LINKAGE STUDIES OF LATTICE CORNEAL DYSTROPHY TYPE 1

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by

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DEPARTMENT OF HUMAN GENETICS

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LINKAGE STUDIES OF LATTICE CORNEAL DYSTROPHY TYPE 1

BY

SANDRA MARLES

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

ABSTRACT

Lattice corneal dystrophy type 1 (LCD1) is an autosomal dominant, fully penetrant condition characterized by a localized deposition of an, as yet, unidentified amyloid protein in the corneal stroma. LCD1 usually presents in late adolescence with corneal erosions. Slit-lamp examination of affected individuals reveals a network of lattice lines which have been proven histochemically to be amyloid deposits. Ongoing gradual progression of the amyloid deposition ultimately leads to blindness. Treatment, at this stage, is corneal transplantation, although amyloid deposition may recur in the transplanted cornea.

A large Belgian-Canadian kindred of 93 members, of whom 37 were affected with LCD1 and 15 were at-risk for developing LCD1 were studied. Kivlin *et al* (1984) reported a weakly positive lod score of 0.96 between *LCD1* and Haptoglobin *(HP)* on chromosome 16q at a recombination fraction (θ) of 0.17. Therefore, the purpose of this study was to extend the linkage analysis between *LCD1* and *HP,* to determine the combined lod score and to establish whether *LCD1* was within measurable recombination distance from several polymerase chain reaction (PCR)-based (AC)n marker loci also located on chromosome 16q.

Combining our data with those in previously published reports, the overall lod score between *LCD1* and *HP* was 2.46 at a recombination fraction (θ) of 0.20 which was not sufficient to demonstrate linkage to chromosome 16q. Linkage analysis between *LCD1* and a number of loci for (AC)n repeat markers, distal and proximal to *HP*, also did not confirm linkage to chromosome 16q in our family. Haplotype analysis revealed significant differences between related branches of the family. Based on this evidence, chromosome 16q had been excluded as the chromosomal location for the disease-causing gene for LCD1 in our family. This conclusion was supported by the recent publication by Stone *et al* (1994) which reported mapping the disease-causing genes for 3 corneal dystrophies, including LCD1, to chromosome 5q.

ACKNOWLEDGEMENTS

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I would also like to thank Margaret Gibb and Cheryl Taylor for their technical assistance.

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I would also like to thank Dr. M. Ekins and the members of this kindred who participated in this research project. Without their interest in scientific research, this study would not have been possible.

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1.000 INTRODUCTION

1.100 Anatomy of the cornea (Figure 1)

The cornea is a transparent and avascular tissue that is 1 mm thick and 11.5 mm in diameter (Vaughan and Asbury, 1977). It is the main refractive element of the eye with a refractive power equivalent to +43 diopters (Krachmer and Palay, 1991). There are five layers to the cornea which are, from anterior to posterior: (1) epithelium (continuous with the bulbar conjunctival epithelium), (2) Bowman membrane, (3) stroma, (4) Descemet membrane and (5) endothelium. The epithelium has 5-6 layers of cells. Bowman membrane is a clear acellular layer, which is a modified portion of the most superficial aspect of the stroma. The stromal layer accounts for 90% of the corneal thickness and contains nerve endings and fibroblasts which are oriented parallel to the corneal surface but are not intertwined. The endothelium is only a single layer of cells. The avascular cornea receives nutritional components and oxygen by diffusion from the limbal blood vessels. Some oxygen is derived from the atmosphere. The fifth cranial nerve supplies the cornea with the superficial corneal layers (including the stroma) containing approximately 70 sensory nerve fibers making the cornea extremely sensitive to pain. The transparency of the cornea

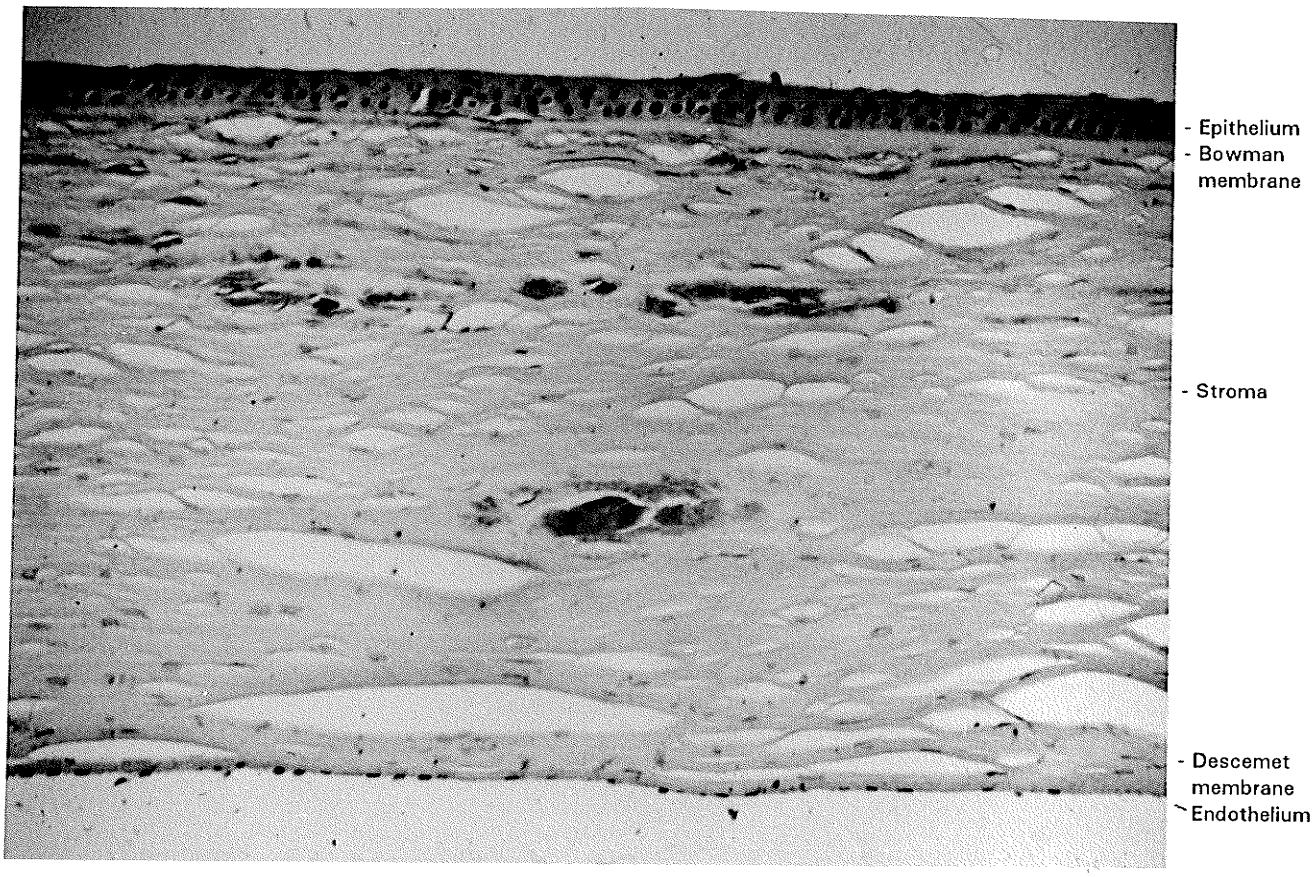


Figure 1. Photograph of cornea from affected patient demonstrating layers of the cornea. Note amyloid deposit in centre of stroma. (From Dr. M. Ekins)

is maintained by three elements: uniform structure, avascularity, and deturgescence. Deturgescence (relative dehydration of the corneal tissue) is maintained by an active Na^+-K^+ pump of the endothelium and epithelium and their anatomic integrity (Vaughan and Asbury, 1977).

1.200 Corneal dystrophies

Corneal dystrophies are a group of primary disorders of the cornea. They are usually unassociated with systemic disease or prior inflammation (Krachmer and Palay, 1991; Waring *et al*, 1978). Secondary inflammation and vascularization may mask the primary dystrophy making diagnosis more difficult. Corneal opacities that are manifestations of systemic diseases, such as mucopolysaccharidoses or other storage disorders, are not classified as dystrophies (Waring *et al*, 1978). Corneal dystrophies are usually bilateral, symmetric and slowly progressive, although unilateral and asymmetric cases have been described. Each corneal dystrophy initially involves a single layer of the cornea and, therefore, the usual classification is anatomic, based on the corneal layer involved. Transmission electron microscopic characteristics provide another way of classifying the corneal dystrophies (Waring *et al*, 1978). Corneal dystrophies may cause severe

visual impairment or blindness. Treatment is symptomatic for there is no cure (Ramsay, 1960). Corneal transplantation may be performed to improve vision, although the corneal dystrophy may recur. Corneal dystrophies, in general, are uncommon and the relative frequencies vary according to the geographic distribution of the affected families (Waring *et al*, 1978).

1.210 Lattice corneal dystrophy

In 1937, Bucklers reported that there were three distinct forms of familial corneal dystrophy: granular, macular and lattice. Granular corneal dystrophy, an autosomal dominantly inherited condition, is characterized by circumscribed discrete axial stromal opacifications separated by clear stroma. The clear areas accumulate eosinophilic deposits with time. The deposits were previously described as hyaline but are now considered to be associated with increased phospholipid, as shown by biochemical analysis and staining with Luxol-fast blue. Immunohistochemical stains revealed reactivity with antibodies against microfibrillar protein at the edges of the deposits (Rodrigues and Krachmer, 1988). Macular corneal dystrophy (MCD) is an autosomal recessively inherited disorder characterized by the gradual accumulation of irregular corneal opacities with indistinct borders. The opacities are central and superficial early in the disease,

then increase in number and spread into the peripheral and deeper cornea; diffuse corneal cloudiness between opacities reaches the limbus. The pattern of the opacities is the most reliable feature that distinguishes this condition from granular corneal dystrophy. Corneal culture studies revealed a defect in the synthesis of keratan sulphate, a proteoglycan, characterized by the addition of large oligosaccharide side chains. The lectin binding patterns of normal corneas and MCD corneas are different. Activity of alpha-galactosidase has been reported to be significantly lower in MCD corneas compared to normal corneas (Rodrigues and Krachmer, 1988).

Biber (1890) was the first to recognize lattice corneal dystrophy which he named "gittrige keratitis". Haab (1899) and Dimmer (1899) noted the hereditary nature of the dystrophy. Fuchs (1925) followed Dimmer's cases, added new cases and described the clinical features. In 1967, Klintworth identified lattice corneal dystrophies as inherited forms of amyloidosis limited to the cornea.

Klintworth (1967) designated this classic inherited form of Biber-Haab-Dimmer corneal dystrophy as lattice corneal dystrophy type 1 (LCD1) (see below, Section 1.220). Lattice corneal dystrophy, Meretoja form, was designated type 2 (LCD2) and lattice corneal dystrophy, Japanese form, has been designated type 3 (LCD3) (Klintworth, 1967). LCD2 was first

described in Finland (Meretoja, 1969) and presents with visual symptoms including decreased vision, corneal erosions and iritis. The condition progresses to blepharochalasis (extra fold of upper eyelid skin), cutis laxa, upper facial paralysis, minor peripheral neuropathy and systemic amyloidosis. This condition is inherited as an autosomal dominant trait (Meretoja, 1969; Rodrigues and Krachmer, 1988). LCD3 was first described in Japan and presents with decreased visual acuity later in life, with no history of antecedent corneal erosions or systemic amyloidosis. The amyloid deposits in the cornea are different from those in LCD1 or LCD2. Inheritance is considered to be autosomal recessive (Hida *et al*, 1987).

1.220 Lattice corneal dystrophy type 1

LCD1 usually presents in later adolescence although there have been reports of symptomatic 2 year olds as well as individuals presenting in the fourth or fifth decade (Ramsay, 1957; Waring *et al*, 1978). Some patients remain asymptomatic. The first symptom is recurrent painful epithelial corneal erosions leading to photophobia, excessive lacrimation and redness lasting 3-10 days. Treatment at this stage is symptomatic utilizing artificial tears, lubricating ointment or patching (Ramsay, 1957; Ramsay, 1960; Tsubota *et al*, 1987;

Rodrigues and Krachmer, 1988). Erosions may precede the typical findings on slit-lamp examination and early in the disease, subepithelial punctate opacities without the typical lattice lines may be found. Slit-lamp examination of affected individuals characteristically reveals a delicate interdigitating network of coalescing dots, dashes and filaments which are refractile on retroillumination and have been confirmed histologically to be amyloid deposits. As amyloid is deposited, the filaments thicken, the dots coalesce and the overlying corneal epithelium becomes irregular causing decreased vision and recurrent corneal erosions. The early central indistinct subepithelial round opacity becomes more dense over time, due to recurrent erosions or ongoing amyloid deposition. In later life, there are fewer corneal erosions, which are less painful as corneal sensation decreases. The diffuse corneal opacity becomes denser and extends to the periphery obscuring the amyloid filaments. Occasional secondary corneal vascularization develops due to the erosion-induced inflammation. Significant visual impairment may be present by the sixth or seventh decade of life. Treatment of blindness is by corneal transplantation. Unfortunately, the amyloid deposition may recur in the transplanted cornea via infiltration from the rim of the original cornea which has been left as an anchor for the new cornea. This process may take a variable number of years, leading to the need for retransplantation (Ramsay, 1957; Waring *et al*, 1978; Tsubota

et al, 1987).

This condition is occasionally mistaken for recurrent primary corneal erosions, herpes simplex, superficial punctate keratitis, and keratoconjunctivitis eczematosa in isolated cases or atypical cases on preliminary examination (Ramsay, 1957; Tsubota *et al*, 1987).

LCD1 is not associated with any systemic signs or symptoms. LCD1 is not part of primary or secondary systemic amyloidosis (Ramsay, 1960; Waring *et al*, 1978; Krachmer and Palay, 1991).

1.230 Pathology of LCD1

Refractile lines, white dots and a central diffuse opacity characterize LCD1. These findings are most often found in the central superficial cornea, leaving the peripheral cornea clear. White dots are occasionally found in the pre-Descemet stroma. The lattice lines are the hallmark of LCD1 and range from small curved flecks to a dense network of irregular ropy cords. The smaller lines may be seen as a random net configuration early in the disease and slowly progress to larger more radially oriented cords that branch toward the central cornea and run obliquely from the

superficial to mid-stroma. Under direct illumination, the lines appear to have irregular margins or a beaded appearance due to the accumulation of small dots within them. The lattice lines fluoresce under ultraviolet light with a wavelength of approximately 365 nm. The intervening stroma is clear early in the disease but gradually becomes more hazy. The white dots appear fluffy and slightly stellate on direct illumination but are refractile on retroillumination. If the dots are the only finding on examination, LCD1 may be mistaken for granular corneal dystrophy. Initially the amyloid deposit of the central diffuse opacity is located in the stroma which also contains collagen fibrils and fibroblasts. In later stages of LCD1, the central white opacity becomes denser, obscuring the lines and dots and extending into deeper layers of the stroma (Ramsay, 1960; Klintworth, 1971; Waring *et al*, 1978).

The hallmark lesion on light microscopy is a fusiform deposit of amyloid (Section 1.300) that displaces collagen lamellae. The number of stromal fibroblasts is decreased; some appear metabolically active with increased rough endoplasmic reticulum and golgi apparatus while others appear degenerated. The deposits are most concentrated anteriorly but may appear in the deeper stroma. These deposits correspond to the lattice lines and dots seen clinically (Waring *et al*, 1978).

1.300 Amyloid

In 1842, Rokitansky reported the "lardaceous liver" found at autopsy in chronically ill patients. Virchow showed, in 1854, that the tissues gave an identifiable colour reaction with iodine and sulfuric acid and he named the deposits amyloid. In 1859, Friedreich and Kekule showed that amyloid deposits were mainly composed of protein.

Amyloid deposits appear amorphous and eosinophilic when stained with hematoxylin and eosin and viewed under light microscopy (Benson and Wallace, 1989). Amyloid deposits are never found in normal tissues (Varga and Wohlgethan, 1988). The deposits are extracellular and therefore displace normal tissue structures causing disruption of cellular function and ultimately cell death. Methylviolet and crystalviolet give metachromatic reactions with amyloid, but Congo red has become the standard for identification of amyloid on histologic sections. Amyloid deposits take up the Congo red dye and demonstrate a characteristic green color when viewed under a polarizing microscope (Benson and Wallace, 1989). The amyloid deposit has a very ordered structure which accounts for the crystalline property of birefringence (Benson, 1991). Collagen is also birefringent in histologic sections but does not bind Congo red and therefore does not have green

birefringence. The fluorescent bioflavin dyes also confirm the presence of amyloid but are not routinely used (Benson and Wallace, 1989). These amyloid deposits may be associated with autofluorescent material that stains with elastin stains but resists digestion with elastase (Pe'er *et al*, 1988).

In 1959, Cohen, Calkins and Spiro reported that amyloid, which was felt to be structureless, was actually fibrillar in nature (Benson and Wallace, 1989). At high resolution electron microscopy, amyloid deposits contain nonbranching fibrils with a diameter of 70 to 100 Angstrom (Å) and indeterminate length. These fibrils appear to consist of two to several parallel subunit filaments. These subunits, which demonstrate helical twisting and measure 25-35 Å in width, may give a beaded appearance to the fibrils (Benson and Wallace, 1989). The substructure of the amyloid fibril has been studied by X-ray diffraction and forms the basis for the beta-pleated sheet model of amyloid fibrils. The beta-pleated sheet structure is extremely stable and imparts resistance to proteolysis and solubilization under physiological conditions (Varga and Wohlgethan, 1988). Although analytical protein chemistry has demonstrated several distinct forms of amyloid, all forms share the same physical properties:

1. polarization birefringence after Congo red stain
2. nonbranching fibrils with a diameter of 70-100 Å
3. twisted beta-pleated sheet (Varga and Wohlgethan,

1988; Benson and Wallace, 1989; Benson, 1991)

Amyloidogenesis, in general, is characterized by proteolysis of a larger protein precursor molecule with subsequent production of low-molecular weight fragments that polymerize and assume a beta-pleated sheet confirmation. These fragments are deposited in an extracellular location. The amyloidogenic proteins may result from overproduction of either intact or aberrant proteins, reduced degradation or excretion of proteins or variant proteins due to underlying genetic mutations (Benson and Wallace, 1989; Stone, 1990).

Chemical characterization of the fibril material was impossible until 1971 when Glenner *et al* were able to solubilize the amyloid fibrils from the tissues of patients with primary amyloidosis and isolate the major subunit proteins. Amino acid sequencing revealed that the subunits were homologous to the variable segment of immunoglobulin light chains (Glenner *et al*, 1971). Amyloid fibrils from patients with secondary amyloidosis were found to be composed of protein ultimately named amyloid A protein (AA). AA was discovered to be derived from an acute phase serum amyloid A (SAA) protein synthesized in the liver (Benson and Wallace, 1989). Amyloid material from patients with hereditary amyloidosis was reported by Costa *et al* in 1978 to be composed of a subunit protein reacting with antiserum to plasma

prealbumin. This observation was confirmed at the structural level by Benson in 1981. Several variants of this plasma protein are associated with hereditary amyloidosis (Benson and Wallace, 1989).

Corneal amyloid deposits demonstrate amyloid P component, a type of amyloid seen in all forms of secondary and some forms of localized amyloidosis. Protein AA has been seen in the amyloid deposit of secondary systemic amyloidosis as well as in patients with familial Mediterranean fever (Mondino *et al*, 1980). Other investigators have been unable to confirm the presence of protein AA in the amyloid deposition of LCD1 (Gorevic *et al*, 1984).

The origin of the amyloid deposit in LCD1 is unknown; there are several precursor proteins possibly involved in the genesis of this condition. Fibroblasts, matrix precursors and collagen predominate in the central, avascular portion of the cornea where the earliest amyloid deposits are located. At least three hypotheses have been reported to account for the pathogenesis of amyloid deposition in LCD1. The first hypothesis suggests that corneal fibroblasts, which are known to synthesize and secrete collagen precursors, also secrete amyloid fibrils. The second suggests that amyloid deposits represent the degradation products of collagen. The corneal collagen is always morphologically intact, even when the

amyloid deposits blend into the contiguous collagen so that a degradative product of collagen has never been established as the source of the amyloid deposit. The third hypothesis suggests that the amyloid is produced elsewhere and brought to the cornea via the limbal blood vessels. The earliest lattice lesions are found in the central portion of the avascular cornea which may imply that the amyloid is not produced elsewhere and transported to the cornea for deposition (Klintworth, 1971; Waring *et al*, 1978). Recurrence of the amyloid deposition after corneal transplantation may imply that the precursor protein is derived from the circulation. However, this recurrence begins in the periphery of the transplanted cornea which is surrounded by a rim of the diseased cornea from which fibroblasts may secrete amyloid into the transplanted corneal periphery. The immunohistochemical characterization of the recurring amyloid using monospecific antisera has not been successful (our data, not shown).

Lectin receptors consisting of extracellular stromal glycoconjugates containing mannose/glucose, sialic acid/N-acetylglycosamine residues, terminal galactose residues and other oligosaccharides have been reported by others (Bishop *et al*, 1991; Freeman, 1992). A panel of lectins, which are ubiquitous, naturally occurring proteins and glycoproteins largely derived from plants, were used to identify these

lectin receptors. The relationship between these receptors, the amyloid deposits and the cellular mechanisms underlying LCD1 is unknown (Bishop *et al*, 1991; Freeman, 1992).

1.310 Hereditary amyloidoses

Hereditary amyloidoses are a heterogeneous group of disorders that range from localized deposition of amyloid at specific sites to generalized systemic involvement. It is hypothesized that factors related to the amino acid sequence of the amyloid precursor protein or the biochemical nature of the tissue in which the amyloid is deposited may determine the affinity to certain organs (Varga and Wohlgethan, 1988).

There is no widely accepted classification for the hereditary amyloidosis syndromes; but one system divides them into systemic and localized forms (modified from Varga and Wohlgethan, 1988):

I. Systemic

A. Neuropathy

1. Familial Amyloid neuropathy type I (Portuguese)
2. Familial Amyloid neuropathy type II (Indiana/Swiss or Maryland)
3. Familial Amyloid neuropathy type III

(Iowa)

4. Familial Amyloid neuropathy type IV (Finnish)

B. Nephropathy

1. Familial Mediterranean fever
2. Nephropathy and urticaria, deafness, limb pain

C. Cardiopathy

1. Progressive heart failure
2. Hereditary atrial standstill
3. Appalachian neuropathy

II. Localized

The neuropathic forms of hereditary amyloidosis are also known as "familial amyloidotic polyneuropathy" (FAP) and all are inherited in an autosomal dominant pattern. FAP type I was assigned to the Portuguese, Japanese and some Swedish families who usually presented with lower extremity neuropathy. FAP type II has been described in the Indiana/Swiss family and Maryland families in whom the usual presenting feature was carpal tunnel syndrome. In an Iowa kindred, FAP type III was associated with nephrotic syndrome and and peptic ulcer disease. FAP type IV was initially described in Finnish families who had a distinctive cranial neuropathy (Benson and Wallace, 1989).

FAP types I-II are associated with variants of plasma prealbumin (transthyretin). Plasma prealbumin is synthesized in the liver constitutively as a single polypeptide chain of 127 amino acids and circulates as a tetramer composed of four identical monomers. Each monomer has eight beta pleated sheet configurations arranged in an antiparallel form in two planes, a structure which may predispose the protein to amyloid fibril formation. Two binding sites for thyroxine are located in the central "channel". Prealbumin also binds retinol binding protein which likely binds to the outside of the tetramer. Prealbumin is a negative acute phase protein because its concentration decreases during acute or chronic inflammation and in many patients with prealbumin amyloidosis (Benson and Wallace, 1989). The gene for prealbumin, which has been mapped to chromosome 18q11.1-q12.1, is 6.9 kb in length and contains four exons and three introns (Sasaki *et al*, 1985). All of the mutations identified in variant prealbumin proteins are associated with amyloid fibril formation and may alter the surface topography of the molecule allowing aggregation and fibril formation (Benson and Wallace, 1989).

Another classification system for the hereditary prealbumin amyloidoses is based on the biochemistry of the prealbumin variants (Benson and Wallace, 1989). One type of variant prealbumin is characterized by a substitution of methionine for valine at position 30 of the prealbumin

molecule. Most kindreds (Portuguese, Japanese, Swedish, Greek) have been clinically classified as FAP type I. The lower extremity neuropathy is usually accompanied by autonomic and gastrointestinal symptoms; varying degrees of renal and cardiac involvement may be present. Ophthalmologic symptoms of amyloid deposits in the vitreous or "scalloped pupil" deformity have also been described. Although mental function is usually preserved, amyloid deposits may be found in the central nervous system and leptomeninges (Benson *et al*, 1989). In 1985, a case report of an individual with lower extremity neuropathy, diarrhea, impotence and amyloid vitreous deposits was published (Gafni *et al*, 1985). An autopsy performed on his father, who died from similar symptoms, demonstrated amyloid in all major organs, particularly thyroid, kidney, spleen and nerves.

A different prealbumin mutation causes the phenotype of FAP II in the Indiana/Swiss kindred and Maryland kindred. The Maryland kindred does not have vitreous eye deposits. There is a histidine substitution at position 58 of prealbumin in the Maryland kindred.

The Indiana/Swiss kindred has symptoms of carpal tunnel syndrome followed by generalized infiltrative peripheral neuropathy and vitreous amyloid deposits. The Indiana/Swiss family has nearly 100% incidence of the vitreous opacities.

Bowel involvement and sexual impotence in males has also been reported. Cardiomyopathy is the usual cause of death. Affected and presymptomatic carriers of the mutant gene in the Indiana/Swiss kindred have reduced plasma retinol binding protein. Such carriers can usually be identified on this basis, with one study demonstrating 100% concordance. A serine for isoleucine substitution at position 84 of prealbumin could account for the observed phenotypic effects. The area of the molecule around position 84 is involved in retinol binding (Benson and Wallace, 1989; Benson, 1991).

Other substitution mutations causing FAP II described in the prealbumin molecule include: tyrosine for serine at position 77, methionine for leucine at position 111, valine to isoleucine at position 122 (Benson and Wallace, 1989).

FAP type IV was originally described by Meretoja in 1969 and is synonymous with LCD2. Patients may present with amyloid deposition in the cornea which is different from that of LCD1. The lattice lesions are more coarse and extend from the limbus toward the central cornea. These lesions, which may have branches and terminal thickening, involve the mid stroma at the periphery and the more superficial stroma centrally. Edema and opacities may be present in the anterior and mid stroma. Few amyloid deposits occur in the central cornea, in contrast to LCD1 which has deposits throughout the

stroma and a central opacity. Over time, the skin of the forehead and back thickens with typical amyloid deposition and may become dry and pruritic. Blepharochalasis and protruberant lips may result. Patients may develop facial paralysis (mask-like facies) secondary to cranial neuropathy. Nephropathy due to renal amyloid deposition may cause renal failure and, ultimately, death. Other more variable features include polycythemia vera and ventricular hypertrophy. At autopsy, amyloid deposition is widespread but it is very pronounced in the intima and media of arteries and capillaries, glomeruli, perineurium and endoneurium of peripheral nerves and the myocardium. The first reported patients were Finnish but patients of different ethnic origins have since been described (Purcell,Jr *et al*, 1983; Gorevic *et al*, 1991).

The amyloid protein identified in FAP type IV is a monomer with a molecular weight of 9000. It has been identified as an internal degradation product of 7-12 kD of a variant form of the human protein, gelsolin. Gelsolin, which functions as an actin-modulating protein, is synthesized primarily in the muscle and exists in two forms. The cytoplasmic form has a molecular weight of 90,000, and the plasma circulating form has a molecular weight of 93,000. The cytoplasmic and secreted forms are derived from alternative transcription initiation sites and message processing of a single gene mapped to chromosome 9q32-q34. An aspartic acid

to asparagine substitution at position 187 has been confirmed in the studies of affected Finnish-American families (Gorevic *et al*, 1991; Maury *et al*, 1990).

1.400 Gene Mapping

1.410 Candidate Gene Approach

There are at least two approaches, 1) the candidate gene approach and 2) the positional cloning approach which are used to map a disease-causing gene and identify the underlying genetic abnormality. The candidate gene approach (also known as functional cloning) utilizes pre-existing knowledge about the biologic or biochemical basis for the disorder. A candidate gene for a specific condition is one whose protein product has properties which imply that it may be the disease gene in question and may be identified in two ways. If the amino acid sequence of the wild type protein product is known, eg. phenylalanine hydroxylase, the relevant cDNA sequence may be isolated from the appropriate cDNA library expressing this transcript using either antibodies or degenerate oligonucleotides based on the amino acid sequence. Alternatively, when a genetic disorder has been mapped to a particular chromosome, eg. by linkage analysis, a review of the genes known to reside within the physical interval of

interest may identify a gene whose gene product has properties of the mapped disease-causing gene. Such a gene then becomes a candidate gene and may be examined for mutations. There are several examples of human disease genes cloned by the candidate gene approach: rhodopsin and peripherin genes in retinitis pigmentosa, cardiac myosin heavy chain in familial hypertrophic cardiomyopathy, ryanodine receptor in malignant hyperthermia and fibrillin in Marfan syndrome (Collins, 1992; Nora, Fraser *et al*, 1994). Occasionally, related or similar diseases without a known biochemical basis are examined for novel mutations in those genes which could cause the disease in question.

1.420 Positional Cloning Approach

In many single gene disorders, information about the biochemical nature of the genetic defect is not available. In the first step of positional cloning, the gene responsible for the phenotype is mapped to its chromosomal location. After the correct gene has been cloned, the predicted function of the gene product is determined. The positional cloning approach is initiated by collecting appropriate pedigrees in which the disease gene is segregating. Families are studied with a variety of polymorphic markers until evidence of linkage is obtained with at least one or two markers (linkage analysis). Additional fine mapping using additional probes

from that region is undertaken to reduce the interval of interest as much as possible, usually to a region of approximately one million base pairs. Fine mapping is usually limited by the number and position of recombinants in the area of the gene. Once the target interval has been reduced as much as possible, the closest distal and proximal markers are utilized as boundaries to screen YAC (yeast artificial chromosome) libraries or contigs (series of overlapping DNA fragments) of the region of interest to isolate clones for initiating a bidirectional walk. Overlapping clones are isolated to generate a contig in the region of interest. DNA isolated from the clones may then be used as hybridization probes for long range mapping and the identification of additional polymorphic markers. Polymorphic markers (such as microsatellite markers described in section 1.430), isolated from within the boundaries of the target region, can also be used to localize the disease-causing gene more specifically by the detection of linkage disequilibrium or the identification of key recombinants. This target region is then searched for evidence of transcribed sequences, by identifying regions containing a high density of hypomethylated CpG residues associated with the 5′ ends of genes, or by exon trapping, in which an exon is identified by its ability to remain as a mature mRNA after splicing (Hamaguchi *et al*, 1992). Sequences from such target regions are used to screen relevant cDNA libraries. cDNAs which map back to the interval of interest

are then screened for conservation across species by hybridization to DNA of a number of species (zoo blotting). These cDNAs could also be used to screen Northern blots from relevant tissue-specific expression and be sequenced for disease-causing mutations in families of interest. In addition, cosmids isolated from YACs can be sequenced directly (shotgun sequencing) to search for open reading frames using computer programs, such as GRAIL (Lange *et al*, 1988).

Occasionally a visible chromosomal alteration in the karyotype of affected family members may direct the search to a particular chromosomal location. Gross rearrangements in the karyotype, if only present in a small subset of family members, accelerate the process of gene identification. In addition, rearrangements detectable at the Southern blot level are helpful. Using an approach as described above, the genetic defect for cystic fibrosis was identified without the discovery of any genetic rearrangements in affected individuals (Rommens *et al*, 1989; Riordan *et al*, 1989; Kerem *et al*, 1989). A positional cloning approach has been used to identify the genetic defect in such diseases as Duchenne muscular dystrophy (Monaco *et al*, 1986), retinoblastoma (Friend *et al*, 1986), and Wilms tumor (Rose *et al*, 1990). In each of these cases, mapping of the gene was followed by cloning of the gene (Wicking and Williamson, 1991; Collins, 1992; Nora and Fraser, 1994). This approach was also

initiated in our study of LCD1.

1.430 PCR Microsatellite Markers

A large number of PCR (polymerase chain reaction) based microsatellite markers have been identified and been used in human genome analysis. Microsatellites, in general, consisting of approximately 10-50 copies of motifs from 1-6 bp, can occur in perfect tandem repeats, as imperfect or interrupted repeats or together with another repeat type. All eukaryotic DNAs examined, except for yeast, have recurrent, random microsatellites. In humans, 76% of repeat types are A, AC, AAAN, AAN, or AG, in decreasing order of abundance. The repetitive (AC)n sequences can occur, on average, every 30-60 kb and are distributed equally in 5′- and 3′-untranslated regions and in introns (Hearne *et al*, 1992). These repeats often exhibit size polymorphisms which are readily detected by PCR-amplifying small fragments of DNA containing the repeat and then resolving the products of the amplification electrophoretically (Weber and May, 1989; Litt and Luty, 1989). The mutation rate of microsatellites is approximately $5x10^{-4}$ to 10^{-5} which allows for their use in linkage analysis with disease mutations and in forensic applications (Hearne *et al*, 1992). Microsatellites of PIC (polymorphic information content) > 0.7 are the most helpful

in linkage analysis because the parents are more likely to be heterozygous at the loci of interest and segregation of the alleles would be observed and scored in the offspring. Highly polymorphic microsatellites allow more families to be fully informative (Hearne *et al*, 1992). Dinucleotide alleles may demonstrate additional "stutter" bands after electrophoretic analysis, which may represent truncated products resulting from mispairing of the template and the newly synthesized strand during elongation across a dinucleotide tract or from a combination of mispairing and a terminal transferase-like activity of the *Taq* polymerase. Several techniques, such as reducing the number of PCR reaction cycles or fluorescently labelling the PCR primers, have been utilized to improve the resolution of the gels (Hearne *et al*, 1992).

1.440 Linkage analysis

Genetic linkage is defined as the tendency for two or more non-allelic genes or DNA markers to be inherited together more often than is expected by independent assortment, because they are located close to each other on the same chromosome (Risch, 1992). When the loci are farther apart, crossing over between homologous chromosomes at meiosis creates new combinations of alleles. The frequency of recombination,

defined as the recombination fraction, θ, can vary from 0 to 0.5 and increases with the distance between loci. The amount of recombination that occurs between loci is an indirect measure of the distance in centiMorgans (cM) between the two loci. If the loci are far apart on the same chromosome, or on separate chromosomes, the probability of recombinant and parental chromosomes is equal (θ= 0.5), indicating absence of linkage or independent assortment. Recombination events along a chromosome that has multiple, closely spaced markers can be used to localize a disease gene to within a region of DNA (Risch, 1992), which may also lead to the construction of a genetic map (Lathrop *et al*, 1984).

The lod score method for linkage analysis, originally described by Morton (1955), was used in this study. In this approach, an odds ratio, L, is determined by the ratio of the probability of observing the distributional pattern of the two traits in a set of families under the hypothesis of linkage, at a particular θ (the alternative hypothesis), to the same probability under the hypothesis of no linkage, θ=0.5 (the null hypothesis). The decimal logarithm of L, the lod score, is reported at several standard values of θ, and the maximum lod score is obtained graphically. If meioses are phase-known, the number of recombinants (R) and nonrecombinant (NR) children can be determined; if phase-known, the number of children of each type is represented (z score). The lod

scores for the same loci analyzed in several kindreds can be added together without bias, because the lod score analyses are based on a sequential test. Such a sequential test allows continued accumulation of data until the required levels of significance are reached to prove or disprove the hypothesis. A maximum lod score of 3.0 (odds ratio of 1000:1) is required to establish linkage and implies that the posterior probability of linkage is 95% and the posterior probability of no linkage or false positive probability is 5%. Testing multiple markers across the genome before a lod score of 3.0 is obtained increases the posterior probability of linkage, although the increase is minimal until many markers have been tested (Risch, 1992). When the total lod score is between -2.0 and 3.0, the results are equivocal and more data are required.

Informative matings demonstrate clear segregation of the alleles at two loci, which occurs when at least one parent in the nuclear family is heterozygous at the two loci of interest. When clear segregation cannot be seen, the mating is noninformative. Although phenotypes of grandparents, grandchildren or partially examined individuals may not be used directly in the calculation of lod scores, they may aid in defining the genotypes of parents in the nuclear family of interest. Analysis of grandparents may be required to establish the phase of doubly heterozygous parents. Phase

known families are more informative than phase unknown families (Maynard-Smith *et al*, 1961).

Any disorder to be studied by standard linkage analysis should be single-gene and should be inherited in a dominant or recessive (autosomal or X-linked) pattern (Risch, 1992). LCD1 conforms to an autosomal dominant pattern with an incidence and prevalence much higher in the kindred than in the general population. Vertical transmission through several generations is demonstrated and offspring of affected individuals are each at 50% risk of developing symptoms of LCD1, regardless of sex of the affected parent or at-risk offspring. The gene causing LCD1 is assumed to be 100% penetrant in individuals aged 25 years or greater in this kindred, and there is no evidence in the pedigree to suspect reduced penetrance, such as an apparently unaffected parent having an affected child.

1.450 Linkage analysis for LCD1

The linkage analysis performed by Kivlin *et al* (1984), combined with a previous study by Hammerstein *et al* (1975), gave a weakly positive lod score with haptoglobin (*HP*) of 0.96 at a recombination fraction of 0.17. These results, which were based on a total of 54 individuals of whom 28 were affected, also excluded close linkage with 14 blood group loci.

1.500 Aims

In the present study, a large Belgian-Canadian kindred was studied with the following specific aims:

1. to extend the linkage analysis between *LCD1* and *HP* performed by Kivlin *et al* (1984) and Hammerstein *et al* (1975) and to calculate the overall combined lod scores from all data sources available
2. to determine if *LCD1* is within measurable recombination distance from one or more RFLP marker loci or one or more PCR based (AC)n marker loci on the long arm of chromosome 16.
3. to attempt to identify the chromosomal assignment of *LCD1* from the analysis of this large kindred.

2.000 MATERIALS AND METHODS

2.100 The Pedigree

The large Belgian-Canadian kindred with LCD1 that was the focus of this study, has been published in part by Ramsay

(1960) and is presented in Figure 2. Members of this kindred are now followed regularly by one ophthalmologist, Dr. Marilyn Ekins, a Winnipeg corneal specialist. When an individual at 50% risk of inheriting the LCD1 disease gene has a normal slit-lamp examination after the age of 25 years (in this family), the individual is considered to be unaffected (Dr. Ekins, personal communication). In such a case, it is assumed that their affected parent has transmitted the normal *LCD1* allele to that individual. Currently, Dr. Ekins follows 41 affected individuals and 40 at-risk individuals from this pedigree. An additional 20 at-risk individuals have not yet been assessed. Since 1981, 20 patients have received 25 corneal grafts and several individuals await transplantation.

2.200 Sample collection

After informed consent, heparinized blood was drawn for haptoglobin typing, DNA extraction, and blood group serology on 93 individuals in this large multigeneration kindred. There were 41 samples from unaffected family members (older than 25 years of age) or their unrelated spouses, 37 samples from affected family members and 15 samples from at risk individuals. Plasma samples were frozen at -30°C within several hours of collection.

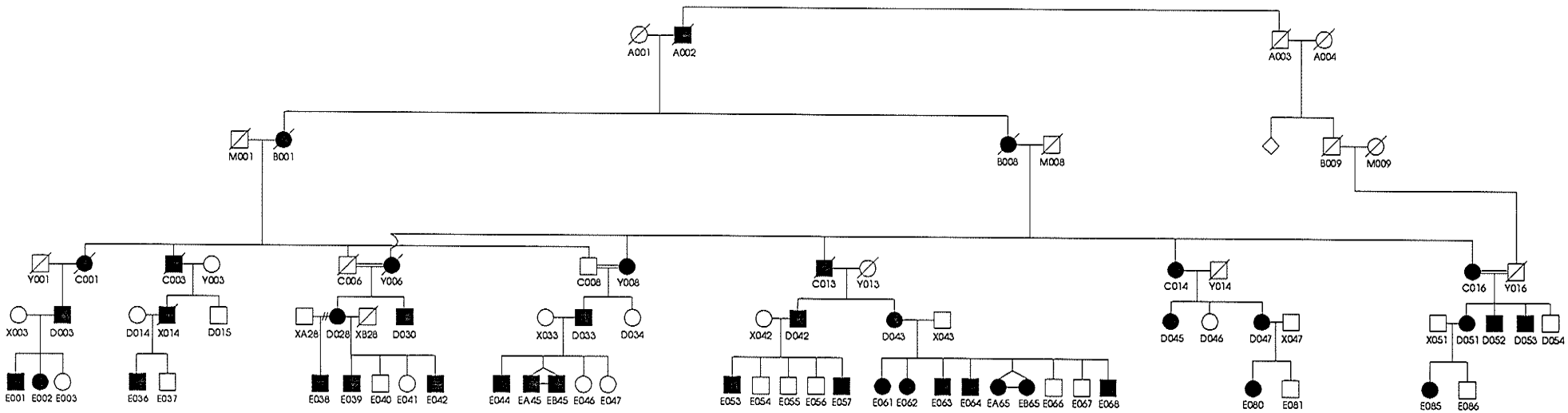


Figure 2. Pedigree of the LCD1 kindred under study. Solid symbols represent affected individuals; open symbols represent unaffected

2.300 Molecular Methods

Individuals in the Rh Laboratory (Winnipeg, Manitoba) separated each whole blood sample into its components which were then distributed to the participating laboratories. Red cells were tested for blood group polymorphisms by Dr. Teresa Zelinski and associates (Rh Laboratory). Linkage analysis between the blood group loci and *LCD1* were then determined using the Mark III computer program (Cote, 1975). The buffy coat was saved for DNA extraction from the lymphocytes using the standard protocol from the Molecular Diagnostic Laboratory of the Alberta Children's Hospital (Calgary, Alberta). An equal volume of buffered phenol (approximately 4 ml) was added to the buffy coat and mixed well, manually, for ten minutes. The mixture was then centrifuged at 2000 rpm for 5 minutes (International Clinical Centrifuge) to separate the organic and aqueous phases. The same phenol extraction was repeated again with the aqueous layer. The aqueous layer was extracted in the same way with chloroform:isoamyl alcohol (24:1) and then centifuged again under the same conditions. The upper aqueous layer was saved and 1/10 volume of 4M ammonium acetate was added and well mixed. An equal volume (aqueous layer and ammonium acetate) of absolute isopropanol was added to precipitate high molecular weight DNA. This DNA was washed with 70% ethanol and was allowed to air-dry. The DNA was redissolved in 1 ml of low TE buffer (TRIS 10 mM, EDTA 1 mM,

pH 8.0). The dissolved DNA was quantitated spectrophotometrically at 260 nm and the purity estimated by measuring the ratio of the absorbance at 260 nm to the absorbance at 280 nm. Pure DNA had a ratio of 1.8. A ratio of < 1.8 indicated contamination with protein or phenol, while ratios of > 1.8 indicated contamination with RNA. After the DNA samples were determined to have been purified, they were stored at 4°C.

Haptoglobin phenotypes were determined following starch gel electrophoresis of plasma saturated with a red cell lysate using the buffer system of Poulik (1957) and staining with guaiacol. Gels were prepared and run by Dr. Phyllis McAlpine (Department of Human Genetics, Winnipeg, Manitoba) and analyzed by myself.

The large scale plasmid preparation for isolation of probes for Southern hybridization was based on the protocol in Sambrook, Fritsch and Maniatis (1989). Two plasmid preparations were set up for the isolation of two probes: ACH207 (American Type Tissue Collection, Rockville, Md) which defines the locus *D16S4* and Hp2α (American Type Tissue Collection, Rockville, Md) which defines the locus haptoglobin *(HP)*. Two cultures each containing 500 ml of LB broth and bacteria carrying the appropriate vector containing the probe insert were set up. The appropriate antibiotic required to

complement the resistance conferred by the plasmid was also added. The cultures were incubated overnight at 37°C with vigorous shaking. Aliquots of 100 ml were removed from each culture and were incubated at 37°C with vigorous shaking for about 2.5 hours, until the OD_{600} was 0.6. Each aliquot was added to 500 ml of LB broth containing the appropriate antobiotic and was shaken at 37°C for 2.5 hours and then chloramphenicol was added to each flask in a final concentration of 200 μg/ml. The cultures were allowed to incubate overnight under the same conditions. The overnight cultures were centrifuged at 5000 rpm and 4°C for 10 minutes (Beckman J2-21, rotor JA-10), washed with 100 ml of cold low TE buffer (TRIS 10 mM, EDTA 1 mM, pH 8.0) and then recentrifuged again under the same conditions. The pellet was suspended in 12 ml of glucose buffer (50 mM glucose, 25 mM TRIS-HCl pH 8.0, 10 mM EDTA) which contained 40 mg of lysozyme and then incubated at room temperature for 10 minutes. The cells were then lysed in 27.6 ml of 1% SDS in 0.2N NaOH and swirled manually on ice for 15 minutes. Fourteen ml of potassium acetate (3M potassium, 5M acetate, pH4.8) were added to the mixture to precipitate the high-molecular weight RNA, protein and chromosomal DNA. The contents were left on ice for 30 minutes and then centrifuged for 20 minutes at 10,000 rpm at 4°C (Sorvall RC2-B, Rotor SS34). The supernatant was removed and divided into 4 equal amounts, placed in smaller containers and extracted with equal volumes of

phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was separated by centrifugation for 5 minutes at room temperature and at 6000 rpm (Beckman J2-21, rotor JA-10). Cold isopropanol (0.6 volumes) was then added and the solution was allowed to precipitate overnight at -20°C. The precipitated DNA was centrifuged at 10,000 rpm for 20 minutes at 4°C to form a pellet (Sorvall RC2-B, rotor SS34). The pellet was air-dried and then resuspended in low TE buffer (TRIS 10 mM, EDTA 1 mM, pH 8.0). The plasmid was purified using the Qiagen kit and their protocol without modification (Qiagen Inc., Chatsworth, CA).

A digest reaction was set up to confirm the presence of the insert of interest in the plasmids. The 10 μl reaction contained 1 μl of the appropriate restriction endonuclease, 1 μl of the appropriate buffer, 2 μl of plasmid and 6 μl of nanopure water. The reaction was incubated at the appropriate temperature and then boosted with 1 μl of the enzyme. The reaction was left overnight under the appropriate conditions and then electrophoresed on an agarose gel the following day. An agarose gel was made with a 1% solution of electrophoresis grade agarose in TAE (0.04 M TRIS-acetate, 0.001 M EDTA, pH 7.9). The 40 ml volume was boiled until the solution was clear, after which it was allowed to cool to about 55°C, when ethidium bromide (10μg/ml) was added for a final concentration of 1μg/ml. The molten agarose was then poured into a 7.5 cm

X 10 cm mould, a 12 tooth plastic comb was inserted and the mould was allowed to harden. The gel was placed in an electrophoresis tray and covered by 2 cm of TAE buffer. The first lane of the gel was loaded with 250 ng of *Hind*III digested lambda DNA (molecular weight marker II, Boehringer Mannheim, Laval, Quebec) and one other well was loaded with the sample. Electrophoresis was performed at about 85 V for about 40 minutes in room temperature. The gels were then photographed and examined for the presence of the insert of interest.

A larger scale reaction was performed to isolate the insert from the plasmid for later use in experiments as a probe. A 50 μl reaction containing 5μl restriction enzyme, 5μl appropriate buffer, 5μl plasmid and 38 μl nanopure water was prepared. The reaction was treated in the same manner as previously described and then loaded onto a 20 cm X 20 cm 1% agarose gel made as described above but in a volume of 200 ml. Electrophoresis was performed at room temperature at about 54 V for about 3 hours, until the dye had run at least two thirds of the length of the gel. The bands were illuminated under long ultraviolet wavelengths and the bands of interest were cut out. The insert was purified using the Gene Clean kit and their protocol without modification (Bio 101 Inc., Vista, CA). The insert was then ready for use as a probe.

The Southern blotting technique is the standard procedure for analyzing specific DNA sequences after digestion by restriction endonucleases. The DNA fragments were separated on the basis of size by agarose gel electrophoresis (Southern, 1975).

A 30 μl reaction containing 10 μl of genomic DNA (about 10 μg), 15 μl nanopure water, 3 μl appropriate enzyme buffer, and 2 μl of restriction enzyme (purchased from Boehringer Mannheim, Laval, Quebec; Pharmacia, Baie D'Urte, Quebec) was made and allowed to digest at the appropriate temperature and for the appropriate time.

The agarose gels were made, and the samples were loaded, according to the method described above. Following electrophoresis, the DNA was transferred to Zeta-Probe GT membrane (Bio-Rad Laboratories, Richmond, CA) by the Southern transfer method (Southern, 1975), which was modified according to the manufacturer's protocol. Specifically, the membrane was pre-wet in nanopure water and then soaked in 10X SSC (1.5M sodium chloride, 0.15M sodium citrate) at room temperature for 15 minutes. The agarose gel was placed on the filter paper, DNA wells down and the membrane was then applied to the gel. Two more sheets of filter paper were moistened with 10X SSC from the transfer tray and applied over the membrane. A thick layer of absorbant towels were placed on top and DNA transfer

proceeded for approximately 18 hours. The membrane was then rinsed and shaken for 15 minutes at room temperature in 2X SSC and allowed to air-dry after which it was sealed in a plastic bag containing prehybridization solution (0.5M Na_2HPO_4, 0.5M EDTA, pH8.0, 20% SDS) warmed to 65°C and was stored for several hours at 65°C.

DNA probes were labelled using the Klenow polymerase I (Pharmacia, Baie D'Urte, Quebec) method. Twenty-five nanograms of each probe were boiled for 2 minutes and chilled on ice for 5 minutes, then added to 11.75 μl appropriate enzyme buffer, 1 μl Klenow polymerase I, 2.5 μl α ^{32}P dATP and 2.5 μl α ^{32}P dCTP (NEN). The mixture was incubated at 37°C for 50 minutes and then stopped with 0.5M EDTA.

Probes were denatured by boiling with 100,000 cpm of *Hind*III digested lambda DNA and salmon sperm (10 mg/ml) for 10 minutes followed by cooling on ice for 10 minutes. Probes were then added to about 10 ml of prehybridization solution warmed to 65°C and then added to the bag containing the membrane. The membrane was incubated at 65°C overnight and then washed in Church solution (0.4M Na_2HPO_4, pH7.2, 20% SDS) once for 15 minutes at room temperature, and twice at 60°C for 40 minutes. The membrane was air-dried and then sealed in a plastic bag. Subsequently, membranes were exposed to radiographic film (Kodak XAR-5) in an X-ray casette at -70°C

for 1-7 days.

Haptoglobin genotypes were determined from the analysis of both starch gel phenotypes and the banding patterns following Southern hybridization of the Hp2α cDNA probe to genomic DNA. The Hp2α cDNA, obtained as a subclone in the pAT153 vector, was hybridized to *Eco*RI, *Hind*III and *Pst*I digests of genomic DNA to distinguish *HP*2* from *HP*1* alleles as described by Maeda *et al* (1984). *HP*1F* was distinguished from *HP*1S* by *Xba*I digestion of genomic DNA (Maeda *et al*, 1984). Digestion of the genomic DNA by *Xba*I, in the first step of the Southern blotting protocol, was performed as described above except that spermidine (2mM) was added to the digestion reaction after 20 minutes to facilitate the reaction.

The locus *D16S4* (16q22.100-16q22.102) was studied using the probe ACH 207 (American Type Tissue Collection, Rockville, Md). This cDNA was cloned into the vector pBR322 and resuspended in low TE. Genomic DNA samples were digested with *Msp*I and *Taq*I and then Southern blotted and probed, as described in detail previously.

Primer pairs for studying the (AC)n repeat markers were provided by Dr. David Callen (Department of Cytogenetics and Molecular Genetics, Adelaide Children's Hospital, North

Adelaide, Australia). The loci, probes and primer sequences were, respectively, as follows:

1. *D16S289*- 16AC7.46 forward 5'CACCACTTATCATTTCTTCCAAGCTGTG3'
   reverse 5'AGTTGGAGGAAGAGAAGCAG3'
2. *D16S301*- 16AC6.21 forward 5'GATCCTAAGGACAAATGTAGATGCTCT3'
   reverse 5'AGCCACTTCCCAGAACTTGGCTTCC3'
3. *D16S320*- 16AC8.52 forward 5'AGTCTGAGAGACATCCAGGT3'
   reverse 5'GTGATATCAGTCAGTCCTGT3'
4. *D16S304*- 16AC1.14 forward 5'GTCAGTGCAATGGAGGTAAGAAAAAG3'
   reverse 5'GATCAGATGAGATAGGGCATATTCATGG3'

Each genomic DNA sample was amplified by the polymerase chain reaction (PCR) using the protocol obtained from Dr. Callen's laboratory. A master mix containing the buffer (sterile water, 1M $(NH_4)_2SO_4$, 1M Tris-HCl pH 8.8, 1mM EDTA, 10mg/ml bovine serum albumin (BSA), DMSO) and the cold dNTPs and radioactive α^{32}P dATP and α^{32}P dCTP was aliquoted to each sample DNA being analyzed. Each reaction sample was pre-incubated at 94°C for 5 minutes prior to the addition of *Taq* polymerase. The PCR conditions were 94°C for 1 minute, 60°C for 1.5 minutes and 72°C for 1.5 minutes for a total of 10 cycles. The conditions were then modified to 94°C for 1 minute, 55°C for 1.5 minutes and 72°C for 1.5 minutes for 25 cycles. Following amplification, each DNA sample was subjected to electrophoresis in a 5% denaturing polyacrylamide sequencing gel for 3 hours at 2000 V. The gel was dried and then exposed to radiographic film, for up to five days. For each locus

studied, control samples with previously assigned alleles were included to eliminate the variability of both labeling and enzyme effects which might otherwise alter allele designation (Knowles *et al*, 1992). Alleles were designated numbers (based on Callen's sample gels of CEPH families and control samples), ranging from -12 to +18 depending on the number of additional repeats their amplified fragments contained. The allele assignments were made independently by myself and Dr. Greenberg without prior knowledge of disease status.

Using the same protocol and aliquots of genomic DNA from our family members, data from the following loci were generated in Dr. Callen's laboratory: *D16S318, D16S397, D16S398, D16S450 and D16S522.*

Primers were also obtained for a (AC)n polymorphism found within the gelsolin gene (*GSN*), a candidate gene for *LCD1*. A 125-143 bp DNA fragment encompassing the (AC)n repeat was amplified by PCR according to the protocol described in Wiens *et al* (1992) (Appendix 1). The *GSN* alleles were designated 0-16 as described elsewhere for other (AC)n VNTR microsatellites (Kwiatkowski *et al*, 1991). The allele with the least number of AC repeats was designated 0; the other alleles were designated 2-16 according to the number of additional repeats their amplified fragments contained. Two point lod scores were calculated between *GSN* and *LCD1* using

the Mark III computer program (Cote, 1975).

2.400 Linkage Analysis

All of the loci and probes used to generate polymorphism data are listed as follows:

1. restriction fragment length polymorphisms
    i. *Haptoglobin* (*HP*)- Hp2α
    ii. *D16S4*- ACH207
2. (AC)n repeat markers
    i. *D16S304*- 16AC1.14
    ii. *D16S320*- 16AC8.52
    iii. *D16S301*- 16AC6.21
    iv. *D16S398*- MFD 168
    v. *D16S397*- MFD 98
    vi. *D16S318*- 16AC8.20
    vii. *D16S522*- 16AC80H3
    viii. *D16S450*- 16AC33G11
    ix. *D16S289*- 16AC7.46

The map location and genetic distances based on analysis in CEPH families for the chromosome 16q loci studied in our laboratory and Dr. Callen's laboratory are illustrated in Figures 3 and 4.

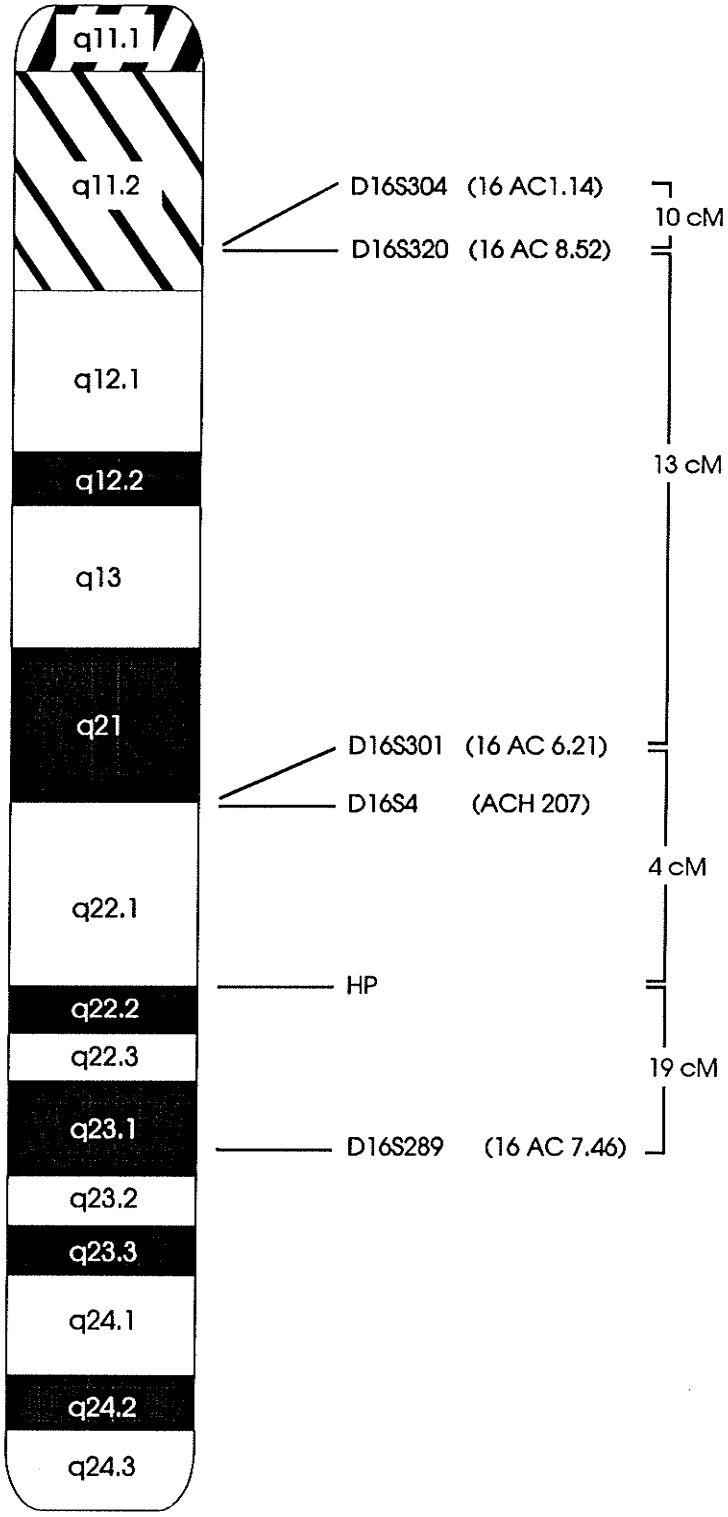


Figure 3. Relative physical and recombination positions of loci (probe) studied on 16q

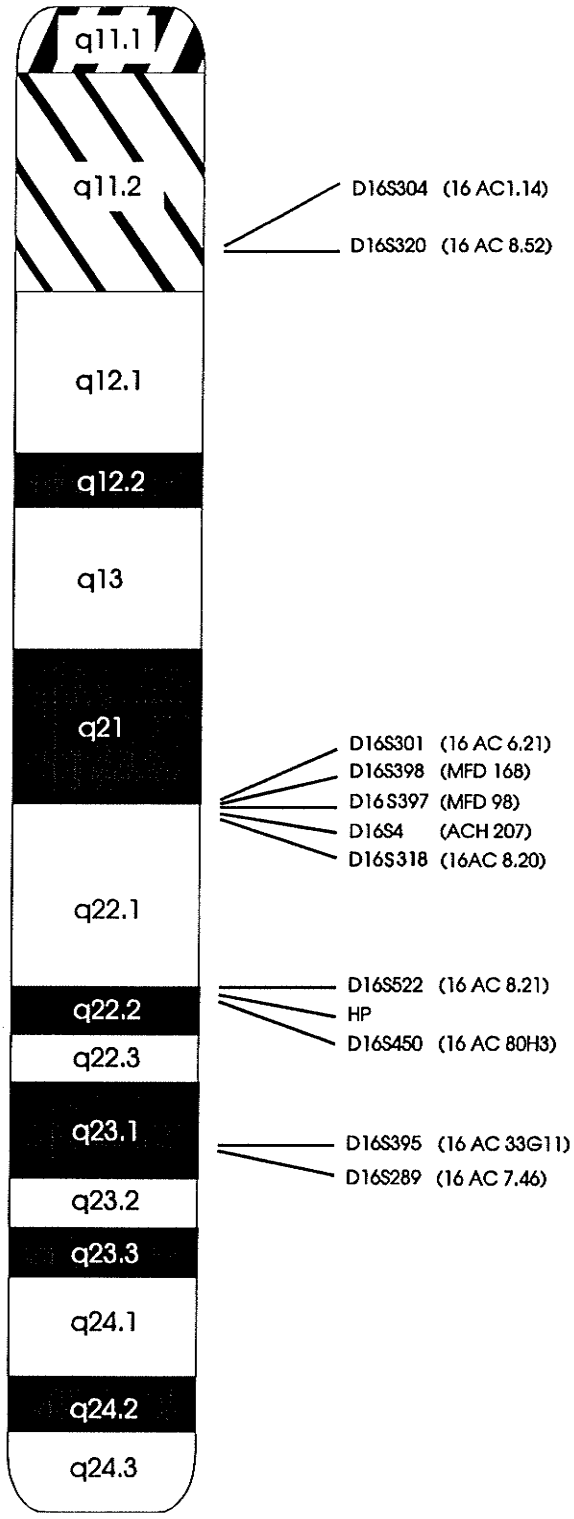


Figure 4. Relative physical positions of all loci (probe) studied on 16q

Two-point lod scores were calculated on the assumption that the LCD1 mutation was fully penetrant and inherited in an autosomal dominant pattern.

The data for each marker were organized into eight nuclear families for ease in the manual determination of 2-point lod scores by linkage analysis. Sex-specified two-point lod scores for fully informative meioses were calculated manually, using tables of published lod scores, at the standard values of θ: 0.05, 0.10, 0.20, 0.30, 0.40 (Smith *et al*, 1961). The sum of the maternal and paternal lod scores was calculated. A total lod score combining the maternal and paternal lod scores was then calculated.

Sylvia Philipps (Rh laboratory, University of Manitoba) used two-point lod score data with the Map83 program which calculates a maximum likelihood map using pairwise recombination (Sherman *et al*, 1984). For this analysis, 2-point lod scores, determined for pairwise combinations of *LCD1* and each marker, in the order in which each appears on chromosome 16q, were tested for goodness of fit. The maternal and paternal lod score data were combined (Philipps, personal communication).

The data for linkage analysis by the MENDEL (Lange *et al*, 1988) and LINKAGE (Lathrop *et al*, 1985) computer programs were

sent to Dr. Ken Morgan (McGill University, Montreal, Quebec). For this analysis, allele frequencies of each marker based on CEPH family data (Dr. David Callen, Department of Cytogenetics and Molecular Genetics, Adelaide Children's Hospital, North Adelaide, Australia) were used.

Haplotypes were constructed manually for each family member, arranged within nuclear families and then were analyzed by inspection.

3.000 RESULTS

3.100 Molecular Markers

3.110 RFLP data

In one nuclear family, the usual assignment of *HP* genotypes (after analysis by starch gel electrophoresis and standard hybridization of HP2α probe to Southern blot of genomic DNA) was impossible. This complication arose because of the presence of a a rare subtype of *HP*2*: *HP*2SS* (Nance and Smithies, 1963).

HP is a tetramer consisting of two α-chains and two β-chains. The α and β chains are generated by cleavage of the

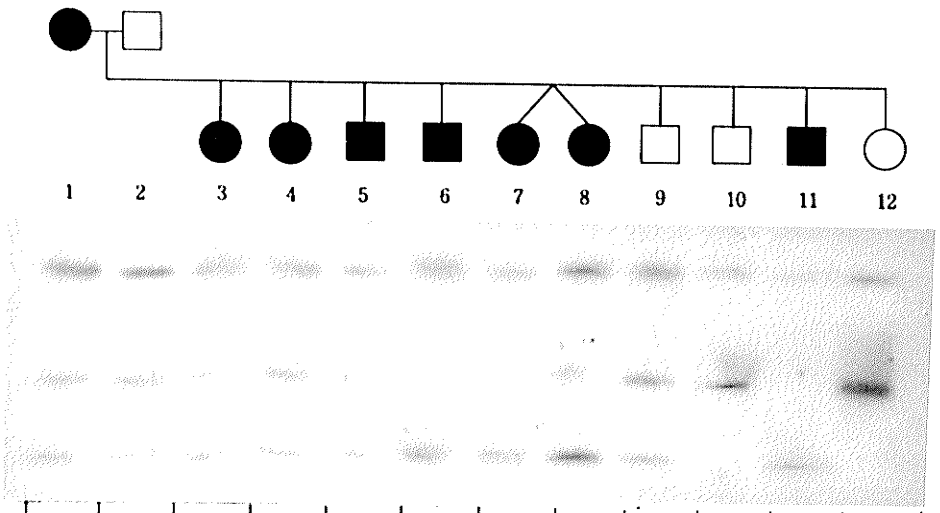
peptide produced by a single mRNA encoded by the haptoglobin locus on chromosome 16q (Bensi *et al*, 1985).

Normal heritable variation of human HP was demonstrated by starch gel electrophoresis of plasma samples by Smithies in 1955 who described 3 common phenotypes: HP1, HP1,2, HP2. These variants are due to the 2 common alpha chain alleles: *HP*1* aand *HP*2*. Additional allelic variation of *HP*1* is due to the *HP*1F* and *HP*1S* alleles. *HP*2* has an internal duplication of 1.7 Kb that includes two of the alpha-chain exons and generates a polypeptide, HP2α, consisting of 142 amino acids compared to the 83 amino acids of the HP1α polypeptide (Maeda, 1992).

Nance and Smithies (1963) suggested that the formation of *HP*2* resulted from the product of a partial gene duplication likely formed as a consequence of non-homologous crossing over within an *HP*1F* and *HP*1S* gene. Protein studies have demonstrated the existence of electrophoretic variants of the HP2α chain with the properties of *HP*2SS* and *HP*2FF* (Nance and Smithies, 1963). The *HP*2SF* allele has never been demonstrated either by DNA sequencing nor protein electrophoresis (Maeda, personal communication). Maeda *et al* (1984) reported that the nucleotide sequence analysis of the most common form of *HP*2* is *HP*2FS* with the 5′ end of the *HP*2* allele resembling *HP*1F* and the 3′ end resembling *HP*1S*.

Homologous crossovers between *HP*1F* or *HP*1S* allele in *HP*2FS/HP*1* heterozygotes can change the usual form of *HP*2* (*HP*2FS*) to three other rare forms: *HP*2SS*, *HP*2FF* and *HP*2SF* (Teige *et al*, 1986). The frequencies of the *HP*2SS* and *HP*2FF* alleles are approximately 2.5% and 1.8% respectively of the total frequency of *HP*2* in the Caucasian population. A more complete discussion is found in the article by Marles *et al*, 1993 (Appendix 2).

Figure 5 shows the autoradiogram with HP phenotypes seen on starch gel electrophoresis, the initial and the revised *HP* genotypes. The father's DNA in lane 2 was heterozygous for the 4.6 and 4.0 Kb alleles and his phenotype determined by starch gel electrophoresis was HP*2/HP*1. His initial genotype assignment was inferred to be *HP*1S/HP*2FS*. The mother's DNA in lane 1, also heterozygous for the 4.6 and 4.0 Kb alleles, was inferred to be *HP*1F/HP*1S* based on her starch gel phenotype of HP1. Assignment of genotypes from the DNA of the offspring in lanes 6-8 and 10-12 was problematic. For example, the son's DNA in lane 9 is homozygous for the 4.6 Kb allele and has a starch gel phenotype of HP1. If the father's genotype is actually *HP*1S/HP*2FS*, his son should have a starch gel phenotype of HP 1,2. Repeat analyses always yielded the same results. Based on knowledge of the phenotypic frequencies of 8 blood group systems in random Manitoba Caucasian males, it was estimated that only 1/360,000



Alleles	4.6/4.0	4.6/4.0	4.6/4.0	4.6/4.0	4.6/4.0	4.0/4.0	4.0/4.0	4.0/4.0	4.6/4.0	4.6/4.6	4.0/4.0	4.6/4.6
Starch gel phenotype	HP1	HP1,2	HP1	HP1,2	HP1	HP1,2	HP1,2	HP1,2	HP1,2	HP1	HP1,2	HP1
Initial genotype assignment	HP*1F/HP*1S	HP*1S/HP*2	HP*1F/HP*1S	HP*1S/HP*2	HP*1F/HP*1S	HP*1S/HP*1S	HP*1S/HP*1S	HP*1S/HP*1S	HP*1S/HP*2	HP*1F/HP*1F	HP*1F/HP*1S	HP*1F/HP*1F
Revised genotype assignment	HP*1F/HP*1S	HP*1F/HP*2SS	HP*1F/HP*1S	HP*1F/HP*2SS	HP*1F/HP*1S	HP*1S/HP*2SS	HP*1S/HP*2SS	HP*1S/HP*2SS	HP*1F/HP*2SS	HP*1F/HP*1F	HP*1S/HP*2SS	HP*1F/HP*1F

Figure 5. Autoradiogram of *Xbal*-digested genomic DNA hybridized to probe Hp2α

Manitoba males could have been the father of all the children in this family, making the likelihood of non-paternity extremely remote (data not shown). If, however, the father were assigned an alternative genotype, *HP*1F/HP*2SS*, the genotypes of his 10 offspring could be assigned without ambiguity as the *HP*1F/HP*2SS* alleles would also yield 4.6 Kb and 4.0 Kb fragments, respectively, on Southern blot analysis. These results are further summarized in Appendix 2.

Table 1 summarizes the results of linkage analysis for the blood group loci, other loci encoding known genes, and *LCD1*.

Six nuclear families with a total of 23 meioses were informative for linkage analysis of *HP* and *LCD1* (Tables 2 and 3). The highest calculated values of these lod scores in the overall total were 1.555 at a θ value of 0.10 and 1.520 at a θ of 0.20. When the Kivlin and Hammerstein lod scores (Hammerstein *et al*, 1975; Kivlin *et al*, 1984) were added to our data, the largest observed lod score was 2.460 at θ=0.20.

Of 42 possible meioses scored using the ACH207 probe (defining *D16S4*), only 5 were informative and there were no recombinants.

Table 1 Lods for *LCD1* and marker loci, encoding known genes

LOCUS	Recombination Fraction				
	0.05	0.10	0.20	0.30	0.40
ABO+	-5.808	-3.628	-1.691	-0.770	-0.267
AU+	-1.184	-0.672	-0.254	-0.087	-0.018
CO+	-0.400	0.00	0.192	0.144	0.046
PGD	-3.605	-2.218	-0.970	-0.378	-0.088
DO+	0.535	0.465	0.318	0.170	0.049
FY+	-0.370	0.053	0.288	0.270	0.157
GSN	-6.301	-3.429	-1.049	-0.20	0.155
JK+	0.072	0.237	0.258	0.159	0.048
KEL+	-2.071	-1.054	-0.258	-0.005	0.024
FUT3+	-0.721	-0.444	-0.194	-0.076	-0.018
FUT2+	-0.131	-0.108	-0.065	-0.030	-0.008
LU+	-0.829	-0.415	-0.107	-0.019	0.00
MET+	-2.092	-1.095	-0.324	-0.068	-0.005
GYPB and GYPC +	-2.410	-1.234	-0.374	-0.100	-0.016
RAF1	0.330	0.283	0.186	0.095	0.026

+ phenotype determined serologically

Table 2 Lod scores between *LCD1* AND *HP*

Segregation Information	Recombination Fraction				
	0.05	0.10	0.20	0.30	0.40
Maternal					
Z1:1	-0.721	-0.444	-0.194	-0.076	-0.018
Z2:0	0.258	0.215	0.134	0.064	0.017
Z8:0	1.929	1.741	1.332	0.868	0.349
Z3:0	0.535	0.465	0.318	0.170	0.049
TOTAL	2.001	1.977	1.590	1.026	0.397
Paternal					
Z3:0	0.535	0.465	0.318	0.170	0.049
Z3:2	-1.442	-0.887	-0.388	-0.151	-0.035
TOTAL	-0.907	-0.422	-0.070	0.019	0.014
OVERALL TOTAL	**1.094**	**1.555**	**1.520**	**1.045**	**0.411**

TABLE 3 Summary lod scores between *LCD1* and *HP*

LOCUS	RECOMBINATION FRACTION (θ)				
	0.05	0.10	0.20	0.30	0.40
HP (this study)	1.094	1.555	1.520	1.045	0.411
HP (Kivlin *et al*, 1984)	0.23	0.48	0.54	0.37	0.12
HP (this study & Kivlin *et al*, 1984 & Hammerstein & Sholz, 1975)	1.324	2.035	2.460	1.415	0.531

3.120 Candidate gene analysis

A candidate gene approach was utilized to investigate the possibility of a mutation in the gelsolin gene as the cause of LCD1. The amyloidogenic protein in FAP IV (which is synonymous with LCD2) is an internal degradation product of a variant form of gelsolin (Maury *et al*, 1990). On this basis, it was hypothesized that a novel mutation in gelsolin could cause amyloid formation in LCD1. Linkage between the genes for gelsolin and LCD1 was studied using a highly polymorphic intronic microsatellite (AC)n repeat in gelsolin. The scoring of 32 informative meioses from 10 nuclear families for linkage gave a lod score of -6.29 at a θ of 0.05 and -3.42 at θ= 0.10, for combined maternal and paternal meioses. These data, which exclude linkage of *GSN* and *LCD1* at θ<0.10, exclude gelsolin as a candidate gene, have been published and are in agreement with negative immunohistochemical staining with anti-gelsolin antibodies (Appendix 1).

The candidate gene approach was also utilized to exclude a novel mutation in the transthyretin (prealbumin) gene as the cause of LCD1. Mutations in this gene are known to cause FAP types I-II. Single strand gel electrophoresis (SSGE) analysis and direct sequencing of exons 2, 3 and 4, containing the coding region of transthyretin did not demonstrate any sequence differences between genomic DNA from LCD1 patients

and controls using published primer sequences (Sasaki *et al*, 1985) (data not published). This genetic analysis was in agreement with the negative immunohistochemical staining with anti-transthyretin antibodies.

3.200 Linkage Analysis Data

In this large kindred, there are 42 possible meioses to be scored. There were 36 informative meioses for the linkage analysis between *LCD1* and *D16S304* (16AC1.14). There were 30 informative meioses for *D16S289* (16AC7.46) (Figure 6), 31 informative meioses for *D16S320* (16AC8.52) and 28 informative meioses for *D16S301* (16AC6.21). The lod scores for each (AC)n marker at the standard recombination fractions are summarized in Tables 4-8. Only weakly positive or negative lod scores were apparent except for *D16S318* (16AC8.20) with a z=1.5 at a θ of 0.01.

The Mark III computer program (Cote, 1975) did not confirm linkage of *LCD1* to *HP*, since the maximum lod score was only 1.555 at a θ of 0.20, which is equivocal (Table 2). Only negative or weakly positive lod scores were calculated for the (AC)n marker loci and *LCD1* (Table 8). The equivocal lod scores for calculated between *HP* and *LCD1* and the other alternating positive and negative lod scores strongly suggests

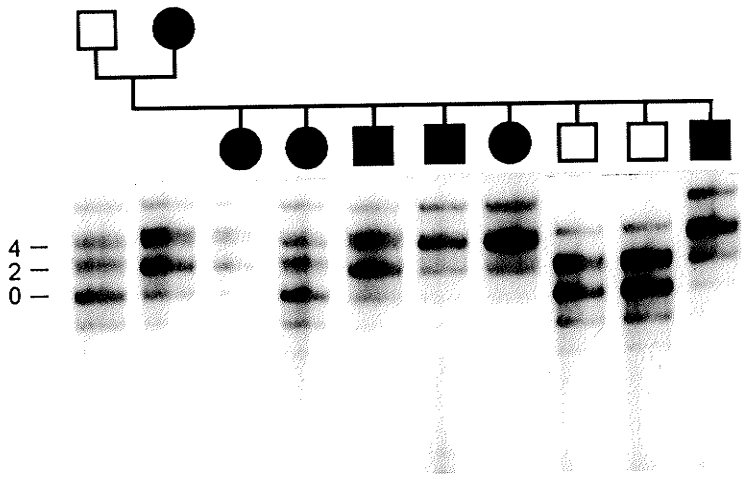


Figure 6. Photograph of (AC)n repeat polymorphism of *D16S289* (16AC7.46) in one nuclear family.

Table 4 Lod scores between *LCD1* and *D16S304*

Segregation Information	Recombination Fraction				
	0.05	0.10	0.20	0.30	0.40
Maternal					
Z3:1	-0.464	-0.229	-0.060	-0.011	-0.001
Z2:0	0.258	0.215	0.134	0.064	0.017
0R:2NR	0.558	0.511	0.408	0.292	0.158
Z5:0	1.093	0.975	0.720	0.436	0.149
Z3:1	-0.464	-0.229	-0.060	-0.011	-0.001
Z2:0	0.461	0.397	0.265	0.138	0.039
Z1:1	-0.721	-0.444	-0.194	-0.076	-0.018
TOTAL	0.721	1.196	1.213	0.832	0.343
Paternal					
3R:1NR	-2.721	-1.842	-0.990	-0.519	-0.212
Z3:0	0.535	0.465	0.318	0.170	0.049
Z2:0	0.258	0.215	0.134	0.064	0.017
Z3:2	-1.442	-0.887	-0.388	-0.151	-0.035
TOTAL	-3.370	-2.049	-0.926	-0.436	-0.181
TOTAL	**-2.649**	**-0.853**	**0.287**	**0.396**	**0.162**

Table 5 Lod scores between *LCD1* and *D16S289*

Segregation Information	Recombination Fraction				
	0.05	0.10	0.20	0.30	0.40
Maternal					
IR:1NR	-0.721	-0.444	-0.194	-0.076	-0.018
Z2:0	0.258	0.215	0.134	0.064	0.017
Z3:1	-0.464	-0.229	-0.060	-0.011	-0.001
1R:1NR	-0.721	-0.444	-0.194	-0.076	-0.018
Z7:1	0.650	0.787	0.730	0.503	0.193
TOTAL	-0.998	-0.115	0.416	0.404	0.173
Paternal					
Z3:2	-1.442	-0.887	-0.388	-0.151	-0.035
Z1:1	-0.721	-0.444	-0.194	-0.076	-0.018
3R:1NR	-2.721	-1.842	-0.990	-0.519	-0.212
TOTAL	-4.884	-3.173	-1.572	-0.746	-0.265
TOTAL	**-5.882**	**-3.288**	**-1.156**	**-0.342**	**-0.092**

Table 6 Lod scores between *LCD1* and *D16S320*

Segregation Information	Recombination Fraction				
	0.05	0.10	0.20	0.30	0.40
Maternal					
1R:1NR	-0.721	-0.444	-0.194	-0.076	-0.018
Z4:0	0.814	0.720	0.517	0.298	0.094
Z8:0	1.929	1.741	1.332	0.868	0.349
Z3:1	-0.464	-0.229	-0.060	-0.011	-0.001
TOTAL	1.558	1.788	1.595	1.079	0.424
Paternal					
Z2:0	0.258	0.215	0.134	0.064	0.017
Z3:0	0.535	0.465	0.318	0.170	0.049
3R:1NR	-2.721	-1.842	-0.990	-0.519	-0.212
Z2:1	-0.721	-0.444	-0.194	-0.076	-0.018
TOTAL	-2.649	-1.606	-0.732	-0.361	-0.164
TOTAL	**-1.091**	**0.182**	**0.863**	**0.718**	**0.260**

Table 7 Lod scores between *LCD1* and *D16S301*

Segregation Information	Recombination Fraction				
	0.05	0.10	0.20	0.30	0.40
Maternal					
Z1:1	-0.721	-0.444	-0.194	-0.076	-0.018
Z2:0	0.450	0.387	0.258	0.133	0.037
Z2:1	-1.052	-0.684	-0.313	-0.125	-0.029
Z8:0	1.929	1.741	1.332	0.868	0.349
1R:1NR	-0.721	-0.444	-0.194	-0.076	-0.018
Z1:1	-0.721	-0.444	-0.194	-0.076	-0.018
TOTAL	-0.836	0.112	0.695	0.648	0.303
Paternal					
2R:2NR	-1.442	-0.887	-0.388	-0.151	-0.035
Z2:0	0.258	0.215	0.134	0.064	0.017
IR:1NR	-0.721	-0.444	-0.194	-0.076	-0.018
Z3:2	-1.442	-0.887	-0.388	-0.151	-0.035
TOTAL	-3.347	-2.003	-0.836	-0.314	-0.071
TOTAL	**-4.183**	**-1.891**	**-0.141**	**0.334**	**0.232**

Table 8 Summary lod scores for *LCD1* and 16q loci

LOCUS	RECOMBINATION FRACTION (θ)					
	0.01	0.05	0.10	0.20	0.30	0.40
D16S304	___[a]	-2.649	-0.853	-0.287	-0.396	-0.162
D16S320	___	-1.091	0.182	0.863	0.718	0.260
D16S301	___	-4.183	-1.891	-0.141	0.334	0.232
D16S398	-15.7	-7.1	-3.8	-1.3	-0.6	-0.7
D16S397	-1.4	0.4	0.9	1.1	0.8	0.4
D16S4	NOT INFORMA-TIVE	NOT INFORMA-TIVE	NOT INFORMA-TIVE	NOT INFORMA-TIVE	NOT INFORMA-TIVE	NOT INFORMA-TIVE
D16S318	1.5	1.4	1.2	0.9	0.6	0.3
D16S522	-11.6	-5.1	-2.6	-0.7	0.0	0.1
HP	-2.737	1.094	1.555	1.520	1.045	0.411
D16S450	-9.7	-4.4	-2.3	-0.7	-0.1	0.1
D16S395	-6.3	-1.2	0.5	1.3	1.0	0.3
D16S289	___	-5.882	-3.288	-1.156	-0.342	-0.092

[a] score not calculated

that the disease-causing gene for LCD1 is not located on chromosome 16q.

The Map83 computer program analysis (Sherman *et al*, 1984) did not provide any evidence for linkage when *LCD1* was artificially located in relation to the known map of chromosome 16 generated by 2-point lod scores (data, this thesis) incorporating the known order from physical data (Dr. D. Callen, personal communication). In fact, the goodness-of-fit chi^2 (observed data compared to expected data) was 0.00-0.03, when at least a score of 3.0 is required for significance.

Dr. Ken Morgan (McGill University) did not confirm linkage between *LCD1* and *HP* using LINKAGE (Lathrop *et al*, 1984) or MENDEL (Lange *et al*, 1988). The maximum lod score was 0.34 at a θ of 0.181, using the allele frequencies calculated using CEPH families (Dr. Callen, personal communication). He also excluded linkage between *LCD1* and *D16S289* for $\theta \leq 0.263$ and between *LCD1* and *D16S320* for $\theta \leq 0.08$. When Dr. Morgan assumed that the only alleles present at a locus were those which were found in the typed family members and, if they were made equally frequent for each locus, then the lod score increased to 1.29 at $\theta = 0.1625$.

3.300 Haplotype Analysis Data (Figures 7-14)

Analysis of haplotypes using the markers at specific loci (*D16S304, D16S320, D16S301, HP, D16S289*) determined for each individual in each nuclear family did not change the order of the markers established by Dr. Callen's laboratory for chromosome 16q. However, there are significant genetic distances between some of the loci (e.g.: 19 cM between *HP* and *D16S289*); and, then, it is not unreasonable to expect recombination between some of the loci studied during meiosis. The analysis of the inheritance of certain haplotypes in this kindred confirmed this assumption. Of 84 haplotypes (42 offspring), 51 haplotypes were inherited in an unchanged fashion, 18 haplotypes could be explained on the basis of one crossing over event, 14 required 2 crossing over events and one haplotype could be explained only by 3 crossing over events.

There are differences in the haplotypes inherited among different branches of the family. For example, individuals D042 and D043 are brother and sister, respectively. Both are affected with LCD1 and the haplotypes (from Figure 7 and 12) segregating with the disease gene appear to be "0,6,-2,2,4" and "0,6,0,1S,4", respectively. Individuals Y008, C014, and C016 are sisters affected with LCD1. Their haplotypes (from Figures 9, 10 and 11) segregating with the disease are "6,0,-

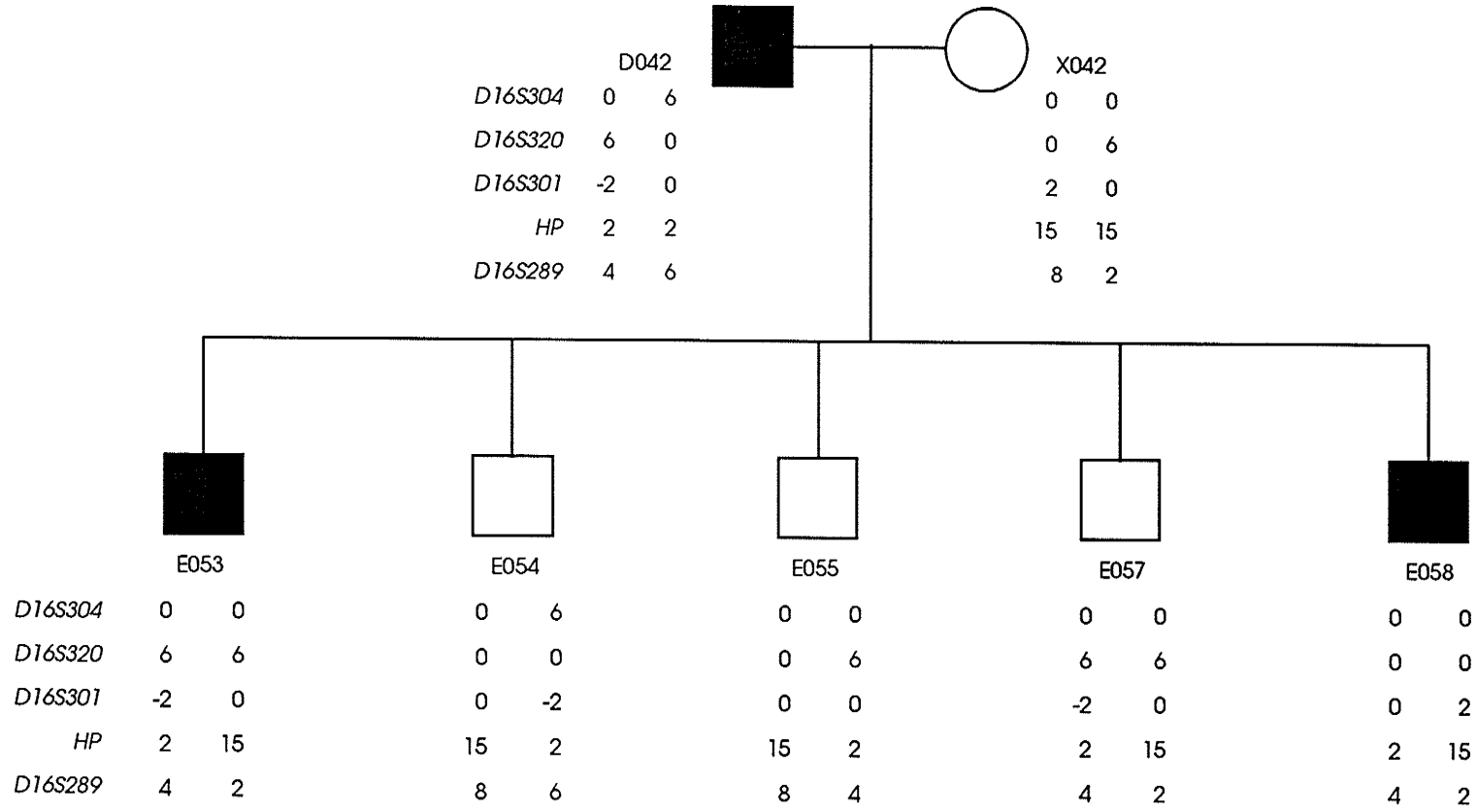


Figure 7. Haplotypes constructed for nuclear family 1 with designated alleles for each locus. Solid symbol indicates affected; open symbol indicates unaffected.

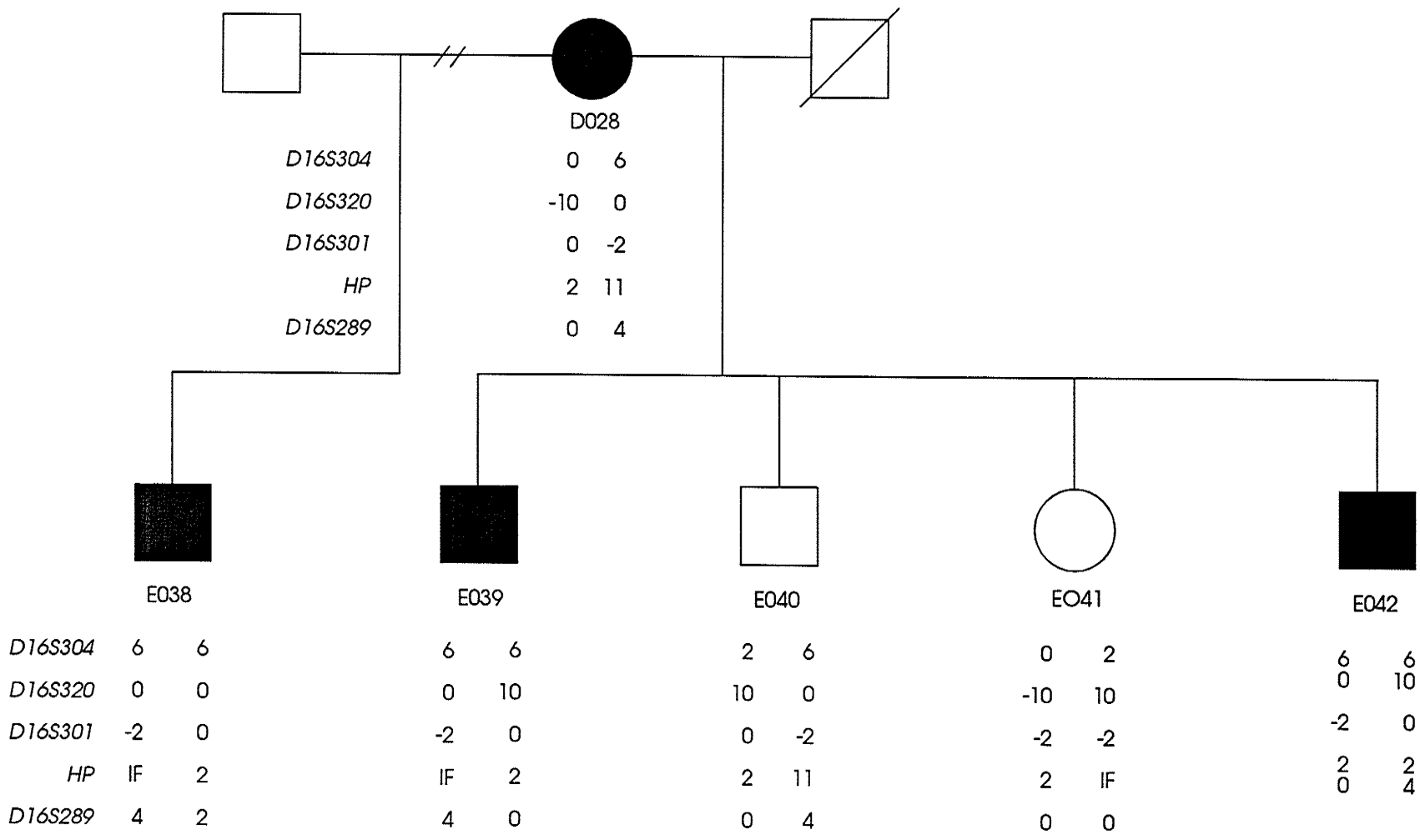


Figure 8. Haplotype constructed for nuclear family 2 with designated alleles for each locus. Solid symbol indicates affected; open symbol indicates unaffected.

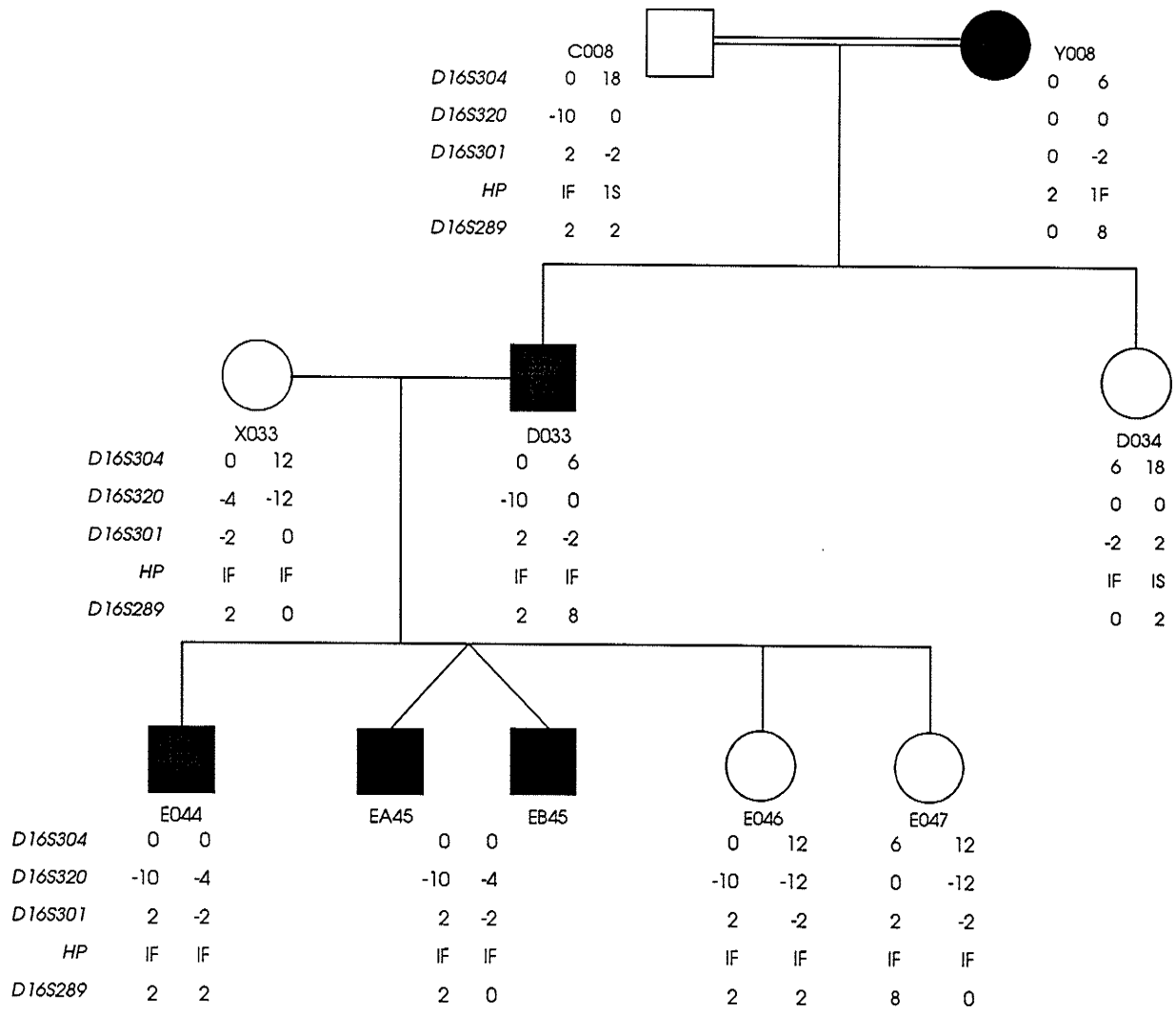


Figure 9. Haplotypes constructed for nuclear family 3 with designated alleles for each locus. Solid symbol indicates affected; open symbol indicates unaffected

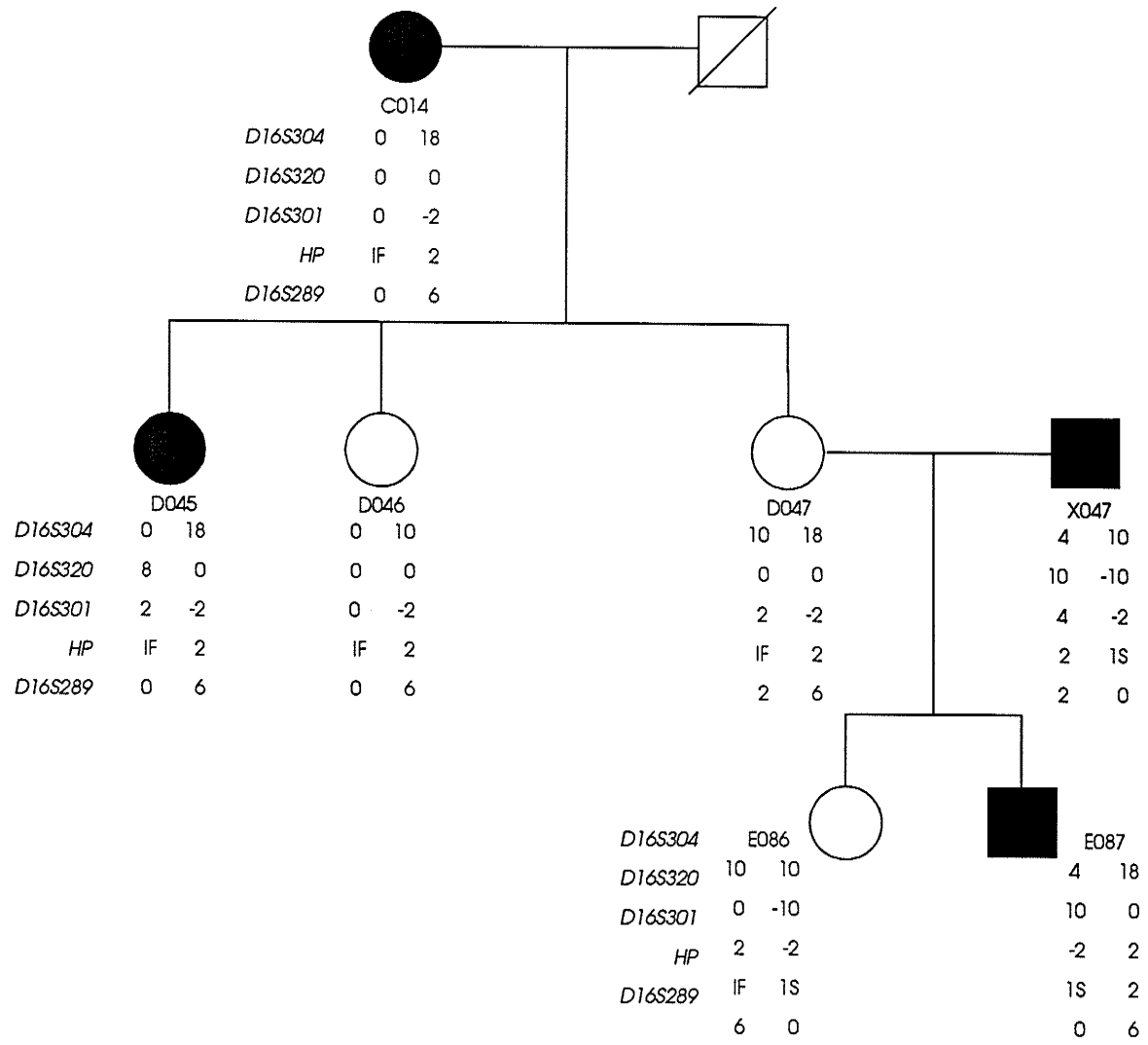


Figure 10. Haplotypes constructed for nuclear family 4 with designated alleles for each locus. Solid symbol indicates affected; open symbol indicates unaffected.

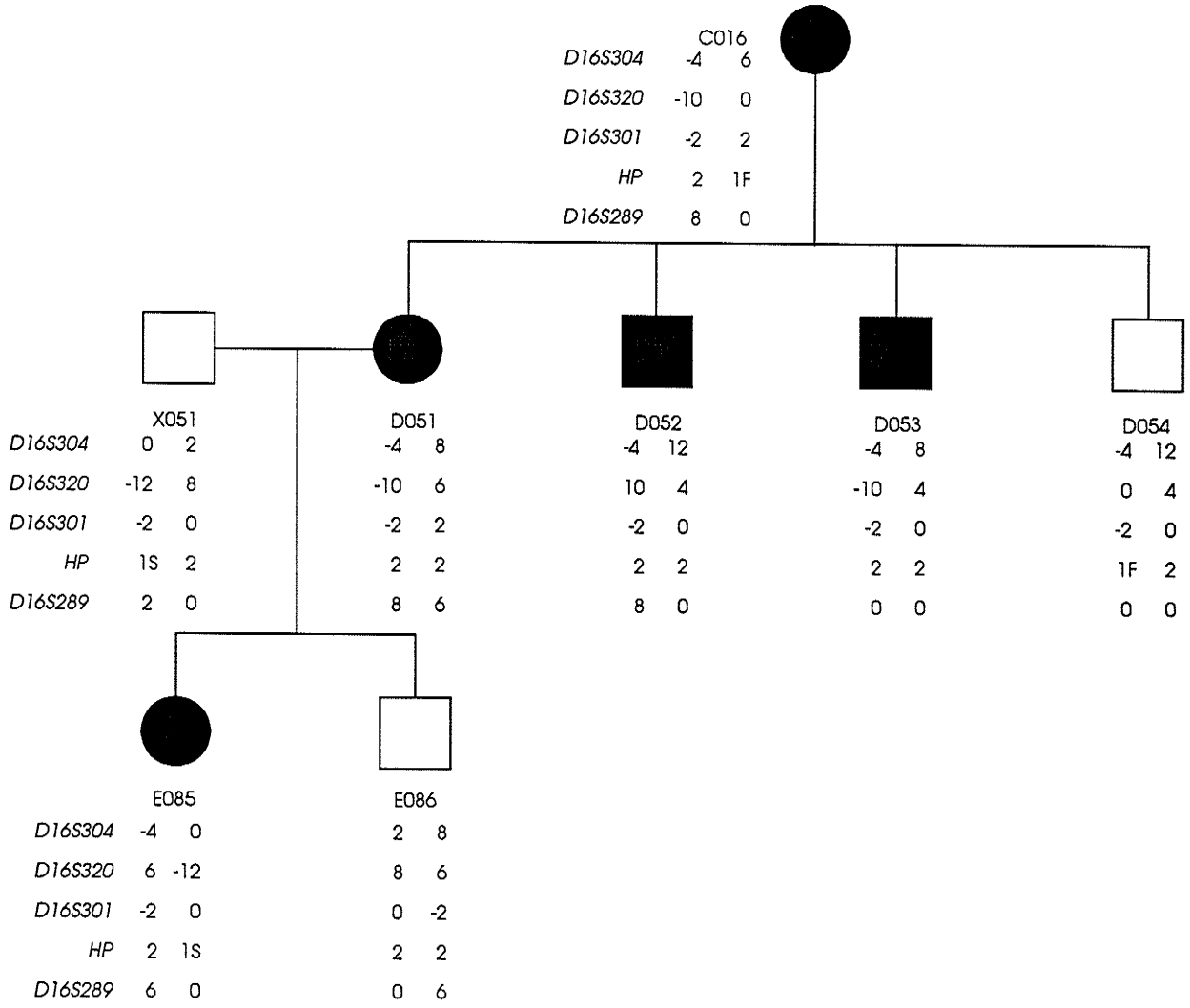


Figure 11. Haplotypes constructed for nuclear family 5 with assigned alleles for each locus. Solid symbol indicates affected; open symbol indicates unaffected.

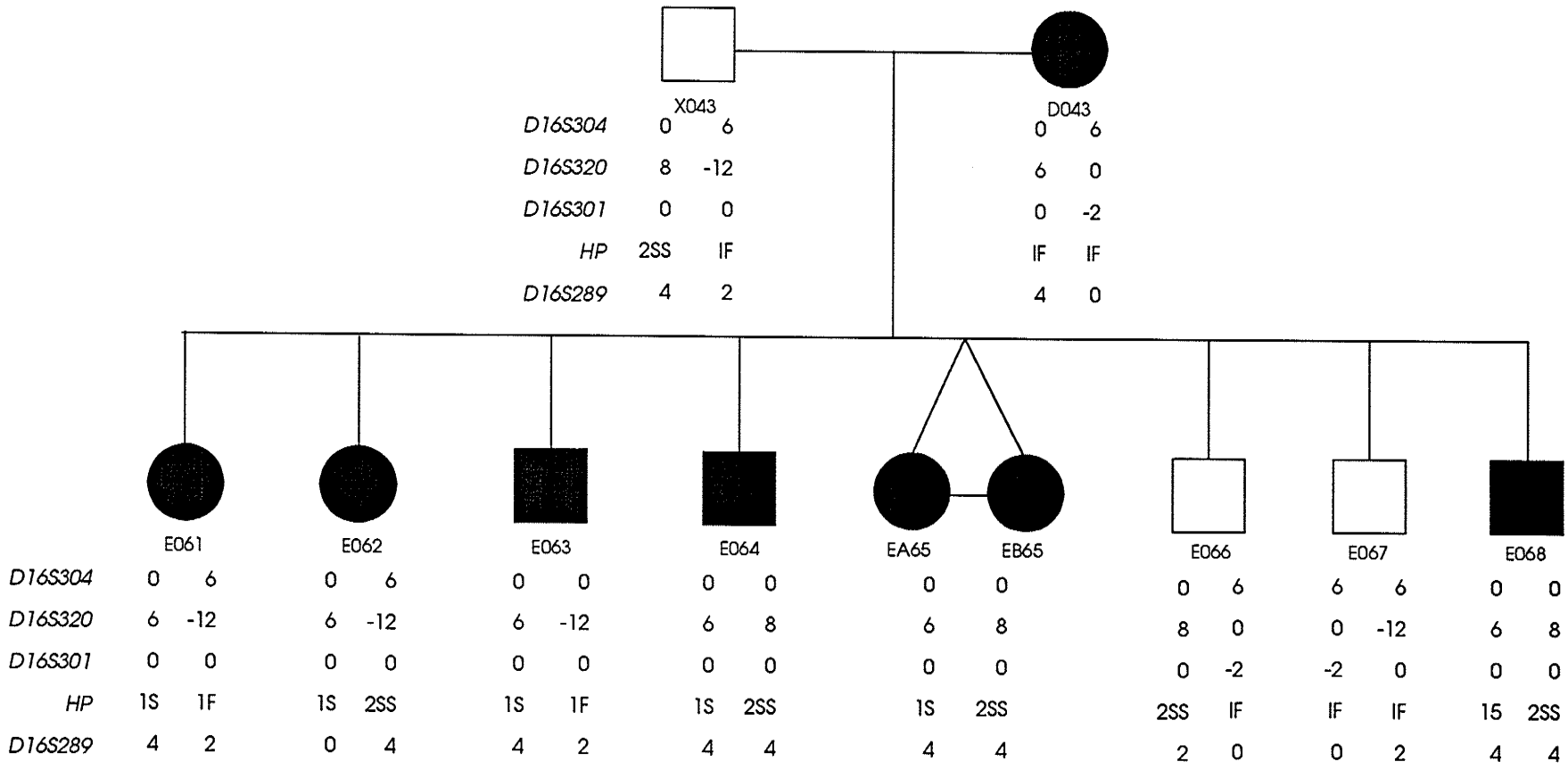


Figure 12. Haplotypes constructed for nuclear family 6 with assigned alleles for each locus. Solid symbol indicates affected; open symbol indicates unaffected

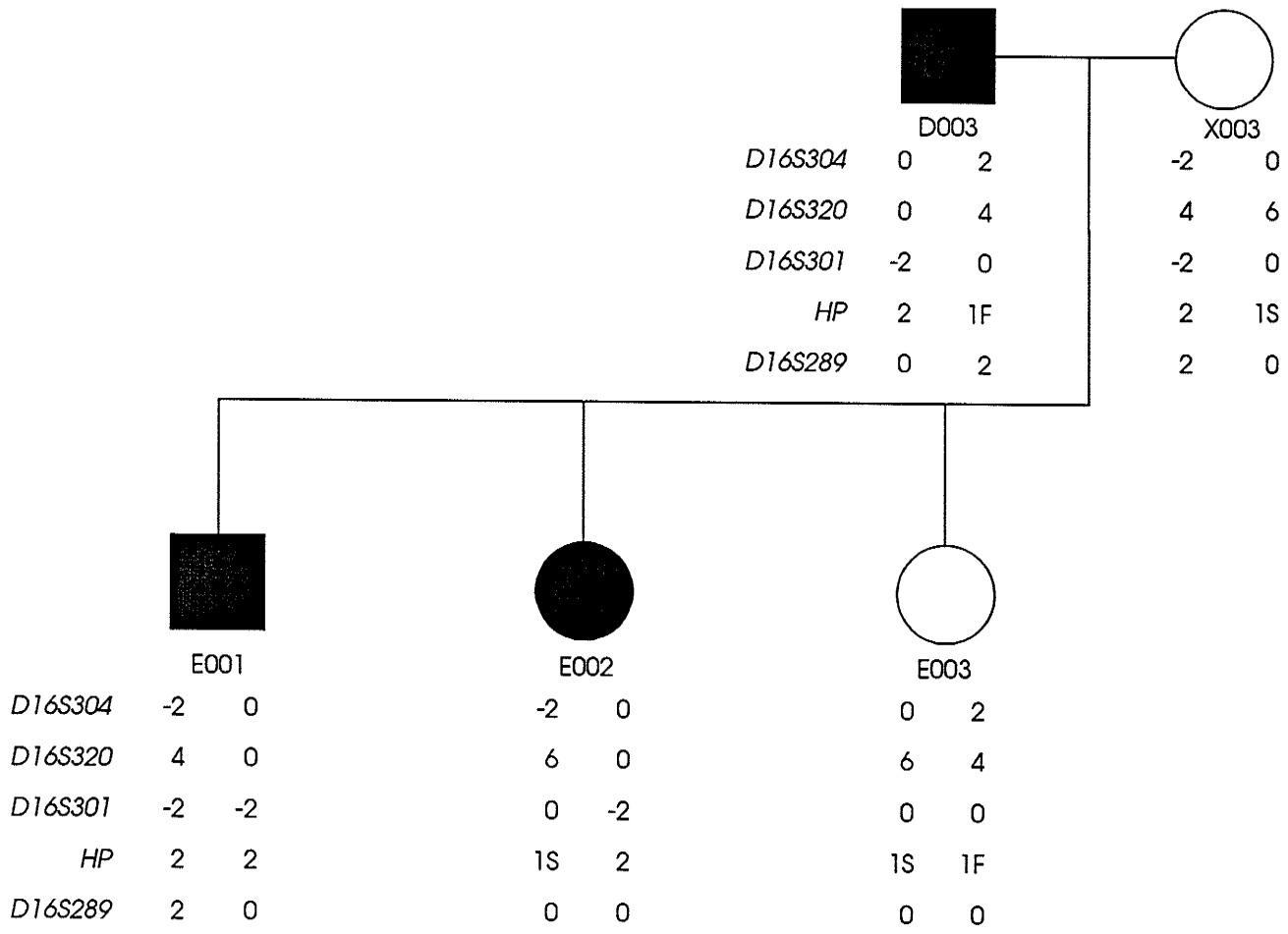


Figure 13. Haplotypes constructed for nuclear family 7 with assigned alleles for each locus. Solid symbol indicates affected; open symbol indicates unaffected.

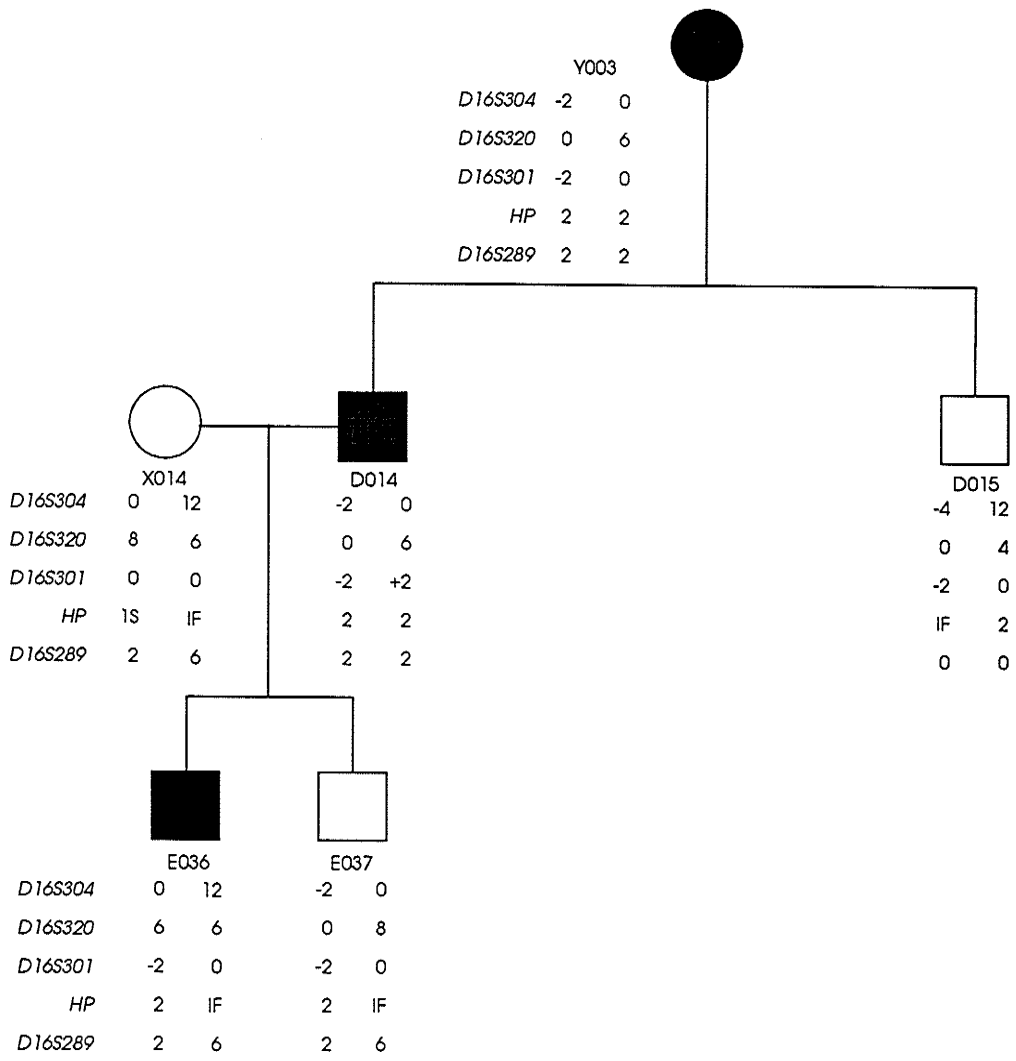


Figure 14. Haplotypes constructed for nuclear family 8 with assigned alleles for each locus. Solid symbol indicates affected; open symbol indicates unaffected.

2,1F,8" and "18,0,-2,2,6" and "-4,-10,-2,2 8", respectively. There are significant differences between these haplotypes, which would not be expected for individuals as closely related as sisters, if the disease-causing gene were located within this region. These differences may also reflect the amount of crossing over that occurs when markers have great genetic distances between them.

3.400 Immunohistochemical Data

Immunohistochemical analysis on serial sections of unstained paraffin embedded histologic sections of our patients' LCD1 affected corneae was performed in two independent laboratories: Dr. P. Gorevic in New York and Dr. C.P. Maury in Helsinki. Although abundant amyloid was present in the specimens, negative immunohistochemical reactions were seen with antibodies to serum amyloid A, prealbumin, apolipoprotein A1 and gelsolin (unpublished data).

Direct chemical analysis of the amyloid deposits was performed by 2-dimensional gel electrophoresis, in collaboration with Dr. R. Aebersold of the Protein Chemistry Laboratory (University of British Columbia). Solubilized proteins were recovered from snap frozen LCD1 corneae obtained at the time of corneal transplantation and from control corneae prepared in an identical way, from the Lions' Eye Bank

(Dr. M. Ekins, Director). The silver-stained 2-dimensional gel patterns from control and affected corneae were complex but no obvious distinct differences between the affected and control corneae were identified (unpublished, data not shown). Two-dimensional gel patterns from ^{35}S labelled proteins from cells cultured from affected and control corneae and secreted into the culture media were also very complex and did not reveal any readily distinguishable differences (unpublished, data not shown). In collaboration with Dr. J. Safneck, Department of Pathology, University of Manitoba, paraffin-embedded cell blocks prepared from cultured affected and control corneal cells were stained with Congo Red and Thioflavin-T. Stainable amyloid was not detected in these specimens, although the cells were proven to be corneal stromal cells, by positive histochemical staining with vimentin antibodies and negative histochemical staining with tokeratin and cytokeratin antibodies (unpublished, data not shown).

4.000 DISCUSSION

As described in sections 1.410 and 1.420, there are at least two approaches utilized to map a disease-causing gene and to identify the underlying genetic abnormality. The only published information available about the possible location of

the disease-causing gene in LCD1 was weakly positive lod scores with *HP* on chromosome 16q (Kivlin *et al*, 1984), as a chromosomal rearrangement associated with the LCD1 phenotype has never been reported. Known amyloid genes were excluded by the immunohistochemical analysis of the amyloid deposits in the corneae of patients with LCD1 removed at the time of transplantation. Linkage analysis between *GSN* and *LCD1* excluded gelsolin as a candidate gene (Appendix 1). Sequence analysis of the exons encoding the mature transthyretin protein did not reveal any differences between genomic DNA of control individuals and LCD1 patients (data not published). Hence, the first step of the project was to extend the linkage analysis reported for *HP* and *LCD1* (Kivlin *et al*, 1984) and to study the possible linkage of *LCD1* to a series of loci on chromosome 16q before initiating a genome-wide search via the positional cloning approach.

4.100 Linkage Analysis

The lod scores that were calculated manually, using lod score tables, for *LCD1* and *HP*, as well as those for *LCD1* and the (AC)n repeat marker loci (*D16S304, D16S320, D16S301, D16S289*) were in total agreement with those calculated by the Mark III computer program (Cote, 1975). This computer program divides a kindred into individual nuclear families, and

calculates two-point lod scores for each family, thereby assuming that each nuclear family of the kindred is independent. Hence, the exclusion from the analysis of individuals linking the nuclear families results in some loss of information. As a result, the Mark III computer program is conservative, particularly when it is run without including any inferred data. It is, however, useful for linkage analysis in certain circumstances, such as situations involving chromosomal rearrangements (P. McAlpine, personal communication). It was expected that the manual calculation of the two-point lod scores would be identical because the process of dividing the kindred into nuclear families and calculating each lod score independently of each other (manually) is the same approach used by the Mark III computer program (Cote, 1975). The lod scores for each family can be summed together for an overall total lod score as well as for a maternal lod score total and a paternal lod score total.

Dr. Ken Morgan (McGill University, Montreal) completed two- point linkage analysis of *LCD1* and the *HP, D16S320* and *D16S289* loci using two linkage programs, MENDEL (Lange *et al*, 1988) and LINKAGE (Lathrop *et al*, 1985). MENDEL analyzes kinship loops automatically; LINKAGE requires input as to which kinship loops are to be broken during the analysis. LINKAGE also estimates the likelihood of location of loci from

multipoint segregation information. For example, in this kindred, two brothers (C006 and C008) married two sisters (Y006 and Y008). The results for MENDEL and LINKAGE were identical for selected values of recombination. The maximum likelihood estimate of the θ between *LCD1* and *HP* was θ=0.181 at a lod score of 0.34 (data not shown), which is much lower than our lod score of 1.555 at θ=0.10. Dr. Morgan excluded linkage between *LCD1* and *D16S289* for values of θ less than approximately 0.263, on the basis of a lod score of -2.08 at a θ=0.2625. Our lod score for this locus pair was -1.022 at θ=0.20. Linkage of *LCD1* and *D16S320* was excluded at $\theta \leq 0.08$ by Dr. Morgan's analysis based on a lod score of -2.32 at θ=0.075 (data not shown). Our lod score for *D16S320* and *LCD1* was -1.091 for θ=0.05 (Table 6).

The discrepancies between the lod scores calculated by the different computer programs may be a consequence of changes in the treatment of the data, differences in the linkage analysis programs or specification of allele frequencies. Three families (D028 X X028 (X XA28), C003 X Y003, C014 X Y014) were not included in the linkage analysis of the Mark III program because in each mating, the alleles of one parent, unavailable for blood sampling, had to be inferred. The programs utilized by Dr. Morgan would have included these families as the program calculates all possible genotypes from the cumulative data using known allele

frequencies. Dr. Morgan then experimentally changed the specification of the allele frequencies, assuming that the only alleles present at a locus were those found in the typed pedigree members and that they were equally. Under these assumptions, the new maximum lod score for *LCD1* and *HP* was 1.29 at θ=0.1625. This manipulation did not, however, significantly alter the results for the other two loci (data not shown).

It is therefore possible that the Mark III program may have overestimated the lod scores obtained for each marker while the LINKAGE or MENDEL programs may have underestimated the lod scores. Regardless of the differences in the numerical values of the lod scores obtained by either method, linkage between *LCD1* and any of the marker loci examined was not detected.

Finally, Dr. Morgan plotted the lod score curves for 2-point linkage analyses of *LCD1* and the three marker loci and these are displayed in Figure 15. Only the lod score curve for linkage between *LCD1* and *HP* has values greater than 0.

Originally, procedures for the estimation of recombination and mapping were based on combining independent two-point linkage data. Assumptions about the complex mathematical relationships between map distance and

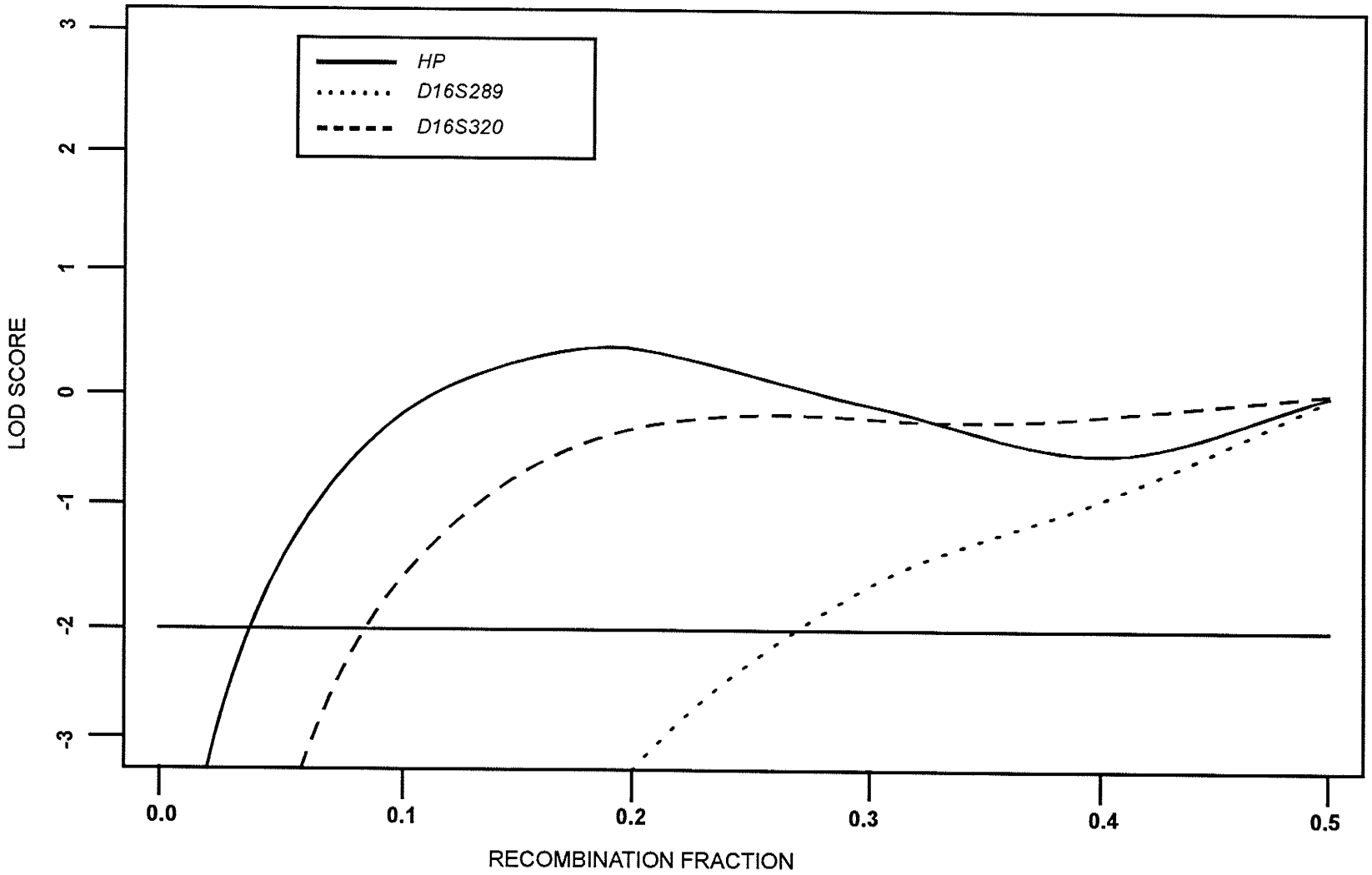


Figure 15. Graphic determination of maximum lod score for *LCD1* and 3 loci on 16q

recombination frequency were also required. Map distance represents the probability of occurrence of crossing-over within an interval, expressed in Morgans, and is additive. Recombination values are not additive because recombination between two loci results from an odd number of crossing-over events in the interval they define. Evidence suggests that crossing-over events do not occur independently although this fact is not usually accounted for in the calculations (Lathrop *et al*, 1984 and 1985).

Multipoint linkage analysis is more efficient in estimating recombination because it utilizes information on genetic location from all available sources while accounting for nonindependence. The probability that a family is informative for linkage at one or more loci increases with the number of loci studied. Each informative locus may then provide information on the location of the new marker or disease relative to the existing map; the use of previously estimated recombination rates between markers in multipoint tests may increase power (Lathrop *et al*, 1984). Dr. Callen provided the CEPH family genetic map of chromosome 16q with the map distances in centiMorgans (Figure 3). Unfortunately, significantly positive two-point lod scores (>3.00) were not attained to establish linkage between *LCD1* and any of the marker loci, although the highest lod score for *LCD1* and *HP* (z=1.555 at θ of 0.10) was promising. However, when the

HP:LCD1 data of other investigators were combined, the maximum lod score attained was only 2.46 at a θ of 0.20. No consistent pattern of negative or positive lod scores was observed between *LCD1* and each of the marker loci when they were examined in order of their location on chromosome 16q. Thus, *LCD1* was excluded from the interval between *D16S304* (16q11.2) and *D16S289* (16q23.1) on chromosome 16q on the basis of this linkage analysis.

Multipoint linkage analysis between *LCD1* and the markers on chromosome 16q was not performed since significantly positive lod scores were not obtained at any two-point linkage analysis.

4.200 Recent Report of Map Location on Chromosome 5q

A recently published paper by Stone *et al* (1994), supported our data that *LCD1* was not linked to any of the markers tested on chromosome 16q and that neither gelsolin or transthyretin were candidate genes for *LCD1*. Stone *et al* (1994) studied linkage between polymorphic markers near loci that have been associated with amyloid deposit disorders in previous studies and excluded linkage in eight families, each with some individuals affected with one of three clinically distinct stromal corneal dystrophies. They were able to

demonstrate that the location of the disease-causing gene(s) is on chromosome 5q. Two families studied by Stone *et al* (1994) had typical features of LCD1. An additional two families had granular corneal dystrophy, a condition in which irregular aggregates of hyaline material are deposited in the corneal stroma. Four families with Avellino dystrophy were also studied. Patients with Avellino dystrophy have coexisting deposits of both amyloid and hyaline material in their corneal stroma. Significant linkage of the disease-causing gene(s) to 5q markers (defining a 10 centiMorgan interval) was independently obtained for each of the three clinically distinct corneal dystrophies. The maximal combined lod score for all eight families was 28.6 at θ=0 with *D5S393*: *disease-determining gene*. Only three recombination events were detected with the five closest loci: one with *IL9* and two with *D5S436*. Thus, *IL9* and *D5S436* were considered to be flanking markers.

As none of the 14 known genes whose products are involved in the human amyloid deposit disorders map to chromosome 5q, these corneal dystrophies are, therefore, unlikely to be allelic with any of the previously characterized human amyloidoses (Stone *et al*, 1994).

Stone *et al* (1994) postulated that there may be more than one gene in the interval responsible for the diseases or that

they may be allelic disorders. The different phenotypes of allelic disorders could result from different mutations in the single gene, or the effect of other genes on the expression of a single mutation. Hereditary cerebral hemorrhage and Alzheimer disease are allelic disorders and result from different mutations in *APP*. Amyloid and nonfibrillar deposits of APP may occur in the same patients which is analogous to the occurrence of amyloid and hyaline deposits in the same corneas of Avellino patients.

4.300 Future Directions

Linkage analysis of informative families in the paper by Stone *et al* (1994) was utilized to map the disease-causing gene for LCD1, granular corneal dystrophy and Avellino dystrophy to chromosome 5q. Linkage analysis of the data generated from relevant chromosome 5 markers will be studied in our large kindred. In particular, the maximum combined lod score with the marker *D5S393* closest ($z=28.6$ at $\theta=0.00$) to the disease-causing locus will be assessed using the following primer sequences, forward 5'TTCTACCTGACCTTTCCTCT3', and reverse 5'CATTCCTCATTCCTCATTCC3'. Using the primers, a 208 bp sequence at *D5S393* will be amplified, the (AC)n repeat will be sized and the segregation of this polymorphic (AC)n repeat followed in our family (Weissenbach *et al*, 1992). The

proximal (*IL9*) and distal (*D5S436*) marker loci are approximately 10 cM apart. If linkage between *LCD1* and *D5S393* appears likely, then more markers in the interval of interest would be utilized to localize the disease-causing gene more specifically. We will endeavor to establish a collaboration with Stone *et al* and test new markers generated from the interval of interest in our family. Key recombinants may ultimately help focus the fine localization of *LCD1* leading to the ultimate cloning of the disease-causing gene. Any alterations in such a gene would have to be proven to be disease-causing and not present in the normal population,

It is possible that a single mutation accounts for every case of LCD1 or there may be a number of different mutations, as in cystic fibrosis. If a single mutation is responsible for the LCD1 phenotype, then it will be possible to provide quick preclinical detection of LCD1 in at-risk individuals. Preclinical detection of LCD1 may still be possible even if multiple mutations cause this condition, given that usually only a single mutation segregates in an individual family. Preclinical detection will provide valuable information, such as career planning, for these individuals, but will present new dilemmas for physicians and their at-risk patients, such as the age at which preclinical detection will be offered and how this knowledge may affect or change the lives of the patients.

Identification of the mutation(s) causing the LCD1 phenotype may help us to understand the pathogenesis of LCD1 and its relationship to other amyloid deposit disorders. This knowledge may allow the development of new treatment protocols with the goal of ultimately preventing the accumulation of amyloid in the stromal layer of the cornea in predisposed individuals.

5.000 SUMMARY AND CONCLUSIONS

1. A large Belgian-Canadian kindred with LCD1 was studied by linkage analysis. Weakly positive lod scores of 1.555 at θ=0.1 for *LCD1* and *HP* were obtained by linkage analysis. When these data were combined with previously published results, the overall maximum lod score was 2.46 at θ=0.2 (Hammerstein *et al*, 1975; Kivlin *et al*, 1984), which is equivocal and therefore not sufficient to demonstrate linkage to chromosome 16q.

2. Linkage analysis between *LCD1* and a variety of loci for (AC)n repeat markers did not demonstrate linkage to chromosome 16q.

3. The q11.2-23.1 region of chromosome 16 has been excluded as the chromosomal location for the disease-causing

gene of LCD1 in our family, based on the analyses of lod score and haplotype data. Since Stone *et al* (1994) recently provided linkage data to suggest that the disease-causing genes for LCD1, granular corneal dystrophy and Avellino dystrophy are located on chromosome 5q and may be allelic, our family with LCD1 could provide valuable confirmatory evidence for linkage of *LCD1* to chromosome 5q. Collaboration of our laboratory in the search for the disease-causing mutation(s) would be very exciting, as would studying the pathogenesis of this disorder.

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Exclusion of the Gelsolin Gene on 9q32-34 as the Cause of Familial Lattice Corneal Dystrophy Type I

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Summary

Familial lattice corneal dystrophy type I (LCD1) is a localized form of inherited amyloidosis limited to the corneal stroma. Recently the Finnish form of hereditary amyloidosis with lattice corneal dystrophy has been shown to be due to a mutation in the gelsolin gene ($G^{654}\rightarrow A$; Asp187→Asn). In this paper we exclude the gelsolin gene as the cause of the autosomal dominant form of isolated LCD1.

Introduction

Lattice corneal dystrophy type I (LCD1) is a fully penetrant autosomal dominant corneal dystrophy that primarily involves the stromal layer of the cornea (Waring et al. 1978; Isubota et al. 1987). The disease is characterized by recurrent corneal erosions causing pain, photophobia, and redness, and it usually progresses to severe visual impairment by the fourth to seventh decades of life. Although the underlying genetic abnormality and the pathogenesis of LCD1 are not known, some important observations have been made. Slit-lamp examination of affected individuals reveals a delicate interdigitating network of coalescing dots, dashes, and filaments, usually beginning in the central portion of the corneal stroma. Histological examination of affected corneas have confirmed the presence of amyloid consisting of small congophilic deposits with apple-green birefringence in polarized light (Klintworth 1971). Immunohistochemical analysis has not, to date, characterized the basic component of the amyloid fibril in LCD1 (Mondino et al. 1980; Gorevic et al. 1984).

The amyloidoses are a large heterogeneous group of both localized and systemic disorders that may be classified clinically and by histopathological identification of the protein subunit of the extracellular amyloid fibril (Benson and Wallace 1989; Benson 1991). In hereditary systemic amyloidosis, the most frequently encountered subunit protein is plasma prealbumin (transthyretin) (Benson 1991). In the subgroup of familial amyloidotic polyneuropathies, the Finnish syndrome (type IV FAP or FAF) is characterized by lattice corneal dystrophy in addition to progressive cranial neuropathy and systemic amyloidosis (Meretoja 1969; Boysen et al. 1979; Purcell et al. 1983; Darras et al. 1986). The slit-lamp appearance of thickened and radially oriented lines seen in FAF corneas differs from that seen in LCD1 (Meretoja 1972). The amyloidogenic protein in FAF has been identified as an internal degradation product of gelsolin (GSN), an actin-modulating protein (Ghiso et al. 1990; Maury 1990). A single amino-acid substitution at GSN residue 187 has been demonstrated in amyloid fibrils isolated from patients with FAF (Ghiso et al. 1990; Maury 1990). This amino acid change resulted from a single-nucleotide guanine-to-adenine transition at nucleotide position 654 ($G^{654}\rightarrow A^{654}$) identified in genomic DNA, and it presumably represents the disease-causing mutation in FAF (Levy et al. 1990; Maury et al. 1990).

Herein we describe the results of a linkage analysis performed between the LCD1 gene (*LCD1*) and the GSN gene (*GSN*) by using a $(GT)_n$ polymorphism found within *GSN*. The population that formed the

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basis for this study is a large Manitoba kindred of Belgian descent in which LCD1 is known to be segregating. Because a mutant GSN protein is involved in the pathogenesis of the lattice corneal dystrophy associated with FAF, it was important to evaluate *GSN* as a likely candidate gene for LCD1.

Subjects and Methods

Family Studies

The LCD1 kindred being studied is a large multigeneration Manitoba kindred of Belgian descent (fig. 1). This kindred was initially described by Robert Ramsay in 1958 (Ramsay 1958). The mode of transmission is consistent with autosomal dominant inheritance with full penetrance. Additional pedigree and clinical information will be reviewed elsewhere (S. Marles, unpublished data). All affected and at-risk individuals are now being followed by one ophthalmologist (M.B.E.). All affected individuals have been symptomatic and/or have demonstrated characteristic corneal changes on slit-lamp examination by age 25 years. There is neither known history nor objective signs of any associated systemic illness— specifically, neurological, cardiac, or renal. Sixteen individuals have received a total of 21 corneal transplants in the past decade. Characteristic histopathological changes of amyloid deposition have been confirmed in all corneas studied.

DNA Methods

DNA was extracted from heparinized blood samples obtained from 92 kindred members including 37 affected individuals, 23 unaffected individuals, 17 unaffected spouses, and 15 at-risk family members, as described elsewhere (Greenberg et al. 1987). Linkage between *GSN* and *LCD1* was studied by using a highly polymorphic intronic microsatellite $(GT)_n$ repeat in

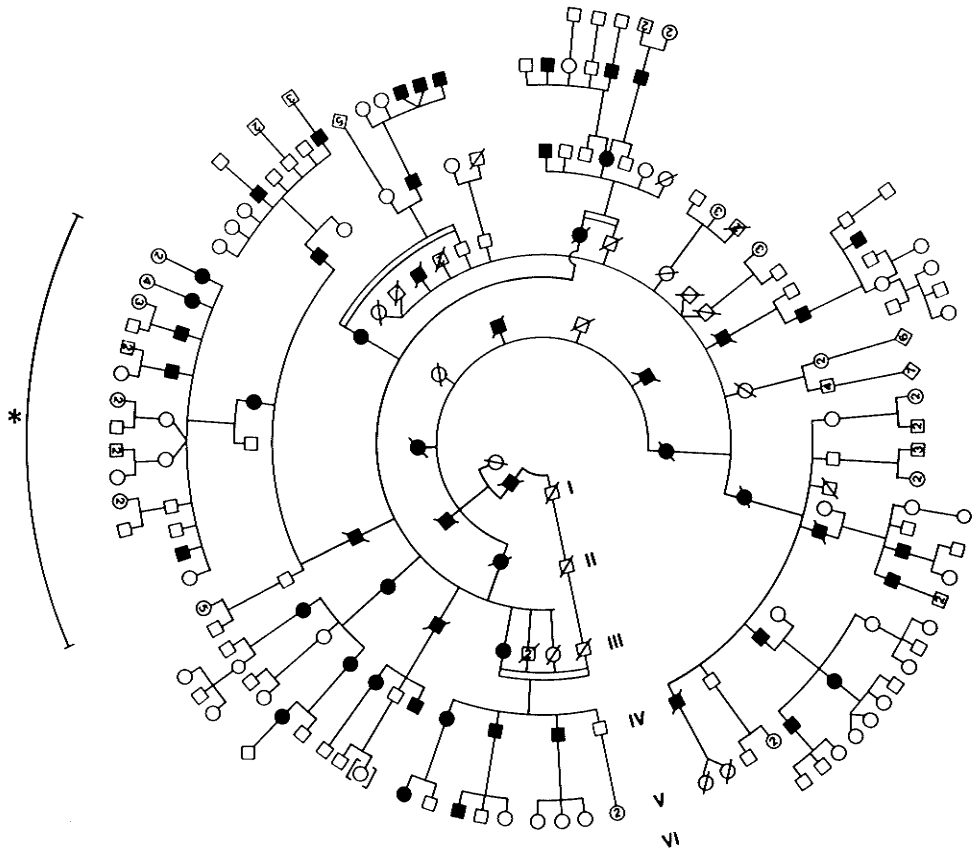

Figure 1 Manitoba pedigree with LCD1. □ = Unaffected male; ○ = unaffected female; ■ = male affected with LCD1; and ● = female affected with LCD1. A slash (/) indicates that the individual is deceased. An asterisk (*) indicates the portion of pedigree shown in fig. 2.

GSN. A 125–143-bp DNA fragment encompassing the $(GT)_n$ repeat was PCR amplified (Saiki et al. 1988) according to the following protocol: The PCR program included an initial denaturation at 94°C for 1.5 min; 25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 40 s; and 72°C for 4 min 20 s. Each reaction was carried out in a total volume of 10 μl consisting of 2 μl dNTP mix (1 mM dATP, 1 mM dGTP, 1 mM dTTP, and 12.5 μM dCTP), 0.4 μl oligonucleotide primer-pair mix (100 ng/μl each), 0.08 μl α-^{32}P dCTP (NEG-013H; 3,000 Ci/mM; NEN) 1 μl 10 × reaction buffer (Perkin Elmer Cetus), 0.05 μl Amplitaq (Perkin Elmer Cetus), 4.47 μl ddH_2O, and 2 μl genomic DNA (1:300 dilution of leukocyte DNA preparation). Two microliters of the PCR product was mixed with 3 μl of formamide stop solution, was heated at 80°C for 5 min, and was loaded on a 7 M urea, 6% polyacrylamide sequencing gel. After approximately 2 h at 2,000 V, the gels were dried and exposed to X-ray film for 1–24 h. The *GSN* alleles were designated 0–16, as described elsewhere for other $(GT)_n$ VNTR microsatellites (Kwiatkowski et al. 1991). The allele with the fewest number of GT repeats seen was designated "0," and the other alleles were designated 2–16 according to the number of additional bases that their amplified fragments contained. The allele assignments were made without prior knowledge of disease status. To date, a total of 12 alleles with a PIC value of .76 and a heterozygosity of .70 have been described at the *GSN* locus (Kwiatkowski and Perman 1990).

Linkage Analysis

Two-point LOD scores were calculated between *GSN* and *LCD1* by using the Mark III computer program (Côte 1975). Inheritance in this family is consistent with autosomal dominant transmission with full penetrance. Thirty-two informative meioses from 10 nuclear families (3 paternal and 7 maternal) were scored for linkage. Only confirmed affected individuals or those unaffected individuals older than 25 years were included in the linkage analysis.

Results and Discussion

Figure 1 shows the pedigree of the participants in this study. There are 37 living individuals who are clinically affected with LCD1 as assessed by clinical and slit-lamp examinations. Figure 2 depicts the informative polymorphism in the length of amplified DNA in one affected nuclear family. This $(GT)_n$ polymorphism displays Mendelian codominant inheritance. The youngest daughter in this nuclear family is not

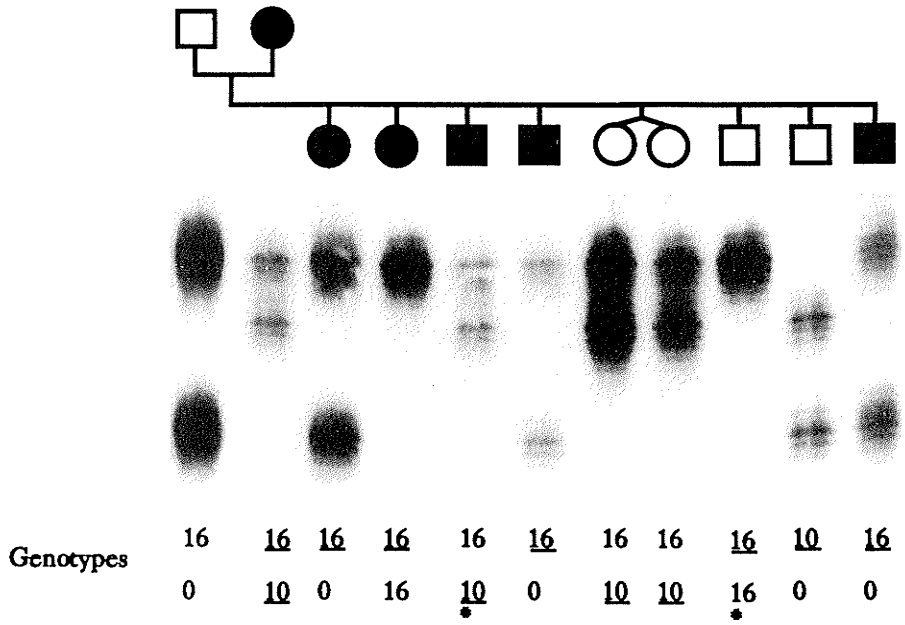

Figure 2 $(GT)_n$ repeat analysis in one nuclear family. Mendelian codominant inheritance of alleles is evident. In a given lane, each single darkest band with several fainter surrounding bands represents amplification of a single allele differing in size by a multiple of a single repeat. Pedigree symbols are as in fig. 1. An asterisk (*) indicates recombinants.

Table 1

Lod Scores for Linkage between *LCD1* and *GSN*

Segregation Information	No. of Families	No. of Meioses	Lod Score for θ of .05	.10	.20	.30	.40
Paternal.....	3	10	−1.34	−.60	−.04	.13	.12
Maternal	7	22	−4.95	−2.82	−1.009	−.25	.03
Total	10	32	−6.29	−3.42	−1.049	−.12	.15

included (fig. 2); since she is 18 years old and ophthalmologically normal, she cannot be scored in the linkage analysis. The results of two-point linkage analysis between the loci for *LCD1* and *GSN* are shown in table 1. The LOD score is −6.29 at a recombination fraction (θ) value of .05 and is −3.42 at θ = .10, for combined maternal and paternal meioses.

The genetic basis for LCD1, an autosomal dominant corneal stromal dystrophy, is unknown. A mutation in *GSN* appears to result directly in an amyloidogenic GSN molecule, a fragment of which polymerizes to amyloid fibrils that deposit in FAF (Levy et al. 1990; Maury 1990). Our study investigated *GSN* as a candidate locus for LCD1. Of 32 informative meioses scored for linkage, 11 obligate recombinants were seen (fig. 2 and data not shown). This results in strongly negative LOD scores between *GSN* and *LCD1* (−6.29 at θ = .05). The genomic region region of *GSN* encompasses only 70 kb of DNA (Kwiatkowski et al. 1988), and our LOD score of −2.00 at a θ = .15 makes the possibility of 15% intragenic recombination virtually impossible. This genetic evidence thus excludes *GSN* as a candidate gene in our kindred. Our study also provides strong evidence that the LCD1 locus is not located within 10 cM of *GSN*. These results are further supported by our observations that immunohistochemical analysis with anti-GSN antibodies (Maury 1991) completely failed to stain the amyloid deposits in the corneas from our affected LCD1 patients (data not shown).

These results indicate that the pathogenesis of the corneal dystrophy seen in LCD1 is not due specifically to the GSN fragment but that other amyloidotic proteins can also deposit in the cornea. FAF is characterized by a systemic distribution of amyloid deposits in the cornea. FAF is characterized by a systemic distribution of amyloid deposits in addition to corneal deposits (Meretoja 1969; Purcell et al. 1983; Darras et al. 1986). LCD1, on the other hand, appears to have amyloid deposition localized solely to the stromal layer of the cornea (Klintworth 1971). It then follows that the localized nature of the amyloid deposition in LCD1 might be of a different origin than are the systemic deposits seen in FAF. A search for mutations in other candidate genes, including prealbumin (transthyretin) and apolipoprotein-AI, as well as the isolation and characterization of the corneal amyloid from LCD1 corneas, are in progress.

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human genetics

APPENDIX 2

Identification of an uncommon haptoglobin type using DNA and protein analysis

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Abstract. The inherited variations in haptoglobin phenotypes are attributed to the homozygous and heterozygous combinations of three common autosomal alleles: *HP*1F, HP*1S* and *HP*2. HP*1F*and *HP*1S* encode polypeptides that differ by two amino acids at positions 51 and 53. The formation of *HP*2* is postulated to have resulted from a breakage and subsequent reunion event at non-homologous positions of two *HP*1* alleles. The most common form of *HP*2* is *HP*2FS* in which the 5′end of *HP*2* resembles *HP*1F* and the 3′ end resembles *HP*1S*. Homologous crossing over between *HP*2* and either an *HP*1F* or *HP*1S* allele in *HP*2/HP*1* heterozygotes can change the usual type of *HP*2* to three other forms: *HP*2SS, HP*2FF* or *HP*2SF*. We describe a nuclear family in which the uncommon genotype *HP*2SS* in one parent caused initial confusion in assigning genotypes to the rest of the nuclear family. The data demonstrate the need for a cautious approach when deducing haptoglobin genotypes from molecular analysis alone.

Introduction

Haptoglobin, synthesized in the liver, is secreted into the plasma where it complexes with hemoglobin. Haptoglobin is believed to prevent the loss of heme at the level of the kidney tubule and to aid in the reabsorption of hemoglobin in the liver (Raugei et al. 1983). Haptoglobin is a tetramer consisting of two α-chains and two β-chains. The α- and β-chains are generated by cleavage of the peptide produced by a single mRNA encoded by the haptoglobin locus on chromosome 16.

Haptoglobin (HP) is postulated to have evolved from a common ancestor to the serine protease family. Normal heritable variation of human HP was demonstrated by starch gel electrophoresis of plasma samples by Smithies in 1955 who described three common phenotypes: HP1, HP1,2 and HP2. These varients are due to two common α-chain alleles: *HP*1* and *HP*2*. The phenotypic differences observed by starch gel electrophoresis are attributed to differences in rates of migration of tetramers generated from HP polypeptides. Homopolymers $(HP1\alpha, HP\beta)_2$ in *HP*1/HP*1* individuals migrate as a single fast band (HP1), whereas polypeptides in *HP*2/HP*1* and *HP*2/HP*2* individuals, respectively, form various heteropolymers that migrate as a series of slower bands (HP1,2 and HP2). Additional allelic variation of the *HP*1* allele is due to the *HP*1F* and *HP*1S* alleles. Their designation is based on fast (F) and slow (S) migration during electrophoresis. These alleles encode polypeptides HP1Fα and HP1Sα, respectively. They differ only by two amino acids at positions 52 and 53, aspartic acid and lysine in HP1Fα and asparagine and glutamic acid in HP1Sα[3]. The *HP*2* allele has an internal duplication of 1.7kb that includes two of the α-chain exons. The HP2α polypeptide consists of 142 amino acids, while the HP1α polypeptide has 83 amino acids (Maeda 1992).

Nance and Smithies (1963) suggested that the formation of the *HP*2* allele resulted from the product of a partial gene duplication likely formed as a consequence of non-homologous crossing over within an HP*1F and HP*1S gene. Maeda et al. (1984) concluded that the breakage and reunion event was at non-homologous positions within the fourth and second introns of two HP*1 genes. Maeda et al. (1984) demonstrated that the nucleotide sequence analysis of the most common form of the *HP*2* allele is *HP*2FS* with the 5′ end of *HP*2* resembling *HP*1F* and the 3′ end resembling *HP*1S*. Homologous crossovers between *HP*1F* or *HP*1S* allele in *HP*2FS/HP*1* heterozygotes can change the usual form of *HP*2 (HP*2FS)* to three other rare forms: *HP*2SS, HP*2FF, HP*2SF* (Teige et al. 1986). This is illustrated in Figure 1. The frequencies of the *HP*2SS* and *HP*2FF* alleles are approximately 2.5% and 1.8%, respectively, of the total frequency of *HP*2* in the Caucasian population. Protein

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DNA in this nuclear family of interest. The HP phenotypes seen on starch gel electrophoresis (not shown) are recorded at the bottom of each designated lane as are the initial and revised *HP* genotypes. The father in lane 2 is heterozygous for the 4.6- and 4.0-kb alleles. His starch gel phenotype is HP1,2. Initially his genotype was inferred to be *HP*1S/HP*2FS.* Mother in lane 1, also heterozygous for the 4.6- and 4.0-kb alleles, was inferred to be *HP*1F/HP*1S* based on her starch gel phenotype of HP1. Assigning genotypes to the offspring in lanes 6–8 and 10–12 proved problematic. The son in lane 10 is homozygous for the 4.6-kb allele and has a starch gel phenotype of HP1. If his father were truly *HP*1S/HP*2FS,* his starch gel phenotype should be HP1,2. Similarly the son in lane 11 is HP1,2 on starch gel but is homozygous for the 4.0-kb allele on Southern blotting. Repeat analyses yielded the same results. Based on our knowledge of the phenotypic frequencies of eight blood group systems in random Manitoba Caucasian males, we estimated that only 1 in 360,000 Manitoba males could have been the father of all the children in this family. This makes the likelihood of non-paternity extremely remote. (complete data available upon request)

Alternative assignment of the father's (lane 2) genotype resolved the difficulty. If he is assigned the genotype *HP*1F/HP*2SS,* which is indistinguishable on Southern blot analysis from *HP*1S/HP*2FS,* the genotypes of his ten offspring can be assigned without ambiguity.

Discussion

The data demonstrate the potential difficulty in deducing haptoglobin genotypes from Southern analysis of *Xba*I digests alone. The most common form of the *HP*2* allele is *HP*2FS* in which the 5′ end of *HP*2* resembles *HP*1F* and the 3′ end resembles *HP*1S.* The occurrence of *HP*1F* sequence within an *HP*2FS* allele accounts for the finding that *HP*2FS* and *HP*1F* alleles both generate bands at 4.6 kb after *Xba*I digestion of genomic DNA.

In this nuclear family, if the father in lane 2 is assigned the more uncommon genotype of *HP*1F/HP*2SS,* the banding pattern would be indistinguishable from the initial incorrect assignment of *HP*1S/HP*2FS.* In this case, the *HP*1F* allele generates a 4.6-kb band and the *HP*2SS* allele gives a 4.0-kb band, the latter being identical to a *HP*1S* allele. This *HP*1F/HP*2SS* genotype now allows for the assignment of his offspring's genotypes without conflict. Unfortunately blood samples this father's parents or grandparents are not available for study, and polypeptide subtype analysis by gel electrophoresis is deemed impractical. Future analysis of the *HP* genotypes in his grandchildren will be pursued.

The possibility of alternate forms of the *HP*2* allele arising from homologous crossovers in *HP*2FS/HP*1* heterozygotes should always be considered when discrepancies exist between haptoglobin genotypes assigned from *Xba*I-digested DNA and from *HP* phenotypes seen on starch gel electrophoresis. It is thus necessary to confirm suspected rare subtypes by family study, as in this case, or by protein analysis or DNA sequencing.

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