype
2	Available	Use original phenotype
3	Unavailable	Use simulated phenotype

In practice, as the phenotype was rarely in doubt, all availability coding was either 0 or 2, simply depending on whether marker data had been generated from that particular individual.

A flowchart of the general interaction of the data files and programs in data simulation is shown (Figure 3).

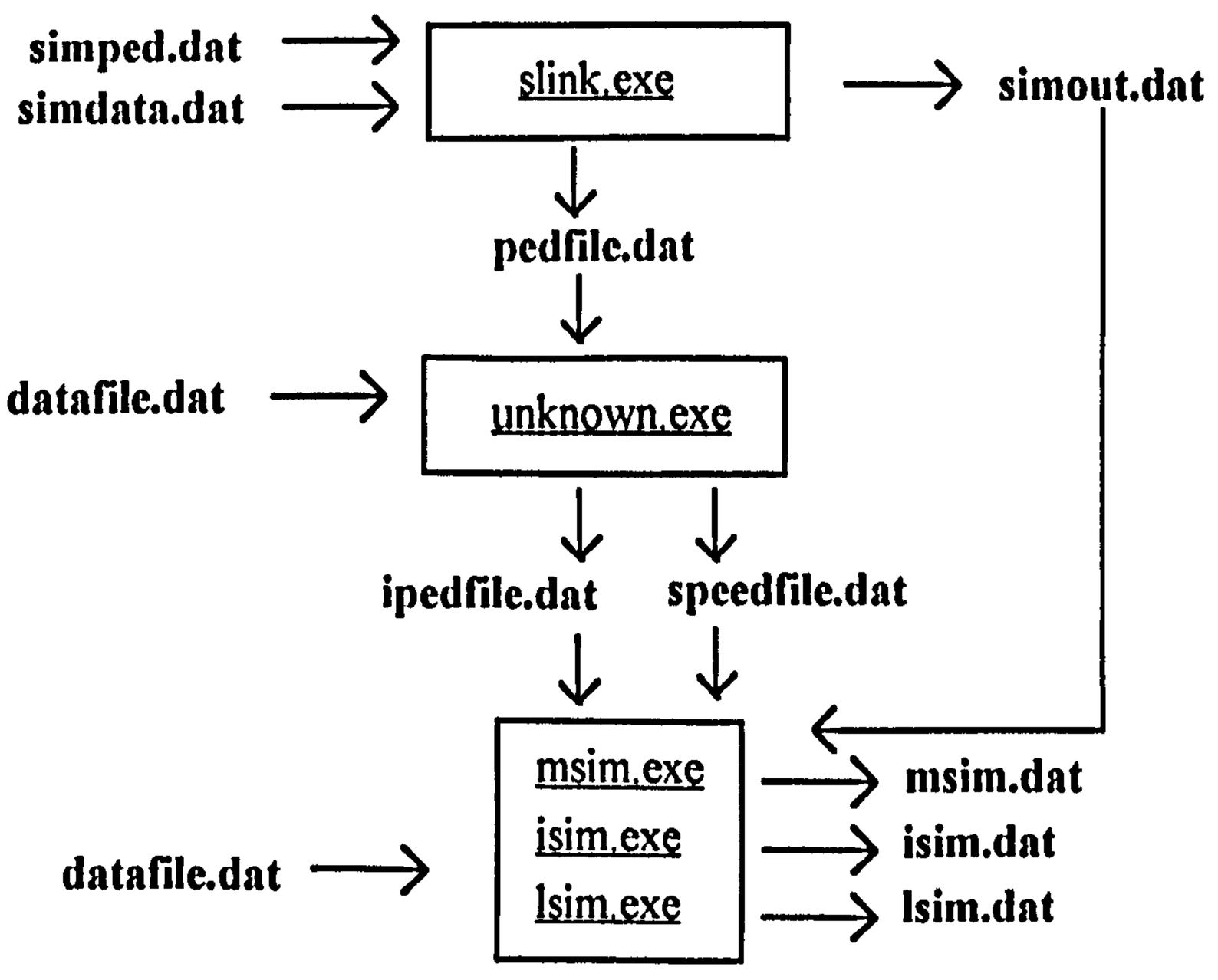


Figure 3.
Flowchart of the Simulation Programs
(executable files correspond to capitalised program names in text)

The "true" linkage situation is determined by the simdata.dat file which dictates the relationship between the simulated markers and the disease locus. The initial generation of the linkage data by the SLINK program was in accordance with the desired value of α, the proportion of linked families. In the case of single family analysis, α was either set to 0.0 or 1.0. Linkage data for 500 or 1000 pedigrees were simulated under linkage or non-linkage in accordance with known genotypes where appropriate. These families are analysed in accordance with the datafile.dat specifications which are bone fide linkage data files, describing the values of theta at which linkage is to be tested. Any of the three programs MSIM, LSIM or ISIM can be used to analyse the data and after processing the files for unknown data. Each has a corresponding LINKAGE counterpart; MLINK, LINKMAP and ILINK and are thus used for estimation of lod with fixed theta, estimation of the maximum likelihood positions of one locus over a map of linked loci and the estimation of theta by iteration. The corresponding output files summarise the data in terms of mean, standard deviation of lod score and maximum and minimum values. In order to avoid statistically analysing $-\infty$ it is as well not to set theta to zero in the analysis between disease and marker for unlinked families. Although due to the penetrance parameters set in the liability table $-\infty$ is never attained in these analyses as no completely recombinant event is ever scored. The SLINK package of programs are written in Turbo Pascal and can be recompiled to suite particular analyses in the same way as the LINKAGE programs. The constants which are subject to change prior to compilation are analogous to those described above for the LINKAGE programs.

2.5.2 Combining Linkage and Simulation Studies to Detect Linkage in Pedigrees Insufficient to Generate a Lod Score of 3.0 or More

One of the limitations of linkage analysis applied is the assessment of significance of two-point lod scores falling below the accepted criterion for linkage (3.0) or of multi-point data with linked markers. Does a lod value approaching 3.0 represent linkage in families with poor statistical power or does it represent the chance co-segregation of marker and disease gene? One approach to this problem is the use of simulation studies. Simulation programs allow the random generation of linkage data for a given family (or set of families) structure within the limitations set by the parameters defining the disease locus (e.g., disease allele frequency, number of alleles) and markers (e.g., heterozygosity). Such data generated for several hundred data families with the structure of the actual families available can then be analysed by the linkage programs in the normal way. The output data is statistically analysed to examine the distribution of the resultant lod scores. The simulated data can be generated under "true" conditions of linkage or under "true" conditions of non-linkage. The percentage of generated lods which fall above or below any given criteria can then be used as a measure

of false negatives and false positives. For instance, for a given set of families with linkage data simulated under non-linkage, the proportion of family sets generating a lod score above 3.0 is a measure of the false positive rate. Similarly, the false negative rate can be ascertained by the proportion of lod scores falling below -2 from the data set generated under conditions of "true" linkage. Furthermore, the distribution of lod values generated in this way approximates a normal distribution and p values can be ascribed to any lod value generated from actual linkage data. A ratio of probabilities of the alternative hypotheses (i.e., likelihoods that the family is drawn from a linked or unlinked population) can be easily derived from these probabilities. Thus, the significance of any actual lod value can be assessed.

[In general, the discriminative power of a family can be estimated by a discriminative function, Δ, where

$$\Delta^2 = (\mu_{linked} - \mu_{unlinked})^2/\text{variance of lod within groups}$$

where μ = mean lod scores (adapted from Armitage, Berry, 1987) . A pooled estimate of variance from both groups is acceptable when the number of replicates is high. The higher the value of Δ the higher the discriminative power of the family. Typical families analysed in this thesis had a Δ value of 20-30, whereas a family with $\mu_{linked} = +2.0$, SD = 2.0 and $\mu_{unlinked} = -5$, SD = 2.0 has a Δ of 78 for 1000 simulations in each group. F19, chapter 5 had a Δ of 36 compared to F172, which was poorly discriminative with a Δ of 24, chapter 7].

This method was employed in assessing several early onset families (F19, F172, F139, F144) as linkage information was obtained. The significance of positive lod scores was evaluated and this enabled detection of linkage to the *βAPP* locus far below the accepted criterion of 3.0.

A refinement of this method is the generation of simulated data on untyped individuals but with known genotypes entered. This refinement limits the amount of variation that can be randomly generated and so mimics the actual linkage problem more closely. This is particularly important with respect to the allele segregating with the disease. The frequency of this allele has a large impact on the maximum lod score obtainable. When there was no linkage data available, the marking allele is randomly chosen in accordance with the frequencies of all the possible alleles. When linkage data are known and a particular allele appears to segregate with the disease, this allele is fixed as the marking allele and all other linkage data are generated randomly. This is a particularly important refinement due to the effects discussed in **2.3.2**, above.

CHAPTER 3

Segregation of a Missense Mutation in the ß-Amyloid Precursor Protein Gene with Familial Alzheimer's Disease

3.1 Abstract

Two-point linkage analyses of chromosome 21 markers of low information content in a single pedigree suggest linkage to the ß-amyloid precursor protein (ßAPP) gene. Sequencing of this gene reveals a point mutation creating an endonuclease restriction site, which is linked to the disease. A screening protocol detects the mutation in affected members from a second small pedigree from the US.

3.2 Introduction

Previous linkage analyses of familial Alzheimer's disease (AD) suggested the presence of a locus on chromosome 21 in some families (St George Hyslop *et al.*, 1987). The proposed AD locus was thought to be close to the *βAPP* gene (Tanzi *et al.*, 1987). However, recombinants between the *βAPP* gene and the AD locus had been reported (Schellenberg *et al.*, 1988; Van Broeckhoven *et al.*, 1987) which excluded *βAPP* (if genetic homogeneity was assumed) as the site of the mutation causing familial AD. Genetic analysis of a large number of AD families had demonstrated that the disease was most likely heterogeneous (St George Hyslop *et al.*, 1990). In particular, families with late onset AD had not shown linkage to chromosome 21 markers (St George Hyslop *et al.*, 1990; Pericak-Vance *et al.*, 1988) and only some families with early onset AD did show linkage to chromosome 21 markers (Schellenberg *et al.*, 1988; St George Hyslop *et al.*, 1990; Goate *et al.*, 1989).

These data suggested non-allelic genetic heterogeneity within early onset familial AD (Schellenberg *et al.*, 1988; St George Hyslop *et al.*, 1990). To avoid the problems that heterogeneity posed for genetic analysis, an analysis of the co-segregation of AD and markers along the long arm of chromosome 21 was undertaken in a single large family (F23). Once linkage was suggested, direct sequencing revealed a point mutation in the *βAPP* gene. This mutation causes an amino acid substitution (Val→Ile) close to the carboxy terminus of the ß-amyloid peptide (henceforth referred to as Aß). The mutation creates a *Bcl*1 endonuclease restriction site, enabling rapid screening in other samples by pcr amplification of exon 17 and digestion. Screening other cases of familial AD in this way revealed a second unrelated family

in which this variant occurred. There was insufficient statistical power in the family samples supplied from F372 to generate significant lod scores, however the finding of a second family with this mutation lent further weight to the suggestion that this mutation was disease causing.

3.3 Materials and Methods

3.3.1 Family Material

The F23 pedigree structure is shown in Chapter 4 Figure 1a. The clinical assessment is detailed in the Materials and Methods and a detailed report of affected individuals is described in Chapter 4. The clinical assessment was completed blind to the genetic data.

3.3.2 Linkage Analysis

Modestly polymorphic RFLP markers spanning 55% of the physical and genetic length of the long arm of chromosome 21 were analysed in Family 23. These markers, at the loci (from centromere to telomere) D21S16, D21S13, D21SI, *βAPP*, D21S17, D21S156 and D21S167 (Warren *et al.*, 1989) (see Figure 2), cover the genetic distance in which the AD locus had been proposed to occur, with the exception of the pericentromere. Two-point linkage analyses are shown between the disease and each of the polymorphic loci and AD (Table 1). Liability classes, phenocopy rates and mode of inheritance were attributed as described in materials and methods chapter. Allele frequencies used for the loci D21516 D21513, and D21S1 have been calculated from unrelated individuals in a British population. Allele frequencies used for the other polymorphisms are as previously reported 12~l6. The allele frequency used for the *Bcll* polymorphism was 0.01, which was the highest frequency predicted from non-detection of the polymorphism in 200 normal chromosomes (99% confidence [1-(1-confidence interval)$^{1/200}$]). The following loci were not informative within the pedigree: D21580, D21852, D21511, D21S8, D21S111 and D21582.

3.3.3 Mutation Screening (see Materials and Methods)

Figure 1 illustrates the results of the screening protocol pcr and endonuclease digestion employed to detect the Val→Ile mutation in F372.

3.4 Results and Discussion

The two point linkage analysis of AD in F23 and chromosome 21 RFLP and microsatellite markers is shown in Table 1. Several of the markers were completely uninformative (S13 Taq2, S80 (AC)n) or partially informative (S52 HindIII) within the family. The two microsatellite markers D21S156 and D21S167 were informative having a much higher heterozygosity value. At theta = 0.0, they generate the largest (and negative) lod scores in the family, reflecting recombination between marker and disease. Small positive lod scores were generated at S16, S13, S1/S11 and a larger one with the Eco RI polymorphism at *βAPP* (Z_{max} = 2.61 at θ = 0.0). The data were consistent with *βAPP* being the causative gene in this family, although markers covering a much larger genetic area showed positive lod scores with the disease. Multi-point analysis did not produce a lod score of greater than 3.0 due to the large genetic distance between markers. [A multi-point analysis of S13 Taq1, S1Bam H1 and *βAPP* EcoR1 gave a maximum lod of 2.98 at zero recombination with *βAPP*]. The lod scores at *βAPP*, together with its candidate status, suggested it should be sequenced in this family. In addition, another condition hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) (Levy *et al.*, 1990), was known to be due to a mutation in exon 17 of *βAPP*. This mutation leads to precocious deposition of Aß in cerebral vessels. It was possible, as Aß was central to the pathology of both conditions, that they were allelic variants. The *βAPP* genes were therefore sequenced in a number of affected and unaffected individuals beginning at exon 17.

Sequencing of exon 17 revealed a C to T transition at base pair 2,149, causing a valine to isoleucine change at amino acid 717 (transcript *βAPP*770; Yoshikai *et al.*, 1990). The substitution creates a *BclI* restriction site which allows detection of the corresponding polymorphism within the PCR product. Screening the family and running the disease against this polymorphism demonstrated co-segregation with AD (Z_{max} = 3.41, θ = 0.0). Screening by PCR of 100 unrelated, normal individuals from the same population as the AD pedigree, and 14 cases (9 families) of familial late onset disease, failed to detect this substitution. Screening of 18 cases (16 families) of early onset familial disease revealed the *BclI* restriction site in two affected individuals from a second family (Family 372) in which linkage between AD and chromosome 21 markers had previously shown small positive lod scores (Pericak-Vance *et al.*, 1988) (D21S16, Z_{max} = 0.6, θ = 0.0; D21S1, Z_{max} =0.9, θ = 0.0). Affected individuals from both families with the mutation were also genotyped for the rare EcoRI polymorphism detected by the 3' end of the *βAPP* complementary DNAs; this probe detects a polymorphic site in the intron between exons 17 and 18. In Family F23, the disease (and the mutation) co-segregates with the rare allele, which is present in only 5% of the normal population. In contrast, affected individuals in Family 372 do not share this allele, but are

homozygous for the common allele. This suggests that the two pedigrees are unlikely to be related to each other, as the markers are separated by less than 20 kilobases. The genetic data show that the disease locus is linked to the missense mutation in F23, but does not prove causation. However, failure to detect this polymorphism in 200 normal chromosomes supports the contention that it is a the general population has an upper limit of 0.01 (with 99% confidence).

Table 1. Two Point Lod Scores Between AD and 18 Chromosome 21 Markers in F23

Marker	Allele Frequencies	Recombination Fraction Between Marker and AD in F23				
		0.00	0.01	0.05	0.1	0.2
AW8-1J	0.24 0.76	0.046	0.04	0.018	-0.00	-0.01
S16 XbaI	0.67 0.33	1.38	1.37	1.30	1.19	0.91
S13 Taq I	0.65 0.35	1.77	1.75	1.63	1.46	1.1
S13 Xba/EcoRI	0.34 0.66	1.26	1.23	1.127	0.99	0.71
S13 Taq 2	0.90 0.10	0.0	0.0	0.0	0.0	0.0
S80 (AC)n	0.02 0.01 0.84 0.13	0.0	0.0	0.0	0.0	0.0
S52 Hind III	0.48 0.52	0.10	0.1	0.08	-0.14	-0.12
S52 Bgl II	0.80 0.20	0.19	0.18	0.16	0.125	0.07
S1 Msp I	0.35 0.65	-0.27	-0.26	-0.21	-0.16	-0.8
S1 BamHI	0.33 0.67	1.99	1.96	1.85	1.69	1.3
S11 Taq I	0.32 0.68	-.45	-.42	-3.4	-0.25	-0.13
S11 EcoRI	0.17 0.83	-.45	-.43	-.35	-0.26	-0.13
βAPP Bgl II	0.99 0.01	-.07	-0.07	-.05	-0.04	-0.02
βAPP Eco RI	0.95 0.05	2.61	2.57	2.39	2.15	1.6
***βAPP* BclI**	**0.999 0.001**	**3.41**	**3.35**	**3.1**	**2.79**	**2.13**
S17 Bgl II	0.57 0.43	0.43	0.48	0.58	0.61	0.51
D21S156	see below	-6.31	-4.29	-2.73	-1.76	-0.77
D21S167	see below	-8.02	-3.44	-1.88	-1.17	-0.49

Bold print shows the linkage analysis of the *βAPP* BclI polymorphism generated by the presence of the exon 17 mutation.

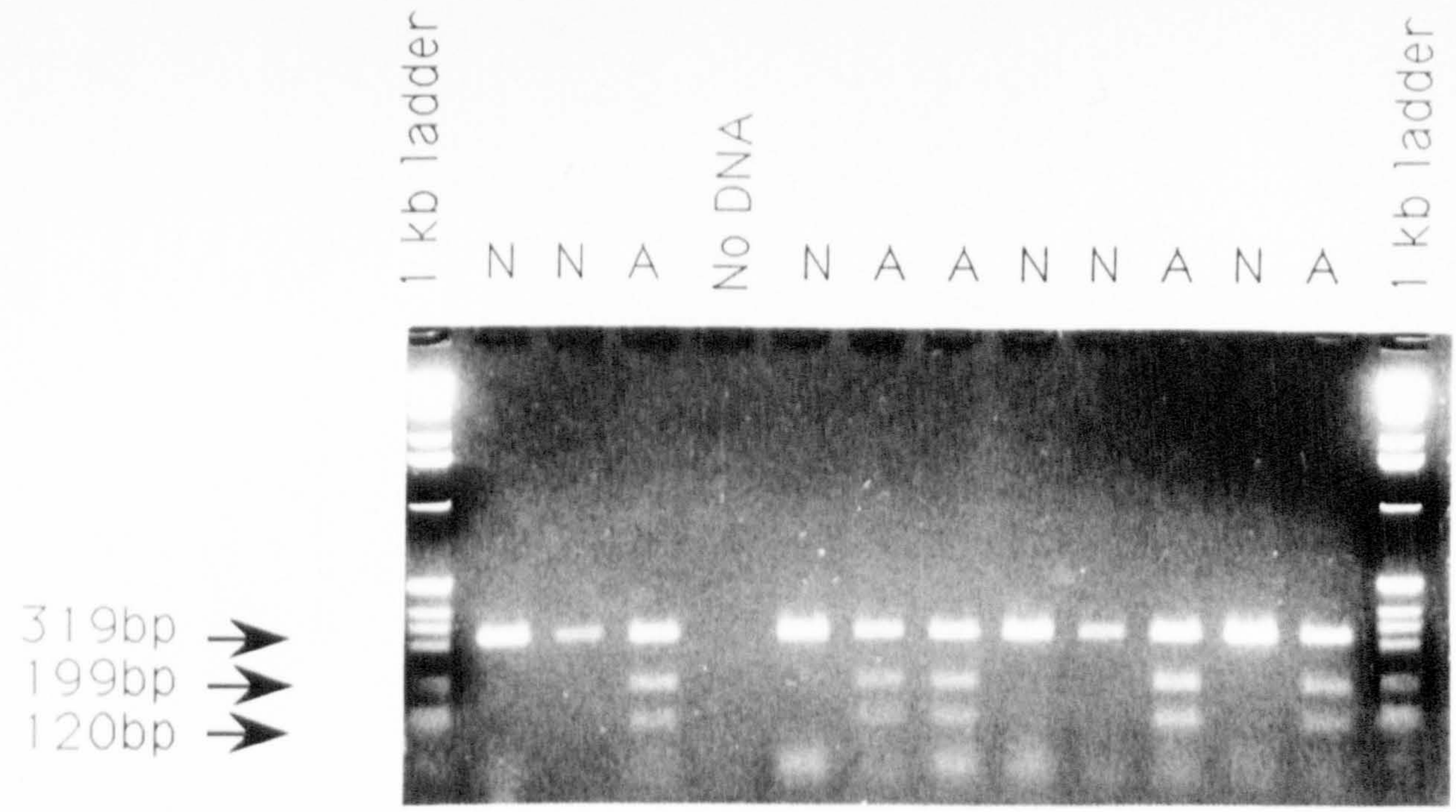


Figure 1. *BclI* digests of the exon 17 PCR product from unaffected (N) and affected (A) individuals in an early onset AD family. *BclI* digests were carried out at 50°C for 2-4 h then electrophoresed in 3% agarose and stained with ethidium bromide. Size markers (indicated in base pairs) are shown in the outside tracks of the gel.

A subsequent analysis of the other exons and the promoter region of *ßAPP* in this family (Fidani *et al.*, 1992) revealed no other mutations which further suggests the substitution described has a functional significance and is not a harmless polymorphism. The subsequent detection of a second family with this mutation and of other families with this mutation strongly supports this contention.

The fact that this mutation was found in only one other early onset family sample at the time of the initial screen suggested either allelic heterogeneity or genetic (locus) heterogeneity might operate in early onset familial disease. This supported a similar view emerging from the linkage analysis of a large number of early onset families, also suggesting genetic heterogeneity (St George Hyslop *et al.*, 1990).

The demonstration of heterogeneity in familial AD suggests that many apparent recombinants in previous analyses (Goate *et al.*, 1989) may have resulted from analysis of families without a mutation on chromosome 21 genes. Alternatively, it was feasible that a second chromosome 21 gene causes some cases of familial disease. Given either of these possibilities a logical strategy was to screen individual pedigrees for linkage to *βAPP* and other chromosome 21 markers. These analyses are the subject of Chapters 5-8.

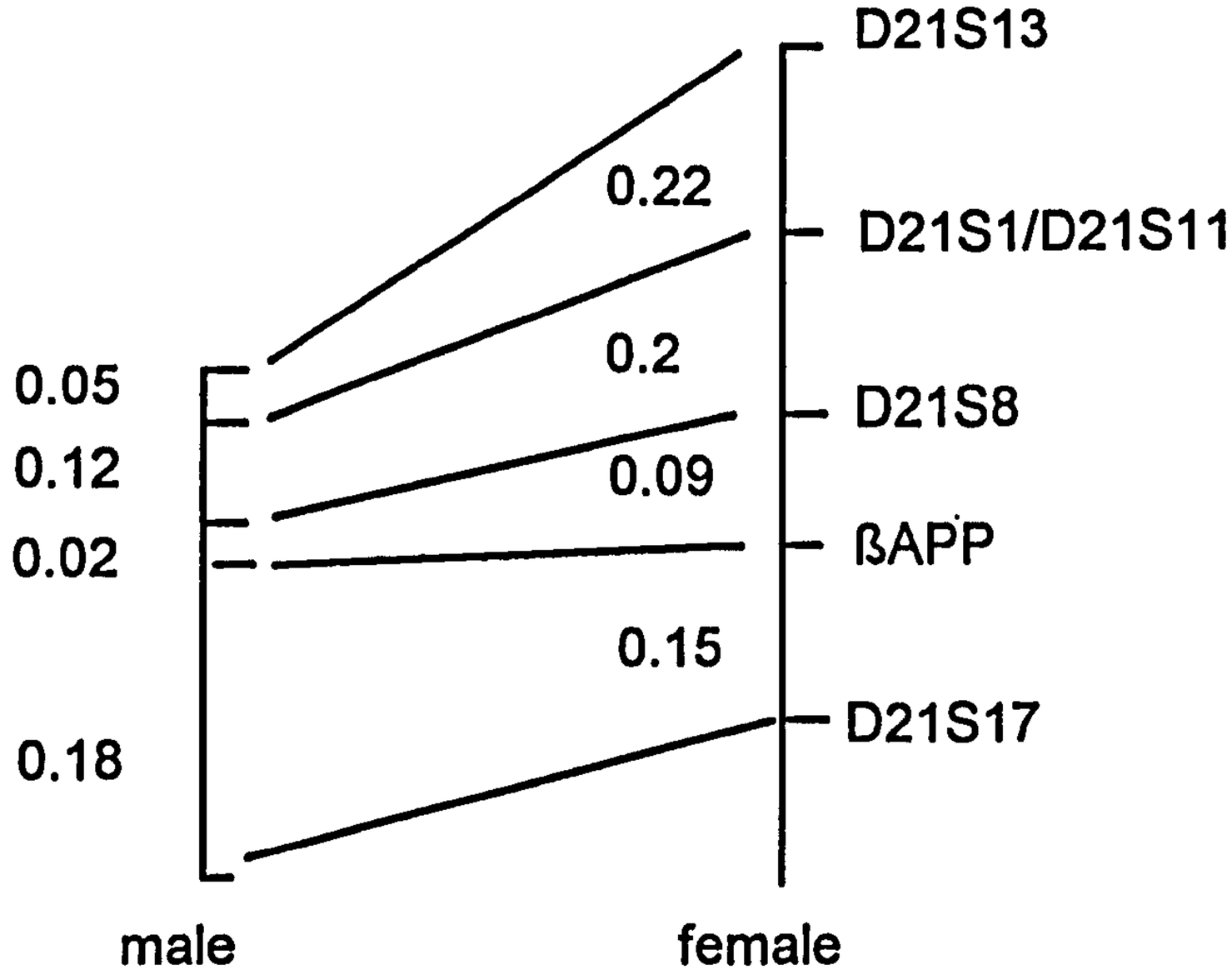


Figure 2. A genetic map of chromosome 21 around the *βAPP* locus. Distances are in centiMorgans.

CHAPTER 4

Clinical Features of Alzheimer's Disease in Pedigrees with the Codon 717 Val→Ile Mutation in the ß-Amyloid Precursor Protein Gene

4.1 Abstract

There are no prominent and reliable clinical features which differentiate between early and late onset disease, familial or sporadic, although some, such as myoclonus have been suggested (discussed in Chapter 1). The definition of clinico-genetic correlates would be advantageous for several reasons; for researchers seeking aetiologically homogeneous subgroups; to provide clues to pathogenic mechanisms; to aid in the differential diagnosis of early onset dementias and provide useful adjuncts to genetic counselling. This chapter examines the clinical features of 16 cases of early onset Alzheimer's disease (AD) with the Val→Ile mutation. This subtype of the disease fulfills NINCDS criteria for probabale or definite AD and in this limited sample no single clinical feature demarcates this form of the disease. However, several features previuosly noted to occur in early onset familial disease also occur commonly in these cases.

4.2 Introduction

The genetic analysis of one early onset pedigree (F23) described in the last chapter resulted in the detection of a point mutation within the ß-amyloid precursor protein (ßAPP) gene at codon 717 predicting a valine to isoleucine substitution in the ßAPP product. Subsequent screening revealed four other pedigrees, detailed in this study, in which this mutation co-segregates with AD (Hardy *et al.*, 1991; Naruse *et al.*, 1991; Yoshioka *et al.*, 1991). In addition, one other pedigree (Tor3) with this mutation has been described (Karlinsky *et al.*, 1992) with detailed clinical, neuropsychological and neuropathological data. Tor3 is discussed below in comparison to the findings in the families in this study. The five families examined here with the mutation were identified in Britain (1 family), the United States (1 family) and Japan (3 families). The mutation has not been reported in the general population of any of these countries (Hardy *et al.*, 1991; van Duijn *et al.*, 1991a; Chartier Harlin *et al.*, 1991a; Naruse *et al.*, 1991). On this basis alone it seems most likely that this mutation is pathogenic. In all cases of these mutations there is complete co-segregation of the *βAPP* mutation with early onset AD, providing statistical evidence that these mutations are pathogenic. The clinical features and limited neuropathology of AD in these families with the *βAPP* 717 Val→Ile mutation are presented.

4.3 Methods

4.3.1 Clinical Assessments

British Sample. Family F23 was assessed in accordance with the schedule described in Chapter 2, Materials and Methods. The pedigree structure is shown in Figure 1a.

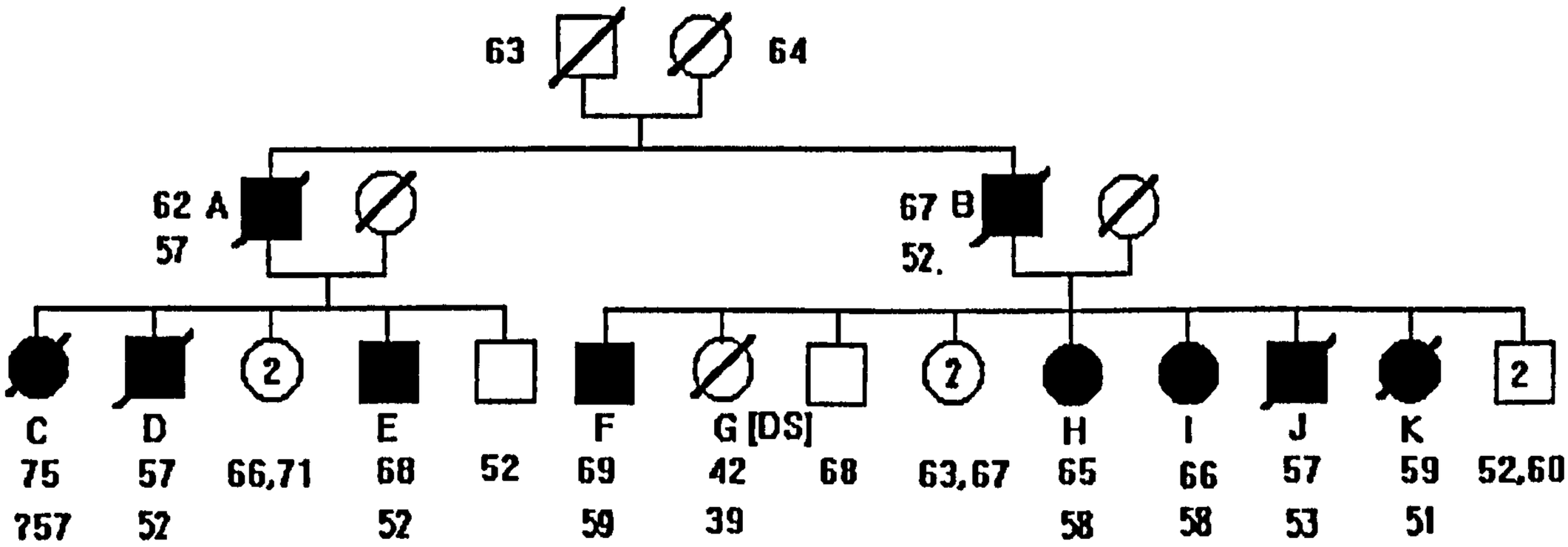


Figure 1a
Family 23

Japanese Sample. (Osaka cases, Figure 1b; Niigata cases, Figures 1c and 1d).

The Osaka cases L and M were subject to non-research clinical assessment which included psychiatric, neurological and minimal psychometric (mini-mental state) evaluation. CT and SPECT imaging was performed. Of the Niigata cases, case O was subject only to neurological examination but case P was comprehensively assessed. The evaluation for case P included the WAIS and MMSE. Additional psychometry evaluating dysphasia, praxis, gnosis, writing and reading skills was employed.

U.S. Sample. Family 372 (Figure 1e) was identified from a genetic linkage study of AD as one which was likely to have a lesion on chromosome 21. The recruitment and assessment (described elsewhere, (Pericak-Vance *et al.*, 1988) ensured a diagnosis of NINCDS probable AD or definite AD in the two autopsied individuals. The ascertainment protocol and clinical assessment methods do not differ significantly from those of the British sample.

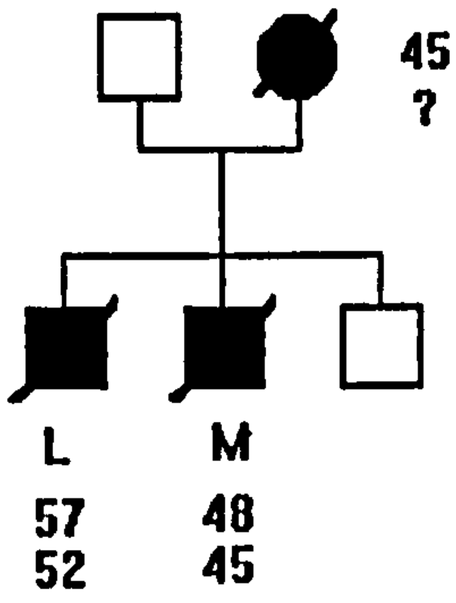


Figure 1b
Osaka family

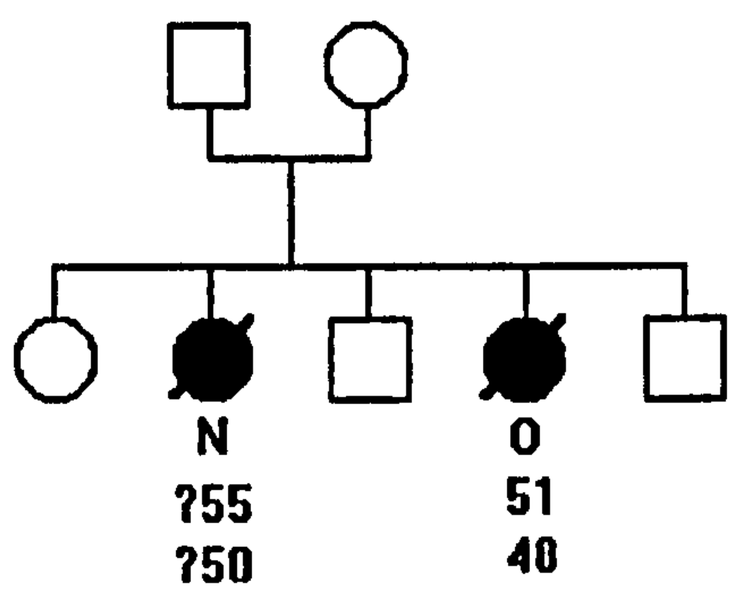


Figure 1c
Niigata: family 1

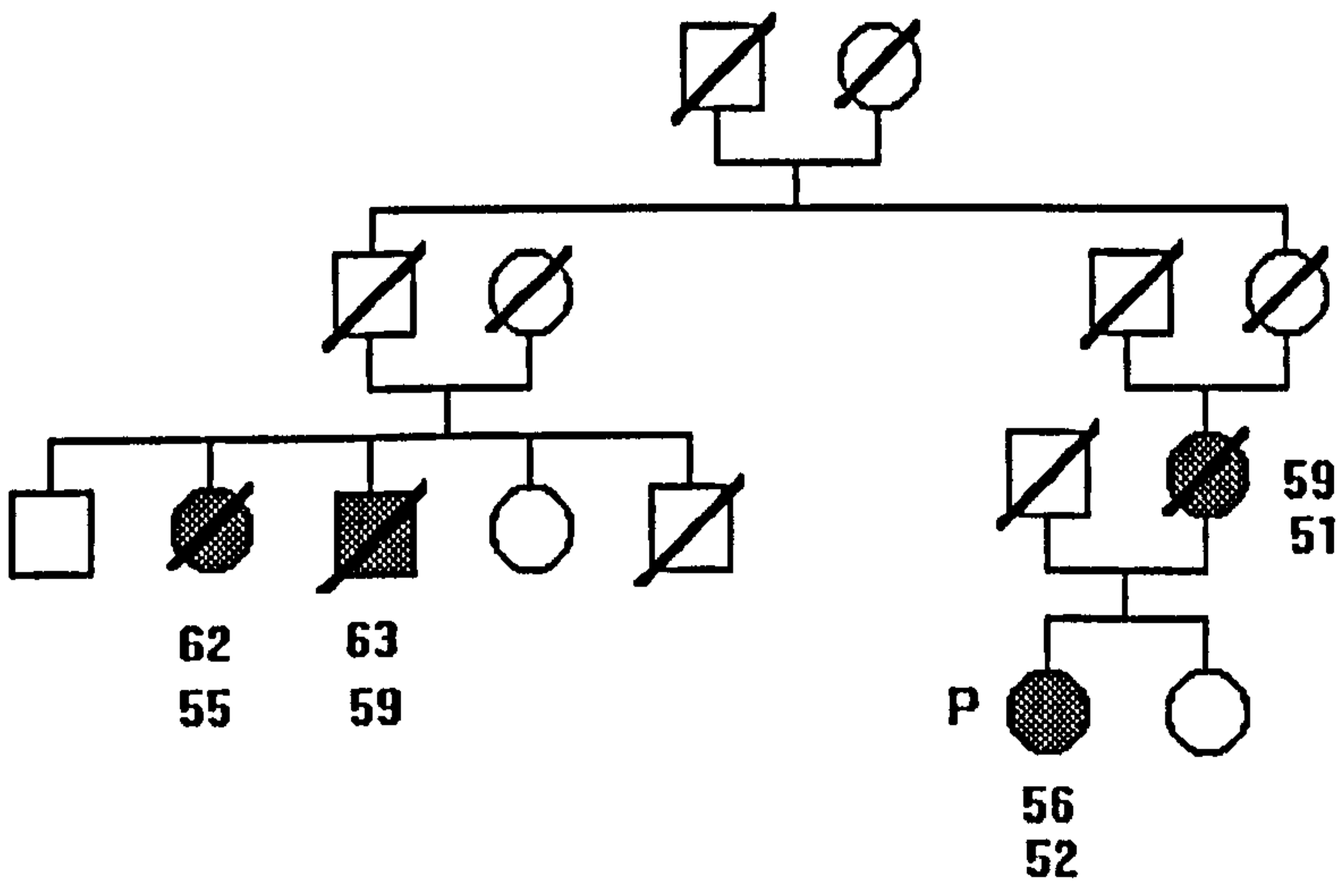


Figure 1d
Niigata: family 2

4.3.2 Neuropathology Methods

British Sample. Blocks of tissue were taken from the frontal, temporal, parietal and occipital lobes, from the basal ganglia and the nucleus basalis of Meynert, from the cerebellar vermis and hemisphere, midbrain (two levels), pons (four levels), and medulla oblongata. Sections were stained with haematoxylin and eosin, luxol fast blue and cresyl violet and congo red and impregnated with silver according to Marshland and Glees (Marshland *et al.*, 1954), and Gallyas (Gallyas, 1971). Immunocytochemistry using antibodies against amino acids 12-28 of the A4 protein (provided by Prof. B.H. Anderton, Department of Neuroscience, Institute of Psychiatry, London, UK), ubiquitin and GFAP (both DAKO) was carried out using the avidin-biotin method.

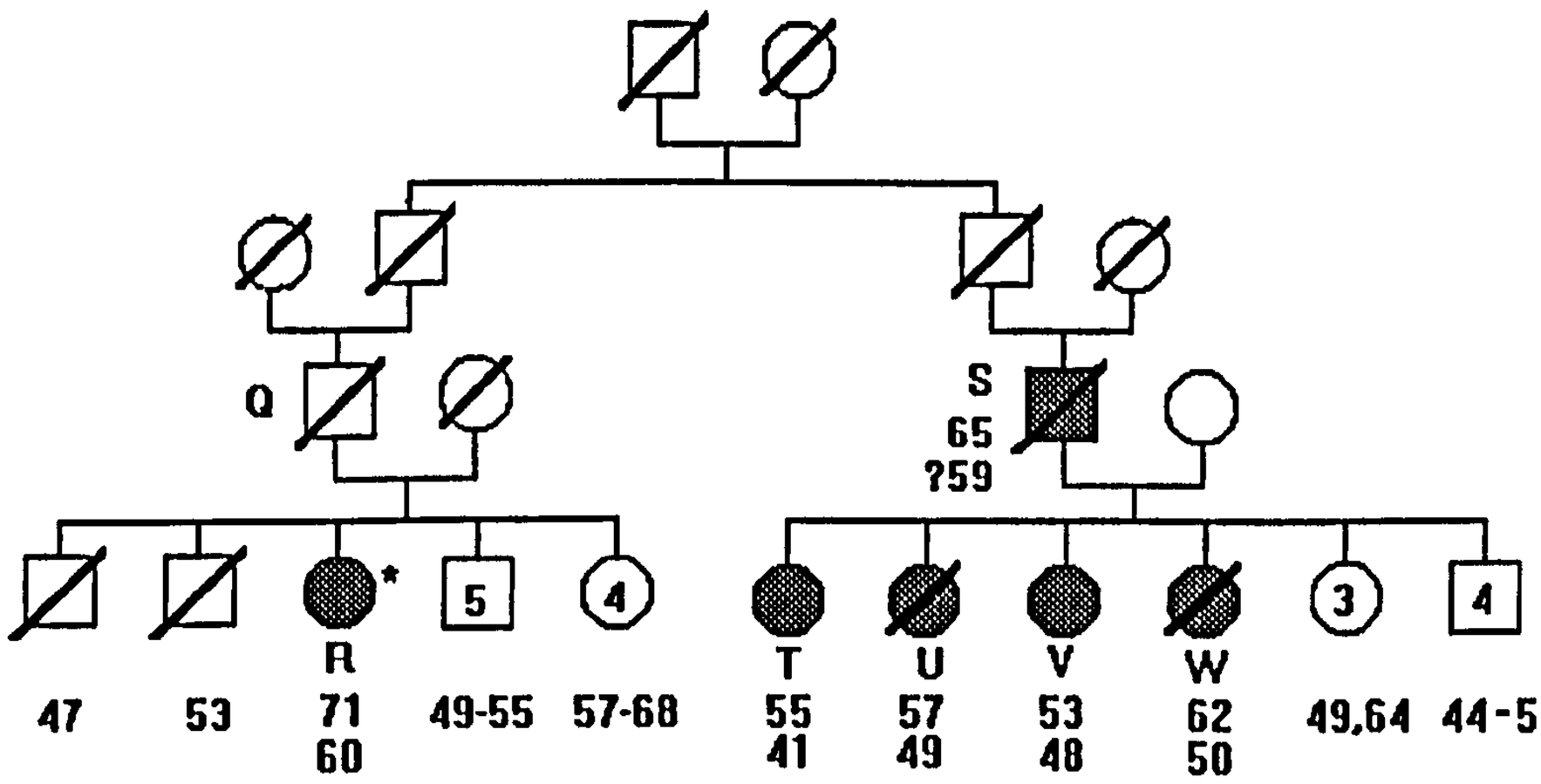


Figure 1e Family 372

Japanese Sample. Blocks of tissue from the frontal, parietal, temporal and occipital cortices, and (in one case) the corpus striatum. Sections were stained with haematoxylin and eosin, luxol fast blue and congo red or thioflavin S.

U.S. Sample. Blocks of tissue were taken from the frontal, temporal, parietal, occipital lobes, from the basal ganglia and the nucleus basalis of Meynert, from the amygdala (one case), from the hippocampal formation and adjacent structures, and from the midbrain. Sections were stained with haematoxylin and eosin, luxol fast blue, microwave King silver stain, Hedreen's quick silver method and either congo red or thioflavin S.

Immunocytochemistry using antibodies against the A4 protein (kindly provided by Dr E. Koo, Harvard University), ubiquitin (Chemicon, Temecula, NY, USA) and intraneuronal and extraneuronal neurofibrillary tangles (SMI 34 and SMI 310, Sternberger Monoclonals Inc., Baltimore, MD, USA) was performed using the avidin-biotin method.

4.4 Results and Discussion

4.4.1 Diagnosis

The affected individuals with this mutation conform to the clinical and neuropathological criteria for either probable or definite AD (McKhann *et al.*, 1984). The phenotype described here, is then the first molecularly defined type of AD. Detailed case reports are given in Appendix 4A.

4.4.2 General Clinical Picture (Tables 1a, 1b and 1c)

In all affected cases there was a gradual onset and smooth progression with no dramatic changes until late in the disease. There is consistency in the pattern of symptoms between cases. Initially, there is a period of cognitive decline beginning with loss of memory for recently acquired information accompanied by loss of concentration and a failure of performance at visuospatial tasks. This progresses to include disorientation for time, loss of memory for remote information, loss of language function with a marked decrease in word fluency and impaired verbal abstract thinking. Dyscalculia was noted as a prominent early feature. As the disease progresses, loss of insight occurs in all cases. Later, there is visual agnosia, aphasia and apraxia with accompanying loss of self-care and hygiene. Later still, there is motor disturbance in the form of hypertonicity, myoclonus, seizures and gait disturbance and the release of primitive reflexes. There is also loss of mobility (with increased flexor tone) and extreme weight loss. Towards the end of the disease process, cerebro-vascular accidents also occur. An interesting feature of the disease is the frequent premorbid complaints which often result in repeated presentation to a general practitioner. In some cases, the presenting complaint is localising (anosmia, Case K) but in others it is not (vertigo, Case J). Complaints by relatives of personality change (preceding cognitive change by several years) are common (Cases C, E, K). Apathy, withdrawal and other affective symptoms are frequent in this premorbid stage and after the onset of cognitive change but do not persist as the illness progresses. Of note also is the occurrence of thyroid disease coming to attention after the onset of the cognitive changes and not occurring in non-affected individuals (Cases F, K).

Table 1a. Clinical Features of F23

ID	Age of Onset	Precognitive Features	* Early Features	Late Features	Psychiatric Features	Tone	Myoclonus	Other
A	57		Dyscalculia, Dyspraxia		Depressive features +			CVA
D	52		New skill acquisition					
E	52				Depressive features +			
F	59					+	+	CVA, Thyroid disease
H	58		Dyscalculia, Dyspraxia					
I	58	None	New skill acquisition		Depressive features +			
J	53	Vertigo		Dysgraphia Dysphasia,	Depressive features ++, Aggression, Somatic complaints			
K	51	Anosmia, LOC, Headaches				+	+	CVA, Thyroid disease, Seizures

* Early = < 3 years post onset
LOC = loss of consciousness
CVA = cerebrovascular accident (clinically diagnosed)

Table 1b. Clinical Features of Japanese Cases

Family	ID	Age of Onset	Early Features	Late Features	Psychiatric Features	Myoclonus	Seizure
Os	L	52	Prosopagnosia Dyscalculia Language dysfunction	Echolalia		+	
	M	45	Comprehension Dyscalculia	Echolalia	Paranoid Ideation Aggression	+	+
Nii 1	O	48	Disorientation			+	+
Nii 2	P	52	Constructional apraxia		Depressive features +		

Os = Osaka
Nii = Niigata

Table 1c. Clinical Features of F372

ID	Age of Onset	Pre-cognitive Features	Early Features	Late Features	Psychiatric Features	Tone	Myoclonus	Seizure	Other
R*	60		Expressive dysphasia Dyscalculia	Aphasia Apraxia					
T	41		Parkinsonism	Proso-pagnosia	Paranoid idcation	+		+	
U	49		Dyscalculia				+	+	
V	48		Dyscalculia			+			
W	50	Syncope Headache	Dyscalculia		Depressive features +	+	+		CVA

*Non-APP mutation
CVA = cerebrovascular accident (clinically diagnosed)

4.4.3 The Occurrence of Specific Clinical Features

Pre-cognitive features occurred in three cases. These cases presented with unexplained disequilibrium and loss of consiousness. Only one of these cases went on to have documented seizures making unlikely that these early disturbances reflected early electroencephalic abnormalities.

Amnesia. All of the 16 cases with the Val→Ile mutation detailed here presented with amnesia as the first cognitive feature. This is the common presenting cognitive feature of many other early onset familial AD cases and has been documented in large AD pedigrees (Karlinsky *et al.*, 1992; Karlinsky *et al.*, 1991; Sadovnick *et al.*, 1988). Memory loss for recently acquired information is generally regarded as the cardinal neuropsychological feature of AD. This is completely born out in the mutation cases examined here and in the literature generally. In fact, if memory loss is not a prominent and early feature then competing diagnoses should be strongly considered.

Apraxia and agnosia were also prominent in all cases generally within four years of onset.

Dyscalculia was a prominent early feature in 7/16 cases. Two other studies of early onset disease noted dyscalculia as an early feature in some cases (Karlinsky *et al.*, 1991; Nee *et al.*, 1983).

Myoclonus as discussed (Chapter1) has been nominated as a demarcating feature of early onset disease and has previously been noted in familial disease (Frommelt *et al.*, 1991; Martin *et al.*, 1991; Bird *et al.*, 1989a; Lowenberg, Waggoner, 1934; Feldman *et al.*, 1963; Cook *et al.*, 1979). Myoclonus was a prominent feature in the Val→Ile cases occurring in 7/16 of them.

Seizures have been noted before in early onset familial disease (Fukutani *et al.*, 1989; Lowenberg, Waggoner, 1934; Cook *et al.*, 1979; Feldman *et al.*, 1963; Sadovnick *et al.*, 1988; Bird *et al.*, 1989b; Martin *et al.*, 1991) and have been associated with myoclonus as in one study where the largest proportion of those patients who go on to develop seizures had early myoclonus (Clark *et al.*, 1991). Another study found a higher prevalence of seizures in familial disease in those cases where myoclonic jerks also occurred (Frommelt *et al.*, 1991). Clearly though they can and often do occur independently (Romanelli *et al.*, 1990). Five of sixteen cases had seizures, generally late in the course of the disease. Of these, all but one had had myoclonus earlier in the course of the disease.

Psychiatric features were common especially depressive symptoms (6/16). Aggressive personality change sometime occurred (2/16). Depression has been infrequently noted before in early familial AD cases (Bird *et al.*, 1989b). However, frank depression and depressive symptoms are common in AD in general (Burns *et al.*, 1990) and it is likely that these features have simply not been sought in some studies.

Clinical stroke occurred in 25% of the cases reported here. Stroke cases are not reported frequently in the literature of AD - probably because prominent early stroke precludes the diagnosis in life. However, approximately 20% of all AD cases have arteriosclerotic changes at autopsy severe enough to cause dementia (Tomlinson *et al.*, 1970). Few of the published studies of early onset familial AD include such data (Bird *et al.*, 1989a).

Thyroid disease has been previously noted in association with AD (Heston *et al.*, 1981) although the mechanism underlying this phenomena has not been elucidated. The temporal relationship of the onset of dementia and thyroid disease is imporant. Clearly, dementia following the occurrence of thyroid disease might be an example of "myxoedematous madness" (Asher, 1949), the most important metabolic differential diagnosis of AD.

The occurrence of phenocopies (Case R, F372). In all members of F23 and the small Japanese families there is complete co-segregation of the *βAPP*717 mutation with the disease or with some of those at risk for the disease. However, genetic analysis of pedigrees with common diseases such as AD is complicated by phenocopies (examples of the phenotype not

caused by the major gene). This problem is illustrated here by the occurrence of Case R in F372 who does not have a mutation in the *βAPP* gene. In this case, the age of onset is greater (60 vs mean of 49.4) than for other family members. In the absence of the identification of a pathogenic mutation, Case R would have been scored as a "recombinant" with *βAPP*. Under these conditions, genetic analysis would have incorrectly suggested that the disease locus was distinct from the *βAPP* gene. Apart from the age of onset being higher than for other family members, there is no clinical data to suggest that this individual has a different aetiology for the disease than her cousins. In fact, she shares the prominent early feature of dyscalculia with them. A further affected family member in a more distant branch of the family in F372 (not shown in pedigree figure) does not have the mutated *βAPP* gene. This person too has an age of onset considerably higher than the mean age of the *βAPP* 717 Val→Ile carrying members.

4.4.4 Age at Onset and Age at Death (Table 2)

Familial AD cases with a large range of onset have been screened for the *βAPP*717 Val→Ile mutation. All five families with the mutation have broadly similar ages of onset despite their differing genetic backgrounds and geographical locations. These are (years) 54.9 in F23 (SE = 3.1, n = 10), 49.4 in F372 (SE = 6.4, n = 5) and 51.5 for the Japanese families combined (SE = 4.2, n = 8). The mean age of onset for all individuals with the mutation is 52.7 years (SE = 4.7). Thus, the age of onset for cases with the mutation is towards the upper end of the conventional split between early and late onset AD. The rate of the disease progression is variable even within a single family as some individuals appear to have a protracted course (Case E). Figure 2 shows that for two individuals, K and I, there is a very similar occurence of major symprtoms, in contrast to Case E where a prolonged period of memory loss was finally accompanied by other cognitive change. This suggests that in contrast to the age of onset which seems highly determined by the single genetic lesion (van Duijn *et al.*, 1991c), progression is influenced by other factors. In F23 and F372 the mean survival from first cognitive sign to death was 9.2 and 8.7 years respectively. In the case of the Japanese families mean survival was 5 years. The reduced survival times in the Japanese sample may reflect a greater rate of progression, later diagnosis or differences in nursing practice.

4.4.5 General Pathological Features, Pathogenesis and Occurrence of Lewy Bodies

The neuropathology of the cases are generally typical of very severe AD with numerous plaques and tangles (Figure 3) with modest congophilic angiopathy (Table 3). The occurrence of cortical Lewy bodies in Case K is of particular interest. In these families there is a defined cause of the disease. Thus, despite the identity of the genetic mutation

responsible for the development of the phenotype, there are important differences in the pathologic features at autopsy. The findings emphasise that specific neuropathology in AD, does not define a specific molecular aetiology a relationship previously noted in some cases of prion dementia (Collinge *et al.*, 1990). Lewy bodies and Aß deposition can occur together in dementing illness (Lennox *et al.*, 1989; Dickson *et al.*, 1989; Hansen *et al.*, 1990; Gibb, Lees, 1989; Perry *et al.*, 1990; Hansen *et al.*, 1989; Bierer *et al.*, 1990; Yamamoto, Imai, 1988) and the relationshhip between the two is obscure. However, in these special cases of AD (with *ßAPP* mutations) when Lewy bodies occur with Aß deposition, they, like tangles, are secondary to this deposition. While persons with the mutation develop neuropathology typical of AD there may be additional uncharacteristic features but there are no pathological findings which are diagnostic for this form of the disease.

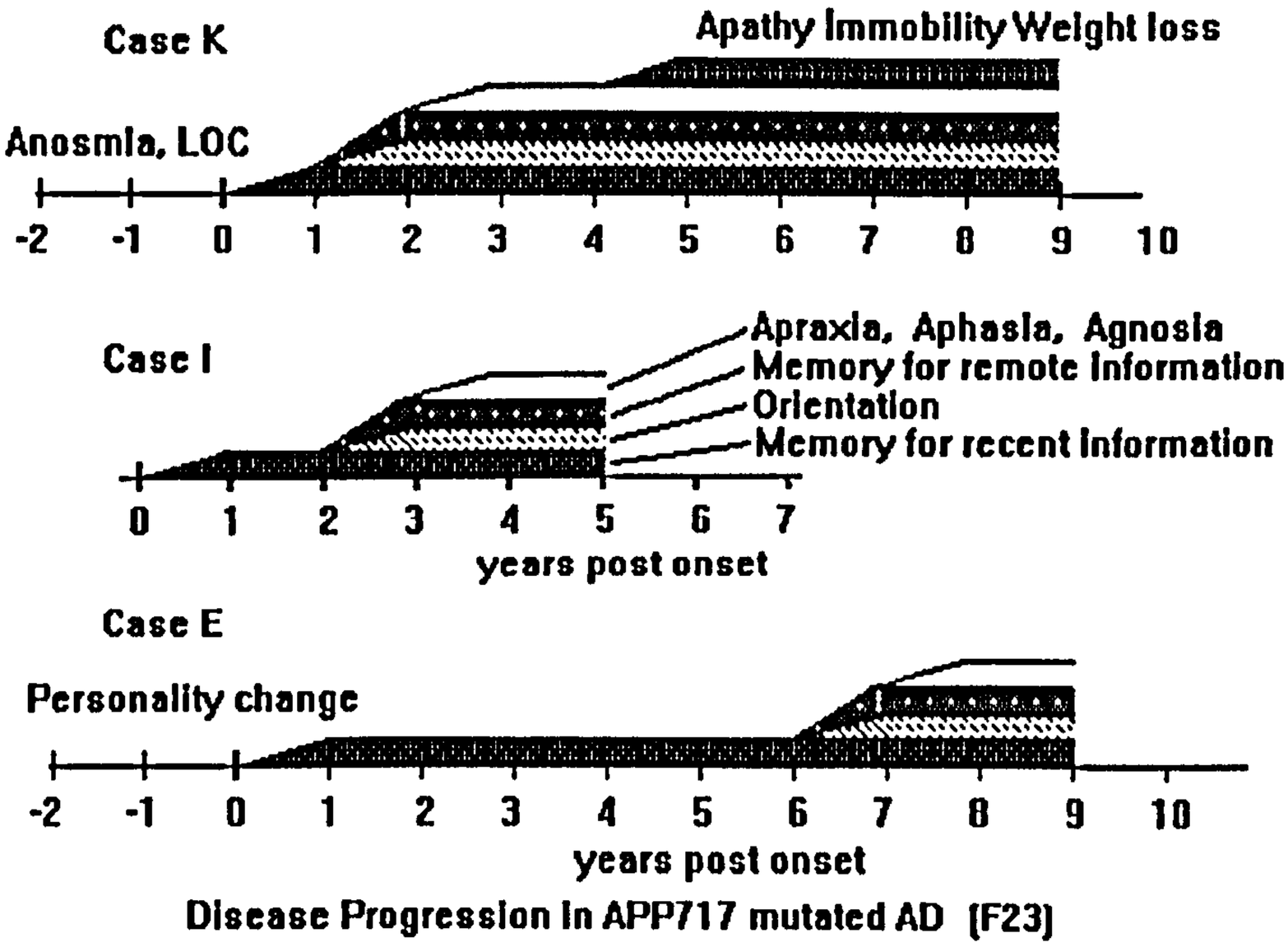


Figure 2. The Rate of Progression of AD in 3 Family Members from F23.

Table 2. Age at Onset, Death and Survival (Years)

	Age at Onset			Age at Death			Survival		
	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range
F23	54.9	3.1	51-59	62.8	7.1	57-75	9.2	5.9	4-18
Jap	51.5	4.2	51-59	56.4	5.5	48-63	5.0	1.9	3-8
F372	49.4	6.4	41-59	61.3	4.0	57-65	8.7	3.1	6-12

Table 3. Neuropathological Features of Autopsy Cases

Family	372	372	F23	OSAKA	NIIGATA Family 1
Case	W	U	K	L	O
Brain weight (gm)	–	–	900	1360	1060
Frontal	>1OOP/xlO 19T/x20	>1OOP/xlO 17T/x20	55P/mm 15T/MM	7P/x20 8T/x20	P+++ T+++
Paricta1	>1OOP/xlO 16T/x20	>100P/x10 18Tx20	23P/mm 8T/mm	8P/x20 9T/x20	P+++
Temporal	>100P/x10 3T/x20	94P/x10 5T/x20	28P/mm 20T/mm LB++	7P/x20	P+++ T+++
Occipital	>100P/x10 24T/x20	–	36P/mm 6T/mm	6P/x20 5T/x20	P+++
Hippocampus CAI	13P/xlO 63T/x20	5OP/x1O 1OOT/x20	-	1OP/x10	T++ GVD
CA2	GVD 1/x40 3T/20	6P/x1O 8T/x20	–	–	–
CA3	8P/x1O 22T/x20	24P/x1O 8T/x20	–	–	–
CA4	26P/x10 13T/x20 GVD 1/x40	20P/x10 17T/x20 GVD 1/x40	–	–	–
Insula	>1OOP/xlO 3T/x20 ?LB	73P/x1O 1T/x20	–	–	–
N basalis of Meynert	47T/x10	–	–	–	–
Amygdala	>1OOP/x1 30T/x20	>1OOP/x10 44T/x20	–	–	–
Corpus striatum	O	O	–	–	P+ T+
Thalamus	O	–	–	–	–
Hypothalamus	O	O	–	–	–
Substantia nigra	LB1	O	–	LB1	–
Cerebellum	–	–	–	–	AA
LB	Y	–	Cortical	Y	N

Key: /x10 = counts per x10 field
P = Plaque
T = Tangle
GVD = Granulovacuolar degeneration
LB = Lewy body
O = Not detected
- = No information/not examined
AA = Amyloid angiopathy

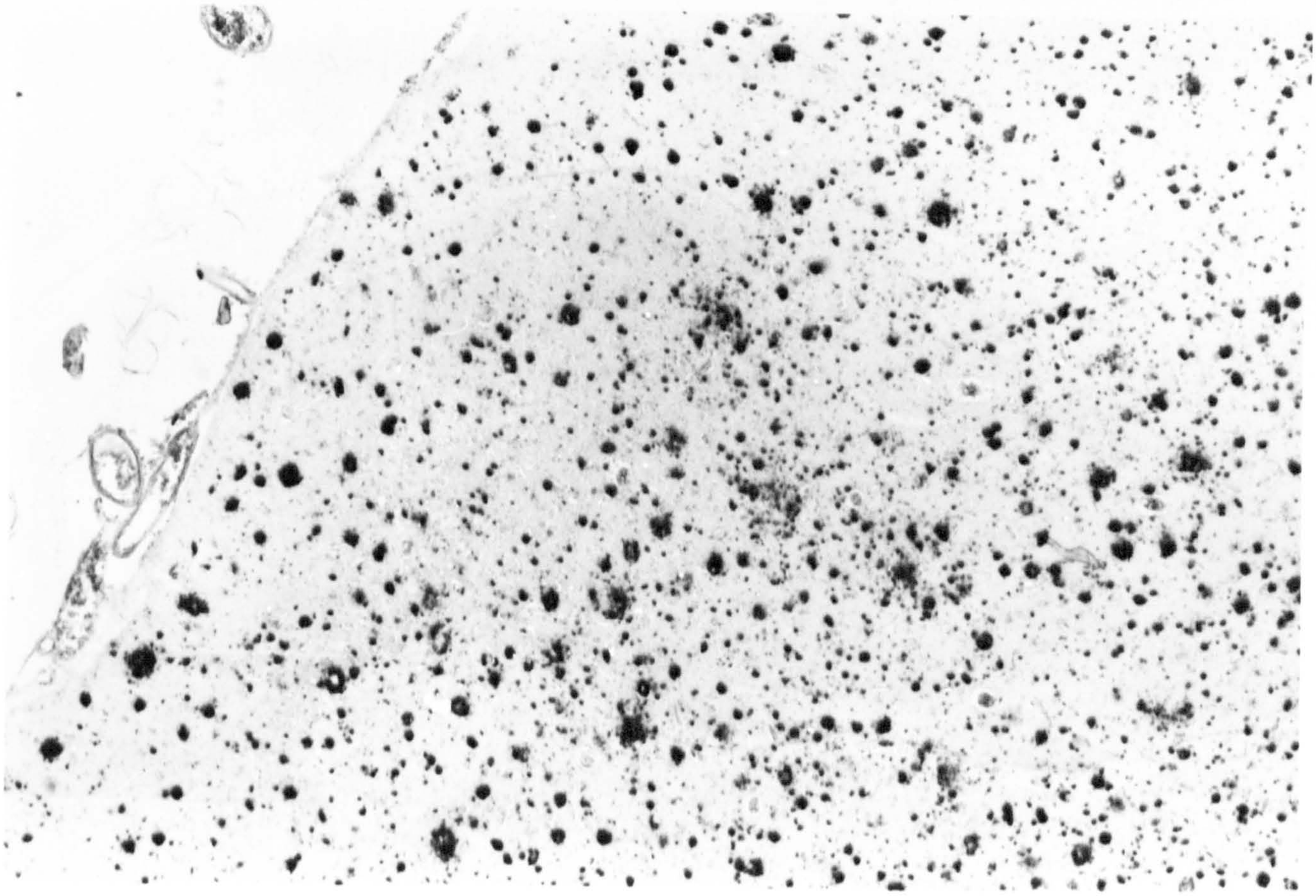


Figure 3a. ß-Amyloid Protein Deposits in Frontal Cortex in Case K, Family 23 (x40).

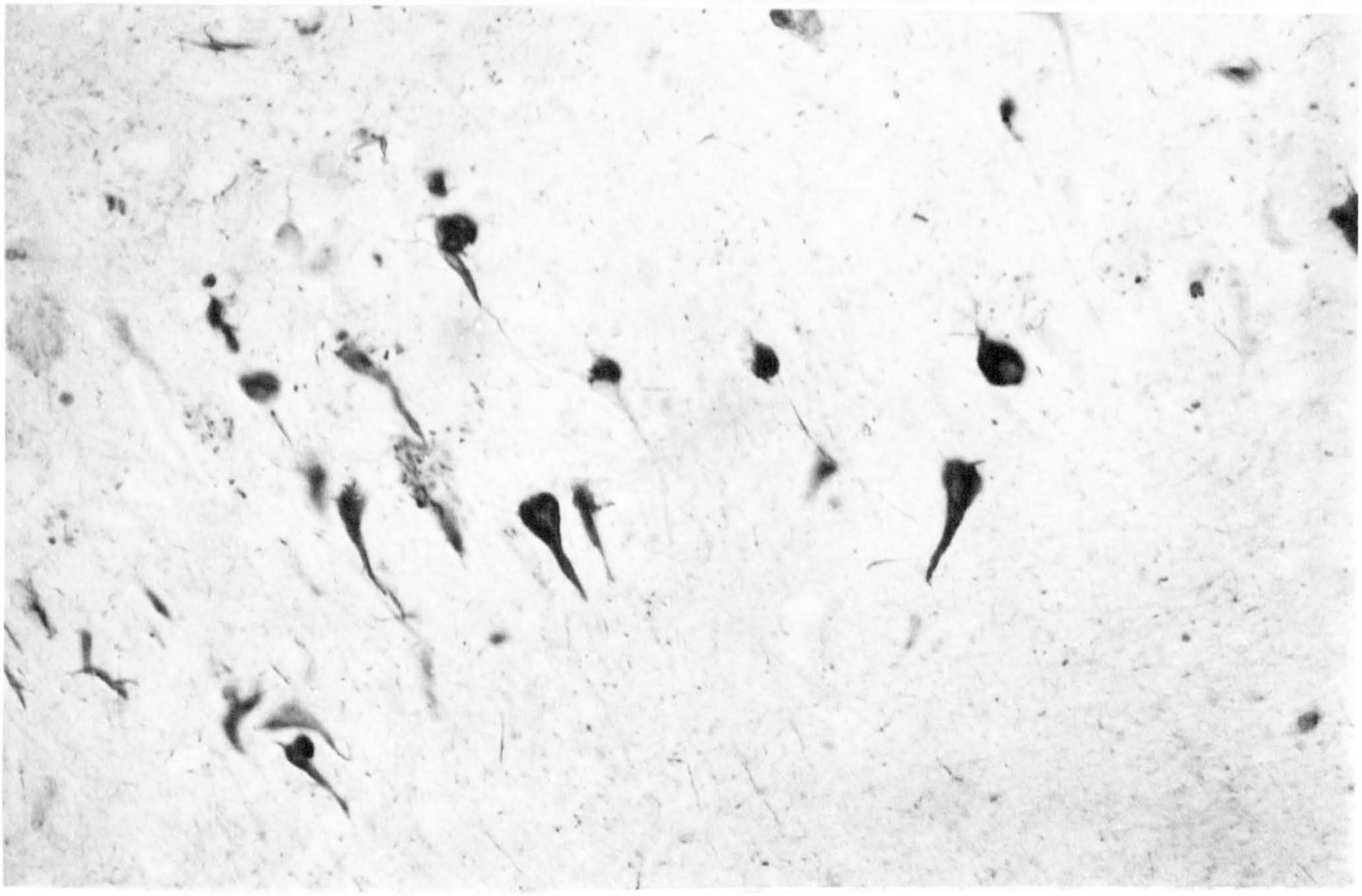


Figure 3b. Ubiquinated Tangles in Hippocampus (x250) in Case K, Family 23.

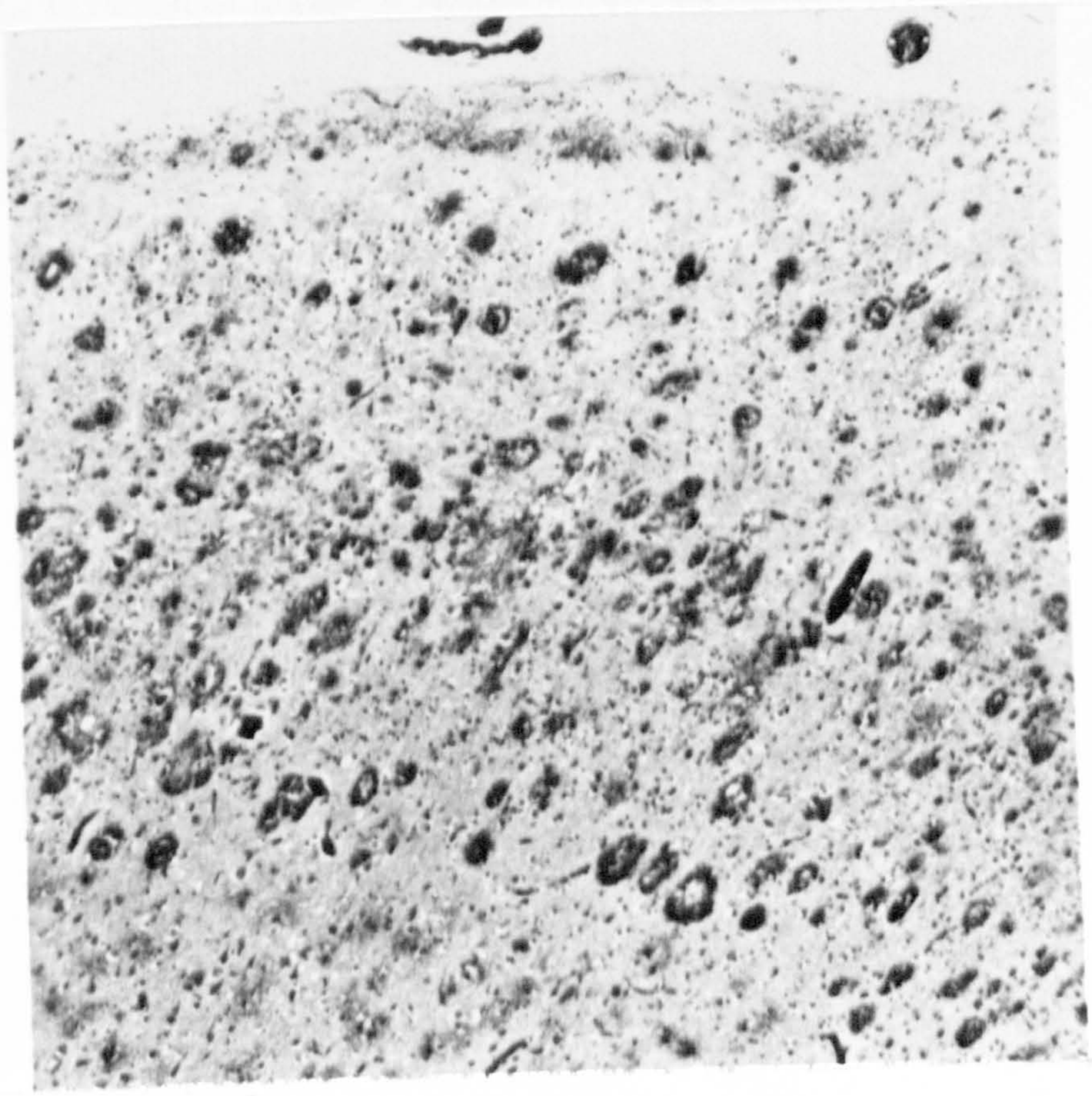


Figure 3c. Silver-Stained Plaque in Superior Temporal Gyrus (x52) in Case U Family 372.

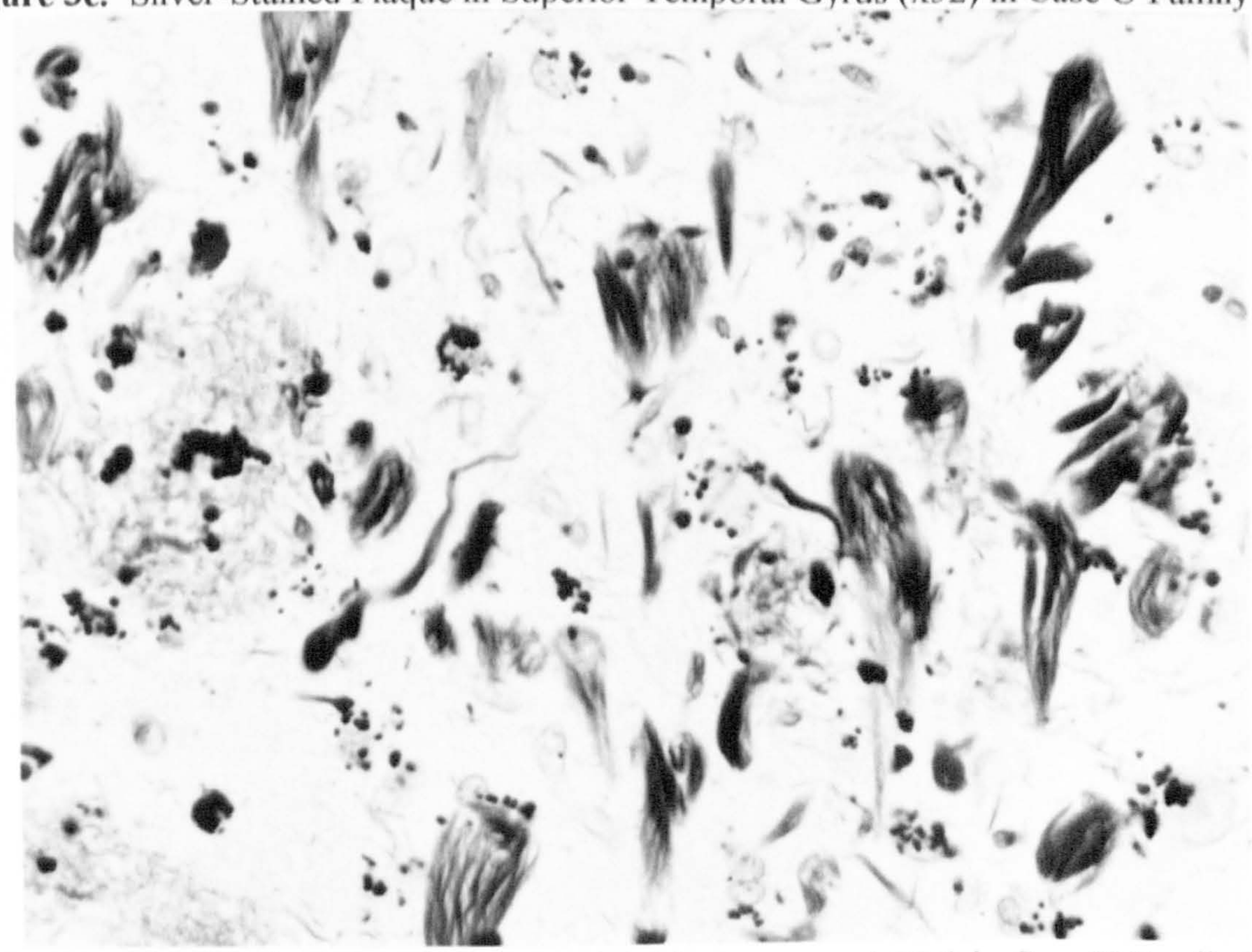


Figure 3d. Silver-Stained Tangles in Area C1 Hippocampus (x325) in Case U Family 372.

4.4.6 Comparison with Tor3

The recent description (Karlinsky *et al.*, 1992; Karlinsky *et al.*, 1991) of the disease onset and progress in a family recruited for molecular and genetic studies in Toronto (Tor3) is most useful for comparison with the families described above - all having the same mutation. This family is known to have emigrated from the British Isles 200 years ago and therefore could be related to either F23 or F372, although there is no genetic or geneologic evidence for this. The mean age of onset for Tor3 is 47.6 years (SE 3.0 years, N = 11) which is significantly younger than F23 ($t = 5.4$, $DF = 19$, $p<0.001$) but not significantly different from the mutation carrying affected individuals in F372 ($t = 0.76$, $DF = 14$, $p<0.5$). Age of onset tends to be constant between even distantly related individuals in early onset familial AD (Chapter 9, (van Duijn *et al.*, 1991c)) which would suggest that Tor3 is more likely to be related to F372 than F23. For the families described above and Tor3 the first cognitive symptom reported in all cases was memory disturbance. There were no reported pre-cognitive signs in the affected Tor3 cases. Late pyschotic features were noted as were seizures but surprisingly no other neurological features were reported in the Tor3 cases. However, the authors point out that the method they employed to collect retrospective clinical information may have missed these and other features of the disease. For two cases in Tor 3 as with cases in F23 and F372 detailed neuropsychology is available. The Tor3 cases showed a pattern of early memory loss (symptom year 1) deterioration in attention (symptom year 2). Later deficits in concept formation were noted (symptom year 5). There are then no obvious neuropsychological contrasts between the Tor3 cases and the cases described here. Neuropathologically the two Tor3 cases examined as with those described here were generally typical of AD but having quite severe plaque and neurofibrillary tangle formation. No Lewy bodies were described in the cortex or elsewhere in the two Tor3 AD cases. In summary, the Tor3 data concurs with the present conclusions that (i) the clinical features in all cases of the disease with the *βAPP*717 Val→Ile mutation are consistent with the diagnosis of AD; (ii) the specific clinical features although generally typical of AD can vary from case to case even within the same family; (iii) no particular clinical feature aside from age of onset appears to occur with this form of the disease. It remains to be seen whether the pre-cognitive early signs of the disease described in some of the cases above are peculiar to early onset familial AD.

Chapter 4 Appendix 4A

Clinical and Neuropsychological Profiles and Neuropathological Descriptions of Affected Individuals

Family 23 (Cases A→K)

Case A. This man was the father of E and D. He was a hairdresser but started to neglect his business in his late 50's. Cognitive decline was noted in the form of poor memory and dyscalculia. There were periods of tearfulness associated with the loss of complex skills like piano playing. He became immobile and died from a cardiovascular accident. However, there had been no previous medical history of stroke or vascular disease.

Case B. This man was the father of the main sibship. Details of his illness are sketchy but there was cognitive decline from aged 52y and he died in his mid 60's having been immobile and unable to care for himself for many years.

Case C. This woman was the sister of D and E and had a cognitive decline which was protracted and accompanied by insight and preceded by personality change. Exact dating of the age of onset is not possible but it was clear that in her late 50's she had problems remembering short lists and often repeated herself. She died from cardiac disease. The diagnosis of AD cannot be made retrospectively in this case and she was entered onto the linkage analysis as unknown.

Case D. This man was a brother of E. Cognitive decline became apparent when he could not adapt to computerisation at work at the age of 52y. He was subsequently made redundant at the age of 53y when forgetfulness and repetition of phrases were noted. A rapid decline ensued. He was disorientated in time and place at 55y and spoke very little. He no longer recognised his own family at 56y and died the following year from pulmonary embolism by which time he was profoundly demented.

Case E. This man, who is the cousin of cases I and K, had no significant previous medical history. He was of average intelligence and was a storeman and parts manager for most of his working life. He was well known in this job because of his remarkable memory for the code numbers of replacement parts kept in the store. At 52y he began repeating phrases and questions. He was apathetic and withdrawn with poor concentration and there was loss of libido. Since these features are also characteristic of depressive disorder, dating the onset of

the dementia is difficult but certainly by the age of 57y, several family members noted deficiencies in his memory for recent events. Two years later he had to retire, since he was unable to learn to use new equipment in his store.

His first assessment took place at 64y. On examination he was mildly disorientated for time (4/6) but not place (5/5). His language comprehension was normal (8/8) as was his expression (23/25). Verbal fluency was moderate (4/6). His abstract thinking was unimpaired (10/10). Memory for remote information was good (6/7) but despite normal registration (3/3), there was no uncued recall (1/14). Recognition of previously presented visual information was 50% correct (3/6). Attention and concentration were good (7/7). There was no evidence of dyspraxia. His visual perception, and perception of objects from unusual angles were normal and there was no dyscalculia. An overall measure of decline showed that he was moderately demented (Blessed 7/28). His predicted premorbid full-scale IQ was 119 with predicted verbal and performance scores of 119 and 116 respectively. His actual scores were; full-scale 83, verbal 93, performance 72.

At the second assessment at age 66y, he was disorientated for time (1/6) and place (2/5). Language comprehension remained normal (8/8) but expression had declined (9/25). Memory for remote information remained good (6/7). Previously presented information was poorly recognised (2/6). Attention and concentration however remained good (7/7). He had dyspraxia for copying and drawing (3/6) and his abstract thinking had declined (5/10). The Blessed score had deteriorated very little between assessments (8/28). Physical and neurological examination remained normal.

Case F. This man was the first born of twins, the other (female) twin dying at the age of 5y from an infection. She had, until her terminal illness, been physically and mentally well. F had lived unremarkably, achieving average academic results at school and enjoyed thirty years of employment with an insurance company working with figures. His cognitive problems began when at the age of 59 he began to repeat the same questions to his spouse and began to forget recently made appointments at work. A diagnosis of Alzheimer's disease was made. His ability to calculate was still good. Six months later, it was thought prudent that he should stop driving as his concentration was beginning to decline. In the following year he began to lose interest in his hobbies and stopped watching TV and reading the newspapers. In the following year it was clear that his remote memory was deteriorating and as he entered the fourth year of the illness he became dysgraphic. By the end of the next year he was developing agnosia and dyspraxia. A year later his ability to look after himself was so impaired that he required full-time nursing care and was admitted to hospital. On examination he was disorientated for time and place and was aphasic but was able to respond

appropriately to simple commands. He was fully ambulant but had persistent restlessness. Later the same year he suffered a sudden onset right hemiplegia attributed to a cerebro-vascular accident, but later recovered to his previous level of functioning. There were several similar incidents during this phase of hospitalisation. A physical examination five years post onset noted flexion of the right arm with increased tone in all limbs and marked gegenhalten. Reflexes were normal with brisk ankle reflexes and bilateral flexor plantar response. There was myoclonus particularly in the upper limbs. He had an orofacial dyskinesia and a positive grasp reflex. He had gait apraxia.

No cytogenetic abnormalities were detected in this case.

Case G. Down's syndrome. This sibling in the main sibship was born when her mother was 42 years old. She never reached a high level of adaptive functioning but was cared for at home until the last few years of her life. She suffered from severe arthritis which was progressive and limited her mobility. She had no psychiatric disorder until she was 39y when she became uncharacteristically untidy and relinquished her favorite pastime of listening to music. In the following three years she became increasingly dependent on the main carer and was able to do less and less for herself. At 40y she developed grand-mal seizures which became so frequent that she was hospitalised. She suffered increasing loss of function and was finally unable to do anything for herself. She died from the complications of immobility at 42y. This in dividual may have carried none, one or two copies of the mutated gene and would be expected to develop Alzheimer pathology in all cases with a respective decrease in onset age.

Case H. This woman had a normal early life, left school at 14y and was an unskilled factory worker for ten years by which time she had married. She had one daughter and was untroubled by illness all her life. Her husband and relatives had noticed forgetfulness from age 58y. In particular, she forgot recently acquired information, such as the day's agenda and shopping lists. In addition, her concentration became poor and she began to make mistakes with her knitting. Eighteen months after this began she forgot birthdays for the first time and was disorientated in time and in new places. She made mistakes with cooking and was unable to learn how to use a new washing machine. As a consequence her husband began to do the housework after one year of her illness. There was a steady decrease in goal directed behaviour and she relinquished her hobbies. There was little change in her mood during this time.

On neuropsychological examination at age 60y, she was mildly disorientated for time (3/6) and place (3/5). Her language comprehension (8/8) and expression (23/25) were intact. She

could recall only a minimal amount of recently presented information [verbal (1/8) and visual (0/6)] after three minutes with no rehearsal but could recognise half of the visual information when re-presented (3/6). Registration was intact (3/3). Her attention and concentration were impaired (4/7). She had a mild dyspraxia for copying and drawing (4/6) but no ideomotor nor ideational dyspraxia. Dyscalculia was present (1/2). She had concrete thinking (5/10) and mildly impaired visual perception of object constancy (4/6) and impaired tactile perception (1/2). She scored very poorly on an abbreviated version of the WAIS with a verbal score of 57 and a performance score of 11. Her premorbid full scale IQ predicted from the NART was 108 with predicted verbal and performance IQs of 107 and 108 respectively.

On general physical examination there was clubbing and neurological examination revealed poor coordination.

Case I. This woman had been fit and well all her life. She had no children, worked as a cash officer and retired at 60y. She was considered particularly numerate by other family members. She began to have difficulty at work at the age of 58y which precipitated her early retirement because she was unable to adapt to new methods in the office and began to make mistakes with figures. In the eighteen months after retirement her memory for recently acquired information began to fail. At 61y she was leading an active and integrated life and did not complain of cognitive symptoms. At this time, she scored 29/30 on the mini-mental state examination but neuropsychological testing revealed evidence of a cognitive deficit. She was poorly oriented for time (3/6) and had an impairment of word fluency (2/6) but no other language dysfunction (comprehension and expression, 27/27). Visual recognition (5/6) and verbal registration (3/3) were normal but recall for recently presented information was severely impaired (2/14). Retrieval of remote information was normal (5/7). There was no evidence of dyspraxia (copying and drawing, writing, ideational, ideomotor: 18/18). Verbal abstract thinking was normal (10/10) as was tactile and visual perception. Dyscalculia was prominent (0/2) despite good concentration (5/7). Estimation of premorbid IQ (NART) was 110 full scale and had dropped to 89 (WAIS) with premorbid verbal and performance IQs of 109 and 110 having decreased to 93 and 87 respectively. The modified WCST showed no excess of perseverative errors (33%) beyond the accepted cut off for frontal lobe defects of 50%. She scored 2/28 on the Blessed scale indicating a very early stage of dementia. General physical and neurological examination were normal.

In the next two and a half years (to age 63y), she became disorientated in new environments, allowed her self-care and personal hygiene to decline and suffered a progressive deterioration in her memory for more distant and over learnt information.

At the time of the second assessment at age 65y (four and a half years after the onset) she had no complaints about her cognitive function, but her spouse had noted, in addition to the above features, periods of particular agitation and confusion. She lacked insight but had occasional episodes of dysphoria when not occupied. She remained disorientated for time and place (2/11) and language comprehension now showed some impairment (3/8) together with impairment of expression: mild nominal dysphasia (2/10), markedly reduced verbal fluency (1/6) and poor picture naming, phrase repetition and writing to instruction (7/9). There was impairment of verbal abstract thinking (3/10). Registration was very poor and uncued recall was absent (0/6) although cued recall was only slightly reduced (5/6). Dyscalculia (0/2), dyspraxia for copying and drawing (1/6), writing (1/3) and ideation (2/4) were present. Visual perception was intact (6/6) as was her appreciation of object constancy (6/8). She scored minimally or zero on all the performance subtests of the WAIS. Verbal subtests were less impaired but nevertheless the verbal IQ was estimated to be below 50. Her overall level of dysfunction as measured by the Blessed scale was 13/28.

No cytogenetic abnormalities were detected in this case.

Case J. This individual was the first-born twin of K. There were no complications of the delivery and both childhood and early adulthood were unremarkable. After leaving school early, he began work and before his premature retirement at the age of 54y had worked for 17y in the same semi-skilled job. His earliest complaint was of vertigo. He had previously been fit and healthy and began to visit his GP regularly at the age of 52y. He began to find it difficult to carry out his work and a year later had clear difficulties with his memory for recently acquired information such as shopping lists. A smooth but rapid loss of this function overlapped with loss of more remote information during the third year post onset. This was accompanied by changes in personality in particular uncharacteristic aggressiveness with abusive language and much later (3y post onset) he would on occasions throw whatever he had in his hand. In the third and particularly fourth year post onset his personal hygiene and self care began to deteriorate markedly. He became dysgraphic but continued to read for another year (although his comprehension was never tested). In the last year he became dysphasic.

His wife was ill during his illness and he had clear features of a major depressive illness which accompanied the cognitive decline. Tearfulness, sleep disturbance and psychosomatic complaints mimicking the symptoms of his wife were common throughout his illness. He had no previous psychiatric history. He continued to complain of vertigo throughout the illness although he never fell. Five years after the onset of the illness he died from a myocardial infarct.

Case K. This lady was a dizygotic twin with an uncomplicated delivery. She had an unremarkable early life, although disrupted by her father's dementia. She was of average intelligence and worked in a cigarette factory up until the time of the birth of the first of her four children. At the age of 42y she had a post-operative (gynaecological) cardiac arrest: otherwise her medical history was unremarkable. After this her husband claimed that "she was not the same". He felt that there were subtle personality changes, with increased apathy but no clinical assessment was undertaken. She saw her GP at the age of 49y complaining of loss of taste and frequent headaches. Anosmia was found on examination. A CT brain scan was normal and the anosmia was attributed to a viral infection. Her cognitive decline apparently began two years later at age 51y when she became depressed and lost weight. As her memory and intellectual function declined, her mood improved. Loss of memory for recent events and information was most marked. The onset and progression were gradual, although there were approximately five episodes of collapse with loss of consciousness early in the illness which were not accompanied by intellectual impairment on recovery. The precise aetiology of these episodes was not established. Two years after the onset (age 53y) her mood was clinically recorded as one of bland euphoria. She was moderately dyspraxic by this time. Physical and neurological examinations were normal except for the anosmia. A repeat CT scan showed moderate cerebral atrophy, and an EEG showed excessive bilateral slow wave activity. TSH was found to be mildly raised. Thyroxine replacement therapy was commenced with no improvement in cognition.

Over the next two years (age 53-55y) she steadily declined with loss of memory for remote events and over learnt information and increasingly severe receptive and expressive dysphasia. She was disorientated for time and place, dyspraxic and required assistance with walking. She was treated with small amounts of a neuroleptic to control shouting and wandering. At the age of 55y, she suffered a sudden onset left hemiplegia which was attributed to a cerebrovascular accident on clinical grounds. Several generalised seizures were recorded over the next six months. For the remaining four and a half years progressive physical deterioration ensued with continued weight loss, stiffness and the development of flexion contractures.

Assessment when she was 57y revealed that she was conscious and responded to her name being called. She showed some sign of recognising her husband but was otherwise mute and apraxic. She was bed bound with incontinence of both urine and faeces and was unable to care for herself. On examination she was very thin with a palpable thyroid gland and a systolic murmur. All limbs were held in flexion with marked increase in tone. There were symmetric crossed quadriceps reflexes and the left plantar response was extensor. There

were marked multifocal myoclonic jerks and positive grasp and pout reflexes. The last two years (57-59y) of her life were characterised by a lessening of her myoclonic jerks and seizure frequency despite absence of medication. Her flexion contraction worsened. Further weight loss occurred during this protracted terminal phase (final weight ca. 30kg). She died from bronchopneumonia.

Neuropathology of Case K. (Table 3, Figure 3a, 3b) The brain weighed 760g and the cerebellum and brainstem 138g. The gross appearance of the brain showed severe atrophy of the cerebral cortex, in particular of the frontal, temporal and posterior parietal lobes. The lateral ventricles were greatly enlarged. The hippocampus was greatly atrophied.

Sections of the hippocampus showed neuronal loss and many neurofibrillary tangles, senile plaques and granulo-vacuoles. Tangles and plaques were abundant throughout the neocortex. Quantitation of this information is presented in Table 3. Tangles were also found in the depleted nucleus basalis of Meynert, the basal ganglia, thalamus, midbrain, pons and medulla. Gallyas's silver impregnation revealed masses of neuropil threads. A few plaques were also seen in the cerebellar cortex. Both Congo red and an antibody to amino acids 12-28 of the A4 peptide revealed amyloid angiopathy. Perivascular mononuclear cells, including pigment-bearing macrophages were occasionally seen. Diffuse plaques, in addition to typical plaques, were demonstrated by the A4 antibody. There was myelin pallor on luxol fast blue cresyl violet stain in the white matter of the frontal lobe, middle and inferior temporal gyri and occipital lobe. Striking astrocytosis, particularly in the grey matter, was noted in the GFAP preparations. Stretches of the cortex showed status spongiosus.

The substantia nigra showed neuronal loss, extra-neuronal pigment in macrophages and in the neuropil, several Lewy and pale bodies, tangles, an occasional Marinesco body and astrocytosis. Similar but milder changes were present in the locus coeruleus. Lewy bodies were also seen in the nucleus basalis of Meynert and various parts of the cortical ribbon. These data are quantified in Table 3.

Unaffected Individuals. All unaffected individuals in the pedigree past or approaching the age at risk had MMSE, Camcog, Blessed, WAIS, Kendrick and WCST scores which were all within the normal range.

Osaka Family (Figure 1b) Case L. There were no episodes of severe illness during the childhood of this patient. He was academically average at school and left to succeed his father in his business of making furniture. The first symptoms of forgetfulness occurred at the age of 52 years old. This was accompanied by poor concentration and dyscalculia. At 53

he developed prosopagnosia which provoked ideas of persecution. His verbal abilities declined with reduced word finding ability. At the age of 54 he became disorientated for place. His mood was bland or euphoric despite the death of his wife. Wandering and incontinence became a problem as the illness progressed. On assessment at the age of 54 he was euphoric but co-operative. He had a nominal dysphasia and jargon aphasia. He was unable to follow simple instructions and repeat phrases and he had dressing apraxia. He was echolalic and perseverative. His procedural memory was intact. He was unable to score on the mini-mental state examination as his comprehension and language function were so impaired. On neurological examination he had a snout reflex and had finger tremor. Brain CT showed diffuse cortical atrophy with no evidence of ischaemic change. SPECT scanning showed reduced cortical blood flow in all areas except the somatic motor sensory areas and the visual cortex. The EEG showed slow primitive wave forms and the occasional theta wave. He was able to remain without constant care for a further six months but at the age of 57 he was bedridden and less physically active. He died from a pulmonary infection complicated by disseminated intravascular coagulation approximately 5 years after the first cognitive sign was noticed. He had myoclonus in the terminal stages of the disease.

Autopsy Findings of Case L. (Table 3) At autopsy the brain was heavier than expected due to swelling secondary to multiple haemorrhage especially prominent in the left frontal lobe and dentate nucleus of the cerebellum. There were many micro-abscesses secondary to septicaemia. There were numerous neurofibrillary tangles and senile plaques in the hippocampus, temporal and frontal lobes. These were of maximum density in the CA1 area of the hippocampus. Free melanin granules and neuronal loss was observed in the substantia nigra. Other forebrain and midbrain nuclei were unaffected. There was no amyloid angiopathy and no evidence of infarction.

Osaka Family Case M. This man had been fit and well all his life and left school with a reasonable academic record to work in the family business. His family noticed at the age of 45 that he was becoming increasingly forgetful. Two years later he was assessed and scored 4/32.5 on the equivalent of the mini-mental state examination. A CT scan showed diffuse cortical atrophy and an EEG showed slow basic activity. He wandered frequently. At the age of 48 he was admitted for a further assessment. He had difficulty comprehending but did answer questions. He was disorientated and dyscalculic. The MMSE score was unchanged. He had ideas of persecution and was behaviourally disturbed as a consequence. He also wandered. There were no abnormal neurological findings and routine blood screening was normal. Brain CT scan showed an increase in diffuse atrophy since the previous scan but no ischaemic changes.

By the age of 49 he was incontinent with poor personal habits. He had echolalia at that age. At 51 he had convulsions of short duration. He became bedridden with myoclonus. He died of acute cardiac failure at the age of 53.

Mother of Cases L and M in Osaka Family. Little is known of the onset of dementia in this woman but it was suspected to be Alzheimer's on clinical grounds. She died of a secondary consequence of the disease at age 45 years.

Niigata: Family 1 Case N. (Figure 1c) There little clinical information on this case. She was known to have developed dementia which was established in her early 50s. She died without autopsy confirmation in her mid-50s.

Niigata: Family 1 Case O. (Figure 1c) At the age of 48 she went to an amusement park with her children and became completely lost. Within a year she was so demented that she was unable to take care of herself at all and required full help with even basic daily living skills such as toileting and eating. Six months later she wandered and was found unconscious in a distant city. After this she became wheelchair bound.

Neurological examination at that time revealed severe dementia, bilateral rigid extremities, hand tremor, exaggerated deep tendon reflexes and positive Babinski signs. The CT showed diffuse cortical atrophy and ventricular dilatation. There were irregular slow waves on the EEG. She was discharged and soon after this she developed myoclonic jerks in the arms and fingers. By this time she was incontinent of urine and faeces and was unable to feed herself. She developed an oral tendency and the myoclonic jerks became worse in the right hand. Grand mal fitting was observed for the first time. At 51 years she was almost bedridden with monologue and emotional incontinence and was admitted for long term care. On examination she had rigidity of her neck and limbs which were held in flexion. The myoclonic jerks persisted and Babinski remained positive. Brown pigmentation was noted in the skin and oral mucosa. The CT showed atrophy and ventricular dilatation and the EEG showed diffuse slow waves. At the age of 51 she developed several infections and died of pneumonia.

Niigata: Family 1 Case O. Neuropathology (Table 3) Autopsy revealed a brain with large ventricles and a weight of 1060 gm. Ammon's horn was relatively spared but there was marked atrophy of the frontal and temporal lobes. There was no gross evidence of infarction or ischaemia. Amyloid angiopathy was noted and in some areas (dentate gyrus) amyloid was spilling out of the affected vessels. Most cortical areas showed very dense plaques and tangles with curly fibres. There was granuolar degeneration with cell loss.

Parents of Cases N and O. There is no information available on the parents of these cases.

Niigata: Family 2 Case P. (Figure 1d) This lady was well until age 52 and worked as a waitress. At this age she started to forget orders. Soon after this she became lost for the first time. As her memory problem was progressive she was admitted to Niigata Hospital for investigation. On admission, age 54, impaired immediate memory and constructional apraxia. She scored 21/30 on the MMSE, had a total WAIS score of 69 with a verbal IQ of 77 and performance IQ of 64. She did not show ideomotor apraxia, limbkinetic apraxia, visuospatial agnosia, tactile agnosia, auditory agnosia, alexia, agraphia, aphasia, nor abnormal behaviour and she was perplexed by her memory difficulties. Neurological examination was normal. Laboratory investigations were normal (thyroid, biochemistry and CSF). CT and MRI scans were normal. The EEG was normal. SPECT revealed decreased blood flow in the parietal, occipital and frontal lobes.

Over the next two years there was rapid and progressive loss of memory for distantly acquired information and within a year she required help with dressing. Her ability to concentrate was lost and she became increasingly apathetic. There was initially a brief period of insight when she was able to recognise her memory problem but this was not sustained. There was no evidence of depression although she was occasionally tearful.

Assessment at age 56 years showed there had been a rapid decline in her cognitive state. In particular she had good comprehension of simple commands and statements but had impairment of abstract thinking. She had no nominal dysphasia nor dyslexia, but was dyscalculic, dysgraphic, and had finger agnosia. She had no ideomotor dyspraxia nor right-left agnosia. Neurological examination remained normal. CT scan at this stage showed widespread cortical atrophy.

Niigata: Family 2 Other Family Members. Few details of the rest of the family are available for Family 2. However, clinically diagnosed dementia was documented in three other cases in this family. The ages of onset and the age at death are shown in Figure 1d.

Family 372 (Figure 1e, Cases Q→W)

Case Q and Other Family Members. There are no details available for Case Q, his father, grandparents or the father of Case S.

Case R. This case does not have the APP717 mutation and is included as an example of a "phenocopy".

This woman, who is a distant cousin to the members of the main sibship had 11 years of schooling. She worked most of her life in unskilled factory work but which she left to take care of her husband at the age of 49. Forgetfulness and difficulties in expressing herself were first noticed by her family when she was 60 years old. She was able to meet her responsibilities for the next two years but at the death of her husband she was unable to make any of the appropriate arrangements. By this time, aged 62, she was dyscalculic. At the age of 64 she scored 10/30 on the MMSE and a CT scan showed ventricular dilatation and cortical atrophy with a normal EEG. She retained fluctuating insight, but at times was agitated.

By age 67 she had lost much of the ability to look after herself, was mildly apraxic for dressing, but was able to manage some simple practical tasks. Neurological examination remained normal except extrapyramidal features which were almost certainly side effects of neuroleptic medication. At 67 she had reduced quantity of speech and a fluent aphasia with a word salad output and a nominal dysphasia. She had reduced verbal fluency on the verbal Fluency Categories Test (comparable to the verbal fluency test of the CAMCOG). Comprehension was impaired for non-verbal tasks. She showed constructional apraxia. She scored 2/30 on the MMSE. In the subsequent year she continued to decline but retained fluctuating insight to her condition.

Case S. Hospitalised in the VA for several years for "arteriosclerosis" from the age of 59, this man died at the age of 65. He was thought by some family members to be demented. Case details are not available.

Case T. This woman was a telephone operator and was admitted to hospital at the age of 41 for investigation of her inability to continue to work. She had a marked bradykinesia with masked facies and waxy rigidity of all extremities and a mildly positive snout reflex. Simple tests of abstraction showed impairment as did tests of concentration and of general over learnt information. There was no evidence of significant depression. Pneumoencephalogram showed mild superficial cortical atrophy and deep cerebral atrophy. All routine blood and CSF tests were normal. At 44 years she developed generalised seizures. At the age of 46 she was very agitated, suspicious and combative in response to paranoid ideation. She was disorientated and had mumbling speech. Treated with neuroleptics, she displayed dystonic posturing. By this time she was completely unable to care for herself and was admitted for nursing care. The progress then was one of a slowly progressive dementia with complete loss of higher cortical functioning and progressive loss of mobility. She remains alive but in a vegetative state 14 years after the presenting symptoms.

Case U. This woman had a modest education and performed semi-skilled work and at time of presentation had been leading an active and full life. At the age of 49 this woman began to complain of frequent and persistent headaches which were investigated and thought to be related to tension. About this time she noticed that her memory was failing. CT at the time revealed very mild generalised atrophy, all other investigations being normal. A few months later at examination her memory for recently presented information was very poor, her concentration was impaired, she had dyscalculia, and some loss of memory for over learnt information. Abstract reasoning was intact and there was no evidence of language disturbance nor any dyspraxia. Seizures were noted from her fifty-fifth year. By this age her ability to recall over learnt information was impaired further and she was disorientated for time and place. Soon after this she began to have "twitching" movements in all limbs. She was increasingly behaviourally disturbed and became less responsive to verbal commands but continued to eat when presented with food. She died at the age of 59 years.

Autopsy Findings for Case U. (Table 3, Figure 3c, 3d) At autopsy, the brain weighed 480 gms. There was severe frontal atrophy and moderate atrophy of the temporal lobe. The parietal and occipital lobes were grossly unremarkable. The plaque and tangle counts per cortical field are shown in Table 3. The middle frontal gyrus showed moderate neuronal loss and gliosis with severe spongiosis in the outer layers. This picture was also seen in the parietal and temporal lobes and in the hippocampus. Modest amyloid deposition was observed in the vessel walls of the nuclei of the diencephalon. Some of these nuclei also showed neuronal loss. The midbrain sections were normal.

Case V. This woman had a high school education and worked as a switchboard operator and then as a seamstress in a factory. At age 48 she noticed she was having trouble remembering messages and two years later her personal physician commented that she could not remember even the simplest things. CT was normal at that time. Over the next few years her memory problems became worse and she stopped reading the newspaper. By the time she was aged 51 her husband had taken over the management of money. At this age also she became lost for the first time. Her ability to accomplish moderately complex but over learnt tasks became impaired. At the age of 52 she could recognise well known faces but could not name them, she was not dyspraxic for dressing and she maintained her personal hygiene. There were no psychiatric symptoms throughout these developments and she was unaware that she had any problems. Neurological examination at age 53 was normal. She scored only 6/30 on the MMSE. An MR scan showed moderate cortical atrophy without significant ventricular dilatation.

More extensive neuropsychological testing at the age of 54 revealed impairments in all areas. She was completely disorientated for time and place. She had no recall of recently presented information but was somewhat better on recognition of previously presented material (12/20 words correctly identified) and was dyscalculic. She was dyspraxic for copying and drawing. She had difficulty following simple instructions. She had a marked nominal dysphasia and was shown to be very impaired in a test of verbal fluency. She scored 6/30 on the MMSE.

In the following year she became more aphasic with constant repetition. There were no significant behavioural problems and she remained generally placid and passive.

Physical examination at the age of 56 was essentially normal. She had reached the lower limits of formal neuropsychological testing, was apraxic and scored 3/30 on the MMSE. She required total daily care. She developed an aversion to seeing her own mirror image.

By the age of 57 years she had developed incontinence. She was frequently agitated with episodes of crying. She had slight cog wheeling when taking neuroleptics. She remains alive in an advanced demented state.

Case W. This woman had no relevant medical or psychiatric history except for a car accident at age 44 when she had a mild closed head injury. At 44 she was investigated after suffering a syncopal attack when she was working as a switchboard operator. No cause was found. At the age of 50 she was hospitalised for the investigation of persistent chronic headaches of one year's duration. This problem coincided with a difficulty in remembering numbers. She had full insight into her memory problem and its significance and this produced a reactive anxiety with depressive features. At this time informal testing revealed that she had good concentration and was oriented for place and person. She was not able to perform simple multiplication. She complained of vertigo and other somatic symptoms. A technetium brain scan was normal although her EEG was said to be non-specifically abnormal.

She was retired as a consequence of her difficulties. At 53 years she lost consciousness for no apparent reason and was investigated again but no cause was found. She continued to complain of headaches. Physical and neurological examination at the time was normal. At age 54 she stopped driving, and at 56 she stopped handling money or dealing with financial issues. In the following year she gave up household tasks and meal preparation. Over this period too, she became progressively more lethargic and at age 57 years there were periods when she was confused, disorientated and wandered. CT scanning demonstrated cerebral atrophy but otherwise routine examination and investigations were normal. From the age of

59 she was dyspraxic for most simple tasks and became disorientated at home. Behavioural problems became prominent with aggression and wandering. Physical examination at age 60 revealed moderate generalised increased tone with cog wheeling in the upper limbs and poverty of facial expression. She had mild myoclonus. A snout reflex was present. Following this evaluation she continued to decline and at one stage had the clinical signs of a CVA. She died age 65 years.

Autopsy Findings for Case W. (Table 3) Autopsy findings revealed mild frontal, parietal and temporal atrophy with moderate dilatation of the lateral ventricles. Microscopic findings revealed high plaque and tangle counts in the cortical fields. There was neuronal loss and gliosis in the nucleus basalis of Meynert and the amygdala, other forebrain nuclei being unremarkable. One Lewy body was observed in the substantia nigra with mild loss of pigmentation.

CHAPTER 5

Genetic Analysis of Early Onset Alzheimer's Disease in a Family with a Codon 717 (Val→Gly) Mutation of the ß-Amyloid Precursor Protein Gene

5.1 Abstract

This chapter reports the identification of an early onset family, Fl9, in which linkage was detected between the ß-amyloid precursor protein (ßAPP) gene and Alzheimer's disease. Simulation studies strongly suggested that the initial apparent linkage was a true positive and direct sequencing of exon 17 in affected individuals from this family revealed a base change producing a Val→Gly substitution, at codon 717. The detection of a second allelic variant at codon 717 linked to the Alzheimer's phenotype lends support to the hypothesis that they are pathogenic mutations. The clinical features of the phenotype are described and do not grossly differ from the Val→Ile phenotype. These results suggest that the codon 717 variants may share a common pathogenetic pathway.

5.2 Introduction

Following the discovery of the codon 717 Val→Ile mutation and the publication of linkage data suggesting that Alzheimer's disease was not an homogeneous disorder (St George Hyslop *et al.*, 1990), the general strategy for detecting additional mutations at the ßAPP locus was to test for linkage at D21S210 with the highly polymorphic dinucleotide repeat sequence probe (GT12) family by family. The problem of poor statistical power to detect linkage in small families was overcome by the use of simulation studies (see Chapter 2).

5.3 Materials and Methods

5.3.1 Clinical

The family was clinically assessed with the protocol details in Chapter 2). The family structure is shown in Figure 1.

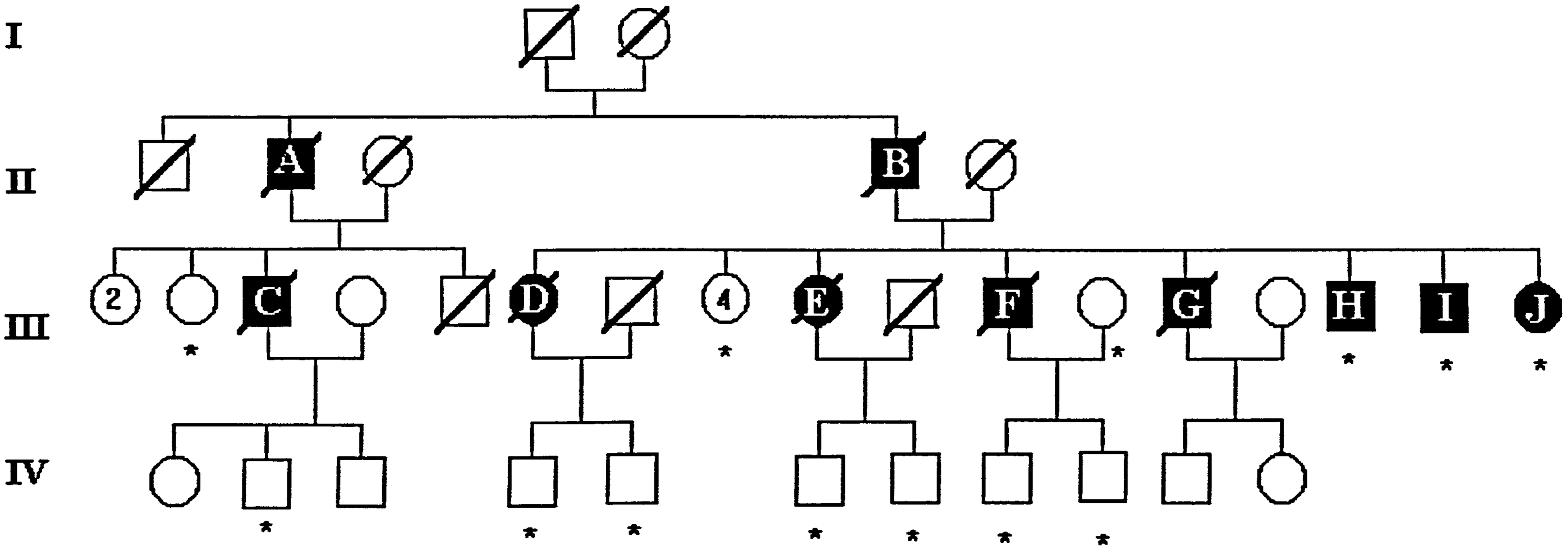


Figure 1 Pedigree structure of Family 19

*** denotes sample availability for linkage study**

5.3.2 Linkage Analysis

Linkage was tested between familial Alzheimer's disease and D21S210 (located close to the ßAPP gene) in pedigree F19 (Figure 1), in which AD was confirmed by autopsy. In order to simplify the analysis the alleles segregating in the family were recoded (see Chapter 2).

Lod scores were calculated with seven liability classes modeling age dependent penetrances from 0.01 to 0.95 with a phenocopy rate of 0.001 and a gene frequency of 0.001 using MLINK from the LINKAGE package.

5.3.3 Simulation

The general method of simulation analysis is detailed in the Materials and Methods section. This analysis had one purpose, to assess the significance of the lod scores generated in this family, with the six alleles that were segregating at the D21S210 locus. Although 16 samples were later available from this family, at the time of the initial linkage and simulation studies there were only 12 samples available. As additional samples became available the analysis was repeated. Simulated data could have been analysed to decide which additional individuals would provide most power to the analysis. However, in this family it was obvious that data from the spouse and children affected individuals in the two main sibships more informative and established phase in some cases.

5.4 Results

5.4.1 Linkage and Simulation

One allele (13) of GT12 cosegregated with the disease in this family. Twelve family members were available for the initial GT12 typing. Lod scores were suggestive of linkage being over 2.0 but below 3.0. Initial simulation studies however, were encouraging (see Appendix 5A); the maximum lod score obtained in 500 families randomly distributed with the 6 segregating alleles (and attendant frequencies) was 1.8, there were 2.6% unlinked families generating a lod over 1.0 but none generating a lod over 2.0 (Appendix 5A). To test whether the family structure and number of samples collected was sufficient to generate a lod score over 3.0 a second simulation study was performed, with random allocation of the six alleles in 500 linked families. In addition to the samples obtained from the 16 individuals marked on the pedigree in Figure 1, an additional 8 family members were included in the analysis, as it was thought that these samples would become available at a later date. Most of the additional family

members added little to the power although the addition of 2 unaffected female members of the smaller sibship and of the implied genotype of a deceased male in the main sibship would have added significantly (about 0.7 of a lod point). The maximum lod score obtained was 3.7 with 15% of the simulated families obtaining reaching a lod score over 3.0 (Appendix 5B). A comparison of Appendices 5A and 5B show that the likelihood that the actual lod score was drawn from the linked rather than the unlinked population was unequivocally in favour of the former. As a consequence of this study little effort was made to collect additional family members and the final typing of 16 individuals yielded an actual peak lod score of 3.02 between GT12 and the disease at a recombination fraction θ, of zero (Table 1).

Table 1. Linkage Analysis Between D21S210 (GT12) and Alzheimer's Disease in F19

Recombination fraction	0.00	0.01	0.05	0.1	0.2	0.3	0.4
Lod score	3.02	2.97	2.75	2.47	1.86	1.22	0.6

The ßAPP gene was therefore sequenced in affected and unaffected individuals in this family starting with exon 17. Direct sequencing revealed a T→G transversion at base pair 2,150 predicting a valine to glycine change at codon 717 of the ßAPP transcript 770. Direct sequencing and single strand conformation analysis (SSCA) of the remaining family members allowed segregation analysis of the mutation with the linked marker GT12 and therefore the disease. The peak lod score between D21S210 (GT12) and the mutation was 3.66 at θ = 0. Three affected and 10 unaffected family members were typed for this analysis.

5.4.2 Clinical Results

The prominent clinical features of the codon 717 Val→Ile cases (derived from the data in chapter 4) and Val→Gly are compared in Table 2. The detailed case reposrts are recorded in Appendix 5C.

5.4.3 Age of Onset

Depression such an early and prominent feature of cases of G and H in F19 that it was not possible to accurately date the onset of cognitive signs. The reminder (N=7) had a man age of onset of 54.7 years (SE 6.6). There was no significant difference in age of onset between the Val→Ile mutations and the age of onset in this family (t=.856, DF=29, p<0.4).

Table 2. A Comparison of the Clinical Features of Val→Ile and Val→Gly Cases

	Val→Ile		Val→Gly	
	Mean	**SE**	**Mean**	**SE**
Age of Onset	52.5	4.7	54.7	6.6
	early sign	late sign	early sign	late sign
Amnesia	16/16		5/5*	
Aphasia	16/16		3/3	
Agnosia	16/16		3/3	
Dyscalculia	7/16		3/3	
Myoclonus	7/16		2/5	
Seizures	5/16		1/7	
Clinical stroke		4/16		0/7
Depressive Features	6/16		4/5	
Personality Change		2/16		2/7
Pre-Cognitive Change	Anosmia LOC, Vertigo Headaches		None	

* The denominator denotes the number of cases assessed for each feature.

5.4.4 Other Clinical Features

The **other key features** noted in early onset AD pedigrees also occur frequently in F19. Amnesia, apraxia, agnosia and dyscalculia occur frequently and early. Myoclonus was noted in 2 of the three cases assessed in detail. Pre-clinical change and stroke did not occur.

5.5 Discussion

Screening for this mutation revealed that it did not occur in any of 24 pedigrees with AD nor did it occur in 52 normal individuals. The occurrence of this mutation was remarkable because it produced a change at the same codon as that previously reported. Direct sequencing or SSCA of other exons did not reveal any other changes in the ßAPP gene in this family, but it is known that SSCA does not detect all single base-pair changes. It remains a possibility that other sequence differences in the ßAPP gene exist between affected and unaffected individuals either in exons not yet examined or sequenced or in the promoter. However, the occurrence of this mutation in a family with histologically confirmed AD, together with the multiple occurrence of the ßAPP717 Val→Ile mutation, strongly support the hypothesis that these mutations are pathogenic. The main features of AD in Fl9 (detailed in Appendix 5C) and families with the ßAPP717 Val→ Ile mutation clinically fulfill NINCDS criteria for probable Alzheimer's disease and neuropathologically fulfill NINCDS criteria for definite Alzheimer's disease. Clinico-pathological features discriminating between these allelic variants have so far not been detected. No other family has been reported with the Val→Gly mutation, therefore the number of cases studied with this mutation remains small.

A third mutation at codon 717 of the ßAPP770 transcript associated with Alzheimer's disease has been reported. The predicted amino-acid change is valine to phenylalanine (Murrell *et al.*, 1991).

Chapter 5 Appendix 5A

Simulation Study to Assess the Lod Score Distribution Attainable Under Non-linkage in F19 with GT12

Six alleles of GT12 were known to segregate in this family. They were recoded 1→6 but retained there expected allele frequencies.

Input Data:

The random number seed is: 23456

The number of replications is: 500

The requested proportion of unlinked families is: 1.000

The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	28
Number of females	14
Number of males	14

There were 16 in category: Marker Unknown; Trait Original

There were 0 in category: Marker Available; Trait Simulated

There were 12 in category: Marker Available; Trait Original

There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data

Linked order of loci: 1 2

True thetas for linked order: 0.000000

Unlinked order of loci: 1 2

True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 3.30000 seconds

Elapsed time = 1624.65000 seconds or 27.08 minutes

Actual proportion of unlinked families: 1.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	-1.642379	2.077348	-5.303446	1.760115

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max

Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	13	2.600
1	Study	13	0.026
2	1	0	0.000
2	Study	0	0.000
3	1	0	0.000
3	Study	0	0.000

Chapter 5 Appendix 5B

Simulation Study to Assess the Highest Lod Attainable Under Linkage in F19 with GT12

Input Data:

The random number seed is: 23000
The number of replications is: 500
The requested proportion of unlinked families is: 0.000
The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	38
Number of females	21
Number of males	17

There were 13 in category: Marker Unknown; Trait Original
There were 0 in category: Marker Available; Trait Simulated
There were 25 in category: Marker Available; Trait Original
There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data
Linked order of loci: 1 2
True thetas for linked order: 0.000100
Unlinked order of loci: 1 2
True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 4.50000 seconds
Elapsed time = 2237.66000 seconds or 37.29 minutes
Actual proportion of unlinked families: 0.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	1.045339	1.160846	-1.254713	3.722492

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	1.697467	1.931607	0.000000	3.8129462

Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	296	59.200
1	Study	296	0.592
2	1	200	40.000
2	Study	200	0.400
3	1	76	15.200
3	Study	76	0.152

Chapter 5 Appendix C

Clinical Features of Affected Members of F19

This family came from a mining town near Sheffield. Many of the men were miners.

Case A

Family sources suggest this individual did not have prominent symptoms until his mid to late 60's. No clinical records were available for this case but memory disturbance was a prominent feature of his illness. He died at age 73 years.

Case B

Little detail was recorded of the early years of the illness of the affected father of the main sibship. He was a collier and became cognitively impaired in his late 50's. He was discharged from work, being a danger to himself and others at age 59 years. Subsequently, relatives were told that his mental problems and behavioural disturbance was due to a pit accident that he suffered. The affected father of the main sibship was admitted for permanent nursing care at age 60 years. By this time he was wandering aimlessly around the ward and was completely disorientated for place and time. He had some behavioural disturbance at this time and was also thought to be depressed. His physical examination was normal at the time of admission. He died after a year of hospitalisation.

Case C

This man was the only affected member of the smaller sibship in generation III. He was beaten on the head as a prisoner of war, but had no other history of head injury. The first sign of dementia was memory deficiency which began at the age of 57. By the age of 63, he had presented with a history of memory loss, apathy, loss of consciousness, aggressive personality change and frontal headaches. At that time he had generalised cortical atrophy on CT scanning. A radioactive brain scan showed an avascular mass in the right vertex of unknown aetiology. He was disorientated for time but not place or person on admission. He had dyscalculia. He had insight into his memory loss even six years after it began recognising that it was likely to be the same condition suffered by his relatives. All routine laboratory investigations were negative except a low B_{12} for which he was treated. On physical examination at that time there were no abnormalities either of the CNS or other systems. The differential diagnosis of cerebral meningioma was raised by the radioscan but a clinical diagnosis of familial dementia was finally made. He had a slow decline and died at age 74.

Case D

The oldest affected female in the main sibship in generation III died with the recorded cause of death as cerebral arterial haemorrhage and presenile dementia. By the time she was 58, she was showing signs of dementia, but had a blood pressure of 200/140 which raised the suspicion of multi infarct dementia. The earliest signs of dementia, recorded at age 54, was memory loss and loss of daily living skills. An EEG compatible with cerebral atrophy ("low to medium voltage" diffuse theta waves). Laboratory tests (including B_{12}) was normal. A psychological report at the age of 57 recorded a borderline subnormal range IQ on the WAIS. Vocabulary was significantly better than her performance IQ. She also complained of severe headaches during the course of her illness necessitating powerful analgesia. She died at age 60 years, from "arterial cerebral haemorrhage" (death certificate).

Case E

This woman was admitted to hospital for full time nursing care in her late 50s. She had suffered progressive memory loss from age 53. She had a history of depression with anxiety. There were periods of unexplained dizziness throughout the early years of her illness. This woman died at age ? early 60s years with a history of presenile onset of dementia. The earliest recorded signs were in the early 50s.

This case was autopsy confirmed as Alzheimer's Disease. The brain weighed 1230gm and there were numerous tangles and senile plaques, with no other obvious pathology. Diffuse Aß deposits were seen throughout the cortex, and there was severe angiopathy.

Case F

This main died at age 62 following pre-senile onset of dementia. Little detail is available for him, as he emigrated.

Case G

Worked as a miner all his life. He retired at age 56 due to illness, which was largely his forgetfulness. Began to have epileptic fits which responded to anti-epileptic medication. Was admitted for full time nursing care at the age of 59 years. By this time he was incontinent of urine at night. He had become aggressive by this time.

Case H

Episodes of misery frequently talks of self harm - shows insight able to describe his memory loss and recognised the same signs and symptoms in himself that he had witnessed in his siblings and father. He was being treated with an antidepressant. The history of dementia was one of progressive and largely smooth decline although day to day variation in

functioning was evident. Emotional lability with confusion and frank panic were prominent features during the decline. After a "mental breakdown" at age 39 he stopped working. This episode was latter attributed to memory problems but was primarily depressive. He began work again and continued up until the age of 54. This job too ended in an episode of depression associated and precipitated by cognitive decline.

In summary, the history of cognitive decline, beginning with memory loss for recently acquired information, was obfuscated by the affective component. This makes the dating of the first cognitive sign very difficult.

He had had a moderate schooling achieving adequately, but left at 15 years to take jobs as a builder, progressing to an apprenticeship in bricklaying, foreman and managerial positions. His previous medical history of childhood tinnitus, duodenal ulceration (requiring surgery) and low back pain were unrelated to his subsequent dementia. During the investigation of dementia, at the age of 50 years he had generalised cerebral atrophy with ventricular dilatation and non focal lesions. Also serum B_{12} and folate were low, requiring treatment.

At the time of his assessment, age 55 years, the physical examination was completely normal including the cardiovascular system except for the presence of myoclonic twitches in his forearms and hands. He scored zero on the Hachinski scale. he scored 11 on the Blessed dementia scale, mostly (6) in the section on changes in personality, interests and drives, which may well have reflected his affective disturbances as much as the cognitive ones. He did score poorly on ability to manage money, orientation in familiar circumstances and inability to remember short lists of shopping items.

Cognitive examination demonstrated very poor recall abilities, recalling 1/10 items after 30 seconds exposure, 3/15 after 45 seconds exposure, 2/20 after 60 seconds exposure and 3/30 after 75 seconds exposure (Kendrick Object Learning Test, KOLT). The repeated items were no better recalled than the new ones in each successive presentation. The CAMCOG battery revealed good orientation for time (6/6), language expression (10/10) and modest definition ability (6/8) , poor word fluency (3/6), good orientation for place (4/5) and good comprehension of simple questions requiring verbal (3/3) and motor responses but not complex ones (2/3). He scored well on phrase repetition. On tests of recall he was (as noted by the KOLT) poor and on the CAMCOG scored (0/6) on recall of previously viewed objects, and 2/6 when asked to chose the items he had previously seen. Registration was good with three new items being repeated correctly on the first attempt. He scored 8/11 on the subtest of retrieval of remote and overlearnt information. His attention and concentration was good as evinced by the correct backward repetition from 20 (all correct) but he scored poorly on

serial sevens (1/5). He was dyscalculic on simple tests of arithmetic and also scored poorly on test of tactile perception, spontaneous or instructed writing, and was poor on tests of abstract thinking. Ideomotor praxis was good (4/4) as was copying and drawing praxis (5/6) and ideational praxis (3/3).

In the examination of the mental state, he showed marked lability of affect, laughing minutes after he had been crying. These episodes of misery were related to insight into the nature and origin of his cognitive difficulties.

Case I

This man had a poor school record, never appearing to be above average academically. He left school early, spent time in the army and finally settled into a job as a furnace man for 27 years. He had smoked 1/2 ounce of tobacco per day all his life.

He suffered a smooth and progressive decline in his intellectual function but some days he was noted to be much poorer in abilities than others. At the time of the first problem, which was memory loss, he was 48 years old. He had a twelve year history of memory loss for recently acquired information. This was first detected when he appeared at work on double shifts as a collier, when he was only expected to a work a single shift. He became unable to manage money early in the illness but remained relatively efficient at other tasks for several years after the onset. At the age of 57 he began to exhibit nocturnal confusion with low mood, tearfulness, loss of appetite and weight loss. Two years later he became completely lost in an unfamiliar place. During these stages of deterioration, he took many jobs and lost them due to his cognitive deficiencies. At the age of 58 he suffered a large MI, preceded by a fit. During routine investigation, a low B_{12} was noted. He had a history of poor circulation with ankle swelling which preceded the MI. However, he had not suffered any sudden neurological deficit which might be associated with stroke. He scored 2 on the Hachinski scale. He scored 8 on the Blessed dementia scale and was able to perform certain simple tasks about the house although he was unable to look after himself.

On physical examination, at the age of 60 years, he had abnormal gait and had generalised hyporeflexia. He had marked cyanosis, with a weak regular pulse which could not be recorded in the pedal arteries.

On the CAMCOG examination, he was completely disorientated for time (1/6) but was orientated for place (5/5). He was able to comprehend the spoken language (6/6) and was able to name common objects (4/4). Verbal fluency was impaired (3/6) and did well on definitions (6/6) and repetition. "Recent" memory was obviously and markedly impaired:

uncued recall (0/6), re-presented pictures (4/6) and retrieval of more distant information was general good (8/11). Registration was good, three new items being registered at the first attempt. He made one error in counting backwards from 20 but failed on the serial sevens (1/5). He had marked copying dyspraxia (0/6), but had intact ideational (3/3) and ideomotor praxis (4/4). He was not dyscalculic on simple arithmetic (2/2) and did well on the test of abstract thinking (7/10). He had good tactile (2/2) and visual perception (9/9).
His perception of the passage of time was also quite good. The KOLT confirmed poor memory scoring well into the dementia range with 2 objects recalled after 30 seconds exposure, 3 recalled after 45 seconds exposure, 4 recalled after 60 seconds exposure and 4 recalled after 75 seconds exposure with no increased recall of repeated objects.

He scored poorly on the NART , indicating a premorbid full scale IQ of 110 with predicted verbal and performance IQs of 109 and 111 respectively. The results of the WAIS-R indicated a current verbal IQ of 76 and a current performance IQ of 65 with a full scale IQ of 70.

Case J

This woman, who was partially paralysed on her left side from birth, became a housewife and until the time of her illness had led an uneventful and generally healthy life. She inexplicably stopped making shopping lists at the age of 45 and started repeating words and phrases and asking the same question several times a day. This was a blatant feature by the time she was 50 years old. At the age of 53 she relinquished her favourite pastime, reading. She was also investigated at this age for fainting which at one time were occurring several times per week.

At age 55 all routine laboratory tests were normal, including CSF. A CT scan showed right sided ventricular enlargement, consistent with her left sided congenital weakness.

At 57, on physical examination, her left hemiparesis was prominent. She had bilateral increased reflexes of biceps and knee jerk (R>L). She exhibited primitive reflexes (pout and grasp) and had myoclonic jerks in all limbs except her left arm.

At the age of 57 the CAMCOG examination revealed that she was poorly orientated for time (0/6) and place (1/5). Her language comprehension was reduced (2/6), but she scored higher in language expression (8/10) although she had marked reduction in the verbal fluency test (3/6). On definitions she scored 2/8. She failed completely on the CAMCOG short term recall test and on recognition of previously exhibited material she scored 2/6. More remote information was also very poorly accessed (2/11). She failed to register three new items after repeated trials and was unable to count backwards at all from 20 (attention/concentration).

She had copying apraxia, ideational and ideomotor apraxia and could neither write to instruction nor spontaneously. She was dyscalculic, and scored zero on tests of abstract thinking visual and tactile perception.

She had undergone a marked change in her personality, previously being loquacious, currently withdrawn and apathetic. There was no evidence of mood disturbance however. As an overall measure of her decline she scored 12 on the Blessed dementia scale.

CHAPTER 6

The Detection of a Novel Pathogenic Mutation for Early Onset Alzheimer's Disease in the *ßAPP* Gene corresponding to the N-terminus of ß-Amyloid

6.1 Abstract

This chapter describes the detection of the presence of a novel mutation in the ß-amyloid precursor protein (ßAPP) gene by linkage analysis in two Swedish families. Despite the subsequent finding of tight linkage between D21S210 (GT12) and this allelic variant at *ßAPP*, the intial lod score did not exceed 3.0 and simulation studies were employed to assess the likelihood of false positive linkage (particularly as the lod score in one family did not exceed 0.5). Subsequent sequencing revealed a double mutation at codons 670 and 671 (*ßAPP* 770 transcript) in exon 16 of *ßAPP*. The mutation is also shown to completely co-segregate with the disease in these two large (related) early onset Alzheimer's disease (AD) families from Sweden. Two base pair transversions (G->T, A->C) from the normal sequence predict Lys->Asn and Met->Leu amino acid substitutions at codons 670 and 671 of the *ßAPP* transcript. This mutation occurs at the amino terminal of ß-amyloid (Aß) and may be pathogenic because it influences the cleavage of *ßAPP* by α -secretase. This lends support to the idea that the AD causing mutations influence the processing of the ßAPP molecule and/or enhance the production of the Aß fragment.

6.2 Introduction

In the search for additional mutations causing AD several strategies are possible. Screening of all eighteen exons and the promoter region of *ßAPP* is an alternative but is time consuming, expensive and irrational if there is no linkage to this locus. On the other hand, if families are so small that linkage is not possible then direct screening might be the only available strategy. [The very small Japanese families with Val→Ile mutations described in Chapter 4 were discovered in this way]. The task of direct screening is much less formidable if only one exon is known or expected to contain mutations. In the case of the large *ßAPP* gene this was not known and there was no *a priori* reason to think that only exon 17 of *ßAPP* would contain mutations. Initial linkage studies were therefore desirable in the two related pedigrees shown in Figures 1a and 1b. In addition routine

screening of exon17 by sequencing showed it was normal in affected members of the family ruling out the Val→Ile, Val→Gly, and Val→Phe as causative. It was therefore rational to test for linkage before any other exons were sequenced.

6.3 Methods

6.3.1 Clinical

Family members were assessed by general clinical work up for dementia. No standard and uniform memory disorder work up was administered to the affected members. However, each of the affecteds scored less than 26 on the mini-mental state examination and were scored as probable AD if they showed deterioration in the capacity to acquire new information and in at least one other cognitive area and there was no other systemic disorder that could account for the dementia syndrome.

6.3.2 Linkage and Simulation Analysis

The same general method of testing for linkage to AD with an informative microsatellite repeat, GT12 (D21S210), close to the *βAPP* gene was employed. Genealogical studies had suggested that the two families (shown in Figures 1a and 1b) were related. It was therefore justifiable to sum lod scores for these two families obtained at the D21S210 locus. The standard linkage analysis was performed (see Materials and Methods). For the mutation analysis a mutant allele frequency of .001 was assumed. Simulation studies were carried out using SLINK. The F144 and F139 structure was coded for availability and diagnostic status and 1000 pedigrees were generated under conditions of linkage and non-linkage. For the initial studies of linked pedigrees all alleles were allowed to segregate with the disease. For the unlinked simulations actual genetic data were known and allele 13 was allowed to mark the disease. These pedigrees were analysed with MSIM from the SLINK package.

6.3.3 Sequence Analysis

Exon 16 was amplified using a biotinylated 5' primer of sequence
GGGTAGGCTTTGTCTTACAG and a 3' primer sequence;
GGCAAGACAAACAGTAGTGG (see references 6 & 9). The PCR reaction was 35

cycles of 1 min 94°C, 1 min 58°C and 1 min 72°C. The biotinylated single strand DNA was recovered using streptavidin coated magnetic beads (Dynal) and was directly sequenced using the sequenase kit version 2.0 (USB) and the non-biotinylated amplification primer as the sequencing primer.

6.4 Results

6.4.1 Clinical

Family 139 and Family 144 (Figures 1a and 1b) are two large Swedish families thought to have a common ancestor. Clinically, all affected family members fulfill NINCDS (McKhann *et al.*, 1984) criteria for probable AD. The mean age of onset is 55 years with a range of 45 to 61 years. The mean duration of the illness is 7 years. Autopsy on one patient revealed generalised atrophy with sulcal widening and mild ventricular enlargement. However, no microscopy was performed. A second autopsy confirmed the diagnosis of AD. The first clinical feature in 12 of the 13 documented cases was loss of memory for recent events. The other case presented with paranoid hallucinations. None of the 13 affected individuals had a history of vascular disease.

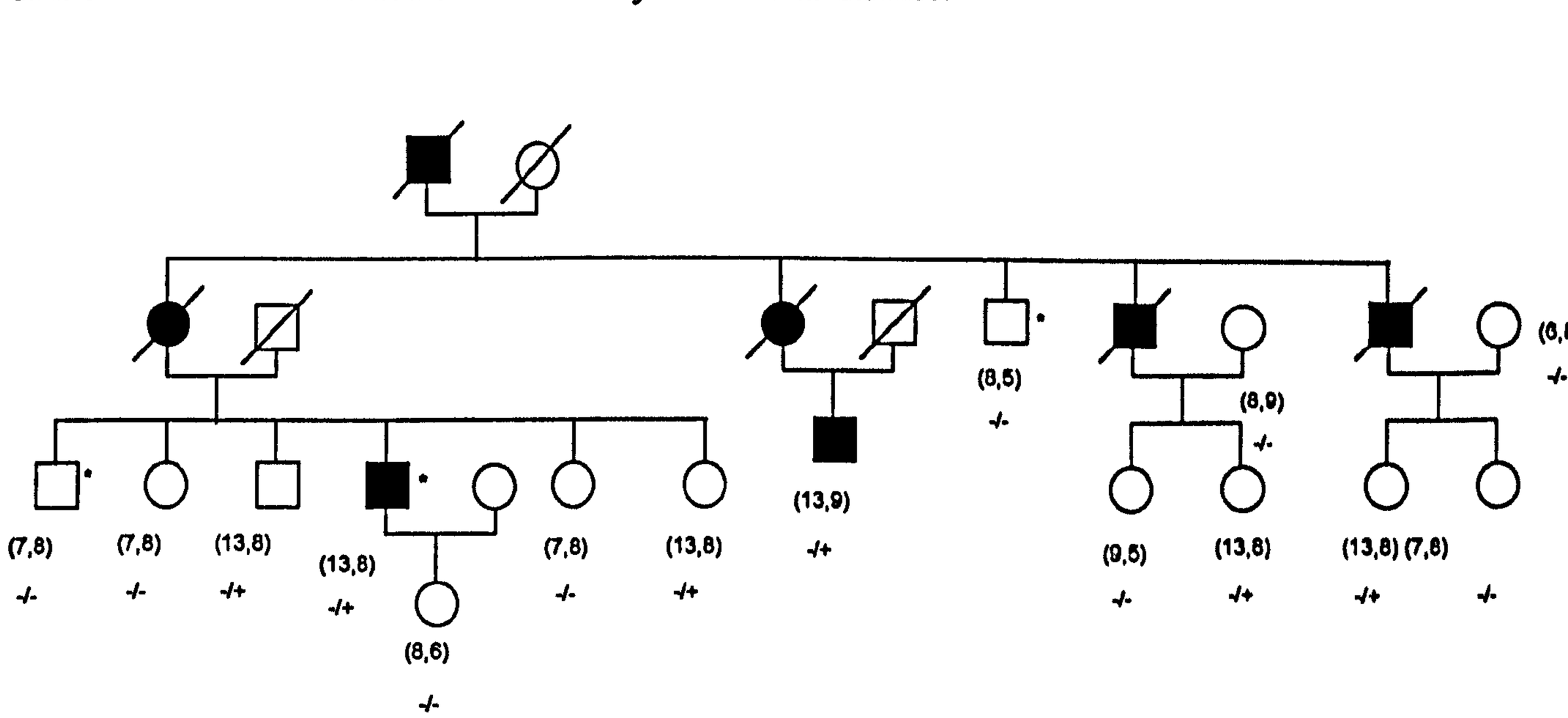


Figure 1a. Segregation of the mutation (-/+) in F144 (GT12 alleles in parentheses).

* APP exons 16 &17 sequenced

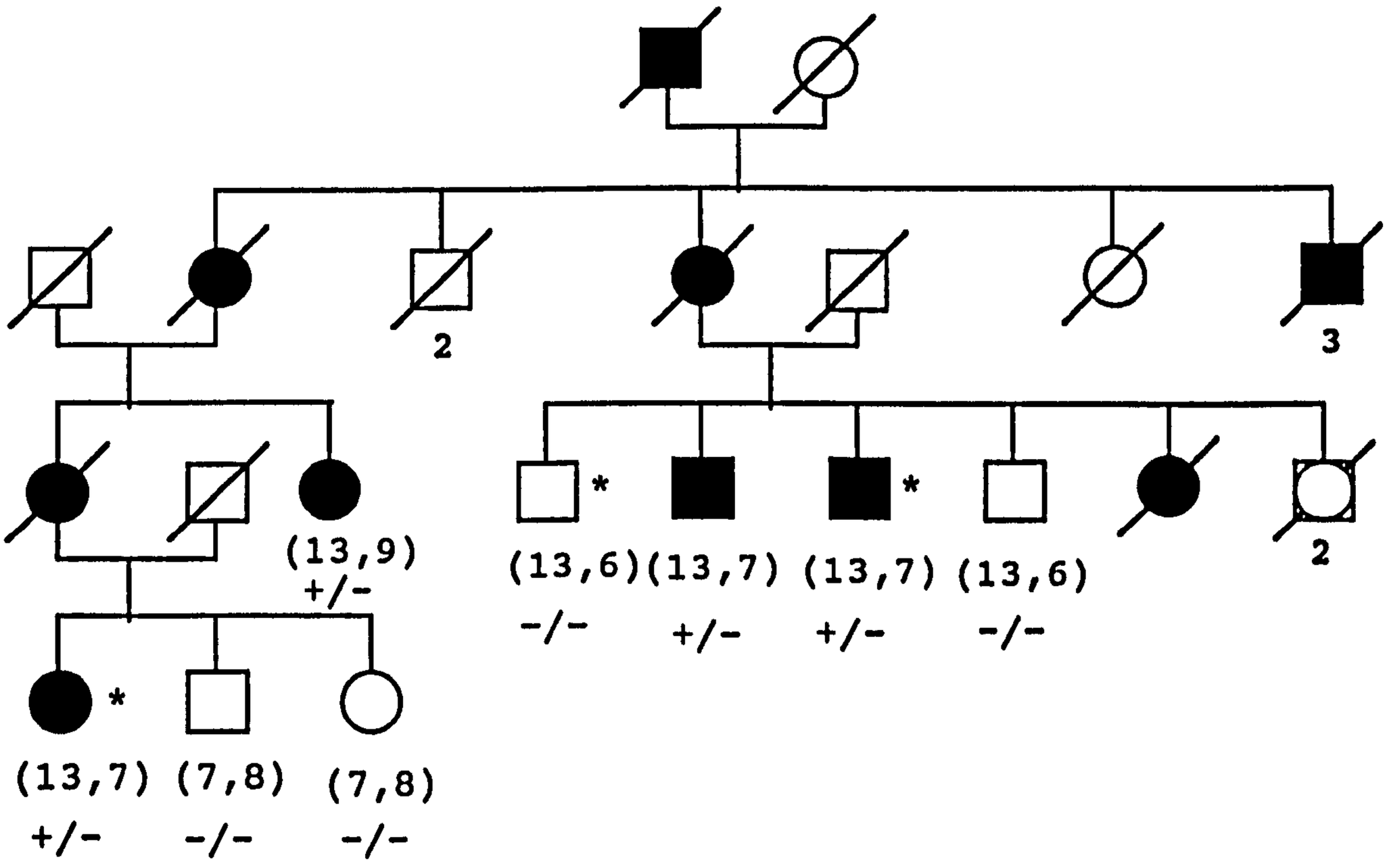


Figure 1b Segregation of the GT12 alleles and the mutation (+)

* APP exons 16 & 17 sequenced

6.4.2 Linkage and Simulation Analysis

The genotypes at D21S210 for family members is shown (Figures 1a, 1b). A lod value of 2.833, with no recombination was obtained between GT12 and AD (Table 1). The score obtained at theta = 0.0 for F144 was highly suggestive of linkage but that for F139 was not. In particular, the low lod score in F139 was shown to result from the poor statistical power of the pedigree structure (vide infra) and two males in the largest sibship (Figure 1b), one of whom was at the peak age of risk and the other of whom was at highest risk for the disease. A rare allele (13) segregated with the disease in both families, (Figures 1a, 1b) (supporting the notion that the two families were likely to be related) and appears to mark the mutation carrying chromosome in F144. [Three individuals below or at the mean age of onset carry allele 13 in F144]. In F139 however, only weak segregation between this allele and the disease was observed due to the presence of allele 13 in unaffected males mentioned above. The detection of allele 13 in these two unaffected individuals high in the liability curve can be explained in two ways. Either there have been two recombinant events between *βAPP* and D21S210 in this part of the pedigree (very unlikely

due to their juxtaposition) or there is a dual source of allele 13 from the parents. This is also unlikely if the allele frequency is as low as reported (.027). The actual lod scores were small and positive and did not increase with greater distance between marker and disease indicating that these alternative probabilities were not in favour of recombination. A possible alternative explanation was that allele 13 was very much more common in the Swedish population than in the previous Caucasian populations for which it had been typed. However, simulation studies of F139 show that it was generally of poor structure to produce high lod scores even when the segregating allele was pre-determined as rare (Appendix 6C). The mean lod produced in 500 simulations was 0.5 with a maximum of 1.2 and only 8% generating a lod greater than 1.00. Although analysis of unlinked families produced an average lod which would exclude linkage by the accepted criteria, almost as many families produced lod scores greater than 1.0. This family then was poorly suited to discriminate between true linkage or non-linkage.

Table 1. Linkage Analysis of GT12 versus the Disease

Ped	Theta					
	0.00	0.01	0.02	0.03	0.04	0.05
F144	2.345	2.301	2.258	2.214	2.169	2.124
F139	0.488	0.469	0.451	0.434	0.419	0.405
Total	**2.833**	**2.770**	**2.709**	**2.648**	**2.588**	**2.528**

However, the results in F144 were quite robust and suggestive of linkage. Analysis of 1000 simulated pedigrees (of identical structure to F144) showed that a lod of 2.35 was likely to occur less than one time in 1000 if the marker and the disease were unlinked (Appendix 6B). Exons 17 and 16 of the *βAPP* gene were therefore sequenced. These exons were sequenced first as these encode the Aß peptide. Two base pair transversions (G->T and A->C) were observed in affected individuals in both families in exon 16 at codons 670 and 671 (*βAPP* 770 transcript). These changes predict lysine to asparagine and methionine to leucine substitutions in the intact protein. In addition, the codon 670

mutation causes the loss of an MboII recognition site. Thus, on digestion of amplified exon 16, two fragments (139bp and 90bp) are observed if the mutation is absent or one fragment (229bp) if it is present. Using this all available members of both F144 and F139 were screened by pcr amplification of exon 16 and digestion with this enzyme. All affected members of both families had lost the MboII cut site (see Figures 1a, 1b). The presence of the mutation was confirmed by direct sequencing of three individuals (see Figures 1a, 1b). Three unaffected individuals, past mean age of onset, were also sequenced at exon 16. [Exon 17 was also sequenced in these six individuals and was normal].

Segregation of the mutation with the disease or those at risk for the disease was obvious by eye (Figures 1a, 1b). Linkage analysis of the mutation (entered as a two allele system with very rare second allele) gave a lod score of 4.36 with no recombination (Table 2). Four individuals who carry the mutation in F144 do not score as recombinant because they are presently low on the age dependent risk curve and are presumably at very high risk for the disease. This analysis in F139 makes it clear that despite the presence of allele 13 in the two males of the largest sibship no mutation is present in either. Allele 13 was inherited in these cases either in a recombinant chromosome or there was a second source of this allele from the unaffected parent. It is possible that the allele frequency of 13 is higher in the Swedish population than the published reference data as there was only a 1:36 chance that allele 13 would enter the family at this point.

Table 2. Linkage Analysis of Mutation versus the Disease

Ped	Theta					
	0.00	0.01	0.02	0.03	0.04	0.05
F144	2.323	2.280	2.236	2.192	2.147	2.102
F139	2.036	1.997	1.958	1.918	1.878	1.838
Total	**4.359**	**4.277**	**4.194**	**4.11**	**4.025**	**3.94**

The co-segregation of the mutation with the disease and the fact that this variant could not be detected in 50 normal chromosomes leads to the suggestion that this mutation is pathogenic of the dementia in these families.

6.5 Discussion

The general method of analysing a highly polymorphic marker tightly linked to a candidate gene in relatively small families was successful in uncovering this allelic variant causing AD. However, several points arise form the analysis, particularly of F139. Firstly, inspection of the pedigree and the simulation results show that this family has few fully informative meiosis and is statistically weak, generating an average lod of 0.5 and a maximum of only 1.2. If the marker had been only a few cM from *βAPP* then the lod score would have been negligible or negative in F139. The screening of the genome in individual families by linkage is then a high risk strategy in families of similar power. In addition, the effects of mis-specification of allele frequencies could result in missed linkages. The lod score in this family and the implication of recombination is critically dependent on the allele frequency of the marking allele (13). Other investigators have noticed similar distortions of lod scores due to frequency assumptions about the co-segregating allele (Knowles *et al.*, 1992), particularly where inferences are drawn about untyped individuals (Ott, 1992). More recently, unpredictable variation in lod score has been observed by varying chromosome 14 marker allele frequencies in early onset AD families (Nechiporuk *et al.*, 1993). In general though, the anticipated effect of decreasing a co-segregating allele frequency will be to raise lod scores in the linked situation. This effect is noted in the simualtion study for F144 (Appendix 6A) where the maximum lod attained was lower than that in the actual linkage analysis. This is because all alleles were allowed to segregate with the disease. If only allele 13 had been set to mark the disease then the distribution would have been shifted to the left. The effect of decreasing the frequency of a non-segregating allele with untyped individuals in F139 is to lower the lod score as the possibility of a second source of the allele is reduced, inferring recombination.

It is also clear from two subsequent screening papers that this mutation is rare in both Swedish and non-Swedish populations of early onset familial and non-familial AD.

This mutation and the codon 717 mutations frame the Aß sequence (Figure 2) suggesting that the mechanism of action may be to enhance the cleavage of this sequence from the parent molecule. The data on mutation mechanism is discussed more fully in the concluding chapter but others have provided evidence for the central role of Aß in AD development by showing that the 670/671 mutation causes a 5-8 fold increase in Aß production in transfected cultured cells.

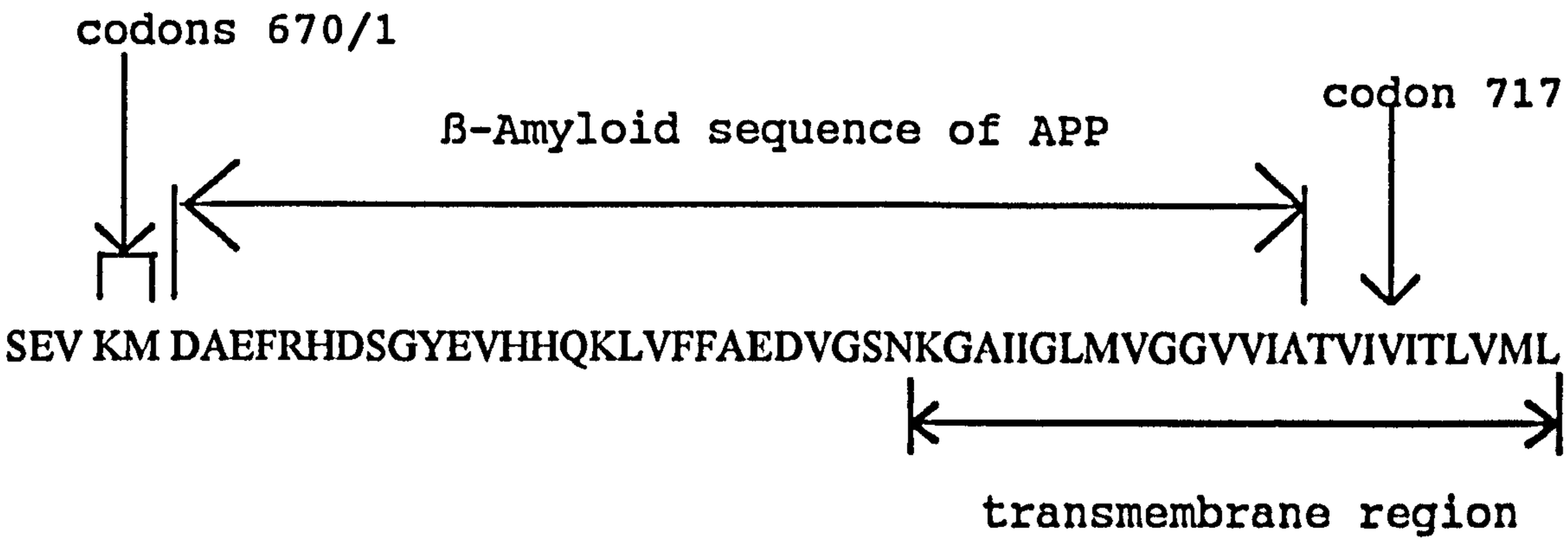


Figure 2. Showing the positions of the codon 670/1 and codon 717 mutations in relation to the ß-Amyloid sequence

E Indicates the site of the HCHWA-D mutation

Chapter 6 Appendix A

Linkage Analysis of 1000 Simulated Linked Families of the Structure of F144

Input Data:

The random number seed is: 12345

The number of replications is: 1000

The requested proportion of unlinked families is: 0.000

The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	24
Number of females	13
Number of males	11

There were 11 in category: Marker Unknown; Trait Original

There were 0 in category: Marker Available; Trait Simulated

There were 13 in category: Marker Available; Trait Original

There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data

Linked order of loci: 1 2

True thetas for linked order: 0.000100

Unlinked order of loci: 1 2

True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 4.83000 seconds

Elapsed time = 1981.88000 seconds or 33.03 minutes

Actual proportion of unlinked families: 0.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.635626	0.747544	1.309676	2.108169

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.734787	0.665179	0.000000	2.131523

Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	183	36.600
1	Study	183	0.366
2	1	13	2.600
2	Study	13	0.026
3	1	0	0.000
3	Study	0	0.000

Chapter 6 Appendix B

Simulation 1000 Unlinked F144 Families and Linkage Analysis. No Lod Score Greater than 2.00 Occurred with GT12 Markers.

Input Data:

The random number seed is: 12345

The number of replications is: 1000

The requested proportion of unlinked families is: 1.000

The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	24
Number of females	13
Number of males	11

There were 11 in category: Marker Unknown; Trait Original

There were 0 in category: Marker Available; Trait Simulated

There were 13 in category: Marker Available; Trait Original

There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data

Linked order of loci: 1 2

True thetas for linked order: 0.000100

Unlinked order of loci: 1 2

True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 3.68000 seconds

Elapsed time = 1781.79000 seconds or 29.70 minutes

Actual proportion of unlinked families: 1.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	-1.035751	1.565266	-6.991036	1.768643

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.093075	0.378095	0.000000	1.775997

Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	13	2.600
1	Study	13	0.026
2	1	0	0.00
2	Study	0	0.00
3	1	0	0.00
3	Study	0	0.00

Chapter 6 Appendix C

Simulation Study of F139: Generation and Analysis of 500 Families of Similar Structure to F139 Under Simulated Conditions of Linkage

The alleles were randomly distributed with the proviso that 50% of families segregated the rare allele 13 with the disease.

Input Data:

The random number seed is: 25678

The number of replications is: 500

The requested proportion of unlinked families is: 0.000

The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	15
Number of females	7
Number of males	8

There were 8 in category: Marker Unknown; Trait Original

There were 0 in category: Marker Available; Trait Simulated

There were 7 in category: Marker Available; Trait Original

There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data

Linked order of loci: 1 2

True thetas for linked order: 0.000100

Unlinked order of loci: 1 2

True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 2.31000 seconds

Elapsed time = 1533.90000 seconds or 25.56 minutes

Actual proportion of unlinked families: 0.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.529400	0.354699	-1.662859	1.231914

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.546801	0.314693	0.000000	1.231914

Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	39	7.800
1	Study	39	0.078
2	1	0	0.000
2	Study	0	0.000
3	1	0	0.000
3	Study	0	0.000

Chapter 6 Appendix D

Simulation Study of F139, Lod Score Distribution Under Non-Linkage

Input Data:

The random number seed is: 29876

The number of replications is: 500

The requested proportion of unlinked families is: 1.000

The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	15
Number of females	7
Number of males	8

There were 8 in category: Marker Unknown; Trait Original

There were 0 in category: Marker Available; Trait Simulated

There were 7 in category: Marker Available; Trait Original

There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data

Linked order of loci: 1 2

True thetas for linked order: 0.000100

Unlinked order of loci: 1 2

True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 3.51000 seconds

Elapsed time = 1817.65000 seconds or 30.29 minutes

Actual proportion of unlinked families: 1.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	-2.232078	2.277617	-7.443192	1.175004

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.141232	0.259670	0.000000	2.094596

Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	6	1.200
1	Study	6	0.012
2	1	1	0.200
2	Study	1	0.002
3	1	0	0.000
3	Study	0	0.000

CHAPTER 7

Exclusion of *βAPP* and Linkage to the Long Arm of Chromosome 14 in the Remaining Early Onset Families

7.1 Abstract

The preceding chapters have highlighted the occurrence of mutations in the ß-amyloid precursor protein (ßAPP) gene causing some cases of hereditary, early onset familial Alzheimer's disease (AD). However, linkage analysis presented in this chapter, of other early onset families with the GT12 marker of *βAPP* demonstrate exclusion of linkage of most of the early onset families in this data set. These data confirm that non-allelic genetic heterogeneityy exists in early onset familial AD. One family additional family, however, F172, was consistent with linkage to *βAPP* and was subsequently found to have a *βAPP*717 Val→Ile mutation. Of the others, all but one were consistent with linkage to markers at the middle long arm of chromosome 14. However, no family showed independent evidence of linkage with two-point analysis and only one family showed independent evidence of linkage on multipoint analysis. Therefore, heterogeneity at this locus must be considered although tests for heterogeneity were not significant.

7.2 Introduction

F23 and F19 in this family data set were shown to be linked to *βAPP* and to have mutations at codon 717 (Chapters 3 and 5). Additional mutations segregating with AD in early onset families were also known to occur at codon 670/671 (Chapter 6) and at codon 692 where the mutation co-segregates with either AD or a phenotype akin to multi-infarct dementia due to heavy deposition of abnormal amyloid in vessel walls (Hendriks *et al.*, 1992). To test for linkage in the other early onset families the segregation of the marker, GT12 at D21S210 was examined, which is tightly linked to *βAPP*. Of the nine remaining families, only one (F126) had a modestly positive lod score at D21S210. Other candidate loci and chromosomes were therefore tested for linkage in these families. Data suggestive of linkage on chromosome 14 had been previously reported in the literature (Weitkamp *et al.*, 1983), particularly in one large pedigree (Nee *et al.*, 1983). In addition, α1-antichymotrypsin (AACT) is a component of plaque cores and a protease inhibitor and has

been proposed as a possible candidate gene for AD (Abraham *et al.*, 1988). This gene had been localised to chromosome 14 (Cox *et al.*, 1990) and was examined for linkage to early onset AD. It was excluded but other chromosome 14 markers are not.

7.3 Methods

7.3.1 Clinical Studies

F74 and F105 were subject to the detailed clinical work up described in Chapter 2. The remaining families had a much shortened work up, consisting of a screening of all members with the mini-mental state examination, followed by examination of clinical notes and phone interviews of the care giver and/or nearest relatives. These data were gathered and examined by two independent clinicians. All affected individuals fulfilled NINCDS criteria (McKhann *et al.*, 1984) for probable or definite AD. Autopsy confirmation was available for some families (Table 1). All unaffected individuals for which DNA was analysed scored normally on the Mini-Mental State screening instrument (Folstein *et al.*, 1975). Age of onset for each affected individual was estimated from the history derived from the patient, primary carers, hospital and general practitioner records.

Table 1. Clinical Details of Early Onset Families

Family number	Mean age of onset	Number of generations	Number of generations	Affecteds (path. confirmed)
53	52	3	5	(1)
74	41	4	7	(2)
105	38	6	14	
121	37	4	6	
126	54	4	4	
134	46	4	6	(2)
148	41	4	5	(1)
168	41	4	6	(1)
172	54	3	10	
206	39	6	7	

7.3.2 Linkage and Simulation Analysis

D21S210, D14S52, D14S43, D14S53, D14S48, AACT and AT data were analysed with MLINK from the LINKAGE package using two-point and multipoint analyses (Lathrop *et al.*, 1984). The chromosome 14 marker was checked by estimating the recombination fraction between D14S52, D14S43, D14S53, D14S48 using the ILINK program. This strategy, is described in the Materials and Methods chapter.

The usual parameters describing the disease locus were used in all linkage analyses; the age of onset distribution was assumed to be normal with no important truncation effects and were used to derive liability classes for unaffected cases using the program written for this purpose (see Appendix 7A). The values of the liability table were as previously described. Published allele frequencies were used (Wang, Weber, 1992). Neutered map distances were taken from published data (Wang, Weber, 1992; Cox *et al.*, 1990).

7.3.3 Linkage Data

A genetic linkage map of chromosome 14 pcr-amplified microsatellites has recently been reported (Wang, Weber, 1992). AC data was generated for D21S210 (GT12), D14S43, D14S53, D14S52 and D14S48 using published primers and conditions. Primers used for the detection of the AACT polymorphism were: 5'-AGAGTTGAGAATGGAGAG-3' and 5'-GTCCACGTGTGTCCCTCG-3', and for the AT gene: 5'-GTGATTCCCCAACCTGAGGGTGACC-3' and 5'-CTGGGCCTTGCCACACAGGCTCTTC-3'. Conditions for both these primer sets were 10 minutes initial denaturation followed by 35 cycles of 72°C for 1min, 94°C for 1min and 55°C for 45sec, in a 50ul reaction volume. The optimum $MgCl_2$ concentration for the AACT primers was 1.5mM and for the AT primers 1.0mM. The PCR product generated by the AACT primers is 155bp which after BstNI digestion gives three fragments of 84,33 and 32bp. The polymorphism changes an alanine to a threonine and abolishes a restriction site resulting in two fragments of 116 and 33bp. The allele frequencies estimated were 0.3 for alanine and 0.7 for threonine.

This locus was analysed by converting a protein polymorphism within the signal peptide of the AACT gene to a PCR detectable polymorphism and developing an SSCP polymorphism within the adjacent anti-trypsin (PI) locus. Overall, two-point analyses of both polymorphisms were negative. These two loci were entered in the linkage analysis as

one locus by setting theta = 0.0 between them, but haplotype frequencies were not calculated.

7.4 Results

7.4.1 Clinical

Table 1 gives details of the mean age of onset and number of generations affected in each pedigree which are shown in Appendix 7D. All affected pedigree members fulfilled NINCDS criteria for probable AD. The autopsied cases fulfilled the criteria for definite AD. More detailed analysis of one family (F74) is given in the next chapter.

7.4.2 Linkage and Simulation: *βAPP* Locus

The results of analysis of AD versus D21S210 is given in Table 2. Family 172 showed a substantial positive lod score the significance of which was further examined with simulation studies. DNA was available for only 9 individuals limiting the maximum lod score obtainable. Four alleles of the GT12 marker segregated in the family. The average lod score from a thousand simulated linked pedigrees with these four markers segregating randomly in F172 was 0.3 (Appendix 7A). The maximum lod obtained was 1.7 with 11.6% of pedigrees exceeding a lod of 1.00. For the unlinked simulation of the rare alleles segregating randomly, a very high maximum lod score was obtained (1.5) although the average was -0.61 (Appendix 7B). The standard deviation was accordingly large and 7% of unlinked families exceeded lod score of 1.00. When the allele frequencies were entered each as 0.25 the average and maximum under unlinked conditions were -0.99 and +1.27 respectively (Appendix 7C). This family then, was poorly structured to discriminate between a positive and negative lod score. However, the generation of linked pedigrees enabled random segregation of the alleles. Thus, any of the four alleles could segregate with the disease and will do so in accordance with their allele frequencies. The family information structure (Appendix 7D) includes unknown married in individuals. The maximum lod score obtainable with common alleles is reduced by these unknown individuals, when the probability of a second source of the marking allele assumes significance. In the actual pedigree the segregating allele frequencies were 0.060000, 0.0270000, 0.0320000, 0.149000. The allele actually marking the disease in the family had a frequency of 0.06. This low frequency allele produced a lod of 1.76 in the real

analysis, higher than the maximum lod generated in the simulated studies, where the more common allele (frequency 0.149) was allowed to mark the disease 2.5 (0.149/0.06) times more commonly. As the lod score was at the extreme of the simulated data this provided good evidence that the *βAPP* gene was linked to the disease. Gene analysis was therefore undertaken; SSCP and direct sequencing showed that this family was another example of a Val→Ile mutation (Fidani *et al.*, 1992).

The remaining families showed a combined lod score of -11.05 with exclusion beyond 10cM on either side of the *βAPP* locus. The only family showing a small positive lod score is F126.

7.4.3 Linkage: Chromosome 14 Locus

The combined AACT/PI polymorphisms together (Table 2) gave exclusion (assuming genetic homogeneity) to 9 centiMorgans. The results of two-point analysis at the other loci are also shown. The accepted criterion for linkage was observed for both D14S43 and D14S53 with early onset AD (Table 2). No family typed for these markers gave any evidence for recombination except F126. This family is the only one in this data set showing any evidence of linkage to D21S210 (Table 2). HOMOG (Ott, 1991) analysis of the D14S43 and D14S53 was negative indicating no evidence of heterogeneity. The multi-point analysis produced a lod score maximum at D14S43 (Z_{max} = 7.8, 0 = 0.0; Figure 1).

7.5 Discussion

These data are consistent with a second major early onset AD locus in the middle long arm of chromosome 14. An overview of this early onset data set suggests that approximately 25% of early onset familial AD are encoded at the *βAPP* locus and the remainder by a gene close to D14S43 and D14S53. In general, there are two methods of isolating the gene once linkage is established; candidate gene approach, examining genes in the linked area, and positional cloning of the linked area. Either of these approaches become easier if the linked area can be reduced. Although flanking markers are identified by this genetic analysis the multi-point map is quite flat over a relatively large genetic distance (Figure 1). Further recombination in additional early onset families (for instance, at D14S43 or D14S53) would narrow this area of linkage. Alternatively, extension of the present

families may reveal recombinants as the apparent absence of recombination at D14S43 and D14S53 may partly be due to incomplete or incompletely informative data. As there is no evidence of heterogeneity a multi-point map of the region was constructed but until homogeneity is established the maximum likelihood position of the disease gene may be misleading. The problems of detecting heterogeneity in small families is discussed in Chapter 1. F126 is a good example, showing segregation with GT12 and partial recombination at D14S43 and D14S53 but not producing a significant test of heterogeneity. Therefore, until a *βAPP* mutation is detected in this family it remains in the collective data set. An additional strategy will be to test for heterogeneity with a larger data set.

A possible candidate gene in the general region, congruous with the central role of amyloid, is the 70 kD heat shock protein gene, HSPA2. The promoter of the *βAPP* gene has a heat shock consensus sequence and ßAPP has been shown to be up-regulated by heat shock (Abe *et al.*, 1991). The genetic localisation of the HSPA2 is unknown and needs to be determined. However, it is of course possible that the gene encoding some other protein interacting with ßAPP is encoded in this area of chromosome 14.

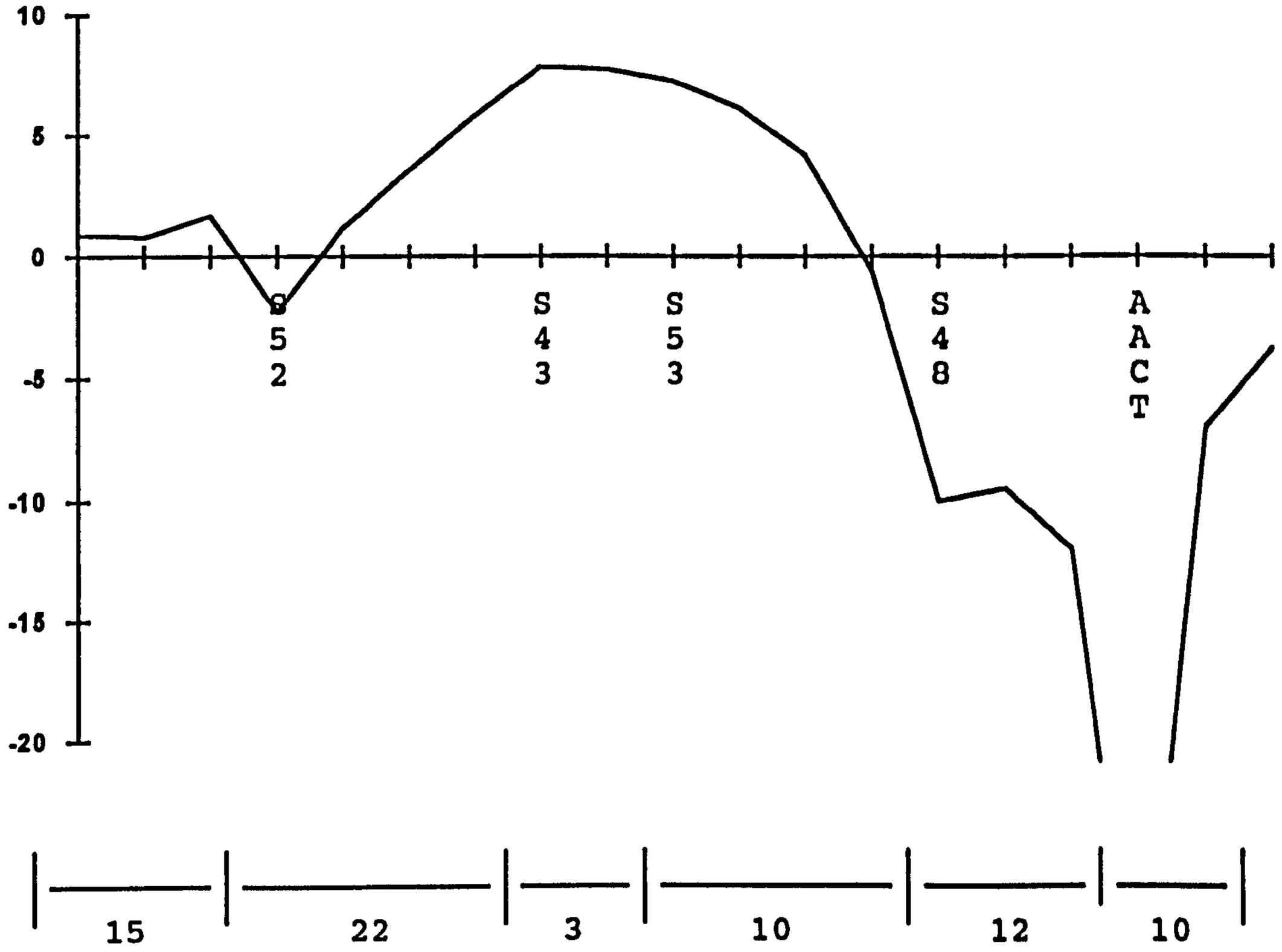
Table 2. Lod Scores for Markers on Chromosomes 21 and 14

Family number	D21S210 recombinant fraction (0)					AACT/AT recombinant fraction (0)				
	0.00	0.02	0.05	0.10	0.20	0.00	0.02	0.05	0.10	0.20
53	-0.91	-0.82	-0.70	-0.52	-0.27	-0.13	-0.12	-0.10	-0.07	-0.03
74	-3.48	-1.79	-1.25	-0.79	-0.31	-5.85	-0.66	-0.31	-0.10	0.03
105	-1.13	-0.99	-0.81	-0.59	-0.29	-0.37	-0.34	-0.30	-0.23	-0.12
121	-0.67	-0.51	-0.35	-0.20	-0.07	-0.11	0.02	0.12	0.19	0.17
126	0.72	0.69	0.64	0.55	0.37	-1.36	-0.98	-0.70	-0.45	-0.20
134	-0.25	-0.22	-0.17	-0.11	-0.05	-0.10	-0.09	-0.08	-0.06	-0.03
148	-0.19	-0.18	-0.16	-0.14	-0.08	0.06	0.07	0.08	0.09	0.07
168	-5.91	-3.34	-2.24	-1.40	-0.60	-14.36	-2.15	-1.39	-0.85	-0.36
206	-0.05	0.40	0.60	0.69	0.59	-12.21	-1.71	-0.99	-0.50	-0.14
Totals	**-11.05**	**-6.76**	**-4.44**	**-2.51**	**-0.71**	**-34.43**	**-5.96**	**-3.67**	**-1.98**	**-0.61**
172	1.76	1.69	1.58	1.40	0.99					

Family number	D14S52 recombinant fraction (0)					D14S43 recombinant fraction (0)				
	0.00	0.02	0.05	0.10	0.20	0.00	0.02	0.05	0.10	0.20
53	0.00	-0.01	-0.02	-0.03	-0.03	-0.03	-0.03	-0.03	-0.03	-0.02
74	-0.10	-0.09	-0.08	-0.06	-0.03	1.57	1.51	1.41	1.24	0.87
105	0.26	0.25	0.24	-0.20	0.13	-0.20	-0.19	-0.16	-0.13	-0.07
121	-0.15	-0.14	-0.12	-0.09	-0.04	0.19	0.16	0.12	0.08	0.03
126	-0.02	-0.01	-0.00	-0.00	0.00	-0.47	-0.39	-0.27	-0.19	-0.08
134	0.00	0.00	0.00	0.00	0.00	0.19	0.19	0.18	0.16	0.12
148	-5.40	-2.10	-1.34	-0.80	-0.33	1.45	1.40	1.33	1.20	0.91
168	0.41	0.39	0.36	0.31	0.21	0.07	0.07	0.05	0.03	0.03
206	0.28	0.29	0.28	0.26	0.18	0.50	0.46	0.41	0.33	0.18
Totals	**-4.72**	**-1.42**	**-0.68**	**-0.61**	**0.09**	**3.27**	**3.18**	**3.04**	**2.69**	**1.97**

Family number	D14S53 recombinant fraction (0)					D14S48 recombinant fraction (0)				
	0.00	0.02	0.05	0.10	0.20	0.00	0.02	0.05	0.10	0.20
53	0.06	0.05	0.03	0.01	-0.01	0.00	0.00	0.00	0.00	0.00
74	0.68	0.18	0.20	0.22	-0.09	-5.80	-0.64	-0.29	-0.08	0.05
105	1.11	0.32	0.29	0.23	0.64	-6.66	-1.18	-0.79	-0.50	-0.22
121	0.12	0.09	0.08	0.08	0.02	-0.41	-0.38	-0.34	-0.27	-0.15
126	-0.53	-0.49	-0.42	-0.32	-0.18	0.00	0.00	0.00	0.00	0.00
134	0.20	0.20	0.20	0.20	0.04	0.31	0.30	0.28	0.24	0.15
148	1.36	1.26	1.19	1.07	0.70	0.32	0.30	0.27	0.22	0.13
168	0.06	0.06	0.06	0.05	0.03	-0.12	-0.09	-0.08	-0.08	-0.06
206	0.35	0.28	0.26	0.24	0.19	0.24	0.19	0.15	0.83	0.09
Totals	**3.41**	**1.95**	**1.89**	**1.78**	**1.34**	**-12.12**	**-1.50**	**-0.80**	**0.36**	**-0.01**

Figure 1. Multi-Point Analysis of Chromosome 14 Markers vs AD in 8 Early Onset Families (F172 not included). Indicated distances are in centimorgans.



Chapter 7 Appendix 7A

Analysis of 1000 Simulated Linked Pedigrees Assuming Four Rare Alleles Segregating in F172 in Accordance with their Frequencies of 0.060000 0.0270000 0.0320000 0.149000

Input Data:

The random number seed is: 12345
The number of replications is: 1000
The requested proportion of unlinked families is: 0.000
The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	19
Number of females	9
Number of males	10

There were 10 in category: Marker Unknown; Trait Original
There were 0 in category: Marker Available; Trait Simulated
There were 9 in category: Marker Available; Trait Original
There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data
Linked order of loci: 1 2
True thetas for linked order: 0.000000
Unlinked order of loci: 1 2
True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 1.48000 seconds
Elapsed time = 1478.21000 seconds or 24.64 minutes.
Actual proportion of unlinked families: 0.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.295123	0.490800	-1.150770	1.694617

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.378805	0.406771	0.000000	1.694617

Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	116	11.600
1	Study	116	0.116
2	1	0	0.000
2	Study	0	0.000
3	1	0	0.000
3	Study	0	0.000

Chapter 7 Appendix 7B

Analysis of 1000 Simulated Unlinked Pedigrees Assuming Four Rare Alleles Segregating in F172 in Accordance with their Frequencies of 0.060000 0.0270000 0.0320000 0.149000

Input Data:

The random number seed is: 12345
The number of replications is: 1000
The requested proportion of unlinked families is: 1.000
The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	19
Number of females	9
Number of males	10

There were 10 in category: Marker Unknown; Trait Original
There were 0 in category: Marker Available; Trait Simulated
There were 9 in category: Marker Available; Trait Original
There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data
Linked order of loci: 1 2
True thetas for linked order: 0.000000
Unlinked order of loci: 1 2
True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 1.53000 seconds
Elapsed time = 1467.77000 seconds or 24.46 minutes.
Actual proportion of unlinked families: 1.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	-0.608118	1.168866	-3.692108	1.463880

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.091569	0.195321	0.000000	1.463880

Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	7	0.700
1	Study	7	0.007
2	1	0	0.000
2	Study	0	0.000
3	1	0	0.000
3	Study	0	0.000

Chapter 7 Appendix 7C

Analysis of 1000 Simulated Unlinked Pedigrees Assuming Four Common Alleles Segregating in the Family in Accordance with their Frequencies of 0.25 0.25 0.25 0.25

Input Data:

The random number seed is: 23456
The number of replications is: 1000
The requested proportion of unlinked families is: 1.000
The trait locus is locus number: 1

Summary Statistics about simped.dat

Number of pedigrees	1
Number of people	19
Number of females	9
Number of males	10

There were 10 in category: Marker Unknown; Trait Original
There were 0 in category: Marker Available; Trait Simulated
There were 9 in category: Marker Available; Trait Original
There were 0 in category: Marker Unknown; Trait Simulated

Results:

Linkage (v4.91) with 2-point autosomal data
Linked order of loci: 1 2
True thetas for linked order: 0.000000
Unlinked order of loci: 1 2
True thetas for unlinked order: 0.500000

Elapsed time for one replicate = 1.48000 seconds
Elapsed time = 1465.08000 seconds or 24.42 minutes.
Actual proportion of unlinked families: 1.000

Average Lod Scores (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	-0.989370	1.404678	-3.940671	1.268353

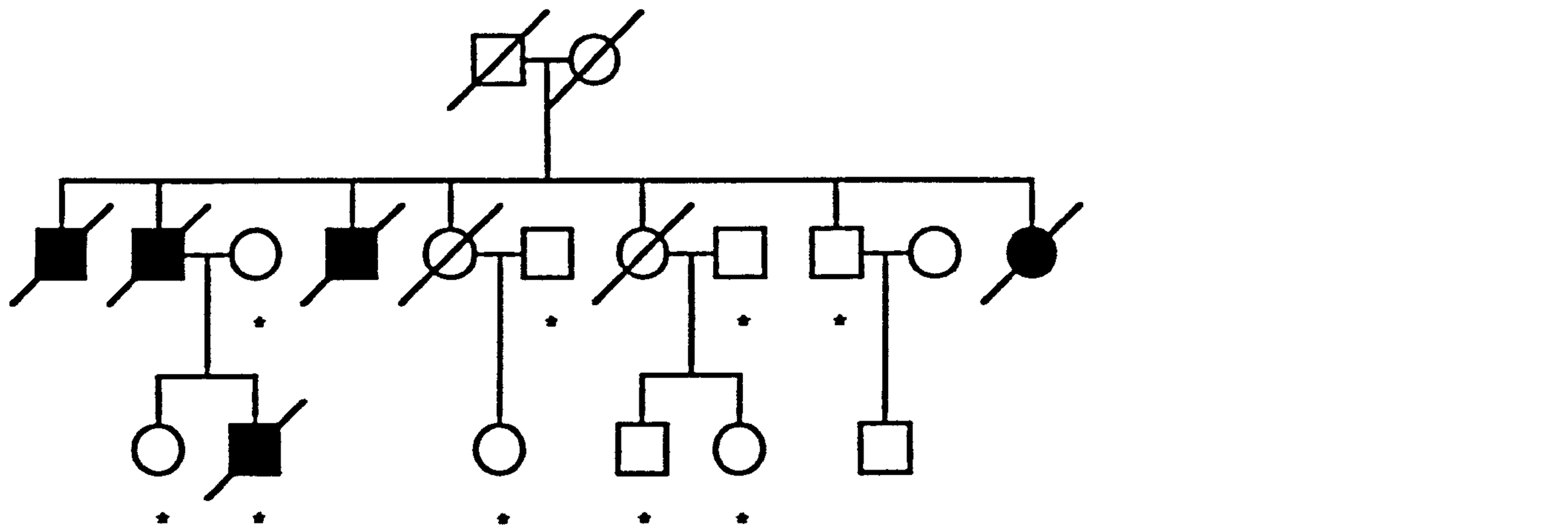
Average Maximum Lod Scores based on quadratic interpolation

Average Maximum Lod Scores Based on Quadratic Interpolation (Theta = 0.0)				
Pedigree	Average	StdDev	Min	Max
1	0.087489	0.196971	0.000000	1.268353

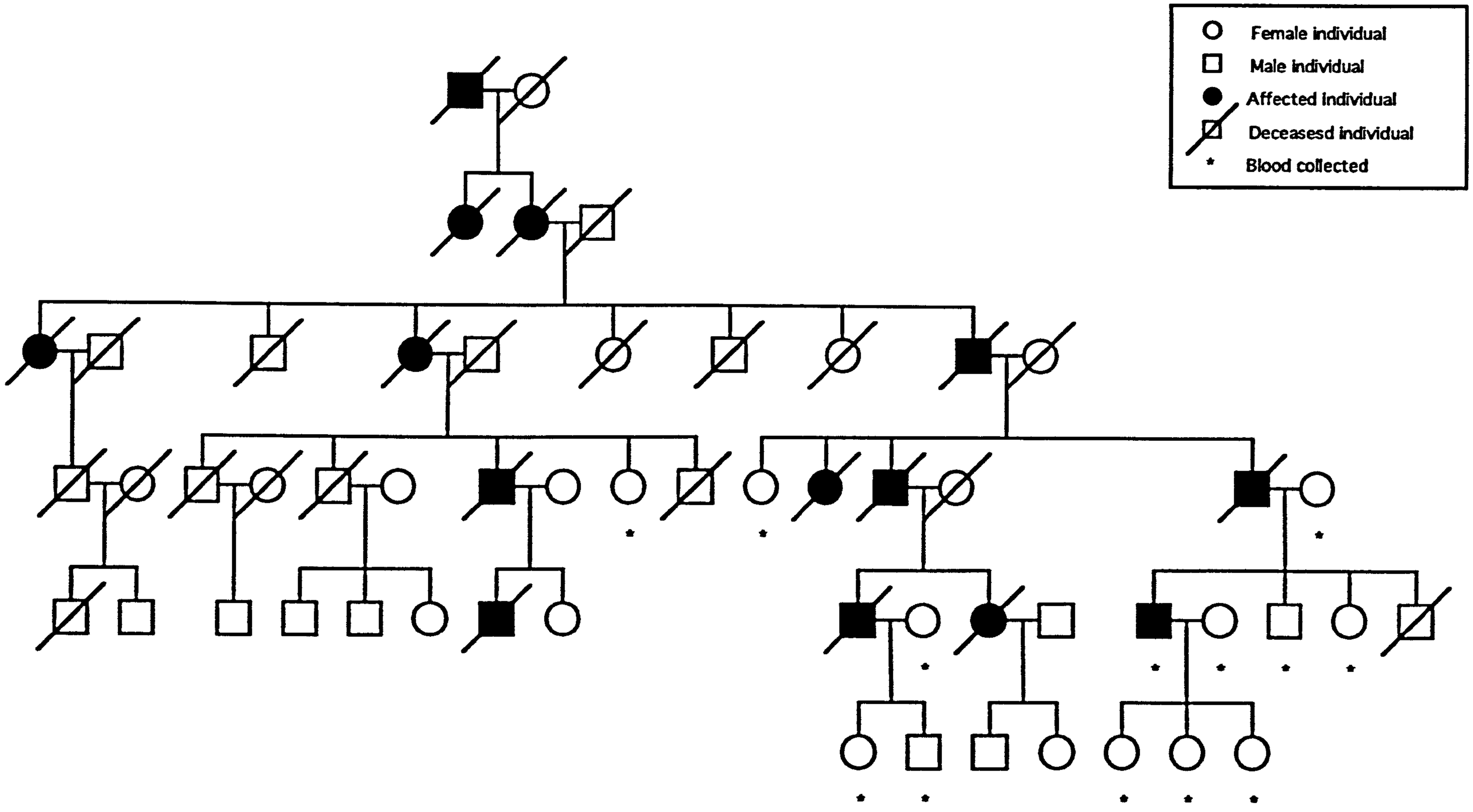
Number of (Interpolated) Maximum Lod Scores Greater Than a Given Constant			
Constant	**Pedigree**	**Number**	**Percent**
1	1	8	0.800
1	Study	8	0.008
2	1	0	0.000
2	Study	0	0.000
3	1	0	0.000
3	Study	0	0.000

Chapter 7 Appendix 7D

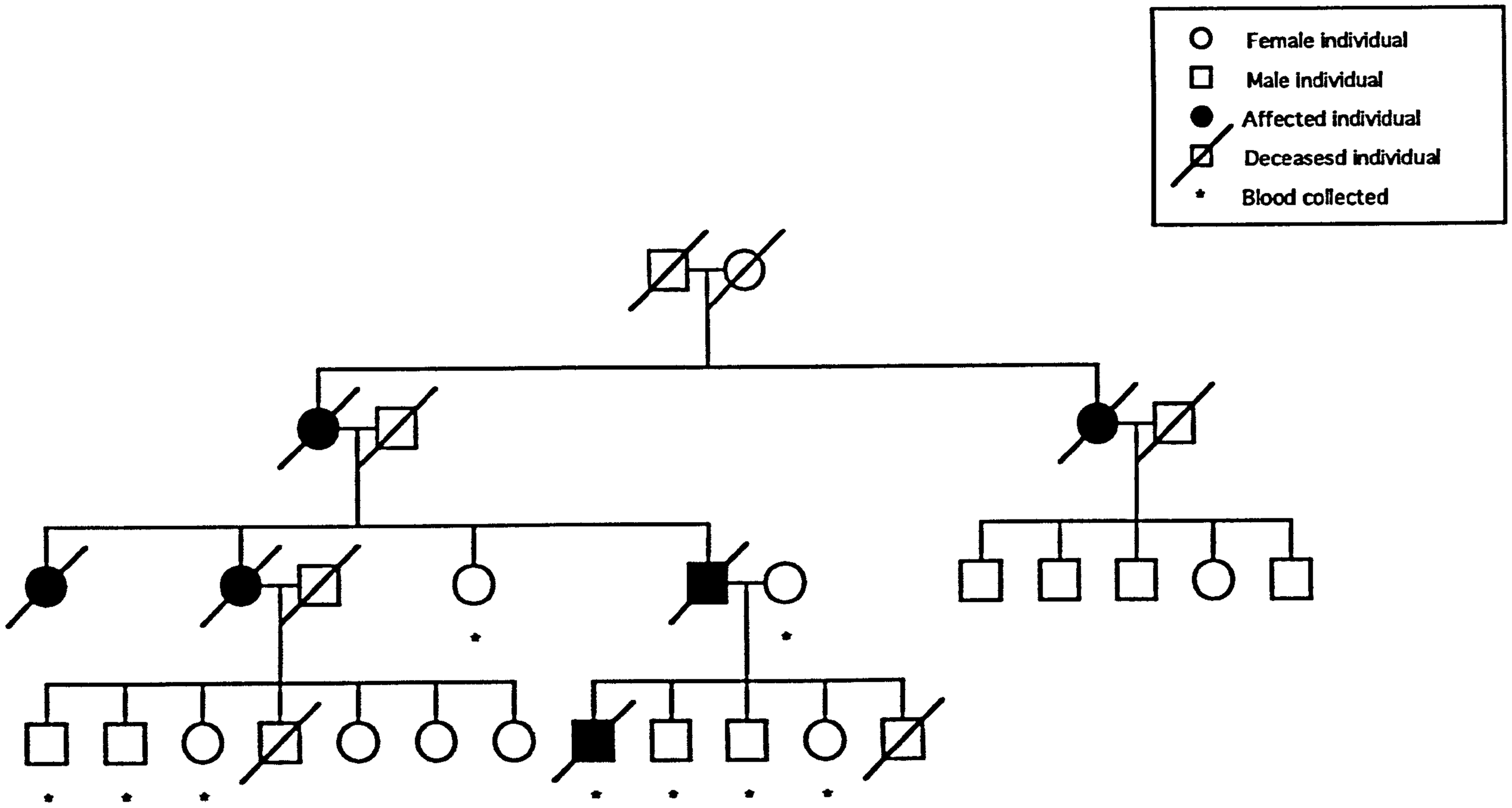
Pedigree Structures


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

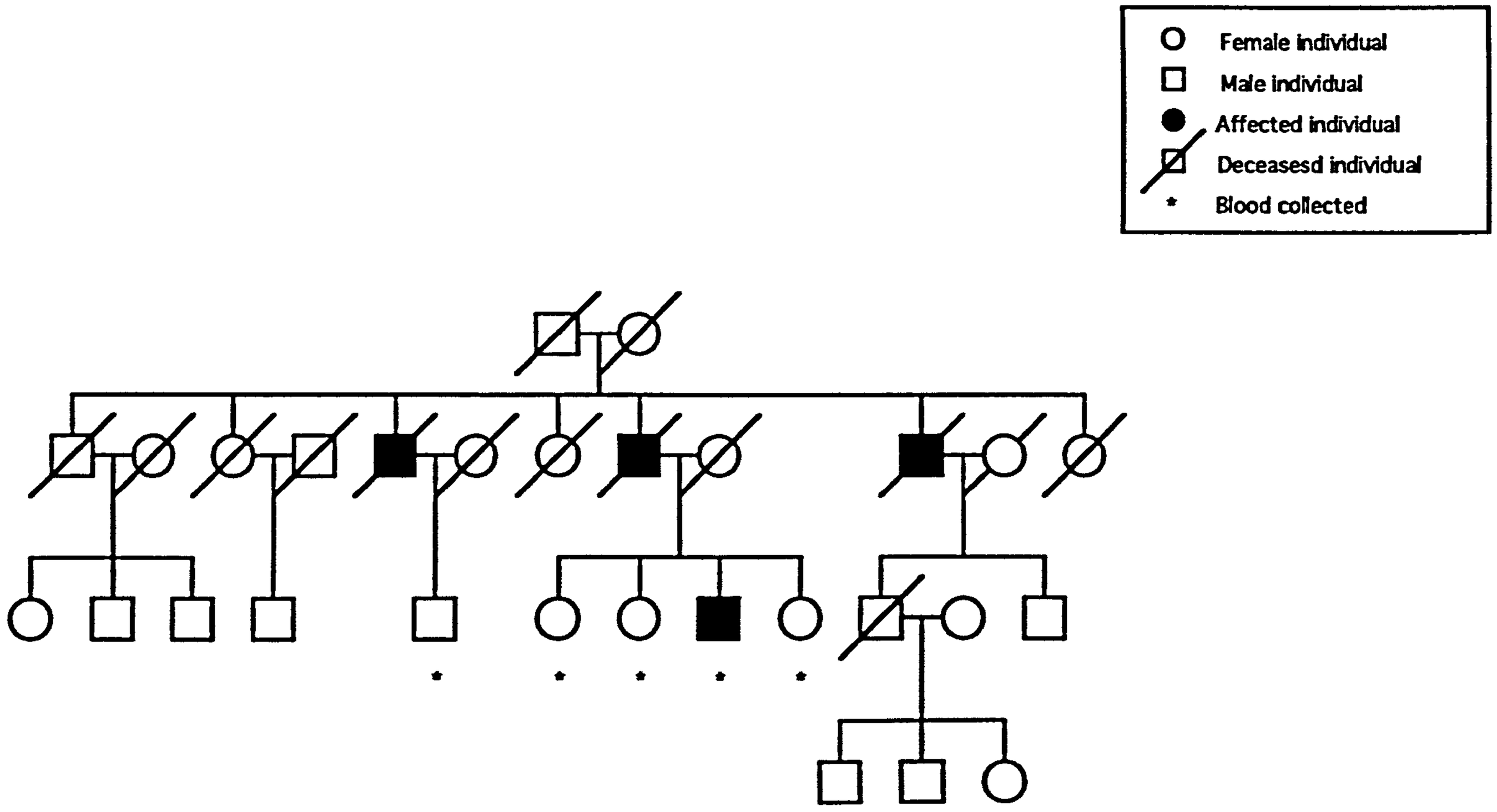
Family 53


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

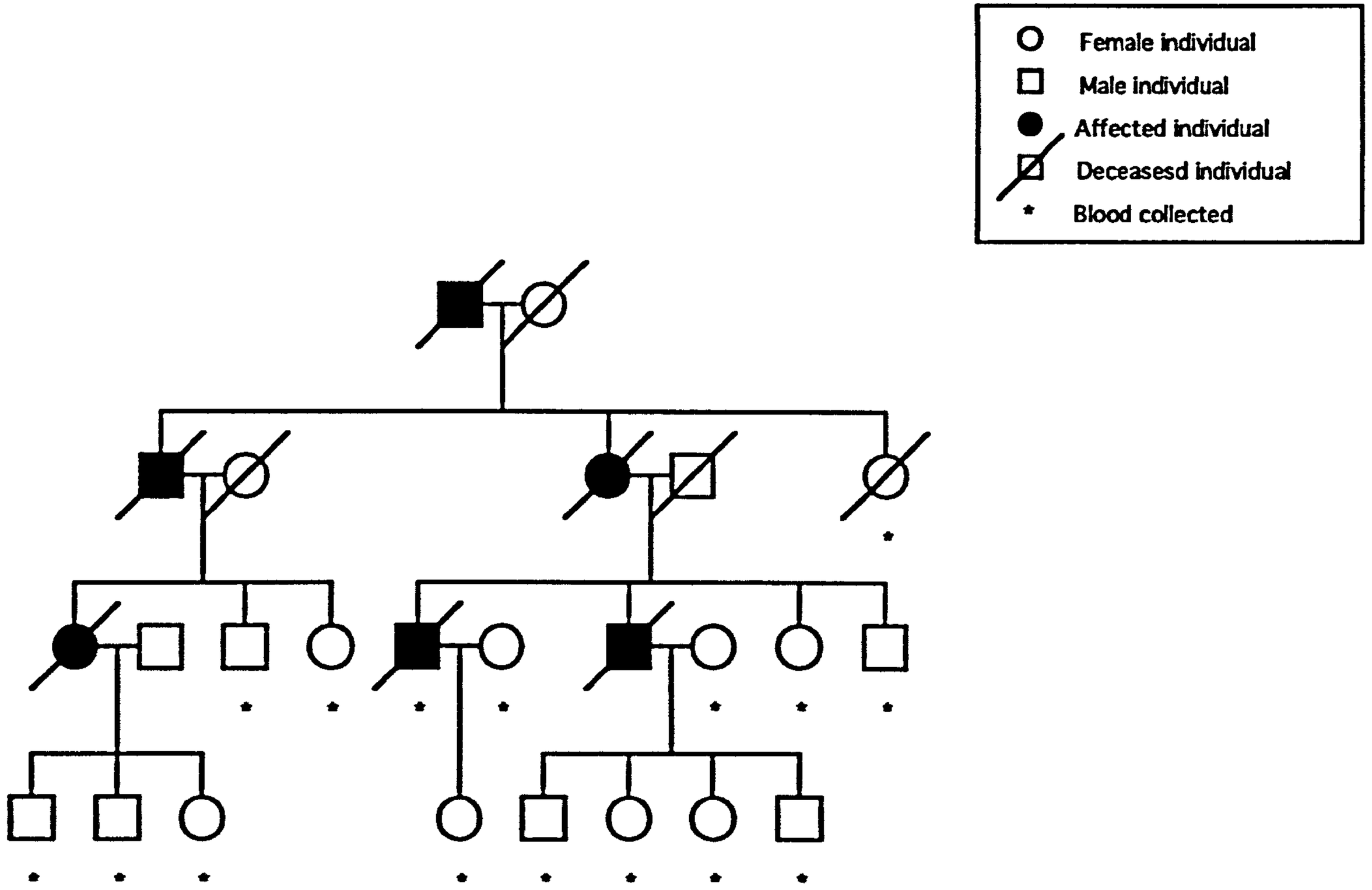
Family 105


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

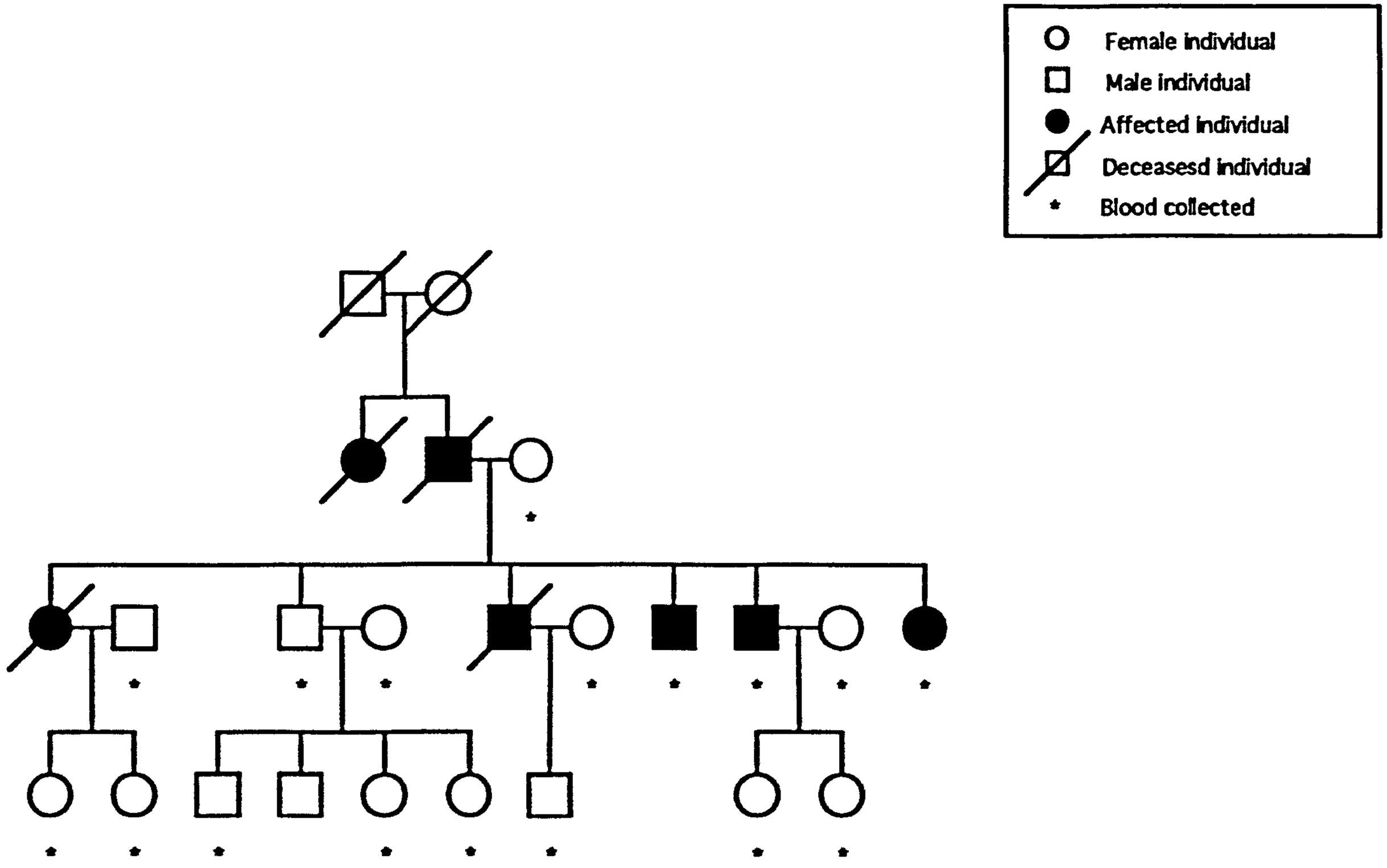
Family 121


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

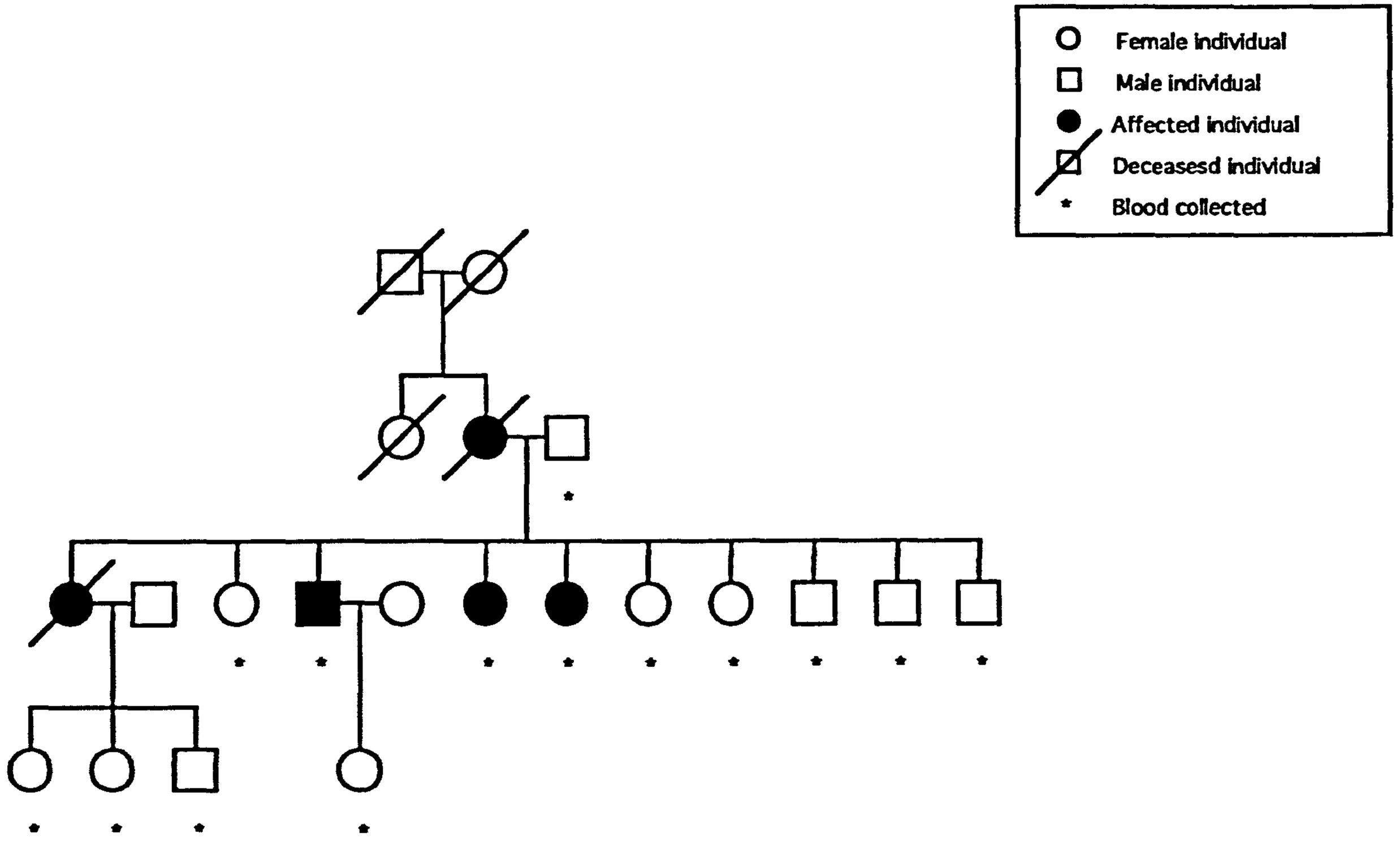
Family 126


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

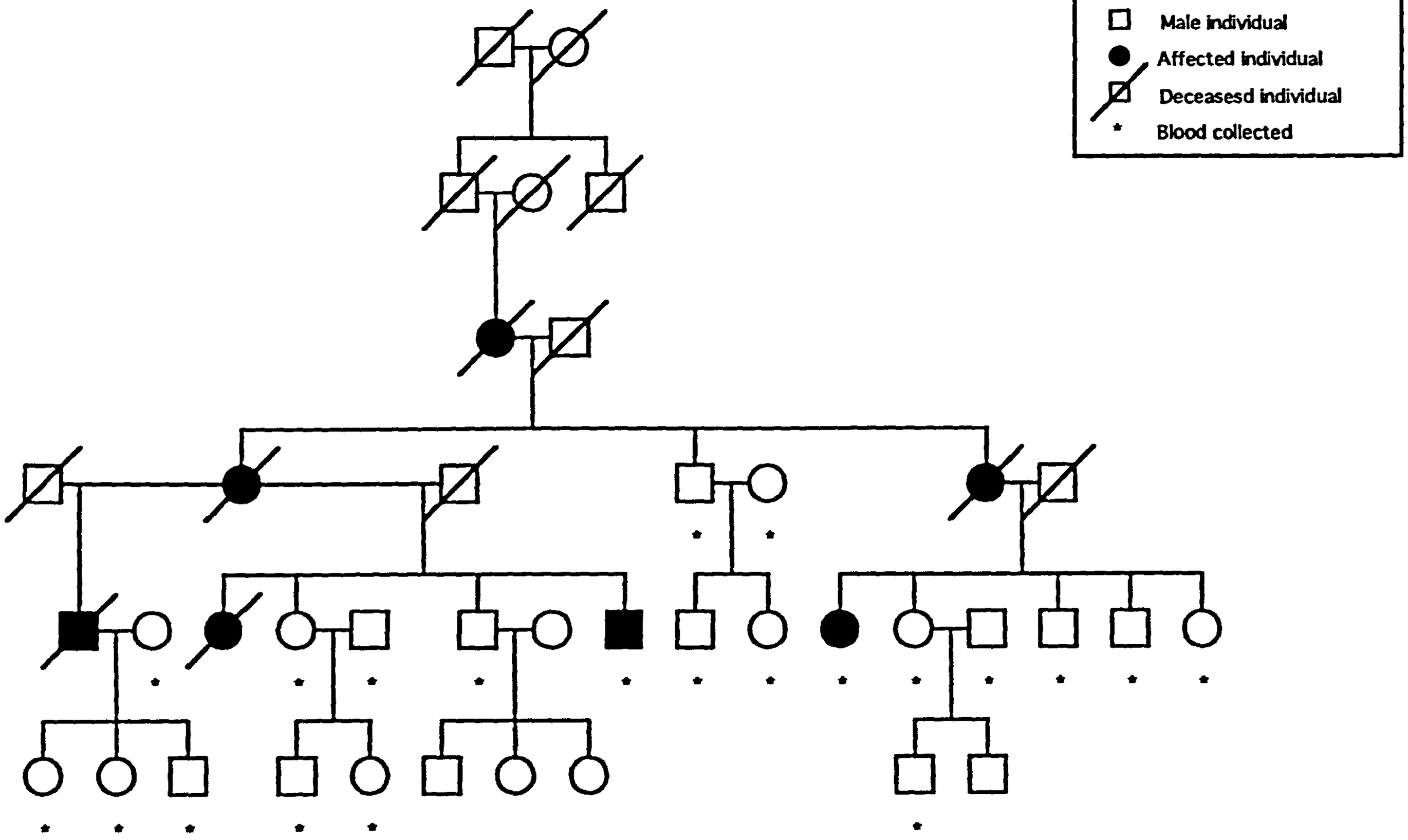
Family 134


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

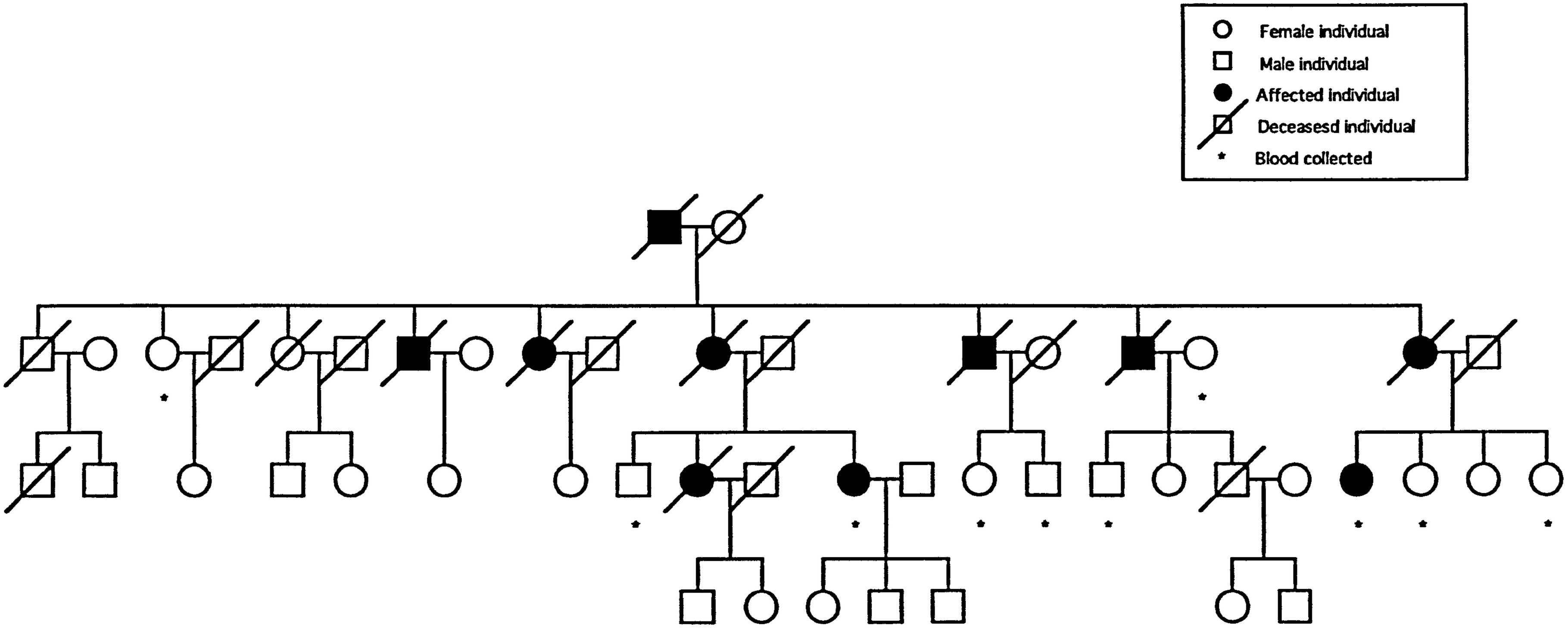
Family 148


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

Family 168


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

Family 206


Female individual
Male individual
Affected individual
Deceasesd individual
Blood collected

Family 172

CHAPTER 8

Clinical Features of Early Onset Familial Alzheimer's Disease Linked To Chromosome 14

8.1 Abstract

Early onset familial Alzheimer's disease (AD) has an autosomal dominant mode of inheritance. Two genes are responsible for the majority of cases of this subtype of AD. The previous chapters have shown that mutations in the ß-amyloid precursor protein (ßAPP) gene completely co-segregate with the disease and there is a second locus for early onset disease on chromosome 14. Here the clinical features and genetic analysis are reported of a pedigree (F74) with early onset AD in which the ßAPP locus does not segregate with the disease but which shows good evidence for linkage on the long arm of chromosome 14. Additional marker data suggest the disease locus is close to D14S43 and D14S77. Genetic data from this pedigree have previously been used in linkage studies using chromosome 21 markers in attempts to localise the defect on this chromosome. The effect of including this family in such analysis contributed to early mis-localisation of the putative chromosome 21 gene. However, apart from age of onset there is no clinical feature that distinguishes between this family and ßAPP717 Val→Ile encoded families.

8.2 Introduction

Since the demonstration of genetic linkage between AD and anonymous DNA markers on the proximal long arm of chromosome 21 there has been confirmation of a disease gene in only some early onset families, others showing exclusion of chromosome 21 loci (St George Hyslop *et al.*, 1987; St George Hyslop *et al.*, 1990; Goate *et al.*, 1989; Schellenberg *et al.*, 1988; Schellenberg *et al.*, 1991). Thus, the assumption of genetic homogeneity was incorrect (St George Hyslop *et al.*, 1990) and this contributed to the mislocalisation of the putative gene in linkage studies. For example, one early genetic linkage study using six early onset families localised the disease gene centromeric to D21S1/D21S11 and in the proximity of D21S13/D21S16 (Goate *et al.*, 1989). Although this analysis lacked data along the long arm of the chromosome, an apparent recombinant event in pedigree 5 of the data set (Family 74: F74) at D21S1/D21S11 excluded this locus and was responsible for the pericentromeric localisation of the putative gene. The pooled linkage data of several centres (St George Hyslop *et al.*, 1990) resulted in a multi-point map which suggested two possible locations of

the putative gene with approximately equal likelihood, one centromeric of D21S13/D21S16 and one telomeric of D21S1/D21S11. This conclusion was unlikely and could be explained by confounding factors of which genetic heterogeneity was most probable. Subsequently, in F23 (Chapter 3) showed significant evidence of linkage to chromosome 21. Screening for the mutation in the other pedigrees has revealed a further 8 families in which the mutation has occurred (Goate *et al.*, 1991; Yoshioka *et al.*, 1991; Naruse *et al.*, 1991; Sorbi *et al.*, 1993). Other mutations at codon 717 have been detected (Chapter 5, Chartier Harlin *et al.*, 1991; Murrell *et al.*, 1991) and a double mutation at the N-terminal of ß-amyloid (Aß) reported in Chapter 6 (Mullan *et al.*, 1992a).

The linkage data analysed here suggests F74 is not linked to ßAPP nor the proximal long arm of chromosome 21. A second major early onset locus has been localised to 14q24.3 (Chapter 7, Schellenberg *et al.*, 1992; St George Hyslop *et al.*, 1992; Van Broeckhoven *et al.*, 1992; Mullan *et al.*, 1992b)). In the previous chapter, analysis of linkage data for this family shows small positive lod scores for chromosome 14 markers which was consistent with overall linkage of families in the data set. Here additional linkage analyses are presented using data from the same region, which in combination with simulation studies, suggests independent linkage of the disease in F74 to that region. F74 then, most likely represents "chromosome 14 Alzheimer's" and is clinically contrasted with the ßAPP mutated Alzheimer families examined in Chapters 4 and 5.

8.3 Materials and Methods

8.3.1 Clinical

Pedigree F74 is shown in Figure 1. The clinical assessment protocol is detailed in the Materials and Methods (Chapter 2).

8.3.2 Marker Data

RFLP detection and analysis was performed as standard as was the generation of amplified (AC) repeat dinucleotide sequences by the polymerase chain reaction (PCR). In the latter case, published primer sequences and conditions were employed.

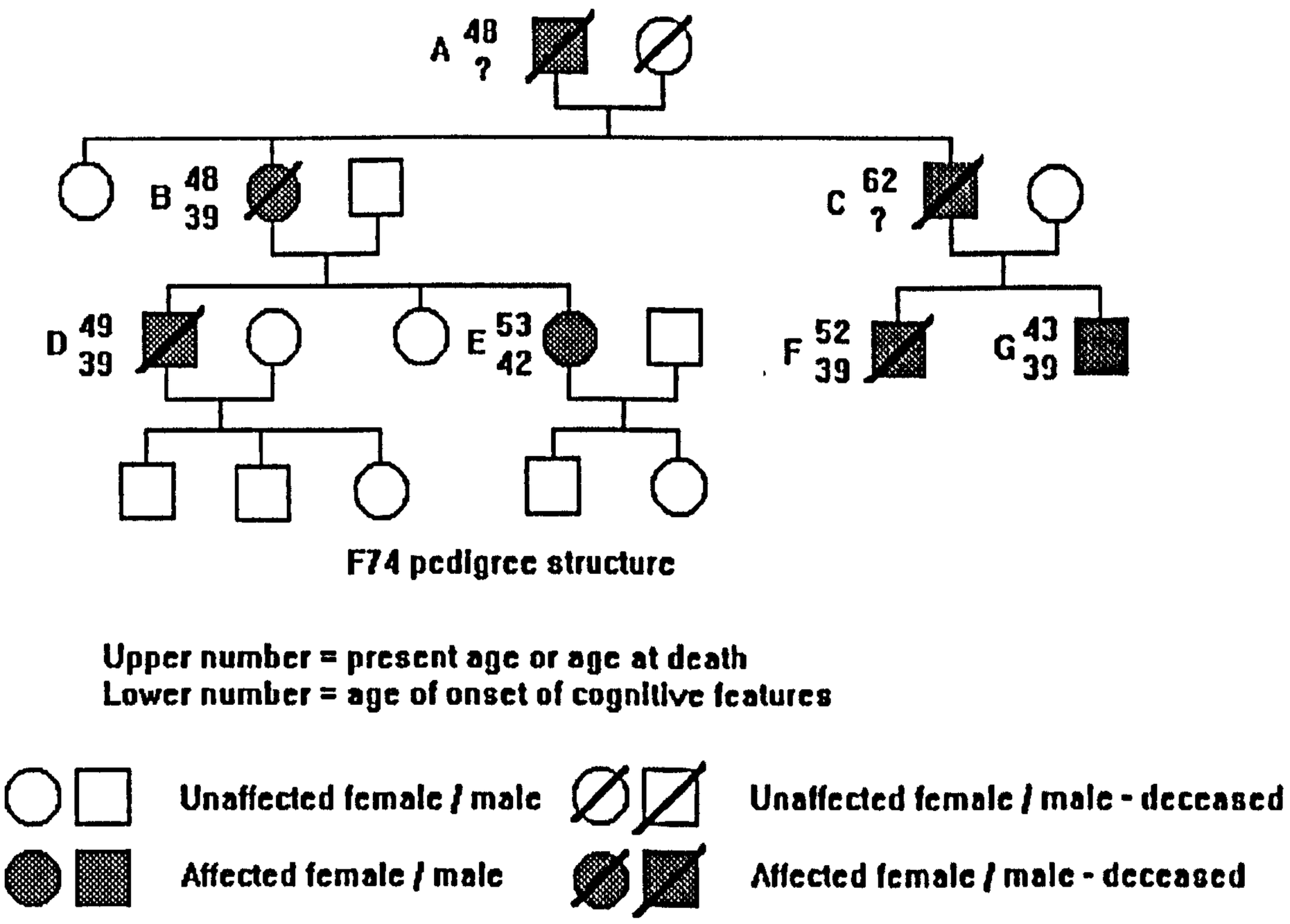


Figure 1. Pedigree F74

The following loci were used in linkage analysis for chromosome 21(Cox, Shimizu, 1990): D21S13 (Taq polymorphism), D21S13 (TaqI and EcoRI PCR polymorphisms) (Stinissen *et al.*, 1990; Stinissen, Van Broeckhoven, 1991), D21S80, D21S52 (HindIII and BglII polymorphisms), D21S1 (MspI and BamH1 polymorphisms), D21S11 (TaqI polymorphism), ßAPP (BglII polymorphism and GT12 and Intron 13 poly A). The following markers were run as pairs with zero recombination between them; D21S13/D21S80, D21S1/S11 and APP/GT12/Intron 13 polyA. However, they were not strictly haplotyped as no haplotype frequencies were calculated.

Linkage analysis for AD in F74 versus the α1-antichymotrypsin locus and other chromosome 14 markers was reported in Chapter 7. Additional linkage data from markers from a more recent short tandem repeat map of the region (Weissenbach *et al.*, 1992) are analysed here.

8.3.3 Linkage Analysis

For the chromosome 21 analysis the MLINK and LINKMAP programs of the LINKAGE (Lathrop *et al.*, 1984) package were used to calculate the likelihood of a disease gene

occurring across the map between D21S13 and ßAPP. The male recombination fractions between loci and the male/female distance ratio were calculated from the concensus CEPH map (Petersen *et al.*, 1991) using the Haldane mapping function. The ratio was calculated as 1:2.72 between ßAPP and S1 and 1:4.36 between S1 and S13 and these values were incorporated into the analyses. The male recombination values between loci ßAPP-S1-S13 were taken as 0.099 and 0.048 and in females as 0.2256 and 0.178. Disease gene frequency, age dependent liability classes, penetrance and phenocopy rates were as described in Chapter 2.

Although the two genetic marker maps (Wang, Weber, 1992; Weissenbach *et al.*, 1992) for the area of interest on chromosome 14 have not yet been integrated, it is clear that D14S77 and D14S43 are very tightly linked (proceedings of International Chromosome 14 workshop, Toronto 1993). In F74 there is no recombination between either of these two markers and the disease nor between each other. The analysis, therefore, multi-pointed the disease with these two markers allowing up to three cM between them.

8.3.4 Simulation Studies

Simulation studies were carried out as standard. F74 structure was coded for availability and diagnostic status and 1000 pedigrees were generated under conditions of non-linkage for marker S43 and S77. The number and frequency of alleles segregating in the simulated families was fixed in accordance with the actual alleles occurring in F74 at each of these loci (three alleles for S43 and six for S77). The simulated pedigrees were analysed in the usual way. The maximum lod score generated under conditions of non-linkage was noted and the number of interpolated maximum lod scores greater than 1, 2 and 3 lod points was calculated.

8.4 Results

8.4.1 Clinical Features

The clinical presentation and pattern of decline is described in detail in the Appendix 8A and in summary in Table 1. Certain points emerge. Firstly all affected individuals fulfill NINCDS criteria for probable AD. In addition, two of them fulfill the criteria for definite AD (cases D and F). The first cognitive feature in every case was the loss of memory for recently acquired information. Pre-cognitive intense and persistent headaches occurred in Case C and accompanied cognitive change in two others (F and G). The age of onset in F74 is 41.3, SE = 4.4. The clinical decline is relentlessly and smoothly progressive in the first few years.

Disorientation for time and place, dyspraxia, dysphasia, dyscalculia all tended to occur prominently and early in the illness. Psychiatric symptoms were common and there was a tendency for the same features to occur (i.e., depression, aggression and paranoia). Neurological features of note as the disease progressed were increased tone, myoclonus, and seizures in some, but not all, cases.

Table 1. Clinical Features of F74

			Prominent Features		Psychiatric Features			Neurological Features		
ID	Age at onset (Death)	Pre-cognitive features	Early*	Late	Depressive signs	Other	Tone	Myoclonus	Seizures	Other
B	39 (48)		Time dis-orientation	Dyspraxia Dysphasia	+					
C	?50 (62)	Headache	Time dis-orientation		+	Aggression		+		
D [1]	39 (49)		Dysphasia			Aggression				
E	42		Place dis-orientation	Dyspraxia Dysphasia	+					Dyskinesia
F [1]	39 (52)		Dyscalculia			Aggression	+	+	+	Headaches
G	39		Apraxia Dysphasia		+	Aggression Agoraphobia Paranoid Ideation				Headaches

* = within three years post onset
[1] Neuropathological confirmation

8.4.1 Linkage Analysis

Two point analyses between the paired loci D21S13/D21S80, D21S1/S11 and APP/GT12/Intron 13 polyA and three non-haplotyped markers (D21S52, D21S156, D21167) are given in Table 2. All two-point data exclude linkage at theta = 0.0 except D21S167.

The multi-point linkage map for the disease gene against the chromosome 21 long arm markers is shown in Figure 2. These data show that the pathogenic locus in this family is not ßAPP and that it is highly unlikely that the disease locus is between ßAPP and the centromere.

Table 2. Two-Point Lod Scores for Paired Loci and Markers on Chromosome 21 for F74					
	Theta				
	0.0	0.01	0.05	0.1	0.2
S13/S80	-2.50	-1.50	-0.80	-0.50	-0.20
S52	-2.50	-1.50	-0.81	-0.52	-0.21
S1/S11	-2.92	-1.30	-0.60	-0.40	-0.20
D21S210/ intron 13 poly A (APP locus)	-4.29	-2.17	-1.32	-0.91	-0.49
D21S156	-3.11	-1.37	-0.69	-0.41	-0.16
D21S167	0.22	0.21	0.19	0.13	0.10

The two-point data for chromosome 14 short tandem repeat markers are shown in Table 3. Table 3 also shows the multi-point results for S43, S77 and the disease which was maximum at 2.4 for theta = 0.0 between the disease and the two loci S43 and S77. This analysis assumes no recombination between S43 and S77. Analysis assuming up to 3.0 cM recombination between these two markers gives a maximum lod score of 2.35 at S43.

Table 3. Two-Point and Multi-Point Lod Scores for AD and Markers on Chromosome 14											
Marker	s52	s42	s43	s53	s48	s77	s71	s76	s61	43 vs 77	AD vs 43, 77
Theta=0.0	-.0	-.19	1.57	-5.6	-5.8	1.57	-.0	0.5	0.1	2.4	2.4

8.4.2 Simulation Studies

The results of the simulation studies for S43 and S77 are shown in Tables 4a and 4b. S43 did not produce a lod score above 1.5 in 1000 simulated pedigrees. The likelihood then, that the actual lod score for this locus are drawn from false positive populations is remote (<0.001, see interpolated data). The S77 simulation study did not produce a lod above 1.5 in 1000 simulated pedigrees but the interpolated data suggested that a lod above 2.0 was possible in 0.5% of cases. It remains unlikely that the actual lod score generated is drawn from an unlinked population (p<.01). Taken with the fact that these two loci are linked, it is even less

likely that they would independently show false positive linkage. Thus, the positive lod scores most likely represent true linkage of the disease locus to these chromosome 14 markers. (The distribution of simulated lod scores under non linkage for S43 and S77 when these two markers are set as linked, could be examined with the SLINK package but preliminary calculations suggested the time required to generate and analyse 1000 pedigrees under this model was prohibitively long on a PC).

Table 4a. The Distribution of 1000 Simulated Lod Scores for D14S43 and D14S77 versus AD in F74 Simulated Under Non-Linkage and Analysed with the Disease Marker Recombination Fraction as Zero

Marker	Average	StdDev	Min	Max
D14S43	-2.402324	2.178145	-7.392478	1.349205
D14S77	-4.930244	3.713158	-12.852224	1.477574

Table 4b. Number of (Interpolated) Maximum Lod Scores Greater than a Given Score

Lod Score	Marker	Number	Percent
1	D14S43	10	1.000
1	D14S77	22	2.200
2	D14S43	0	0.000
2	D14S77	6	0.6
3	D14S43	0	0.000
3	D14S77	5	0.5

8.5 Discussion

There is no evidence for linkage to the ßAPP gene or more centromeric loci on chromosome 21 in this family. In agreement with this finding is the observation that the affected individuals in F74 have normal sequence at exons 16, 17 (which encode the Aß sequence) and the promoter of ßAPP (Fidani *et al.*, 1992). This family has been previously analysed in data sets in an attempt to localise the AD locus on chromosome 21 mixed with families in which APP

is the mutated locus (Goate *et al.*, 1989; St George Hyslop *et al.*, 1990). Thus these present data illustrate that genetic heterogeneity was subverting locus identification.

F74 is one of a number of families with genetic data consistent with linkage to the middle long arm of chromosome 14, Chapter 7 (Mullan *et al.*, 1992b). This finding was in agreement with three other data sets (Schellenberg *et al.*, 1992; St George Hyslop *et al.*, 1992; Van Broeckhoven *et al.*, 1992), suggesting that the chromosome 14 locus is a more common cause of early onset familial AD than ßAPP mutations. The analysis of Chapter 7 did not identify individual families showing linkage. The finding of linkage of S43 and S77 to each other and to the disease is important; for instance, both markers can be used in positional cloning strategies to identify YACs containing the disease gene.

The identification of particular families which are independently linked is an important step because of the possibility of non-allelic heterogeneity at the chromosome 14 locus. At least one early onset family in this data set (F126, Chapter 7) does not show independent linkage to chromosome 14 markers and may be caused by a locus (or loci) other than those on chromosome 14 or 21. Schellenberg has exclusion data for a subgroup of families (the Volga-German pedigrees) which therefore also fall into this category of non-21 non-14 AD.

The identification of families with chromosome 14 encoded AD also allows comparison of the clinical features with ßAPP mutated families. The clinical and neuropathological features of the chromosome 14 families may yield clues to the pathogenesis of this form of the disease. In this regard, contrast with the ßAPP mutated forms of early onset AD may be particularly useful as in these cases ßAPP mismetabolism is very likely to be the cause of the disorder.

The detailed studies of the clinical features of ßAPP mutated families are described in Chapters 4 and 5 and one other Val→Ile family has been described elsewhere (Karlinsky *et al.*, 1992). A systematic survey of additional families in the chromosome 14 group will be necessary but as a preliminary study several features should be highlighted (see Table 5).

1) The age of onset in F74 is 41.3, SE 4.4, significantly lower than those families previously described with ßAPP mutations where the mean age of onset tends to be in the fifties (Delasnerie Laupretre *et al.*, 1983). A comparison with the ßAPP717 Val→Ile family F23 (Terry, Katzman, 1983) for instance (mean 54.9, SE 3.1), shows there to be a highly significant difference ($t = 7.2$, $DF = 14$, $p < 0.001$).

2) Clinically (see Table 5), both the ßAPP mutated families and F74 are typical of AD with insidious progression of cognitive decline beginning with loss of memory for recently acquired

information. Thereafter, there is global deterioration of other cognitive function (Mullan *et al.*, 1993). In both ßAPP mutated families and F74, dyspraxia , dysphasia and dyscalculia are early features. Insight was preserved for longer in F74 than in F23 and was associated with both depression and aggressive responses but depressive symptomatology is common in ßAPP mutated families as is aggressive reaction to cognitive loss.

3) Lewy bodies occurring in the cortex (in abundance) and in the substantia nigra have been found in at autopsy in APP mutated families (Terry, Katzman, 1983), but not in the F74 cases.

4) Of particular interest in F74 is the occurrence of severe headaches preceding the onset of cognitive loss. Pre-cognitive symptoms, including headache, have also previously been observed in the group of ßAPP717 Val→Ile families.

5) A number of features previously highlighted in early onset familial AD are also represented here in both the ßAPP717 Val→Ile families and occur in F74: Myoclonus is common in the both groups and late seizures occurred in one of the myoclonic cases in F74.

In summary, a comparison of the clinical features of the APP mutated families and F74 is more striking for the similarities than the differences. The key features which may be over represented in early onset familial disease (Chapters 1 and 4) do not differentiate between the ßAPP and chromosome 14 linked families (assuming F74 is representative). The exception to this is the age of onset which is explored in more detail in the next chapter.

Table 5. Clinical Comparison of βAPP Mutated Families and F74

	Val→Ile[1]	Toronto 3[2]	Val→Gly[3]	F74
Mean age of onset (SE)	52.5 (4.7)	47.6 (3.0)	54.7 (6.6)	41.3 (4.4)
Pre-cognitive signs	Disequilibrium 3/16	0/11	0/5	Headache 1/6
First cognitive symptom	Amnesia 16/16	Amnesia 11/11	Amnesia 5/5	Amnesia 6/6
Dyscalculia[4]	7/16	3/11	3/3	1/6
Myoclonus	7/16	0/11	2/5	3/6
Seizures	5/16	1/11	1/7	1/6
Clinical stroke	4/16	1/11	0/7	0/6
Depressive features	6/16	Not formally examined	4/5	4/6
Aggression	2/16	1/11	2/7	4/6
Headache[5]	2/16	0/11	2/7	3/6

1 See Chapter 4 and Appendix 4.
2 For the Toronto 3 pedigree(Karlinsky *et al.*, 1992; Karlinsky *et al.*, 1991), the features examined were not equally accessible for all cases but the denominator is given as 11 in all cases. This underestimates the frequency of each feature.
3 See Chapter 5 and Appendix 5.
4 Dyscalculia was regarded as a prominent symptom if it occurred in the first 4 years post onset.
5 Severe persistent headache requiring chronic analgesia.
Note: The denominator indicates the number of affected cases adequately assessed for that feature.

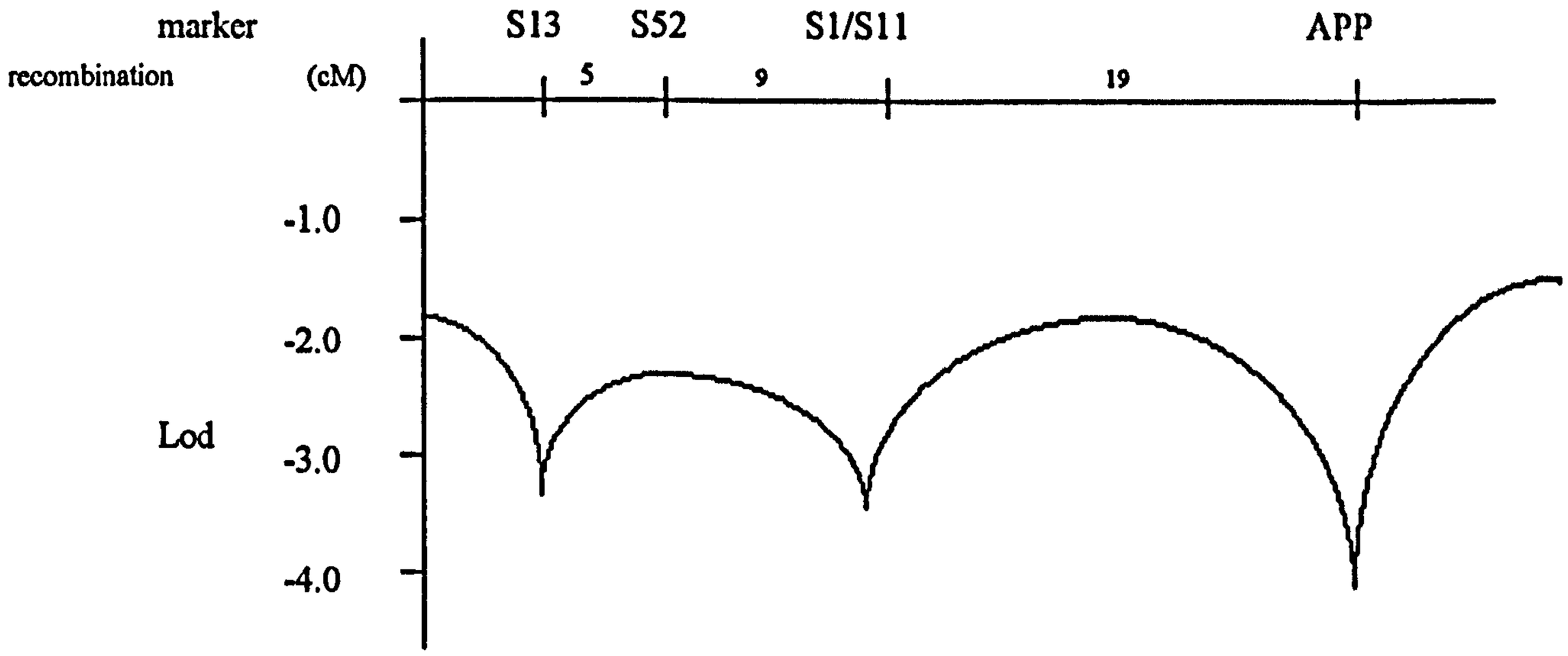


Figure 2. Multipoint map of AD versus chromosome 21 long arm markers

Chapter 8 Appendix 8A

Clinical and Neuropathological Profiles and Neuropathological Description of Affected Individuals in F74

Case A. Family information suggests that this family member had a dementing illness but he was known to abuse alcohol. He died at age 48 years in an institution. His wife died at age 31 years.

Case B. This woman was a housewife and was well until the onset of cognitive problems at approximately 39 years. Two years later she was unable to look after herself and was failing at her household tasks. She became forgetful and disorientated for time. Eighteen months later she was admitted for assessment. She had a well preserved social facade but relied on others for all activities of daily living. On testing, her comprehension was poor, she was dyspraxic and her speech was dysphasic and showed perseveration. She appeared depressed and anxious. There was a slowly progressive decline until death (age 48); no postmortem was carried out.

Case C. This man was the son of A. He grew up in an orphanage, and became a plumber. He complained frequently of cold extremities which were often white. He also complained of severe headaches from his early 40s and took aspirin to excess for many subsequent years. His developed loss of concentration at age 50. Two years later he was disorientated for time and was forgetful. An episode of confusion and vacancy prompted investigation. He became tearful and had aggressive outbursts. Over the next two years he became increasingly apathetic and silent. He developed myoclonic jerks. He lost weight and died of pneumonia at age 62. No postmortem was performed.

Case D. This man developed deficiencies with "short term memory" tasks at the age of 39. Over the next two years he suffered from other intellectual deficits and was assessed at the age of 42. By this age he was co-operative but had a nominal dysphasia with some receptive problems. There were no other neurological signs. A CT scan showed diffuse and extensive cortical atrophy. A brain biopsy confirmed the diagnosis of Alzheimer's disease with senile plaques and neurofibrillary tangles. He continued to deteriorate and became behaviourally disturbed with truculent and aggressive outbursts. He died at age 49: no autopsy was performed.

Case E. This woman first started to have problems with her memory for recently acquired information when she was 42 years old. She became frequently confused and was unable to recognise her daughter's voice. These problems were progressive and within three years she was unable to look after herself and became lost in unfamiliar surroundings. She had insight into her illness and her comprehension remained relatively intact. She became intermittently dysphoric and frustrated. At the age of 45 she developed dysphasia. Jerky movements of her left arm were noted. On examination, she had an oro-facial dyskinesia but an otherwise expressionless face. She had little memory for recently presented information. She was disorientated for time and place, was dyscalculic and mildly dyspraxic and her language difficulties were prominent. She had poor coordination. CT scan showed cerebral atrophy. Throughout her illness she was frequently tearful although she showed no biological signs of depression. The subsequent deterioration was characterised by progressive immobility, incontinence, weight loss and apathy. She remains in this condition 11 years after the onset of the illness.

Case F. This man was the son of C. He had fits as an infant at 18 months thought to be initially due to febrile convulsions. He had an unremarkable school and work record. He had recurrent tubo-tympanic disease which left him deaf in the right ear. He too, often complained of cold extremities. He had an a varicose vein operation at the age of 32 years. From his mid 30s he complained of severe headaches. At the age of 39 he began to require help with calculations. At 42 he began to misplace objects and was forgetful of recently learnt information. He lost a succession of jobs as a result of his difficulties. From the age of 42 he began to have periods of altered consciousness which were thought to be absence seizures. At age 45 a CT scan showed a low density lesion in the right frontal lobe "consistent with an area of infarction". An EEG was diffusely abnormal with an excess of slow waves most marked on the left sided. Neuropsychology six months later recorded a verbal IQ of 77 and performance IQ of 87 and a relative loss of language function was noticed. At 47 years he had grand mal seizures which increased in severity and frequency. At age 48 years he had a cerebral biopsy which revealed numerous plaques and tangles. The diagnosis of AD was made. He developed involuntary jerks at the age of 49 particularly in his arms. At 51 he remained conscious, alert and responding to verbal commands. Tone was increased in all limbs and reflexes were exaggerated with extensor plantar responses. He had myoclonic jerks. He stopped walking at the age of 52. Myoclonic movements persisted. Some months before his death he developed meningitis. He became moribund with flexion contractures of all limbs. There were slow rhythmic movements of the eyes. He died of bronchophopneumonia at age 52.

Examination of the brain showed considerable atrophy and swelling. There was a large organising haematoma in the right frontal and fronto-parietal white matter. There was nothing abnormal in the midbrain and brainstem. The operative biopsy was evident as a 1cm oval defect in the right antero-frontal middle gyrus.

Microscopy revealed dense widespread plaque in all cortical areas. There were also abundant neurofibrillary tangles, Hirano bodies and cells showing granulo-vacuolar degeneration. These changes were also seen in the basal nucleus, the locus ceruleus and the substantia nigra. Congophilic angiopathy in modest abundance was also observed. Examination of the cerebellum showed plaque and tangle deposition especially in the vermis. The white matter was atrophied and gliotic especially around the haematoma.

Case G. This man, the son of C, had an unremarkable early life and subsequently had an average school and work record. He became proficient as an electronics engineer. He had no significant medical history but used to complain from an early age of feeling excessively cold. At the age of 39 his memory for recently acquired information began to fail and he was unable to meet the demands of his job. At the age of 42 he complained of severe headaches and of "feeling weird". At 42 he began to have marked difficulty concentrating and developed a dressing apraxia. He developed nominal dysphasia and he began to perseverate. He had to give up his work as a consequence. At the age of 43 his orientation and his recall of overlearnt and remote information remained good. Psychological assessment at ages 42 and 43 showed a verbal IQ of 72 and 75, respectively, and at the second assessment he had a verbal IQ of 58 and a performance IQ which was unmeasurable. His estimated premorbid IQ was 115. The initial assessment recorded global intellectual decline with severe nominal dysphasia. From the age of 41 he had a number of psychiatric symptoms; agoraphobia; lowered mood with tearfulness, reduced libido and suicidal ideas; aggressive behaviour; paranoid ideation and morbid jealousy. His orientation at age 43 was poor with respect to time and place. His concentration was poor. In addition to the nominal dysphasia, he had impaired word fluency and poor phrase repetition. Comprehension was mildly impaired for spoken commands and unimpaired for simple written commands. He had dyscalculia. Visual perception was intact with good ability to identify simple objects from unusual angles. Retrieval of overlearnt and remote information was good but despite several attempts he could register little new information which he could not recall.

CHAPTER 9

Age of Onset 1: Analysis of Variance

9.1 Abstract

The variance in age of onset was examined in 19 families multiply affected by Alzheimer's disease (AD). In the early onset pedigrees (mean age of onset <60 years), 78% of the variance in age of onset was explained by differences between families and almost none was explained by gender or maternal versus paternal inheritance. In the late onset families (mean age of onset >60 years), 60% of the variance in age of onset was explained by between family differences and neither gender nor paternal versus maternal inheritance was important. In neither group was age of onset observed to decrease in subsequent generations (i.e., no anticipation effect). These findings suggest that in contrast to the late onset families, the age of early onset familial disease is determined largely by family specific factors. As the mode of transmission of the disease in early onset families is autosomal dominant the single gene defects are likely to be largely responsible. In the late onset families, age of onset is likely to be determined by several, as yet unidentified factors.

9.2 Introduction

It is now clear that AD is aetiologically heterogeneous (St George Hyslop *et al.*, 1990) despite a generally homogeneous phenotype (preceding chapters). However, the genetic analysis has been aided by a key clinical feature approximately correlating with aetiology; age of onset. In particular, two genetic loci correlate with early onset disease and a putative third correlates with late onset disease. ß-amyloid precursor protein (ßAPP) gene mutations and the unidentified gene on chromosome 14 are responsible for the majority of early onset familial cases. It has been observed that age of onset tends to be constant within families of early onset cases (see the families referenced in Table1, Chapter 1). This study replicates the finding of constancy of age of onset in early onset pedigrees and validates it against an epidemiologically based sample to exclude the possibility of selection bias operating on pedigrees chosen for linkage studies. In both biased and unbiased samples the largest component of variance of age of onset is attributed to between rather than within family differences. In this study the origin of the variance in relation to gender and paternal versus maternal inheritance of the condition is examined in both early and late onset pedigrees.

9.3 Materials and Methods

Details of the families from the **British sample** are given in Table 1. All families were referred to the linkage project either as a result of notification by clinical colleagues or as self-referral (by a well member of the family) via the Alzheimer's Disease Society. The NINCDS criteria were used as an initial screen to confirm the presence of a slowly progressive neurodegenerative familial dementia due to probable or definite (neuropathologically confirmed) Alzheimer's disease. More detailed clinical work up followed including: a) gathering of clinical notes from hospital and GP sources, b) interviewing relatives and carers, and c) a clinical work up as detailed in Chapter 2 for some families (those identified in Table 1). Age of onset estimation was determined by averaging data from (a), (b) and the patient, where reliable. In this way the diagnosis of dementia was confirmed and causes of dementia other than AD were excluded whenever possible. Four of the early onset pedigrees and two of the late onset pedigrees have histological confirmation of Alzheimer's disease. Age at onset was taken as the appearance of first cognitive change and was generally loss of memory for recently acquired information. Both early and late onset families were included in this sample (see Table 1). The number of generations affected is restricted here to those for which reliable clinical information exists. Most pedigrees have anecdotal information for one or two more generations.

The **Belgian** sample comprised AD/A and AD/B, two large early onset AD pedigrees described elsewhere (Martin *et al.*, 1991). The age of onset for these autopsy confirmed families was taken as a consensus estimate of the first memory disturbance.

The **Dutch sample** was selected by proband with onset before age 60 years. These probands were all the cases diagnosed in two areas of the Netherlands between the years 1980-87 (Hofman *et al.*, 1989); in the families of 16 patients there were at least three affected persons in two generations. The method of determination of age of onset did not differ significantly from that of either of the other two samples.

In total, age of onset information was available for 139/214 individuals (67%).

The analysis of the variance in age of onset by family, gender and paternal versus maternal inheritance (Armitage, Berry, 1987) was determined using a statistical package (Statistical Package for the Social Sciences). As all but one of the sibships in the late onset families had "inherited" the illness from an affected mother no analysis of variance was possible with gender of predecessor so a direct comparison of maternally versus paternally transmitted families was made (Students' t-test). The possibility of an anticipation effect on age of onset

between subsequent generations was also examined. To examine whether age of onset changed over generations, the difference in age of onset was calculated between all possible combinations within a family. Thus, first through to eighth degree relatives were compared in this way.

Table 1. Details of Early and Late Onset Families

Family	Mean Age of Onset	Number Affected	Number of Pathologically Confirmed Cases
British Sample			
14	60	3	
15	52	3	
23	55	14	1
32	54	6	
34	44	3	1
53	52	5	
74	41	6	2
75	51	10	
105	38	7	
121	37	4	
126	54	4	
127	46	7	
Belgian Sample			
AD/A	35	38	11
AD/B	35	24	6
Dutch Sample			
1005	60	9	
1025	56	3	
1034	63	4	
1049	55	4	1
1066	41	9	2
1068	55	4	
1070	59	4	
1072	60	6	1
1085	57	4	
1097	56	3	
1100	39	7	
1104	53	6	
1125	53	5	
1230	55	6	
1264	61	4	
1270	58	4	

9.4 Results

Tables 2 and 5 show the four and three way analysis of variance of age of onset for the early and late onset pedigrees respectively. For the **early onset group** practically none of the variance is attributable to the effects of gender or the sex of the affected parent, nor is there any interaction between these and the main source of variation, namely, familial membership. The latter variable accounts for 72% of the total variance. In the epidemiological Dutch sample 67% of the variance was due to differences between families (Table 3), suggesting there is little bias in the selection process of those families ascertained for linkage studies. For all early onset families combined, Table 3 shows that 77% of the variance is derived from between family differences, emphasizing the influence of the very narrow age of onset in the Belgian pedigrees. Unaffected siblings of the proband are at risk for AD and since siblings tend to have similar ages, bias may occur towards limited variation in age of onset. Upon exclusion of siblings of the probands, there was little change in results for the Dutch sample (intra-class correlation 0.63, $p< 0.005$). Table 4 shows that the age of onset tends to remain constant even in seventh and eighth degree relatives who share no more than $1/126^{th}$ and $1/256^{th}$ of their genes in common. There was a trend towards the differences in age of onset to become smaller as the genetic distance widened. This is mostly due to the fact that the largest families also tend to be the youngest (especially AD/A and AD/B) and that the variance within such families is the lowest of all.

TABLE 2. Analysis of Variance of the Age of Onset of Early Onset Families Derived Solely from the British Sample

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F	Significance of F
Main effects	1588.4	9	176.5	11.1	<.0001
Family	1301.3 (72%)	5	260.3	16.4	<.0001
Gender	.6	1	.6	.04	.84
Generation	90.1 (5%)	2	45.1	2.8	.09
Paternal/ maternal inheritance	16.3	1	16.3	1.0	.33
Explained	1588.4	9	176.5	11.1	.0001
Residual	222.2 (12%)	14	15.9		
Total	1810.6				

Table 3. Analysis of Variance of the Age of Onset of All Early Onset Families

Families	Source of Variation	Degrees of Freedom	Mean Sum of Squares	P value	Intraclass Correlation
All Families	Between	29	407.2	0.0005	0.77
	Within	109	24.46		
Population-Based Sample	Between	15	184.91	0.0005	0.67
	Within	42	25.11		

Table 4. Differences (SD) in Age of Onset Between Sibships by Degree of Relationship

Degree of Relationship (Genetic Distance)	Belgian Families	Dutch Families	British Families	All Families
First (1/2)	6.0 (4.4)	4.8 (3.3)	5.9 (5.8)	5.4 (3.2)
Second (1/4)	4.0 (3.2)	7.6 (3.0)	3.8 (3.6)	4.9 (3.7)
Third (1/8)	5.5 (2.8)	5.0 –	3.0 (2.3)	4.1 (2.7)
Fourth (1/16)	4.2 (2.7)	–	1.7 (2.9)	3.5 (2.9)
Fifth (1/32)	3.3 (2.3)	–	–	3.3 (2.3)
Sixth (1/64)	4.7 (3.8)	–	–	4.7 (3.8)
Seventh (1/128)	4.7 (3.2)	–	–	4.7 (3.2)
Eighth (1/256)	3.3 (2.7)	–	–	3.3 (2.7)

In the **late onset group,** differences in gender of the affected individual account for a negligible amount of variance and most of the total variance (64%) can be attributed to between family differences when gender and generation are included in the analysis. Direct comparison of maternally versus paternally transmitted families revealed no differences in the age of onset ($p > 0.1$). The generation effects are not significant in either early or late onset groups indicating that no anticipation effect occurs between generations in these pedigrees.

9.5 Discussion

These data show that there is more intra-familial similarity of age of onset than inter-familial similarity in both early and late onset disease but that the effect is more striking with the former. The analysis of the population sample indicates that this finding is not due to ascertainment bias, which might be expected. Although age of onset data is likely to be subject to large errors, it is the most robust clinical marker for subdividing the disease by aetiology (see next section for instance). What are the intra-familial factors which determine age of onset? The analysis of Chapter 8 strongly suggests that the disease is predicted solely by the presence of either a *βAPP* or a chromosome 14 gene mutation and it may be that much of the inter-familial differences can be accounted for by the presence of either one or the other of these mutations. This is supported by the analysis of the next section where the difference in mean age of onset between early onset families correlates with their genetic aetiology. As all these families have subsequently been shown to be either *βAPP* mutated families or chromosome 14 linked families it is reasonable to assume that these genes might largely determine the age of onset. This is explored in the next section and it appears to be the case that the average age of onset is set by the genetic mutation. The differences in mean onset age between families within the chromosome 14 group (for instance AD/A, mean age of onset 35 and F74, mean age of onset 41) may be due to environmental factors or may reflect allelic heterogeneity or other genetic factors. However, there is little difference in mean onset age for allelic variants at the *βAPP* locus (see next section) which might suggest that background genetic or environmental effects are important.

In the case of the late onset families there may be factors other than single genes operating. The unexplained intra-familial variance (Table 5) is about three times greater in the late onset families compared to the early onset ones (35% vs. 12%). This probably reflects more complex aetiology in the former group, or possibly that the same factors operate in both groups but have a larger impact in the late onset families as they operate for longer. In addition, given the frequent sporadic occurrence of the disease, the presence of sporadic cases in families otherwise determined by genetic causes is likely to occur commonly. This too, is likely to be reflected in a diverse spread of onset ages within single families. The impact of the *APO E* locus on age of onset in late onset cases has recently been revealed and is discussed in the concluding chapter. The lack of influence of gender related features and the absence of evidence for genetic imprinting in the late onset families also emerge in this analysis.

Table 5. Analysis of Variance of the Age of Onset of Late Onset Families Derived Solely from the British Sample

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F	Significance of F
Main effects	962.6	11	87.5	4.1	.002
Family	951.2 (64%)	9	105.7	5.0	.001
Gender	14.9	1	14.9	0.7	.4
Generation	52.6 (4%)	1	52.6	2.5	.1
Explained	962.6	11	87.5	4.1	.002
Residual	512.2 (35%)	24	21.3		
Total	1474.8	35	42.1		

The results of the early onset family are of interest for two further reasons. Firstly, it is intriguing to speculate on the mechanism by which an inherited lesion does not show its effects until the mid 50's or 40's (depending on the genetic defect). Several investigators have speculated on the delayed onset of AD in general, and the dominant mutations might fit into these hypotheses. For instance, the idea that ßAPP is involved with "neuronal sprouting" in the mid 50's allows that the *βAPP* mutations do not reveal themselves their mismetabolising effects until sprouting occurs. Secondly, these data are of value in genetic counseling; the small intra-familial variance allows accurate prediction of onset age for those at risk and can be used as an adjunct to genetic risk calculations.

Age of Onset 2: Clinico-Genetic Correlates In Early and Late Onset Familial Alzheimer's Disease

9.6 Introduction

The analysis of the previous section suggests that taken as a single group age of onset in early onset disease is quite family specific. This section examines the age of onset within the early onset familial group in relation to the genetic origin of the disease (*βAPP* mutated or chromosome 14 linked).

9.7 Materials and Methods

The determination of age of onset is as previously described in Chapter 2 and the data are analysed by an unequal variance two sample t test (Armitage, Berry, 1987).

9.8 Results and Discussion

The distinction between early and late onset disease has been arbitrarily defined - and is more often a matter of clinical convenience (in accordance with retirement age for instance) than a reflection of aetiology. Late onset familial disease (with a mean age of onset over 65) has been shown not to be linked to chromosome 21 markers, and specifically not to the *βAPP* locus (St George Hyslop *et al.*, 1990; Schellenberg *et al.*, 1991; Pericak-Vance *et al.*, 1991). However, in the course of linkage analysis with early onset disease it has become clear that age of onset correlates with genetic aetiology. Of the data set of 12 early onset families suitable for linkage analysis, 3 families (F19, F23, F172) have *βAPP* mutations (Table 5, Chapters 3, 5, 8). Genetic linkage analysis of all but one (F126) of the remaining 9 families is consistent with a locus at chromosome 14 (Chapter 8). A comparison of the age of onset of the *βAPP* mutated families versus those consistent with a locus on chromosome 14 shows there to be a significant difference (Table 6). Moreover, analysis of the family not showing linkage to chromosome 14 (F126) shows it to be indistinguishable in age of onset from the *βAPP* mutated families but significantly different from the families consistent with a locus at chromosome 14 (Table 6). These data show that the *βAPP* mutated families in this data set have a higher age of onset than those families with mutations elsewhere. This observation does not appear to be confined to mutations at the codon 717 locus. The mean age of onset of two related Swedish pedigrees with APP codon 670/671 mutations is in the same range (55 years, Chapter 6). It remains to be shown whether the gene (on chromosome 14) or others are responsible for the very early form of the disease.

Table 5. Clinical Details of Early Onset Families

Family Number	Mean Age of Onset	Number of Generations	No. Affecteds Included in Linkage Analysis (Path. Confirmed)
19	52	3	12 [b]
23	55	3	12 (1)[a]
172	54	3	7 [a]
53	52	3	3 (1)
74	43	4	7 (2)
105	38	6	5
121	37	4	4
126	54	4	4
134	46	4	6 (2)
148	41	4	6 (1)
168	41	4	5 (1)
206	39	6	6

[a] APP Val → Ile
[b] APP Val → Gly

This further division of the early onset group (<65 years) by age of onset may distinguish the two groups by pathogenesis, as yet unidentified clinical features and prognosis. However, the clinical features of individuals with the *βAPP*717 Val→Ile mutation have been described (Chapter 4) but no striking feature differentiates them from those families likely to be linked to chromosome 14 (Chapter 8). These age of onset data suggest that the mutations in the chromosome 14 gene result in a more virulent molecular pathology than that associated with the *βAPP* mutants.

Table 6. Comparisons of Mean Age of Onset Between *βAPP* Mutated Families and Families Consistent with a Locus at Chromosome 14

	APP Mutated Families	'Ch14 Linked' Families	DF	t	p
Mean	53.95	42.46			
SE	6.57	5.6	63	7.48	<.0001

	F126	'Ch14 Linked' Families	DF	t	p
Mean	54.33	42.46			
SE	7.8	5.6	42	3.46	<.005

	F126	APP Mutated Families	DF	t	p
Mean	54.33	53.95			
SE	7.8	6.57	25	.092	ns

CHAPTER 10

Conclusions: The Contribution of the Genetic Analysis of Familial Alzheimer's Disease to a General Theory of Molecular Aetiology

10.1 Introduction

Of the neuropsychiatric disorders, few have received so much attention as a consequence of the advent of molecular biology, as Alzheimer's disease (AD). It is perhaps not surprising that the molecular biology of AD is largely the molecular biology of the ß-amyloid precursor protein (ßAPP). Not surprising because, in a broad way, much circumstantial evidence for the aetiology of AD inculcates ßAPP. Firstly, the characteristic pathology includes the deposited 4kD ß-amyloid peptide (Aß) derived from the ßAPP molecule. Secondly, the pathology found in cases of trisomy 21 (three copies of ßAPP per cell) is AD-like and the ßAPP gene mutations described here cause AD (Chapters 3, 5 and 6). The mutations in the ßAPP gene linked to AD suggest two ideas; changes in the function or processing of the ßAPP molecule can be sufficient to cause to the disease and analysis of the functional changes associated with the mutations may shed light on the disease process. It has previously been proposed that ßAPP mismetabolism leads to Aß deposition which in turn triggers the disease process. The relationship between ßAPP mismetabolism and Aß production has yet to be determined - clearly establishing the normal metabolism is key and is reviewed here. That the Aß derivative is central and primary remains hypothetical. The studies of early onset AD showing linkage to chromosome 14 (Chapter 7) in families with a generally lower age of onset suggests the protein product of the gene has a more virulent molecular pathology than the ßAPP mutations. This gene product might directly influence ßAPP metabolism and thus precipitate the disease via ßAPP or may act through an independent mechanism of which Aß deposition is an epiphenomenon. The same reasoning can be applied to the recent finding of an association with the *APOE* locus in late onset disease. The influence of the latter locus on disease onset and progression are future areas of research.

10.2 Molecular Genetics

Taken as one disease entity, AD does not lend itself to classical molecular genetic analysis. Successful reverse genetic analysis, resulting in the isolation of causative genes has hitherto been confined to the analysis of disorders inherited in a simple Mendelian way. We now clearly know that AD is aetiologically heterogeneous (St George Hyslop *et al.*, 1990);

although it is highly familial, in many late onset families it does not always occur in a pattern consistent with autosomal dominance. Despite this complex actiology, the genetic analysis of this condition has yielded two genetic loci (for early onset disease) and a putative third (for late onset disease). AD in the early onset families reported in Chapters 3 through 8 is transmitted as an autosomal dominant condition with complete but age dependent penetrance. As described in Chapter 3, the discovery of the first locus predisposing to early onset disease was achieved by a combination of genetic analysis of a candidate chromosome (21) and the subsequent analysis of a candidate gene (*βAPP*). The discovery of other missense variants (described in Chapter 5 and by Murrell and colleagues (Murrell *et al.*, 1991)) in the same gene emphasised the pathogenic nature of these mutations and strongly suggested that changes in the ßAPP molecule alone can be sufficient to cause the disease. Although the ßAPP or Aß molecules must be central to the disease process in these cases, before any extrapolation can be drawn to the common case, an important question immediately arises; is the AD observed in these rare early onset familial cases typical of the more common form of late onset disease? From the clinical features reviewed in chapters 4, 5, and 8 several themes emerge;

1) The AD observed in these cases all fulfill NINCDS criteria for probable or definite AD in life or at autopsy respectively;

2) Other than age at onset (Chapter 9) there is no other prominent clinical feature than demarcates the ßAPP group from the chromosome 14 group (Chapter 8);

3) Both early onset groups show features previously identified as common in early onset familial and non-familial disease, but no single feature occurs only in early onset disease.

These observations require extension to the remaining ßAPP mutated families and to other chromosome 14 linked families but imply a common early molecular pathway for these two non-allelic variants.

In addition, for all practical purposes there is nothing that distinguishes late and early onset forms of AD at the clinical or neuropathological levels, although clearly the molecular mechanisms between the early onset groups and the late onset group are different as they have different aetiologies. Again, this argues for a common pathway. Whether the ßAPP molecule or its derivatives is always the gateway for this pathway remains to be determined.

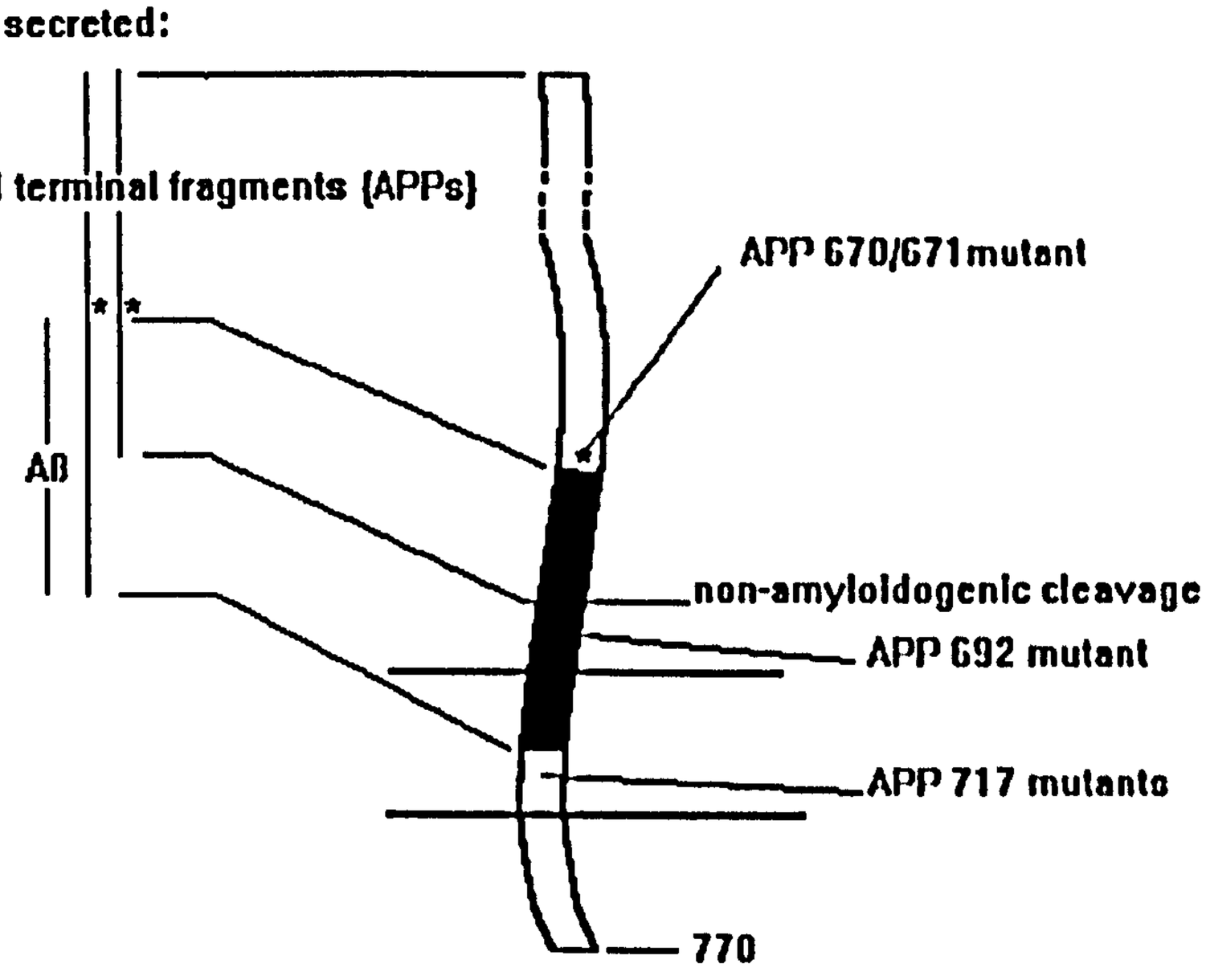
10.3 The ß-Amyloid Precursor Protein

The *βAPP* gene was cloned in 1987(Tanzi *et al.*, 1987; Goldgaber *et al.*, 1987) and localized to chromosome 21. The ßAPP product is a transmembrane glyco-protein, the function of which is unknown. At least five transcripts of the gene are produced, three of which are common and are translated into precursor proteins with 695, 751 and 770 amino acids (Palmert *et al.*, 1990). The *βAPP* gene is expressed in nearly all tissue from an early age and throughout life but to high levels in neurons and glial cells. Aß is a variable 39-42 amino acid peptide cleaved from the mother molecule at residues outside of the N terminus and within the transmembrane domain (see Figure 1). The later cleavage site presents an immediate puzzle to researchers as clearly an intramembranous cleavage in an intact membrane is difficult to envisage. This conundrum led to early (and incorrect) speculation that Aß was a product of ßAPP in only damaged membranes. The ßAPP isoforms, which are expressed in the CNS, all contain the Aß sequence, that portion of the ßAPP molecule that is deposited in all cases of AD. A substantial amount is now known about the <u>normal</u> processing of ßAPP. To summarize a complex area, ßAPP may be cleaved extracellularly to secrete large N terminal fragments (APP_S) (see Figure 2). An important site of extracellular cleavage is between residues 16 and 17 of the Aß sequence precluding the formation of Aß (Esch *et al.*, 1990). The cellular site and sequence of cleavages leading to Aß formation are not yet clear but it is released normally from cells into media and CSF in soluble form (Haas, 1992a; Shoji *et al.*, 1992). Potentially Aß bearing fragments are found in the endosomal lysosomal pathway (Haas *et al.*, 1992b) but Aß may be produced and secreted by other pathways associated with the Golgi or early endosomes (Figure 2). However, Aß has not so far been detected intracellularly and its site of production remains elusive.

10.4 Genetic Linkage Analysis of ßAPP

The localization of the *βAPP* gene to chromosome 21 and the observation that cases of trisomy 21 develop AD like pathology (Mann, Esiri, 1989), led naturally to the idea that the gene for familial AD <u>was</u> the *βAPP* gene. This was refuted by linkage analysis papers showing non-co-segregation of genetic markers of *βAPP* with AD (Van Broeckhoven *et al.*, 1987). In other words, within some families, inheritance of the *βAPP* gene from an affected parent did not correlate with the development of the disease. A large combined study of late and early onset families did demonstrate linkage to chromosome 21 markers (St George Hyslop *et al.*, 1990). More specifically, the analysis revealed two peaks of linkage to chromosome 21 in the early onset families. By contrast, late onset families were excluded from loci on this chromosome. The literal interpretation of the early onset linkage result was

that there were two genes predisposing to the disease. However, owing to the mathematical relationship between linkage of a marker (measured in lod scores) and genetic distance, a lowering of linkage (by including unlinked families) can lead to an apparently increased genetic distance between the marker and disease gene.



The APP molecule: main secretory products and sites of the mutations causing AD (the 692 mutant causes either AD or a cerebral angiopathy)

Figure 1.

This was a likely interpretation of the observed early onset results, i.e., that some families were linked to chromosome 21 and others had causative loci elsewhere. In response to this idea, the linkage strategy described in this thesis was instigated. This was a successful strategy and immediately resulted in genetic linkage data that were consistent with *βAPP* being the causative gene in some early onset families. After the detection of linkage in F23, sequencing the *βAPP* gene revealed a mutation only in the DNA derived from affected or at-risk individuals (Goate *et al.*, 1991). The mutation, a single nucleotide base pair substitution results in the incorporation of isoleucine rather than valine (Val→Ile) into the ßAPP molecule. This finding was rapidly followed by linkage and sequencing of F19 (Chapter 5) where a valine to glycine change is predicted in the ßAPP peptide. One other mutation at the same codon (717 of the 770 transcript) in the *βAPP* gene was reported and predicts a valine to phenylalanine change (Murrell *et al.*, 1991). A screening protocol devised to identify the Val

→Ile mutation uncovered another early onset family, F372, described in Chapter 4. Subsequently, 7 other families have been identified worldwide with this mutation (Naruse *et al.*, 1991; Yoshioka *et al.*, 1991; Mullan *et al.*, 1992; Karlinsky *et al.*, 1992; Sorbi *et al.*, 1993). Clinically these families do not have any consistent features which differentiate them from AD of different aetiology, except age of onset (Chapters 5 (Mullan *et al.*, 1993) and 9). The *βAPP* 717 Val→Ile families present and progress classically and fulfill NINCDS criteria for probable AD in life and definite AD at autopsy (Mullan *et al.*, 1993). A further mutation in *βAPP* has been noted at codon 692 which co-segregates with either AD or a phenotype akin to multi-infarct dementia due to heavy deposition of abnormal Aß in vessel walls (Hendriks *et al.*, 1992). This demonstrates that the allelic variants of *βAPP* can have quite diverse clinical consequences. A third site of mutation, at codons 670/671, has been discovered at which mutations co-segregate with only early onset AD. This double base-pair mutation which has been identified in two Swedish pedigrees is rare (Houlden *et al.*, 1993) and causes the disease to onset in the mid 50s like the ßAPP717 mutations.

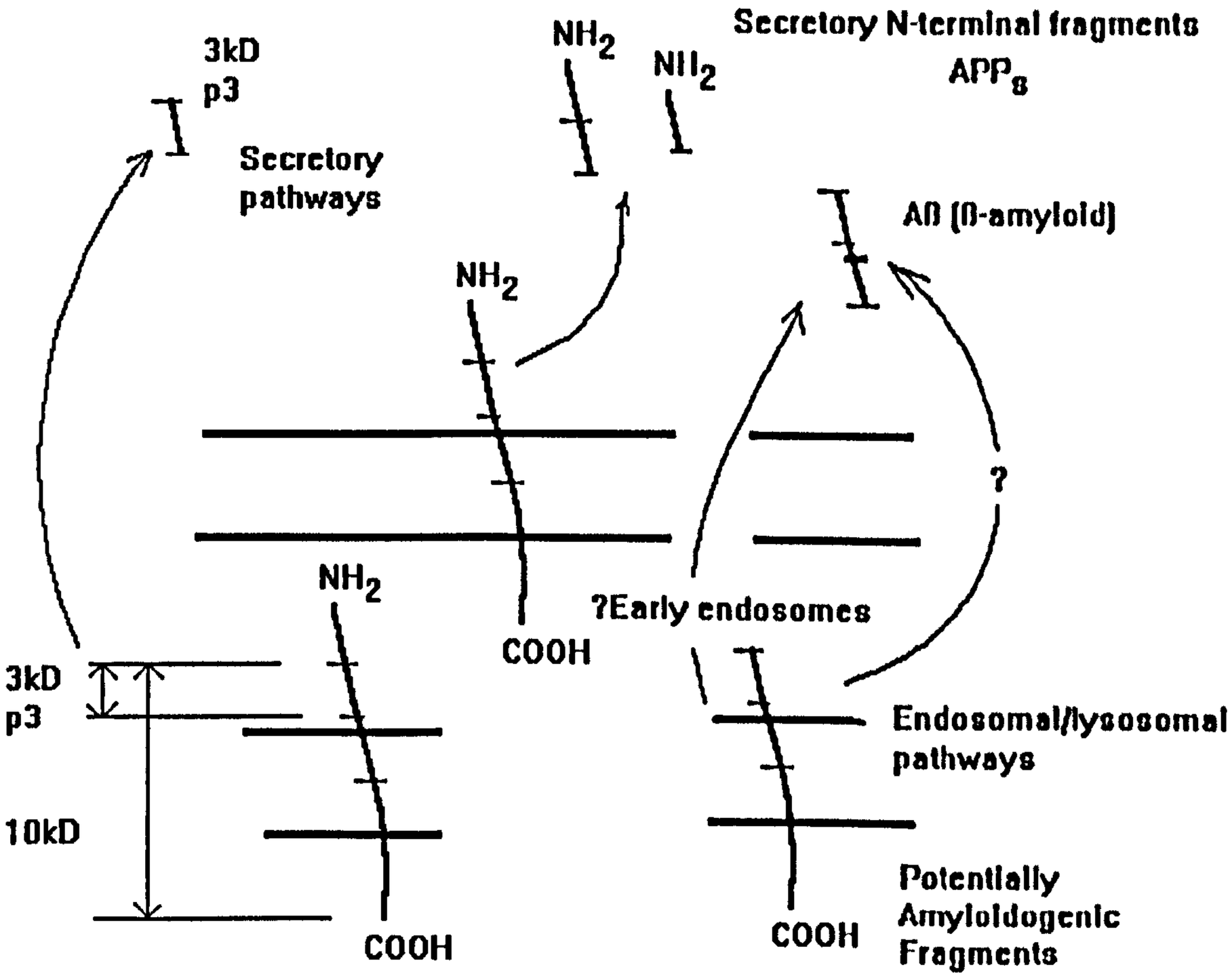


Figure 2. Main routes of processing of the ßAPP molecule

10.4.1 A Retrospective Look at the Genetic Analyses of AD

The analysis of AD had been instructive not only for its successes but also for its errors. Three analyses deserve comment, the lessons from which are applicable to other complex disorders.

10.5.1 Initial Chromosome 21 Linkage

The initial analysis of pedigrees showing linkage at chromosome 21, by Hyslop et al (St George Hyslop *et al.*, 1987), shows two point lod scores below 3.0 but in excess of 4.0 for multi-point analysis in four families combined (FADs 1-4). More recently, these families were the subject of a report of linkage to chromosome 14 (St George Hyslop *et al.*, 1992). In this latter analysis the lod scores are in excess of 8.00 for these families combined. If the original report of linkage to chromosome 21 does not reflect an underlying predisposition then a chance linkage has occurred to the long arm of the chromosome to which true linkage was later confirmed in other families. However, the authors suggest that this may not be a chance linkage result and invoke an epistatic model to explain the linkage to both chromosome 21 and 14 in the same families. But, taking the βAPP mutation families as one group, the combined likelihood of complete co-segregation of βAPP with the disease is in the order of 100 million to one in favor. Moreover at least two large family data sets show that those families which are consistent with a linkage to chromosome 21 are not consistent with linkage to chromosome 14 and vice versa (analysis of Chapter 8 and that of Schellenberg and colleagues (Schellenberg *et al.*, 1992)). Epistasis seems an unlikely explanation and perhaps these results reflect the difficulties of working with very large and statistically powerful families in conditions where the genetic parameters are to some extent indeterminate. The Belgian pedigrees AD/A and AD/B have also previously been shown to have small positive lod scores to chromosome 21 markers but again much more significantly positive lod scores with chromosome 14 markers. However, at the time that the chromosome 21 data were available it was concluded that at least in AD/A the disease was due to a gene centromeric on chromosome 21 (Van Broeckhoven *et al.*, 1988). It was assumed that small negative lod scores in the related AD/B family were due to poor informativeness of markers. Many poorly polymorphic markers were run in the chromosome 21 analyses of both these data sets and it is possible that some selectivity of results has occurred with analyses and that multi-point analysis using all the available data would have excluded linkage. It is also likely that more polymorphic markers, had they been available, would have excluded linkage along the whole long arm of chromosome 21.

10.5.2 The Exclusion of the ßAPP Gene as a Candidate

The analysis of pedigrees not showing linkage to the ßAPP locus led to the hypothesis that ßAPP was not "the" familial AD gene. Given that it was assumed that there was linkage to chromosome 21 (above) it was concluded that another locus existed on this chromosome, and that this was more centromeric (probably around D21S13/S16). Included in one analysis, were families that later were shown to be linked to the chromosome 14 locus (AD/A and AD/B) but also two late onset families (Van Broeckhoven *et al.*, 1987). Included in a second analysis were the other families noted above (FADs 1-4) which were later shown to be highly linked to the chromosome 14 locus. The inclusion of late onset families or families with a causative locus on another chromosome naturally lead to the exclusion of ßAPP. The genetic analysis and sequencing of ßAPP in F23 thus clarified the existence of heterogeneity. The pooling of late and early onset families for linkage would now not be acceptable practice, as we now know they have different aetiologies. At the time these analyses were performed, this was not known and clinically there were no gross distinguishing features between the AD/A and AD/B pedigrees (Martin *et al.*, 1991) and the late onset families. Nor were there *a priori* reasons to suppose that F23 and FAD 1-4 and AD/A and AD/B were non-allelic variants although a comparison of age of onset between FAD 4 (which is independently linked to chromosome 14), and AD/A and AD/B on the one hand and F23 on the other would have shown very significant differences in the mean age of onset (FAD 4, 43 years, AD/A, AD/B 35 years and F23, 55 years). A further mislocalisation of the putative AD gene occurred when mis-diagnosed families and both non-ßAPP and ßAPP mutated families were included in the same analysis (Goate *et al.*, 1989).

10.6 The Mechanism of Action of the ßAPP Mutants

Over two years after the discovery of the first *ßAPP* mutation it is still not known by what mechanism mutations at codon 717 cause AD. The site of the mutations, just outside the C-terminal end of the Aß molecule (see Figure 1), suggests that processing of Aß from the ßAPP molecule might be disrupted in mutated ßAPP molecules. There are no data indicating that the codon 717 mutations cause AD by inducing the overproduction of Aß; an idea derived from the presumed overproduction that occurs in individuals with an additional copy of ßAPP (Down's syndrome) in which AD pathology is frequently observed. An alternative explanation is that the mutation may result in increased translation of APP messenger RNAs, causing Alzheimer's disease by a route analogous to that believed to occur in Down's syndrome (Tanzi, Hyman, 1991). The ßAPP717 mutations could operate by destroying an iron-responsive element between base pairs 2,131 and 2,156. There are potentially other mutations

that would disrupt this structure, many of which would be silent at the at the protein level; but no silent changes have yet been reported. The iron responsive element is part of a stem-loop structures believed to modulate gene translation by altering mRNA stability (Klausner, Harford, 1989). However *in situ* hybridization suggests that amounts of ßAPP mRNAs are not grossly altered in the brain of an individual with APP717 Val→Ile (Harrison *et al.*, 1991). It seems unlikely then that mRNA translation is affected by these mutations. The fact that the mutations can convert Val to either Gly or Ile suggests that neither side-chain hydrophobicity nor bulk are responsible for the pathogenicity of these mutations. Aß molecules isolated from the brain of a patient with Alzheimer's disease show N- and C-terminal heterogeneity (Esch *et al.*, 1990; Glenner, Wong, 1984), suggesting that the amyloidogenic pathway involves either sequence-specific proteolysis followed by exopeptidase activity (creating end-heterogeneity), or that it is not sequence-specific. The deposition of Aß depends partly on its length and it remains possible that these mutations cause the production of slightly elongated Aß by inhibiting the C-terminal nibbling to residues 39-42 of Aß after the initial C-terminal cleavage.

By contrast, the codon 670/671 mutation does cause the overproduction of Aß, at least in cultured cells transfected with copies of the mutant gene (Citron *et al.*, 1992; Cai *et al.*, 1993). The mutation in this case which occurs at the N-terminus of Aß (see Figure 1) must enhance the cleavage of Aß from ßAPP. This mutation is instructive and is consistent with the notion that an excess of Aß is either disease causing of itself or is a marker for the disease process. By what mechanism is unknown, but the study of transgenic animals carrying this human mutation is likely to provide the answer. For instance, the observation of the early stages of the disease process in transgenic animals with this mutation will clarify the relationship (in terms of neuropathology and molecular pathology) between Aß deposition and the other features of the disease. There is every likelihood that the codon 670/671 mutation will produce an excess of Aß *in vivo* and a transgenic model would therefore allow direct validation (or refutation) of hypotheses promoting Aß deposition as germane to disease development.

However, it should be noted that entirely consistent with the present data is the hypothesis that the production of Aß (even the enhanced production) is an epiphenomenon of some other neurodegenerative process associated with the remaining fragments of the ßAPP molecule after Aß cleavage has occurred. Interesting data pertaining to this possibility is discussed below. The question of whether or not Aß is the prime molecule causing degeneration might be answered if the basic functions of it and the parent ßAPP molecule were known. These data are currently accruing. The idea that Aß has a normal physiologic function is supported by the analysis of CSF and the media of cultured cells showing its normal production (Seubert *et al.*, 1992; Haass *et al.*, 1992a; Shoji *et al.*, 1992). This contrasts with the notion that the

formation of any Aß is necessarily pathogenic. As Aß is normally soluble it has been suggested that insoluble Aß is required to induce neurodegeneration. What then determines Aß solubility or conversely, precipitation? Four factors, *in vitro* at least, govern Aß solubility; its concentration, length (longer fragments are more insoluble), pH, and the presence or absence of other molecules (Burdick *et al.*, 1992). As an aside, the molecular pathogenicity of at least one *ßAPP* mutation seems likely related to a change in solubility. The mutant form of Aß which occurs in hereditary cerebral haemorrhage with amyloidosis - Dutch type (HCHWA-D) seems to bind to the wild type of Aß and cause precipitation of both in cerebral vessels leading to a pathology distinct from AD. Research into the factors determining solubility would be accentuated if a direct link were established between deposited Aß and neurotoxicity. Research in this area has yielded contradictory results.

There is some persuasive evidence that Aß is neurotoxic - the addition of synthetic Aß to cultured cells resulted in cell death either directly (Yankner *et al.*, 1990) or indirectly (Koh *et al.*, 1990; Mattson *et al.*, 1992). *In vivo* addition of Aß to cortex has produced conflicting results - some laboratories observing Aß deposition and toxicity (Kowall *et al.*, 1992) (but rarely pathology reminiscent of neurofibrillary tangles) and others observing no effect (Clemens, Stephenson, 1992; Games *et al.*, 1992; Podlisny *et al.*, 1992; Stein-Behrens *et al.*, 1992). Several experiments lend credence to the idea that Aß aggregation is required for neurotoxic effects, this phenomenon being associated, for instance, with "*in vitro* aging"(Pike *et al.*, 1991). The requirement of aggregation for neurotoxicity might explain the conflicting evidence for the neurotoxicity of Aß generated by several labs performing essentially the same experiment. In a review of the data, Price et al (Price *et al.*, 1992) point out that the results of five *in vitro* investigations did not show a consistent pattern of toxicity and suggest that transgenic animals may provide more reliable models of the disease. The findings in this crucial area remain temporarily discrepant.

Reports of large amounts of Aß deposition without neuritic plaque or tangle formation in psychometrically normal cases(Delaere *et al.*, 1990), support the idea that other factors control the relationship between Aß deposition and the subsequent development of tangles. However, the example of Down's syndrome seems clear enough. From the study of AD pathology in DS brains autopsied at different ages, there is a progression of pathology (Mann, Esiri, 1989). Essentially, Aß deposition is followed by neuritic plaque formation and then tangle formation and cell death. The available data suggests that this progression is an inevitable consequence of aging in DS. A natural hypothesis is that the same pathological spectrum seen in DS would inevitably occur in the normal aged individuals with Aß deposits or plaque if they lived long enough. One consequence of this hypothesis for molecular genetics is that in late onset disease we should be looking for factors that accelerate the

disease, i.e., bring forward the age of onset or rate of progression. Such a risk locus may have been identified - the *APO E* gene.

10.7 A Second AD Gene on Chromosome 14

It has been known for some time that the ßAPP mutations account for only a small percentage (less then 10%) of the early onset familial cases. The remaining families also show segregation of an autosomal dominant gene. Progress in the Human Genome Project has expedited the search for a second early onset AD gene and has culminated in the approximate localization of the gene by four independent groups. Schellenberg et al (Schellenberg *et al.*, 1992) demonstrated linkage to the middle long arm of chromosome 14. Their linkage data divides their families into two groups; a genetically related group of families, the Volga Germans, showing no linkage to any of the chromosome 14 markers tested; eight out of nine non-Volga German families of mixed genetic origin were consistent with linkage to at least one of three genetic markers in the region. Three other groups (St George Hyslop *et al.*, 1992; Van Broeckhoven *et al.*, 1992; Mullan *et al.*, 1992) have similar data in their own diverse family sets. The fact that four groups have independent data for linkage at this site is convincing evidence that a locus exists on the long arm of chromosome 14. However, in contemplating further fine genetic localization of the gene, the problem of heterogeneity raises its head. Two groups of families have been identified which are not linked to either ßAPP or markers on chromosome 14 (Schellenberg *et al.*, 1992; Lannfelt *et al.*, 1993). Essentially, researchers face the same situation that the chromosome 21 linkage data presented over the last five years. If locus heterogeneity exists in this data set then linkage to chromosome 14 markers will be obscured by those families linked elsewhere. Age of onset correlates with genetic aetiology in the data set examined in this thesis, and this may provide a way of identifying chromosome 14 linked families. However, the general approach to localizing this gene will be to test for genetic heterogeneity, further define the genetic map in homogeneous subgroups, and apply positional cloning strategies. An alternative approach will be to screen candidate genes in the area such as the 70kD heat shock protein gene, HSPA2, and c-fos. The α1-antichymotrypsin (AACT) gene at the distal long arm of chromosome 14 has already been excluded. (AACT is known to be a component of the neuritic amyloid plaque). Many other candidates are suggested from studies of the molecular pathology of AD (see the work on neurofibrillary tangles or calcium homeostasis, below, for instance).

10.8 Late Onset Disease, Chromosome 19 and the APO E Gene

Data suggestive of linkage or association of late onset familial disease to chromosome 19 markers have been reported since 1987. However, it seemed likely from the outset that late onset familial disease would not be due to a single gene operating as a Mendelian dominant. One group detected an association with the *APO CII* gene on chromosome 19 (Schellenberg *et al.*, 1987) in the approximate area that others subsequently reported linkage (Pericak-Vance *et al.*, 1991). The lod score method of linkage analysis has generally strict implications about the relationship of gene and disease unless the parameters are deliberately manipulated to model complex modes of inheritance. Negative or equivocal findings of lod score linkage do not necessarily imply non-linkage but may also reflect mis-specification of genetic parameters. Other methods of analysis (non-parametric) allow the detection of contributory loci where the relationship between variation in DNA and disease may not be one to one. Using such methods, markers on chromosome 19 have produced positive linkage results in late onset families, suggesting complex inheritance of predisposition (Pericak-Vance *et al.*, 1991). Some of the markers examined are linked to the *APO E* gene, relatively close to *APO CII*. The *APO E* gene and its products are well characterized (Davignon *et al.*, 1988). Genetic polymorphism at the *APO E* locus results in three common alleles ε2, ε3 and ε4. Correspondingly, three common isoforms of APO E exist. E-3 is the commonest, E-2 is less common and is associated with protection against atherogenesis. The ε4 allele has previously been recognized as a risk factor for hypercholesterolaemia and coronary artery disease (CAD). Roses and colleagues examined one affected person per family and took as controls the grandparents of large genetic reference pedigrees (CEPH). They found an allele frequency for ε4 of 16% in the controls compared to 50% in the probands from the families (Strittmatter *et al.*, 1993a). A review of historical controls show that there is rarely an allele frequency for ε4 in the general population over 18%(Davignon *et al.*, 1988). A previous examination of the *APO E* locus in well octagenarians revealed a low frequency of the ε4 allele, suggesting the *APO E* locus does influence morbidity and mortality (Davignon *et al.*, 1986). In an examination of late onset sporadic AD cases of AD, 64% have one ε4 compared to 31% of normals. It is clear then, from the outset, that given the occurrence of the ε4 allele in normals does not act as an autosomal dominant in these late onset families. Rather, if the findings are replicated it should be regarded as a genetic susceptibility locus associated with AD within late onset families.

These very important findings need to be clarified and expanded to address the question of whether this locus does confer increased risk of developing AD or whether it advances age of onset in otherwise predisposed individuals. Firstly, there is a simple need of replication both in Caucasian populations and groups with other ethnic backgrounds. One would assume that

if this confers increased risk, it would do so in all populations. Epidemiologically unbiased populations, stratified for age with matched controls are required to determine that the distortion of allele frequencies is not artefactual. For instance, the affect of the risk of CAD conferred by this locus has to be examined. Secondly, if the role of the *APO E* locus is to modify age of onset age there are a number of reference families that could be used to test this hypothesis, for instance those families that have already been shown to have the disease due to *βAPP* mutations or the chromosome 14 gene. The age of onset tends to be constant within all early onset families (van Duijn *et al.*, 1991c) (particularly the chromosome 14 linked families), but variation does occur as does variation in rate of disease progression. An examination of these factors in relation to *APOE* status is needed. Age of onset in late onset families tends to have much greater within-family variation (Chapter 9). This variation should be similarly examined in relation to *APO E* genotype.

Given the risk for vessel disease associated with the *APOE* locus, it is tempting to speculate on the relationship between vessel disease and AD. An alternative explanation to the idea that *APOE* is an independent risk factor for both of these disease is that one is causally related to the other in a subgroup of individuals. A comparison of vascular disease (determined clinically and by scan data, for instance) between those cases with the ε4 allele and those without would begin to address this issue. In addition, the effects of other loci known to be risk factors for vessel disease need to be examined.

10.8.1 Molecular Pathology of APOE

If the *APO E* locus represents and independent risk factor for AD, by what mechanism does it enhance the disease process? One clue may be provided by the localization of APO E to the plaque of AD. Further, it has been noted in one study that the relationship of density and number of the plaque is a function of the *APO E* genotype type. Thus, many of the individuals with the ε4ε4 genotyping have the heaviest staining for Aß in plaque at autopsy (Schmechel *et al.*, 1993). One explanation that has been proposed is that APO E protein acts as a molecular chaperone - aiding the sequestration of Aß into plaque. This idea is supported by the observation that APO E does bind Aß and does so to differing degrees, dependent on the isoform (Strittmatter *et al.*, 1993b). The ε3 isoform forms a dimer with Aß slowly compared to the binding of the ε4 isofrom which is much more rapid. Understanding the molecular mechanism by which APO E enhances disease progression is a major area of current investigation.

10.9 Neurofibrillary Tangles (NFTs)

The other characteristic neuropathological feature of AD, neurofibrillary tangles, has also undergone extensive molecular investigation. It has previously been suggested that Aß was also the main constituent of NFTs. However, the major component of NFTs, paired helical filaments (PHFs) are comprised of the micro tubule associated protein (MAP) tau (Goedert *et al.*, 1988). Aß is also a constituent of NFTs as are other peptides such as ubiquitin. When isolated from brain, tau is usually a mixture of several isoforms. Tau can be phosphorylated, a process that results in conformational change which have been associated with abnormal deposition in AD. Although the majority of NFTs are intracellular structures extra cellular NFTs occur also. There are both conformational differences and structural differences between the intra- and extra-cellular forms.

Differences in phosphorylation status have been noted between tau occurring in normal brain and that of Alzheimer disease brain. Drastic differences in conformation of tau seem due more to the status of particular residues than the overall phosphorylation status of the protein. Phosphorylation status seem to be brought about *in vitro* by certain kinases and not by others (Steiner *et al.*, 1990). One of these enzymes, calcium calmodulin dependent protein kinase, is found in abundance in hippocampal neurons (McKee *et al.*, 1990). It is tempting to speculate, as others have, that primary phosphorylation imbalance is responsible for the production of abnormally phosphorylated tau and subsequent deposition as NFTs.

The role of intracellular calcium levels too, has been explored as a source of neurofibrillary degeneration. Neurofibrillary-like change is caused by elevated calcium levels in cultured cells. This is an attractive finding as the possible role of ßAPP, Aß and other ßAPP metabolites in destabilising calcium homeostasis and increasing cell vulnerability to excitotoxins has also been noted (see below).

10.10 Towards a Comprehensive Theory of AD Pathogenesis

We are a considerable distance from a unitary hypothesis of AD pathogenesis. As the known causes of AD (*ßAPP* mutants and DS) are few and possibly atypical, it is unsurprising that a robust hypothesis explaining the more common forms of the disease is not forthcoming. A direct consequence of these being the only known examples of AD causation is the unitary hypothesis that ßAPP is centrally involved in the aetiology of all cases of AD. One embodiment of this idea, the "amyloid cascade hypothesis" (Hardy, Allsop, 1991), proposes that Aß deposition is the central event leading to the whole gamut of neuropathology. The

hypothesis further proposes that "mismetabolism" of ßAPP leads to Aß deposition. However, as reviewed above there are several key areas of concern and objection:

1) The notion that Aß deposition per se causes all cases of the disease is contradicted by the observations that Aß deposition occurs in normal individuals and does not correlate well with cell death or dementia. Degree of dementia does correlate with plaque count but the best correlation with dementia is NFT count.

2) The causal relationship between Aß deposition and neurofibrillary tangle formation is not known but according to the cascade hypothesis, the former leads to the latter. We should consider that the production of tangles may not follow automatically from the deposition of Aß or at least may proceed at a different pace in some individuals than in others. This implies the existence of factors which modify or permit the production of tangles given the presence of ßAPP mismetabolism. Neither the mechanism by which one induces the other nor the mediating factors are known. For instance, are excess Aß production or deposition mediating? If excess production is a factor is excess of the complete ßAPP molecule sufficient (the Down's finding would suggest it is) or is the overproduction of Aß the critical event (the codon 670/671 mutation is consistent with this)? Perhaps the most reductionist aspect of the "cascade" hypothesis, is that AD is viewed as a cerebral amyloid (Aß) storage disease, the cellular degeneration and death being secondary processes mediated perhaps by over sensitive or otherwise inappropriate immune responses. While this might be the case, it would be imprudent to ignore the possible functional significance of ßAPP and Aß. Such an ubiquitously and abundantly expressed molecule as ßAPP with its highly conserved genetic sequence clearly has a fundamental role perhaps in cell signaling, regulation of cellular processes or trafficking of other molecules. Disturbances in these dynamic processes may lead to the AD pathology rather than the consequences of accumulation of Aß. The data suggesting ßAPP is a neuronal receptor, or is an ion channel, reviewed below, exemplify this alternative viewpoint.

3) The evidence for a direct neurotoxic effect of Aß *in vitro* is controversial and not reproducible by several investigators.

4) Most importantly, though, it is not clear exactly what the cascade is. The AD process occurs late in life and both clinically and pathologically appears "triggered". The idea of a cascade or positive feedback loop does seem appropriate but is not apparent in the linear cascade hypothesis. Perhaps the closest molecular model associated with a degenerative cascade comes from the work of prion disorders where auto catalysis of the prion molecule perpetuates disease progression. Alternatively, in the biophysical realm we might propose that

Aß acts as its own nidus for precipitation. HCHWA-D demonstrates the co-precipitation of abnormal Aß with the normal variant in the vessel walls of affected individuals. However, no such processes have yet been shown to operate in AD. Natural cascades exist in abundance in the cellular regulatory systems, making them more likely candidates for amplification of abnormal metabolism.

It remains possible and consistent with all the data, that the deposition of Aß is not the causative step in AD development. Excess Aß production may be a by-product of some other intracellular process with the critical events for disease process either preceding Aß production or being mediated by its presence. This line of reasoning invariably invokes the question of the function of ßAPP and/or Aß. As noted above little is known of the physiological function of these molecules. One recent intriguing suggestion is that ßAPP is a neuronal receptor mediating its effects via a G protein, G_0 This hypothesis derives from two observations; that ßAPP contains a consensus amino acid sequence common to insulin like growth factor which is known to recognize and bind a G protein; and by the demonstration of binding of G_0 by ßAPP *in vitro* associated with activation of G_0. A further gap in our understanding is the function of G_0 but there are two attractive proposals which might bear on the significance of ßAPP activation in relation to AD pathogenesis. It has been suggested that G_0 is a calcium channel activator thus invoking ßAPP and G_0 as mediators of cell death. Also proposed is that ßAPP binding of G_0 represents a step in the intracellular trafficking of either ßAPP or G_0. As others have suggested, mis-trafficking of the metabolites of ßAPP could lead to abnormal membrane turnover, damage and death. ßAPP contains a consensus sequence in the cytoplasmic tail, known to be present in some cell surface receptors, and associated with signaling for endocytosis. The notion of ßAPP as active at the cell surface has been explored in a different direction. When present in artificial membranes, Aß acts a calcium channel, prompting speculation that this might render Aß neurotoxic (Arispe *et al.*, 1993) (see below).

Clearly the occurrence of Aß deposits and NFTs may be the consequences of imbalances in a single biochemical process. The occurrence of NFTs in other disorders (ALS-Parkinsonian dementia complex of Guam, dementia pugilistica, progressive supra nuclear palsy) argues for malfunctioning of a ubiquitous system. The messengers which regulate cellular functioning are therefore candidates for generating both abnormal Aß processing and the production of NFTs. Protein phosphorylation is an important regulatory mechanism in mammalian brains which contain large amounts of cAMP, cGMP, protein kinases (which add phosphate groups) and phosphorylated proteins. Protein kinase C phosphorylates ßAPP at serine 655 (of ßAPP 695) and phorbol esters increase the large N terminal secretory products of ßAPP presumably by activating protein kinase C. Phosphorylation may also activate the ßAPP cleaving enzymes and thus may exert a regulatory influence on ßAPP processing (Buxbaum *et al.*, 1990).

It has been suggested that abnormal phosphorylation status might lead to extra cellular amyloidosis via a number of mechanisms and therefore the complete spectrum of pathology of AD might be attributed to abnormal protein phosphorylation (Gandy *et al.*, 1990). At this time such encompassing theories are only supported in part.

Calcium homeostasis, as noted above, is an alternative but complementary cellular regulatory system deserving keen attention as a putative central mechanism in AD development. It may be relevant to the possible physiological roles of ßAPP and Aß, to NFT formation and to cell degeneration and death (see Figure 3). The calcium ion is an intracellular messenger in signal transducing pathways but may also mediate cellular degeneration and death, as a result of exitotoxic stimulation by glutamate and other neurotransmitters, especially under conditions of limited energy availability. Loss of calcium homeostasis results in unchecked free radical formation, with deleterious consequences. Significantly, exposure to Aß has been linked to changes in calcium balance (Mattson *et al.*, 1992) but also Ca++ activates protein kinase C, known to process ßAPP (Buxbaum *et al.*, 1990). A circle of deleterious events might occur whereby loss of Ca++ homeostasis adversely affects ßAPP processing which consequently further destabilises Ca++ balance (see Figure 3). In addition, intracellular increases in Ca++ has been associated with an NFT like antigenic profile in cultured hippocampal neurones (Mattson, 1990).

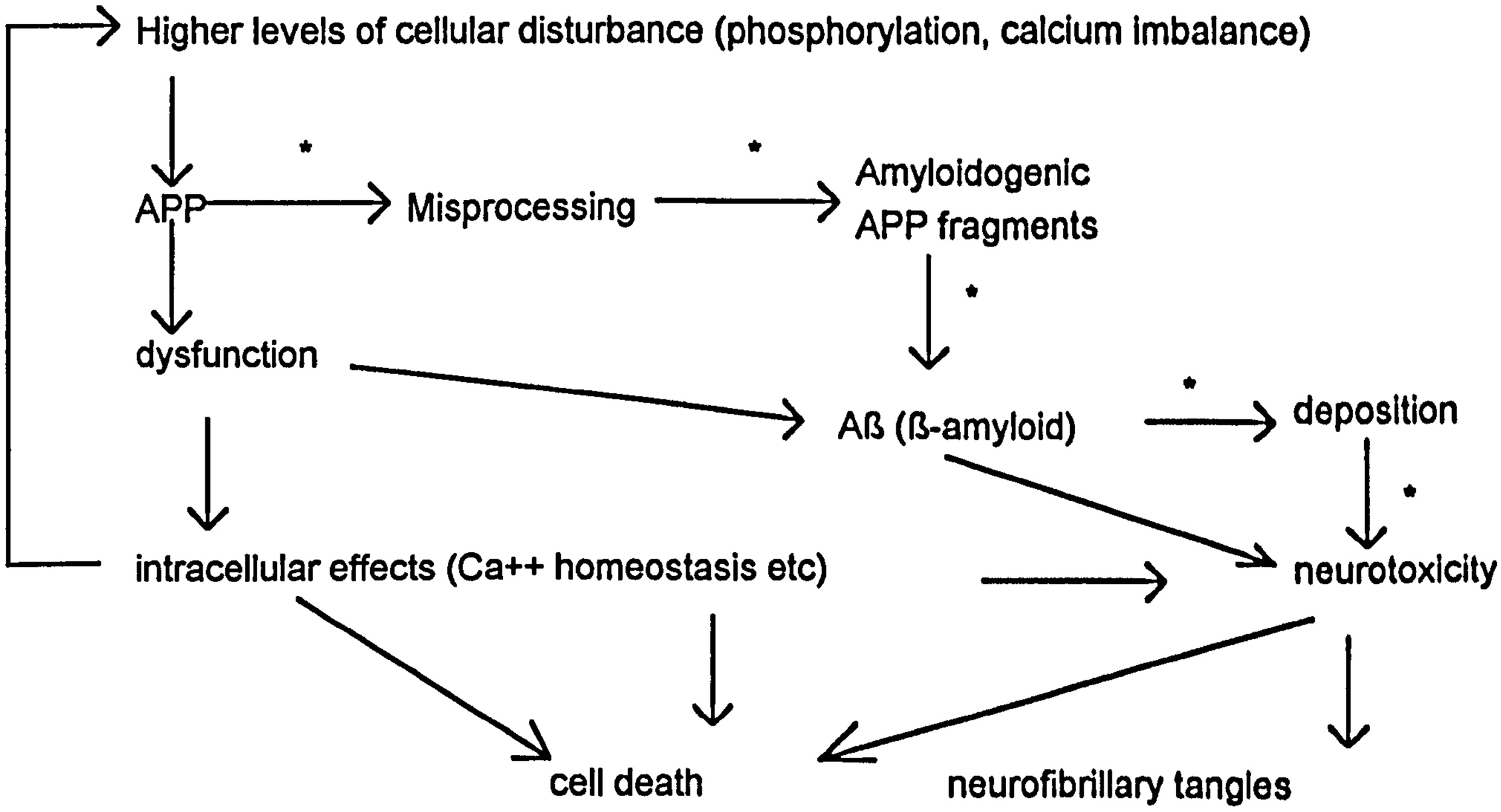


Figure 3. The Pathogenesis of AD

Other known risk factors for AD, such as head injury, known to be associated with increased expression of ßAPP (Roberts *et al.*, 1991) and with excitotoxicity, might be mediated through the same negative cycle. In this scheme of things the deposition of Aß may be associated with indirect toxicity, or may be not be central to the excitotoxic cycle. Aß may operate at the cell surface, influencing Ca++ passage in a manner analogous to that in artificial lipids noted above (Arispe *et al.*, 1993), or by other mechanisms. Other derivatives of the ßAPP molecule may also influence Ca++ balance. In this regard, the finding by Mattson and colleagues (Mattson *et al.*, 1993) of protection by APP_S against calcium dependent cytotoxicity may be key.

10.11 Summary

The importance of ßAPP and Aß in the pathogenesis of AD is likely to differ between subgroups but the function of these molecules may be key to our understanding of the disease. The roles of ßAPP in cellular signaling, transduction and trafficking suggest a disease process which operates via a distortion of the normal metabolic pathways rather than mismetabolism. Further, a pathophysiologic role for soluble Aß may be of more relevance to the disease than the observed Aß deposits. The next wave of information about the role of ßAPP and Aß will come from transfection and transgenic studies of normal and abnormal ßAPP. Independently, a further piece of the puzzle will be added with the cloning of the chromosome 14 gene. Its identification might immediately fill some of the holes in our present knowledge but more likely the same studies required of ßAPP will be required for the chromosome 14 gene. More immediately, the clarification of the role of APO E at a biochemical level will shed additional light on the degenerative process in the more common form of this enigmatic disease.

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ADDENDA

ADDENDUM 1

Allele Recoding Program

```
program Recode; Uses Wincrt, Windos;
const
  ChangeTo1: Pchar = 'C:\progs'; {input directory}
  CDrive: Byte = 3;
  ChangeTo2: Pchar = 'C:\progs';{output directory}
  Imax=50;
var
numloci:integer;
Ans,a,b,c,d,e,f,g,h,i,j,k,l,m,n:integer;
ao,bo,co,dor,eo,fo,go,ho,io,jo,ko,lo,mo,no:integer;
aa,bb,cc,dd,ec,ff,gg,hh,ii,jj,kk,ll,mm,nn:integer;
fid, id, mot, fat, gen, aff, liab, x1,x2, y1,y2,z1,z2,w1,w2,l1a, l1b:integer; {number of columns}
CurDir: PChar;
fromF, ToF,Tosum: text;
namein, nameout,outfilenext,summaryfile: string;
Numread, Numwritten: Word;
buf: array[1..2048] of char;
Anschar,Ans2char,Ans3char:char;
begin
 GetMem(CurDir, 80);
 SetCurDir(ChangeTo1);
 GetCurDir(CurDir, CDrive);
 Writeln('The current directory is ', CurDir, '.');
 Writeln ('What is the name of the input file?');
 Readln (namein);
 Assign (FromF, namein);
 Reset (FromF);
 Writeln('How many marker loci in the input file?');
 Readln(numloci);
 SetCurDir(ChangeTo2);
 GetCurDir(CurDir,CDrive);
 Writeln('The current directory is ', CurDir, '.');
 Writeln ('What is the name of the output file?');
 Readln (nameout);
 Assign (ToF, nameout);
 Rewrite (ToF);
 Writeln ('Working');
  SetCurDir(ChangeTo1);

 Writeln(' ');
repcat
Writeln('    Which locus number do you want recoded?');
Writeln('    { The first [affection] locus = zero}');
Writeln(' ');
readln(Ans);


reset(FromF);
 ao:=0; {original alleles}
 bo:=0;
```

```
co:=0;
dor:=0;
eo:=0;
fo:=0;
go:=0;
ho:=0;
io:=0;
jo:=0;
ko:=0;
lo:=0;
mo:=0;
no:=0;
aa:=0;
bb:=0;
cc:=0;
dd:=0;
ee:=0;
ff:=0;
gg:=0;
hh:=0;
ii:=0;
jj:=0;
kk:=0;
ll:=0;
mm:=0;
nn:=0;
repeat

if numloci=1 then Readln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2);
if numloci=2 then Readln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2);
if numloci=3 then Readln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2, z1, z2);
if numloci=4 then Readln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2, z1, z2, w1, w2);
if Ans=1 then l1a:=x1;
if Ans=1 then l1b:=x2;
if Ans=2 then l1a:=y1;
if Ans=2 then l1b:=y2;
if Ans=3 then l1a:=z1;
if Ans=3 then l1b:=z2;
if Ans=4 then l1a:=w1;
if Ans=4 then l1b:=w2;
if (l1a=1) or (l1b=1) then ao:=1;{original alleles}
if (l1a=2) or (l1b=2) then bo:=2;
if (l1a=3) or (l1b=3) then co:=3;
if (l1a=4) or (l1b=4) then dor:=4;
if (l1a=5) or (l1b=5) then eo:=5;
if (l1a=6) or (l1b=6) then fo:=6;
if (l1a=7) or (l1b=7) then go:=7;
if (l1a=8) or (l1b=8) then ho:=8;
if (l1a=9) or (l1b=9) then io:=9;
if (l1a=10) or (l1b=10) then jo:=10;
if (l1a=11) or (l1b=11) then ko:=11;
if (l1a=12) or (l1b=12) then lo:=12;
if (l1a=13) or (l1b=13) then mo:=13;
if (l1a=14) or (l1b=14) then no:=14;
{counting}
if l1a=1 then aa:=aa+1;
if l1a=2 then bb:=bb+1;
```

```
if 11a=3 then cc:=cc+1;
if 11a=4 then dd:=dd+1;
if 11a=5 then ee:=ee+1;
if 11a=6 then ff:=ff+1;
if 11a=7 then gg:=gg+1;
if 11a=8 then hh:=hh+1;
if 11a=9 then ii:=ii+1;
if 11a=10 then jj:=jj+1;
if 11a=11 then kk:=kk+1;
if 11a=12 then ll:=ll+1;
if 11a=13 then mm:=mm+1;
if 11a=14 then nn:=nn+1;
if 11b=1 then aa:=aa+1;
if 11b=2 then bb:=bb+1;
if 11b=3 then cc:=cc+1;
if 11b=4 then dd:=dd+1;
if 11b=5 then ee:=ee+1;
if 11b=6 then ff:=ff+1;
if 11b=7 then gg:=gg+1;
if 11b=8 then hh:=hh+1;
if 11b=9 then ii:=ii+1;
if 11b=10 then jj:=jj+1;
if 11b=11 then kk:=kk+1;
if 11b=12 then ll:=ll+1;
if 11b=13 then mm:=mm+1;
if 11b=14 then nn:=nn+1;

until eof(FromF);

if ao=1 then a:=1;
if bo=2 then b:=1;
if co=3 then c:=1;
if dor=4 then d:=1;
if eo=5 then e:=1;
if fo=6 then f:=1;
if go=7 then g:=1;
if ho=8 then h:=1;
if io=9 then i:=1;
if jo=10 then j:=1;
if ko=11 then k:=1;
if lo=12 then l:=1;
if mo=13 then m:=1;
if no=14 then n:=1;
{new allele values:}
if n=0 then n:=0 else n:=a+b+c+d+e+f+g+h+i+j+k+l+m+n;
if m=0 then n:=0 else m:=a+b+c+d+e+f+g+h+i+j+k+l+m;
if l=0 then l:=0 else l:=a+b+c+d+e+f+g+h+i+j+k+l;
if k=0 then k:=0 else k:=a+b+c+d+e+f+g+h+i+j+k;
if j=0 then j:=0 else j:=a+b+c+d+e+f+g+h+i+j;
if i=0 then i:=0 else i:=a+b+c+d+e+f+g+h+i;
if h=0 then h:=0 else h:=a+b+c+d+e+f+g+h;
if g=0 then g:=0 else g:=a+b+c+d+e+f+g;
if f=0 then f:=0 else f:=a+b+c+d+e+f;
if e=0 then e:=0 else e:=a+b+c+d+e;
if d=0 then d:=0 else d:=a+b+c+d;
if c=0 then c:=0 else c:=a+b+c;
if b=0 then b:=0 else b:=a+b;
```

```
Writeln('  The following alleles occur at locus ',Ans,' in this data set.');
if ao>0 then writeln('original allele ',ao,' (occurring ',aa,' times) recoded as ',a );
if bo>0 then writeln('original allele ',bo,' (occurring ',bb,' times) recoded as ',b);
if co>0 then writeln('original allele ',co,' (occurring ',cc,' times) recoded as ',c);
if dor>0 then writeln('original allele ',dor,' (occurring ',dd,' times) recoded as ',d);
if eo>0 then writeln('original allele ',eo,' (occurring ',ee,' times) recoded as ',e);
if fo>0 then writeln('original allele ',fo,' (occurring ',ff,' times) recoded as ',f);
if go>0 then writeln('original allele ',go,' (occurring ',gg,' times) recoded as ',g);
if ho>0 then writeln('original allele ',ho,' (occurring ',hh,' times) recoded as ',h);
if io>0 then writeln('original allele ',io,' (occurring ',ii,' times) recoded as ',i);
if jo>0 then writeln('original allele 'jo,' (occurring 'jj,' times) recoded as 'j);
if ko>0 then writeln('original allele ',ko,' (occurring ',kk,' times) recoded as ',k);
if lo>0 then writeln('original allele ',lo,' (occurring ',ll,' times) recoded as ',l);
if mo>0 then writeln('original allele ',mo,' (occurring ',mm,' times) recoded as ',m);
if no>0 then writeln('original allele ',no,' (occurring ',nn,' times) recoded as ',n);
Writeln('  Would you like to save this information to a summary file? (Y/N)');
  Readln(Ans2char);
 if (Ans2char)='Y' then
  begin
    Writeln('Is there already a summary file assigned? (Y/N)');
    readln(Ans3char);
    if(Ans3char)='N' then
       begin
        writeln('  Assign a name to the summary file');
        readln(summaryfile);
        Assign(Tosum,sumnaryfile);
        rewrite(Tosum);
        writeln(Tosum,' ');
        Writeln(Tosum,'  The following alleles occur at locus ',Ans,' in this data set.');
        if ao>0 then writeln(Tosum,'original allele ',ao,' (occurring ',aa,' times) recoded as ',a );
        close(Tosum);
       end
     else
       begin
        append(Tosum);
        writeln(Tosum,' ');
        Writeln(Tosum,'  The following alleles occur at locus ',Ans,' in this data set.');
        if ao>0 then writeln(Tosum,'original allele ',ao,' (occurring ',aa,' times) recoded as ',a );
        close(Tosum);
       end;
   end
  else
begin
reset(FromF);
repeat
 if numloci=1 then Readln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2);
if numloci=2 then Readln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2);
if numloci=3 then Readln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2, z1, z2);
if numloci=4 then Readln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2, z1, z2, w1, w2);

if Ans=1 then l1a:=x1;
if Ans=1 then l1b:=x2;
if Ans=2 then l1a:=y1;
if Ans=2 then l1b:=y2;
if Ans=3 then l1a:=z1;
if Ans=3 then l1b:=z2;
```

```
if Ans=4 then l1a:=w1;
if Ans=4 then l1b:=w2;
if l1a=1 then l1a:=a;
if l1a=2 then l1a:=b;
if l1a=3 then l1a:=c;
if l1a=4 then l1a:=d;
if l1a=5 then l1a:=e;
if l1a=6 then l1a:=f;
if l1a=7 then l1a:=g;
if l1a=8 then l1a:=h;
if l1a=9 then l1a:=i;
if l1a=10 then l1a:=j;
if l1a=11 then l1a:=k;
if l1a=12 then l1a:=l;
if l1a=13then l1a:=m;
if l1a=14 then l1a:=n;
if l1b=1 then l1b:=a;
if l1b=2 then l1b:=b;
if l1b=3 then l1b:=c;
if l1b=4 then l1b:=d;
if l1b=5 then l1b:=e;
if l1b=6 then l1b:=f;
if l1b=7 then l1b:=g;
if l1b=8 then l1b:=h;
if l1b=9 then l1b:=i;
if l1b=10 then l1b:=j;
if l1b=11 then l1b:=k;
if l1b=12 then l1b:=l;
if l1b=13then l1b:=m;
if l1b=14 then l1b:=n;

 if Ans=1 then x1:=l1a;
 if Ans=1 then x2:=l1b;
 if Ans=2 then y1:=l1a;
 if Ans=2 then y2:=l1b;
 if Ans=3 then z1:=l1a;
 if Ans=3 then z2:=l1b;
 if Ans=4 then w1:=l1a;
 if Ans=4 then w2:=l1b;
 SetCurDir(ChangeTo2);
 if numloci=1 then writeln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2);
 if numloci=2 then writeln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2);
 if numloci=3 then writeln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2, z1, z2);
 if numloci=4 then writeln (FromF, fid, id, mot, fat, gen, aff, liab, x1, x2, y1, y2, z1, z2, w1, w2);

until eof(FromF);
 SetCurDir(ChangeTo1);
 Close (FromF);
 SetCurDir(ChangeTo2);
 Close (ToF);
end;
Writeln(' ');
Writeln('The alleles at locus ', Ans,' have been recoded.');
Writeln('  Do you want to recode another locus? (Y/N)');
Readln(Anschar);
  if (Anschar)='Y' then
  begin
```

```
    writeln('  Assign another name to the output file');
    readln(outfilenext);
    Assign(ToF,outfilenext);
    rewrite(ToF);
    Assign(FromF,namcout);
    end;
   until(Anschar)='N';
end.
```

Addendum 2

Summary File of Recoded Data

The following alleles occur at locus 1 in this data set.
original allele 6 (occurring 5 times) recoded as 1
original allele 7 (occurring 10 times) recoded as 2
original allele 9 (occurring 9 times) recoded as 3

The following alleles occur at locus 2 in this data set.
original allele 2 (occurring 3 times) recoded as 1
original allele 3 (occurring 6 times) recoded as 2
original allele 7 (occurring 10 times) recoded as 3
original allele 9 (occurring 1 times) recoded as 4

The following alleles occur at locus 3 in this data set.
original allele 2 (occurring 2 times) recoded as 1
original allele 9 (occurring 22 times) recoded as 2

The following alleles occur at locus 4 in this data set.
original allele 2 (occurring 2 times) recoded as 1
original allele 4 (occurring 4 times) recoded as 2
original allele 6 (occurring 7 times) recoded as 3
original allele 7 (occurring 4 times) recoded as 4
original allele 10 (occurring 1 times) recoded as 5

The following alleles occur at locus 5 in this data set.
original allele 1 (occurring 1 times) recoded as 1
original allele 2 (occurring 4 times) recoded as 2
original allele 3 (occurring 1 times) recoded as 3
original allele 4 (occurring 9 times) recoded as 4
original allele 8 (occurring 2 times) recoded as 5
original allele 9 (occurring 1 times) recoded as 6

The following alleles occur at locus 7 in this data set.
original allele 2 (occurring 19 times) recoded as 1
original allele 3 (occurring 1 times) recoded as 2

The following alleles occur at locus 8 in this data set.
original allele 3 (occurring 1 times) recoded as 1
original allele 4 (occurring 4 times) recoded as 2
original allele 5 (occurring 1 times) recoded as 3
original allele 6 (occurring 4 times) recoded as 4
original allele 7 (occurring 7 times) recoded as 5
original allele 9 (occurring 3 times) recoded as 6

The following alleles occur at locus 9 in this data set.
original allele 2 (occurring 1 times) recoded as 1
original allele 4 (occurring 2 times) recoded as 2
original allele 5 (occurring 11 times) recoded as 3
original allele 6 (occurring 6 times) recoded as 4
original allele 10 (occurring 2 times) recoded as 5

Addendum 3

Liability Class Program

```
program Liability; Uses Wincrt, Windos;
const
  ChangeTo1: Pchar = 'C:\f\early\henjuly\aoo'; {input directory}
  CDrive: Byte = 3;
  days: array [0..6] of string[9] =
  ('Sun','Mon','Tues','Wed','Thur','Fri','Sat');
var
 y,m,d,dow: word;
 CurDir: PChar;
 A, T, n, S, Mn, SE, B1, B2, B3, B4, B5, B6, assyr, age, year  : real;
 Ans, agedob  :char;
 fid,id,filename: string;
 ToF: Text;
begin
  GetMem(CurDir, 80);
  SetCurDir(ChangeTo1);
  GetCurDir(CurDir, CDrive);
 Writeln(' The curent directory is ', CurDir, '.');
 Writeln ('');
 Writeln (' This program determines mean age of onset and');
 Writeln (' attributes liability classes to unaffected individuals');
 Writeln (' based on the assumption of normal distribution of AOO.');
 Writeln ('');
 Writeln ('        ******** Mike Mullan, March 1992 *****');
 Writeln ('');
    Writeln('   What is the family identifier?');
 Readln(fid);
begin
  Writeln('  What is the name of the output file?');
  Readln(filename);
  GetDate(y,m,d,dow);
  Assign(ToF, filename);
  Rewrite(ToF);
  Writeln(ToF, 'This is summary data for ',fid,'.');
  Writeln(ToF, 'Calculated on ',days[dow],', ',m:0, '/', d:0, '/', y:0);
  Close (ToF);
 end;
repeat
 Write ('Enter Age of onset: ');
 Readln (A);
 T:=T+A;
  S:=S+(A*A);
  Append(ToF);
  Write(ToF,' ',A,' ');
  Close(ToF);
  Writeln('Another age of onset? (Y/N)');
  Readln(Ans);
 until (Ans) = 'N';
Write ('How many entries?');
readln (n);
Mn := T/n;
```

```
SE :=Sqrt((S-((T*T)/n))/(n-1));
Writeln('For family   ',fid);
Writeln ('The mean is', Mn);
Writeln  ('And the SE is', SE);
Append(ToF);
   Writeln(ToF,' ');
   Writeln(ToF, 'The mean is', Mn);
   Writeln(ToF, 'And the SE is',SE);
  Close(ToF);
B1:=Mn-(1.07*SE);
B2:=Mn-(0.57*SE);
B3:=Mn-(0.18*SE);
B4:=Mn+(0.18*SE);
B5:=Mn+(0.57*SE);
B6:=Mn+(1.07*SE);
repeat
Writeln ('Enter age(A) or DOB(D) of individual?');
Readln (Agedob);
if  Agedob = 'D'
  then
  begin
  Writeln ('Year these individuals were last assessed? (YY.Y)');
  Readln (assyr);
  Append(ToF);
  Writeln(ToF, 'These individuals were last assessed in ', assyr);
  Close(ToF);
  repeat
  writeln('         Identifier?');
  readln(id);
  Writeln('Year of birth(XX.X)?');
  Readln (year);
  Writeln('For individual   ', id);
  Age:=assyr-year;
if age<=B1 then
  begin
   Writeln ('the liability class is 1');
   Append(ToF);
   Writeln(ToF, 'For individual ', id, ' the liability class is 1');
   Close(ToF);
  end
    else if  (B1<age) and (age<=B2) then
     begin
      Writeln ('the liability class is 2');
      Append(ToF);
      Writeln(ToF, 'For individual ', id, ' the liability class is 2');
      Close(ToF);
end
  else if  (B2<age) and (age<=B3) then
   begin
    Writeln ('the liability class is 3');
    Append(ToF);
    Writeln(ToF, 'For individual ', id, ' the liability class is 3');
    Close(ToF);
   end
    else if  (B3<age) and (age<=B4) then
     begin
      Writeln ('the liability class is 4');
```

```
 Append(ToF);
 Writeln(ToF, 'For individual ', id, ' the liability class is 4');
 Close(ToF);
end
 else if  (B4<age) and (age<=B5) then
  begin
   Writeln ('the liability class is 5');
   Append(ToF);
   Writeln(ToF, 'For individual ', id, ' the liability class is 5');
   Close(ToF);
  end
   else if  (B5<age) and (age<=B6) then
    begin
     Writeln ('the liability class is 6');
     Append(ToF);
     Writeln(ToF, 'For individual ', id, ' the liability class is 6');
     Close(ToF);
    end
     else if  B6<age then
      begin
       Writeln ('the liability class is 7');
       Append(ToF);
       Writeln(ToF, 'For individual ', id, ' the liability class is 7');
       Close(ToF);
      end;
 Writeln ('Another DOB?(Y/N)');
 Readln (Ans)
until (Ans) = ('N');
end
else
if Agedob ='A'
    then
 begin
 repeat
  Writeln('       Identifier?');
  Readln(id);
  Writeln ('Age at last assessment? (XX.X)');
  Readln (age);
  Writeln('For individual   ', id);
 if age<=B1 then
  begin
   Writeln ('the liability class is 1');
   Append(ToF);
   Writeln(ToF, 'For individual ', id, ' the liability class is 1');
   Close(ToF);
  end
    else if  (B1<age) and (age<=B2) then
 begin
  Writeln ('the liability class is 2');
  Append(ToF);
  Writeln(ToF, 'For individual ', id, ' the liability class is 2');
  Close(ToF);
 end
   else if  (B2<age) and (age<=B3) then
    begin
     begin
      Writeln ('the liability class is 3');
      Append(ToF);
```

```
 Writeln(ToF, 'For individual ', id, ' the liability class is 3');
 Close(ToF);
end
 else if  (B3<age) and (age<=B4) then
  begin
   Writeln ('the liability class is 4');
   Append(ToF);
   Writeln(ToF, 'For individual ', id, ' the liability class is 4');
   Close(ToF);
  end
   else if  (B4<age) and (age<=B5) then
    begin
     Writeln ('the liability class is 5');
   Append(ToF);
   Writeln(ToF, 'For individual ', id, ' the liability class is 5');
   Close(ToF);
  end
   else if  (B5<age) and (age<=B6) then
    begin
     Writeln ('the liability class is 6');
     Append(ToF);
     Writeln(ToF, 'For individual ', id, ' the liability class is 6');
     Close(ToF);
    end
     else if  B6<age then
      begin
       Writeln ('the liability class is 7');
       Append(ToF);
       Writeln(ToF, 'For individual ', id, ' the liability class is 7');
                Close(ToF);
               end;
  Writeln ('Another Age?(Y/N)');
  Readln (Ans)
  until (Ans) = ('N');
end;
Writeln ('Another Age or DOB? (Y/N)');
Readln (Ans)
until (Ans) = ('N');
Writeln('For family   ', fid);
Writeln ('The mean is', Mn);
Writeln  ('And the SE is', SE);
Writeln ('End');
end.
```