UNIVERSITY OF STRATHCLYDE

APOLIPOPROTEIN E AND PHYTANIC ACID IN RETINITIS PIGMENTOSA

Submitted by

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This thesis is dedicated to my parents,

husband and children

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Abstract

Retinitis pigmentosa (RP) is a group of inherited progressive retinal degenerations. Various lipid abnormalities have been associated with RP. It has been demonstrated that lipids play an important function in vision. Therefore, a defect in lipid transport may lead to the retinal degeneration found in RP.

Apolipoprotein E is a polymorphic protein, which has three common isoforms E2, E3 and E4. Thus, there are six different phenotypes E2/E2, E3/E2, E4/E2, E3/E3, E4/E3 and E4/E4. A ten-fold increase in the incidence of the unusual apo E2/E2 phenotype in the German RP population was reported. In addition, a four-fold increase in the E2/E2 and an eight-fold increase of E4/E4 were reported in the Scottish RP population. In this study, increased incidence of E2/E2 was not observed. However, there was an increase in the incidence of E4/E4.

Some RP subjects who were suspected of having an apo E variant were investigated by cysteamine modification, SDS-PAGE and apo E DNA sequencing. However, the presence of mutations in these individuals was not confirmed. Therefore, the role of apo E in RP remains uncertain; although there is not a mutation, the dysfunctional apo E2 and E4 isoforms may contribute to the expression of the disease.

Refsum's disease is an autosomal recessive disorder associated with RP. The analysis of phytanic acid levels in two suspected RP patients confirmed the diagnosis. Since than, they have been treated with a low phytanic acid diet and their phytanic acid levels monitored during this project.

The investigation of the involvement of different apo E phenotypes in the transport of phytanic acid showed that the E2/E2 RP subjects have higher phytanic acid level than those with the E3/E3 phenotype. However, recently it was demonstrated in this laboratory that normal E2/E2 people also may have high phytanic acid levels compared to normal E3/E3. This finding may indicate that E2/E2 individuals transport phytanic acid less efficiently, resulting in its accumulation in the plasma.

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Lins da Cunha, M. A., Brzeski, H., Harmmer, H. M. and Converse, C. A. (1997). Apolipoprotein isoforms associated with hereditary retinal degenerations: implication for theraphy. *J. Pharm. Pharmacol.*, **49** (Suppl.4): z.

Lins da Cunha, M. A., Manerikar, N., Converse, C. A. and Brzeski, H. Apolipoprotein E and phytanic acid in retinitis pigmentosa. Presented at Scottish Lipid Discussion Group, Glasgow, June 1977.

Whitehead, J. L., Bell, C., Lins da Cunha, M. A., Converse, C. A., Hammer, H. M. and Hartes, N. E. Identification of a novel rhodopsin amino acid substitution (Ala299→Ser) in an isolated case of retinitis pigmentosa. Submitted for publication.

Introduction

1. Retinitis pigmentosa

1.1 The disease

Retinitis Pigmentosa (RP) is a group of inherited disorders which is characterised by night blindness and progressive loss of visual field as a result of the progressive degeneration of the retina which affects the rod and cone cells and the pigment epithelium layer. Although much research has been done in the field, the pathogenesis of the disease has not been defined. In consequence of this no effective treatment or cure has been found.

RP is a heterogeneous disease in which visual impairment usually manifests as night blindness and loss of peripheral vision. The age of onset of the symptoms differs in the various types of RP from early life to older years. As the diseases progresses, sufferers lose the far-peripheral field of vision leading to the impairment of central vision and in severe cases to blindness.

RP is a syndrome in which different symptoms may be manifest. The symptom of night blindness suggests that rod photoreceptors may be affected in early RP. In addition, degeneration of rod photoreceptors leads to regional visual field loss, whereas some forms of RP present symptoms indicating degeneration of both rod and cone cells. In addition to these symptoms, most RP patients develop cataracts, and some present cystoid macular oedema. Refractive errors, including myopia and astigmatism may be manifested.

Typically, the initial ophthalmoscopic changes are observed in the fundus. Most patients exhibit intraretinal pigmentation, which form a characteristic 'bone spicule' formation. This occurs as a result of the photoreceptor cell degeneration, which leads to the loss of retinal pigment epithelium cells that migrate into the retina. The intraretinal pigment is distributed around the midperipheral fundus where the rod cells are in maximal concentration.

Advanced stages of RP are characterised by attenuated retinal vessels, intraretinal pigment and the waxy pallor appearance of the optic disc.

The diagnosis of RP is based on a clinical examination in which the patient has a history of night blindness and symptoms of visual field loss and pigmentary retinopathy. The diagnosis can be confirmed by the electroretinogram (ERG), which measures the electrical potential in the retina after light excitement. The ERG is an important test in the early diagnosis of the disease especially for asymptomatic patients, who have a normal fundus examination. Most RP patients have an abnormal ERG with diminished rod response and in advanced forms nondetectable rod and cone responses are shown. Finally, the diagnosis can be completed by determining the mode of inheritance by a pedigree analysis of the family.

1.2. Prevalence

The incidence of the RP in the state of Maine (USA) was estimated to be 1 in 5200 (Bunker et al., 1984). In the UK similar results were obtained. In Birmingham, the prevalence was found to be 1 in 4869 (Bundey and Crews, 1984). These results are similar to those determined by Jay in 1978 when it was 1 in 5000.

The prevalence of RP was determined in different countries and found to be 1 in 7000 in Switzerland (Ammann et al., 1965); 1 in 4016 in China (Hu, 1982); and 1 in 4500 in Israel (Merin et al., 1976). The highest incidence of RP was found to be 1 in 1878 in the Navajo Indians (Heckenlively et al., 1981). These results indicate that the prevalence of RP is about 1 in 4000 world-wide and confirm that RP is a principal cause of blindness in the human population.

1.3. The genetic forms of retinitis pigmentosa

RP is a heterogeneous disease which comprises various genetic forms. The classification of the patients by the mode of inheritance is important to the diagnosis and allowed the identification of homogeneous groups of RP. However, the genetic type often is not easily identified, specially when faced with sporadic cases. RP can be classified by the mode of inheritance as autosomal recessive, autosomal dominant, and X-linked. In addition, sporadic (simplex) cases occur without distinct genetic form or evidence of parental consanguinity (although many of these may be autosomal recessive). The distribution of genetic types in different populations is shown in Table 1.

1.3.1 Autosomal recessive retinitis pigmentosa (ARRP)

Autosomal recessive RP is the most common form of RP, estimated to account for 16% to 33% of cases. In this mode of inheritance two copies of an identical gene must be present for the disease to establish itself. Parental consanguinity increases the chance that two individuals transmit the same recessive gene. In fact, when both parents have one autosomal recessive gene, there is a 25% chance for each of their progeny to inherit the disease (Heckenlively, 1988).

Table 1: Prevalence of genetic types of RP in different populations

Population Study	AD	AR	Simplex	X-Linked
USA (Boughman and Fishman 1983)	21.7%	16%	50%	9.0%
England (Jay, 1982)	24.4%	15.5%	52.1%	18%
China (Hu, 1982)	11%	33.1%	48.3%	7.7%

Adapted from Heckenlively, 1988.

Abbreviations:

AD – Autosomal Dominant

AR – Autosomal Recessive

Autosomal recessive RP does not have a specific characteristic. The age of onset ranges from infancy to the fifties or sixties. In ARRP, both rod-cone and cone-rod degenerations are observed. Gene mutations for the rod β-subunit of cGMP phosphodiesterase are found in ARRP (McLaughlin et al, 1993).

There are a number of RP associated syndromes inherited in the autosomal recessive form, in which other organ systems are affected. These syndromes will be discussed in detail later.

1.3.2 Autosomal dominant retinitis pigmentosa (ADRP)

Autosomal dominant RP is considered the mildest form of RP. It is the second most frequent form, accounting from 11% to 25% of cases. It has a later age of onset of the symptoms. The central vision and visual field are conserved until late in life. In fact, the visual field deteriorates later in ADRP compared to X-linked. It also has the mildest loss of visual acuity.

The autosomal dominant form can be classified by family history with a continuous inheritance including male-to-male transmission. The inheritance should be manifested in three or more generations. However, in reduced penetrance forms only two generations could appear. The subclassification of ADRP is based on onset of the disease, gene penetrance and pattern of degeneration: predominant rod versus rod and cone dysfunction. Another criterion used is electrophysiological tests. Various names were given to the sub-groups according to the investigation. Two types were suggested by Massof and Finkelstein (1981) who classified the types I and II. Fishman and colleagues (1985), classified ADRP into four types, according on clinical and electrophysiologic features. Type 1 and 2 are similar to those of

Massof's I and II types. Lyness at al (1985) classified ADRP into two major groups: D (diffuse) and R (regional) types. Type D, corresponding to type I of Massof and Finkelstein, is characterised by diffuse loss of rod function with a later loss of cone function. This suggests that the rod is affected primarily and cone loss is secondary (Lyness at al., 1985). The patient has early onset of night blindness but field losses and retinal pigmentation appear later. Although this type presents diminished rod sensitivity, moderate levels of rhodopsin are present in the retina suggesting that rod structure may continue to function well for some time. On the other hand, Type R or type II of Massof and Finkelstein has a regional loss of both rod and cone function with later onset of night blindness. In this type a correlation is observed between loss of light sensitivity and the reduction in rhodopsin concentration (Kemp et al., 1988). Some families classified as ADRP (R-type) can manifest complete penetrance (CP) or incomplete penetrance (IP) of the disease (Voaden, 1991). About 30% of obligate carriers with reduced penetrance are asymptomatic although they have affected parents and offspring.

The primarily cause of the disease seems to be related to specific gene mutations for proteins and enzymes essential to the visual transduction (Dryja et al, 1991; Humphries et al, 1992). In about 25% of ADRP patients, the causative factor has been attributed to gene mutations. Over 40 different rhodopsin gene mutations have being identified in ADRP patients. In other patients, the combination gene mutation of the two unlinked rod outer segment proteins, peripherin (chromosome 6p) and ROM1 (chromosome 11q), seem to act in a recessive form resulting in digenic retinal degeneration (Kajiwara et al, 1994).

1.3.3. X-Linked retinitis pigmentosa (XLRP)

X-linked RP is considered to be the most severe form of RP. The frequency of XLRP in different countries ranges between 6% to 9% of cases. However, the United Kingdom presents the highest frequency of XLRP, comprising 20% of their RP population (Jay, 1982). This may suggest that the gene has been present in the population for many generations.

XLRP is characterised by early onset of night blindness which occurs in the first decade of life, progressive constriction of visual fields in the second decade developing to blindness by the 3rd (Merin, 1991). Only males are severely affected. It is explained by the fact that the disease is determined by a recessive gene carried on the X-chromosome. Males have only one X-chromosome, so they will be affected if they inherit the X-linked recessive gene from their mother. In addition, the affected males will transmit the gene to all daughters who then have a 50% chance of spreading the gene to each offspring.

X-linked recessive carrier females can be identified by clinical or laboratory investigation. The severity of the retinal degeneration depends on whether or not the defective X-chromosome is activated in the cells of the retina. However the symptoms in carriers are not as severe as in the affected males. Female carriers have a 50% chance of having an affected son and 50% of having a carrier daughter with each childbirth.

There are two forms of XLRP: RP2 and RP3 types. In type RP2, the carrier female show scattered patches of pigmentary degeneration of the retina, whereas type RP3, has a characteristic golden tapetal-like fundus reflex (Weleber, 1994).

Linkage studies indicate that there are at least two gene loci on the X chromosome involved in X-linked RP. Both genes are located on the short arm of X chromosome. Type RP2 is located on Xp11 (Bhattacharya et al, 1984) and type RP3 on Xp21 (Nussbaum et al, 1985).

1.3.4. Simplex and multiplex retinitis pigmentosa

Simplex is the term used when RP occurs as isolated cases with no family history of a similar disease and without parenteral consanguinity. If there is more than one affected in a sibship, then it is classified as multiplex. As can be seen in Table 1, the simplex form comprises the largest type of RP.

There are various reasons that can explain the large frequency of simplex. Many RP patients whose inheritance is insufficiently defined are misclassified as simplex. Some of them may be autosomal recessive. Others may be autosomal dominant or X-linked. In fact, some cases correspond to acquired disease while others are hereditary without a family history of RP. Some cases may be result from new mutations of the RP gene or a multifactorial or polygenic inheritance (Boughman, 1982). This suggests that additional factors, either genetic or environmental, may contribute to the expression of the disease in some cases (Heckenlively et al.,1988).

1.4. Syndromes associated with retinitis pigmentosa

Retinitis Pigmentosa is a heterogeneous disease, which is often associated with systemic disorders. These syndromes have an autosomal recessive inheritance form. There are various syndromes related with RP which are associated with

abnormal lipid metabolism (Table 2). Some of them are not common. Four syndromes will be discussed briefly, and Refsum's disease will be discussed in more detail, due to its importance in this project.

1.4.1. Usher syndrome

Usher syndrome is the most common disease associated with RP. It is estimated that between 6% and 10% of RP patients have this syndrome (Heckenlively, 1988). It is characterized by congenital neurosensory hearing loss and RP. Usher syndrome can be classified in four types. Types I and II are determined by different genetic entities. Type III and type IV differ clinically and may also be caused by different genes (Merin, 1991).

Usher syndrome type I is characterized by night blindness in the first or second decade of life and complete congenital deafness with often incomprehensible speech. Vestibular functions are absent or abnormal. Type I has normal mental development. The retinal changes are typical of RP with visual field loss in the first decade. By the early forties, the patients are extremely affected.

In Usher syndrome type II, the patients have congenital sensorineural hearing loss and normal vestibular function with intelligible speech. The onset of the symptoms is later and night blindness occurs in the second or third decade of life. In addition the vision is preserved until the fifth or sixth decade. Type II is autosomal recessive but it has a different gene than the one for type I (Merin, 1991). The diagnosis of retinitis pigmentosa as part of Usher's disease type I and II can be realized in early life with electroretinography testing. However, no tests are available to classify the carriers of Usher's syndrome types I and II.

Table 2: RP syndromes associated with abnormal lipid metabolism

Syndrome	Symptoms	Lipid abnormality
Refsum's disease	Retinitis pigmentosa Peripheral neuropathy muscle wastage	Raised plasma phytanic acid levels (10-30mg/dl).
Zellweger syndrome	Retinitis pigmentos Hearing impairment Hepatomegaly	Increased levels of long chain polyunsaturated fatty acids in all tissues. Elevated plasma phytanic acid.
Bassen-Kornzweig syndrome (Abetalipoproteinemia)	RP, fat malabsorption, peripheral neuropathy, acanthocytosis	Low cholesterol and triglyceride levels; absence of LDL.
Batten's disease (Ceroid lipofuscinosis)	RP, neurological degeneration	Deposits of autofluorescent lipid pigments in the brain and nervous tissues.
Usher syndrome	RP, congenital deafness	Type IV: increased phytanic acid levels. Decreased plasma arachidonic acid and DHA in some patients

Taken from McColl and Converse (1995)

Usher syndrome type III comprises RP with congenital deafness and absence of vestibular functions. This type has a more severe retinal disease than in type I. Type III is clinically similar to type I except for the vestibular ataxia. It is also called Hallgren syndrome because it was described by Hallgren as a common disease in a large series of patients in Sweden (Weleber, 1994).

Usher syndrome type IV consists of RP with congenital complete deafness and mental retardation.

1.4.2. Bardet-Biedl syndrome

This syndrome is also called the Laurence-Moon-Bardet-Biedl (LMBB) syndrome. It consists of retinitis pigmentosa, mental retardation, obesity, hypogonadism and polydactyly. This syndrome is rare and it is transmitted by autosomal recessive inheritance. The patients present reduced ERGs. Retinal degeneration is the most common feature in this syndrome.

1.4.3. Refsum's disease

Refsum's disease is also called heredopathia atactica polyneuriformes ou phytanic acid storage disease. It was first reported in a Norwegian family by Refsum in 1945. It is a rare inherited condition of lipid metabolism in which the patient is unable to convert phytanic acid to α-hydroxy phytanic acid due to the lack of the enzyme α-hydroxylase (Mize et al., 1969b). This results in the accumulation of phytanic acid in various tissues such as liver, kidney, heart, peripheral nerves, retinal pigment epithelium, and blood (Levy, 1970).

The accumulation of phytanic acid in the plasma has been correlated with several human disorders such as Refsum's disease (Klenk and Kahlke, 1963), and the peroxisomal disorders: Zellweger syndrome, neonatal adrenoleukodystrophy (Stokke et al., 1984), infantile phytanic acid storage disease (Scotto et al., 1982; Poulos et al., 1984),), and rhizomelic chondrodysplasia punctata (Heymans et al., 1985) (Table 3).

Refsum disease is characterised by retinitis pigmentosa, hearing loss, chronic polyneuritis, increased cerebrospinal fluid levels, and ataxia. In addition, features including anosmia, cataracts, pupillary abnormalities, cardiac arrhythimias and skeletal abnormalities may be observed (Gibberd et al., 1985).

Increased levels of phytanic acid have been detected in the plasma, urine, kidney, and liver of patients with Refsum's disease. Toussaint and Dani (1971), in an ocular pathologic study of a Refsum patient, found lipid material throughout all layers of the eye, including the retinal pigment epithelium.

1.4.3.1. Dietary sources of phytanic acid

Phytanic acid is not normally present in human tissues. It is exclusively exogenous in origin, the main sources being especially dairy products and ruminant fats (Britton et al, 1989). Ruminants ingest large amounts of chlorophyll, which are degraded in the rumen by bacteria, liberating phytol. This free phytol is readily converted to phytanic acid (Steinberg, 1995). However, phytol bound in chlorophyll is poorly absorbed in humans (Baxter, 1968).

The polyisoprenoid structure of phytanic acid suggests that it might be endogenously synthesised via the geranylgeranyl pyrophosphate pathway (Glomset

Table 3: Syndromes associated with phytanic acid storage

Syndrome	Characteristic of the disorder
Refsum's disease	α-oxidase deficiency
Zellweger Syndrome	Generalised peroxisomal disorder
Infantile Refsum disease	Generalised peroxisomal disorder
Infantile adrenoleukodystrophy	Generalised peroxisomal disorder
Rhizomelic chondrodysplasia punctata	Peroxisomal disorder with multiple biochemical defects

Adapted from Masters and Crane, 1995

et al, 1990). In fact, in plants, phytol is formed from mevalonic acid by this pathway. However, experiments to demonstrate the biosynthesis of phytanic acid from [2-^{14}C] mevalonic acid in a Refsum's disease patient showed negative results (Steinberg et al, 1965). This suggested that it is unlikely that the phytanic acid accumulated in Refsum's disease originates from endogenous biosynthesis. The importance of the exogenous sources of phytanic acid is empathised by the fact that its elimination from the diet reduces body stores of this compound.

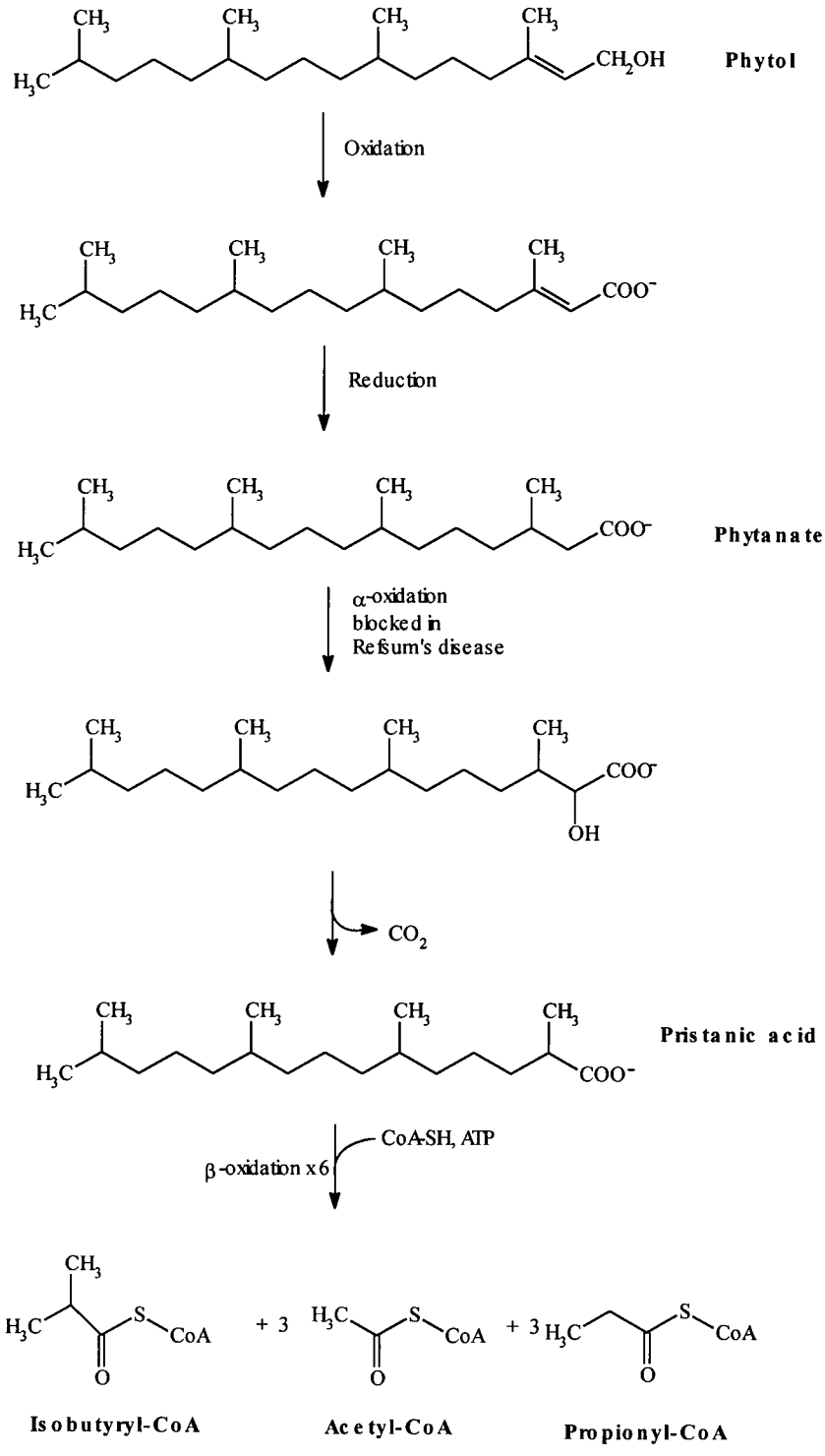
1.4.3.2. The major pathway for phytanic acid oxidation

Several studies were carried out to determine the major pathway for phytanic acid oxidation in human beings and in model animals (Avigan et al, 1966; Tsay et al, 1967 and Mize et al, 1969a). The most important pathway for the degradation of fatty acids is via β-oxidation. However, phytanic acid (3, 7, 11, 15-tetramethyl hexadecanoic acid) cannot undergo straightforward degradation to β-oxidation due to the presence of the methyl group on the C3 which interferes with the normal β-oxidation. Therefore, first phytanic acid undergoes α-oxidation, which involves an initial α-hydroxylation step, followed by decarboxylation yielding pristanic acid and CO_2. Pristanic acid is further degraded by β-oxidation to yield three molecules of acetyl-coenzyme A (CoA), three of propionyl-CoA, and one of isobutyryl-CoA. The schematic pathway for phytanic acid oxidation is shown in Figure 1.

1.4.3.3. Defective oxidation of phytanic acid

Normal animals, including human beings, have a high capacity to degrade phytanic acid and prevent its accumulation even following intake of large amounts.

Figure 1: The major pathway for phytanic acid oxidation



Adapted from Mathews and van Holde (1990)

However, patients with Refsum's disease and generalised peroxisomal disorders such as rhizomelic chondrodysplasia punctata are unable to α-oxidise phytanic acid. Due to this defect, patients with Refsum's disease have increased plasma phytanic acid levels.

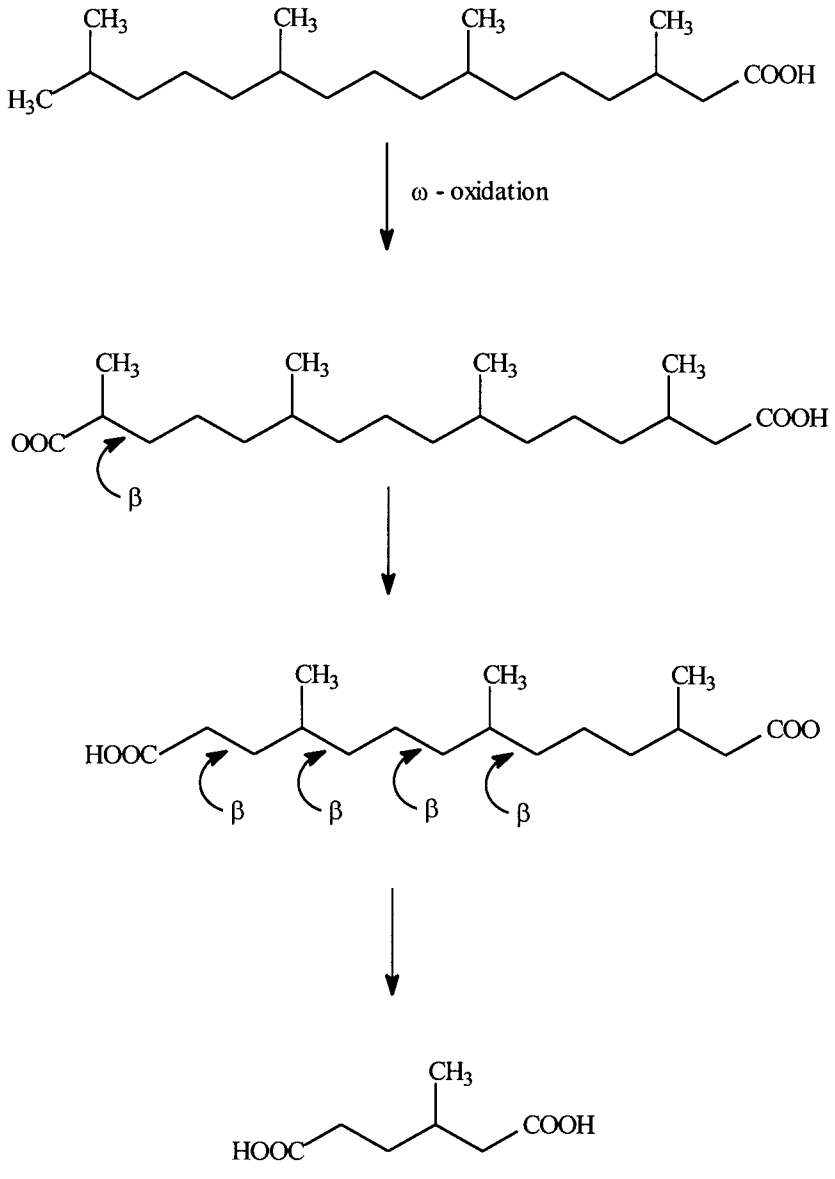
Fatty acid catabolism can also take place by ω-oxidation by initiation from the non-carboxylic end of the chain. The ω-oxidation allows successive β-oxidations to take place from the ω end without interference from the branch methyl group (Steinberg, 1995). In fact, this is the only metabolic pathway for phytanic acid in patients with Refsum's diseases (Billimoria et al, 1982). However, this pathway is limited and can not degrade the total daily intake of phytanic acid, resulting in its accumulation. On the other hand, ω-oxidation can account for the limited rate of phytanic acid oxidation in patients who are defective in the α-oxidation pathway. The ω-oxidation pathway is shown in Figure 2.

1.4.3.4. The subcellular site for phytanic acid oxidation

The major pathway for the degradation of fatty acid is via β-oxidation, which occurs in mitochondria and/or peroxisomes. However, the β-methyl group in phytanic acid prevents its oxidation via β-oxidation. Instead, phytanic acid is α-oxidised to pristanic acid, which is readily β-oxidised.

The subcellular organelle for phytanic acid α-oxidation remains controversial since this process has been described to occur in mitochondria, peroxisomes, and endoplasmatic reticulum. The identification of this organelle is essential for the understanding of the enzyme system involved in its catabolism and the molecular

Figure 2: Schematic pathway for phytanic acid catabolism via ω-oxidation followed by β-oxidation



3-Methyladipic acid

Adapted from Billimoria et al (1982)

defect in the degradation of phytanic acid in Refsum's disease. Earlier studies showed evidence that α-oxidation of phytanic acid to pristanic acid occurs in mitochondria from rat livers (Tsai et al, 1969; Muralidham and Kishimoto, 1984; Skjeldal and Stokke, 1987), monkeys and humans (Watkins et al, 1990). In addition, α-oxidation of phytanic acid is also reported to take place in the endoplasmatic reticulum from rat liver (Huang et al, 1992).

In view of these studies, Watkins and coworkers (1990) proposed that Refsum's disease is probably a mitochondrial disorder. However, the fact that the accumulation of phytanic acid in biogenesis peroxisomal diseases is due to the defective oxidation of phytanic acid, suggests that peroxisomes play a role in the catabolism of phytanic acid. In fact, it was also demonstrated that in human tissues phytanic acid is oxidised in peroxisomes (Singh et al, 1992 and 1993). In order to elucidate these controversial results, it is necessary to determine the enzyme system responsible for the oxidation of phytanic acid.

Fatty acid needs to be converted to its CoA-derivative prior to being transported into mitochondria or peroxisomes for its β-oxidation. Early studies indicated that the phytanic acid α-oxidation involved free phytanic acid rather than phytanoyl-CoA. However, recently, it was demonstrated that phytanic acid is first activated to its derivative phytanoyl-CoA ester prior to α-oxidation (Watkins et al, 1994; Mihalik et al, 1995). The next step involved the conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA via a dioxygenase. This reaction is catalysed by the enzyme phytanoyl-CoA hydroxiylase, and Fe^{++} and 2-oxoglutarate are cofactors (Mihalik et al, 1995). Based on these results a new phytanic acid α-oxidation

pathway was proposed (Jansen et al, 1996) (Figure 3). Phytanoyl-CoA hydroxylase was found to be present in human liver peroxisomes and to be deficient in rat liver peroxisomes and in liver from Zellweger patients. This could explain the fact that phytanic acid oxidation is defective in biogenesis peroxisomal disorders (Jansen et al, 1996). It was recently reported that in liver tissues from Refsum's patients the activity of the enzyme phytanoyl-CoA hydroxylase was not detected, suggesting that Refsum's disease is a peroxisomal disorder (Jansen, et al, 1997).

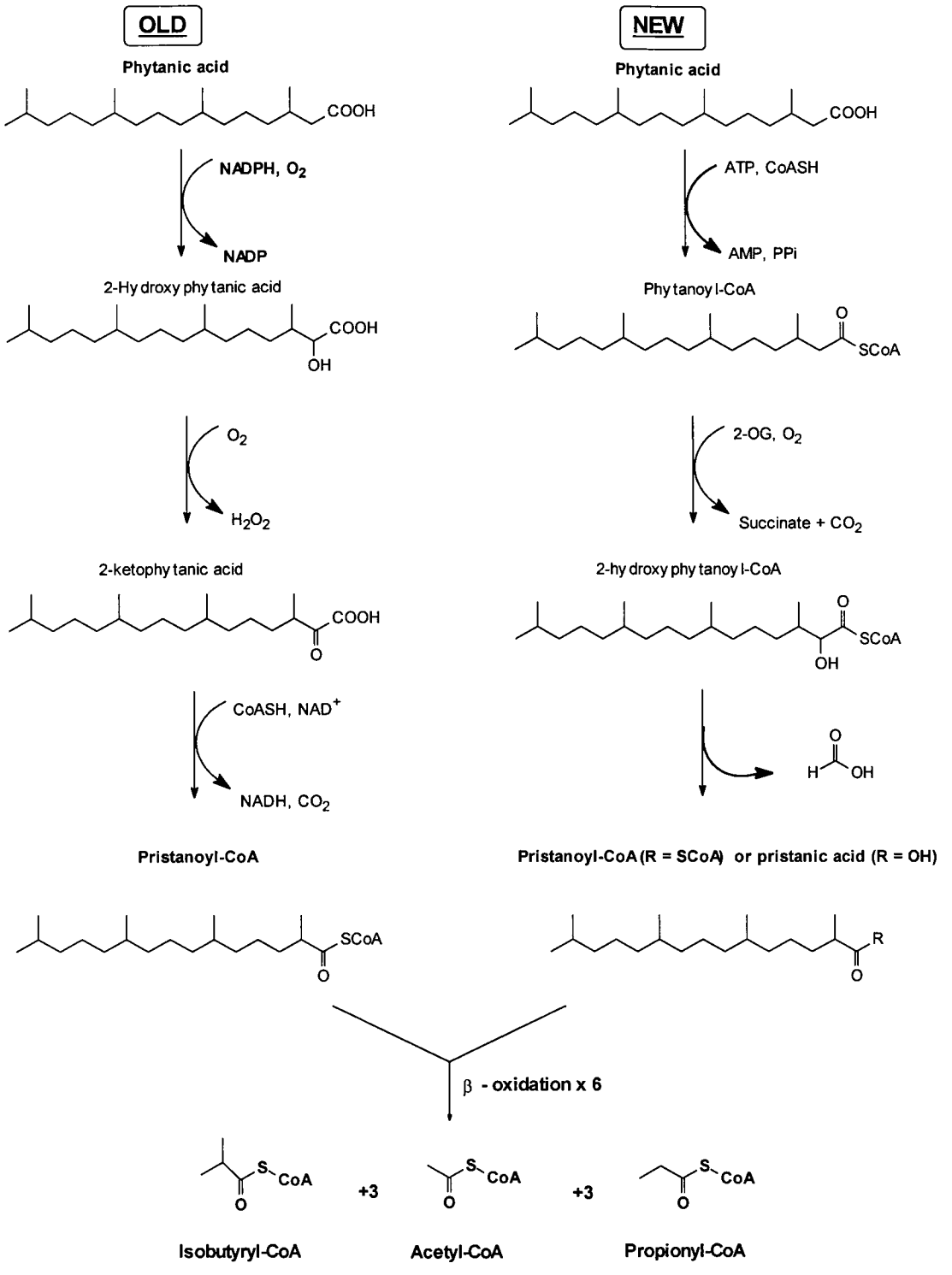
1.4.3.5. Treatment

In humans, phytanic acid is exogenous in origin and dietary phytanic acid is the major source (Steinberg, 1995). Therefore, the elimination of phytanic acid and its precursors from the diet may prevent further accumulation.

The phytanic acid and phytol content of a variety of foodstuffs were analysed (Brown et al, 1993). The amount of phytanic acid in vegetable foods was found to be insignificant and these foods have been included in low phytanic acid diets (Coppack et al, 1988). In the UK diet, the principal source of this fatty acid was found to be foods derived from ruminant animals and fish. They include beef, lamb, and milk and its derivative products from cows, goats and sheep. Fish and fish oils in blended fats were also found to contain phytanic acid (Brown et al, 1993).

Fatty acid catabolism can also be initiated from the non-carboxyl end of the chain by ω-oxidation. In normal people the oxidation of phytanic is so quickly that only a small amount is oxidised by ω-oxidation. In Refsum's disease, it is likely that this is the only metabolic pathway for phytanic acid oxidation. However, ω-oxidation

Figure 3: Comparison between old and new pathways for phytanic acid α-oxidation



Adapted from Jansen et al (1996)

is not capable to metabolise the daily intake of phytanic acid, since its capacity is limited to only 10mg phytanic acid a day and a normal diet is estimated to contain 50-100mg phytanic acid, resulting in phytanic acid accumulation. However, when dietary phytanic acid is kept low (daily intake < 10mg), the ω-oxidation may allow patients to clear their body stores (Billimoria et al, 1982).

Depending on phytanic acid levels, the diet may be started on liquid, phytanic acid free (Gibberd et al, 1979), or liquid/solid, or solid with less than 10mg phytanic acid per day (Claridge et al, 1992). The response may be take months after initiating the diet. In fact, the plasma phytanic acid level falls gradually over a long period (Claridge et al, 1992). Furthermore, the plasma phytanic acid level correlates well with the clinical condition, but does not correspond to the total body phytanic acid content since it is stored in adipose tissues. Therefore, success of treatment depends on the patient maintaining body weight. A loss of body weight leads to release of phytanic acid from tissue stores increasing its plasma levels and causing exacerbation of the symptoms (Berson, 1987; Claridge et al, 1992).

In addition to diet, plasmapheresis may also be used when the patient is severely affected (Gibberd et al, 1979; Harari et al, 1991). Plasma exchange may lower the phytanic acid level rapidly and prevent possible fatal consequences (Claridge et al, 1992). Dialysis has no effect since phytanic acid is bound to lipoproteins (Gibberd et al, 1979).

When the treatment succeeds there is a regression of several symptoms especially improvement in motor conduction velocity, ataxia, and skin manifestations (Claridge et al,1992). The polyneuropathy may be completely reversible (Gibberd et

al, 1979). Improvement in both vision and hearing have not been reported, but it has been observed that the rate of deterioration slows down or there may not be further progression (Djupesland et al 1983).

1.4.4. Abetalipoproteinaemia

Abetalipoproteinaemia or Bassen-Kornzweig syndrome is characterized by fat malabsorption, night blindness, spinal cerebellar ataxia, acanthocytosis and a deficiency in fat-soluble vitamins and essential fatty acids. It also presents as low plasma cholesterol and triglyceride levels due to failure to synthesize apolipoprotein B. The diagnosis is based on a lack of identifiable apolipoprotein B in plasma. In this syndrome patients seem to have an inability to synthesize apolipoprotein B-100 (Berson, 1994), which is the major protein of very low-density lipoproteins (VLDL) and low density lipoproteins (LDL). It also is characterized by an incapacity to synthesize apolipoprotein B-48, which is a major protein of intestinal chylomicrons. In an absence of chylomicrons the patients cannot absorb fatty acids and fat-soluble vitamins (Fliesler and Anderson, 1983). In consequence of this patients have a deficiency of vitamins A and B in plasma with consequent effects on retinal function. In this syndrome, the retinal degeneration may be caused by the absence of LDL and consequently deficiency of lipids and vitamins that are essential for the maintenance of rod outer segments.

Abetalipoproteinaemia can be treated with large doses of vitamins A and E, and essential fatty acid supplementation (Schaefer, 1987). This treatment can prevent the progression of the retinal degeneration and can also reverse the ERG responses to

normal in the early stages. However more advanced stages have not responded to therapy (Berson, 1994).

As can be seen, in both abetalipoproteinaemia and Refsum's disease a defect in lipid metabolism is involved. In fact, large amounts of polyunsaturated fatty acids especially docosahexaenoic acid (DHA), which comprises about 50% of the esterified fatty acids of rod outer segment phospholipids, are found in the rod outer segment disc membranes of the retina (Fliesler and Anderson, 1983). The constant process of outer segment regeneration needs the supply of fatty acids particularly DHA, consequently any disproportion of DHA may be lead to their degeneration. In addition, it has been reported that animals deprived of dietary DHA developed visual dysfunction (Neuringer et al, 1984 and 1986).

The symptoms of retinitis pigmentosa are manifested in the early stages in patients with abetalipoproteinemia and Refsum's disease. Because they are treatable, it is important to consider these syndromes in newly diagnosed patients. In fact, except for abetalipoproteinemia and Refsum's disease there is no treatment for the retinal degeneration found in RP.

1.5. The retina

1.5.1. Structure of the retina

Structurally and functionally, the retina can be divided into two parts: the non-neural retina or retinal pigment epithelium, and the neural retina. The neural retina consists of several distinct layers including photoreceptor cells and various neuronal cells. A schematical diagram is shown in Figure 4.

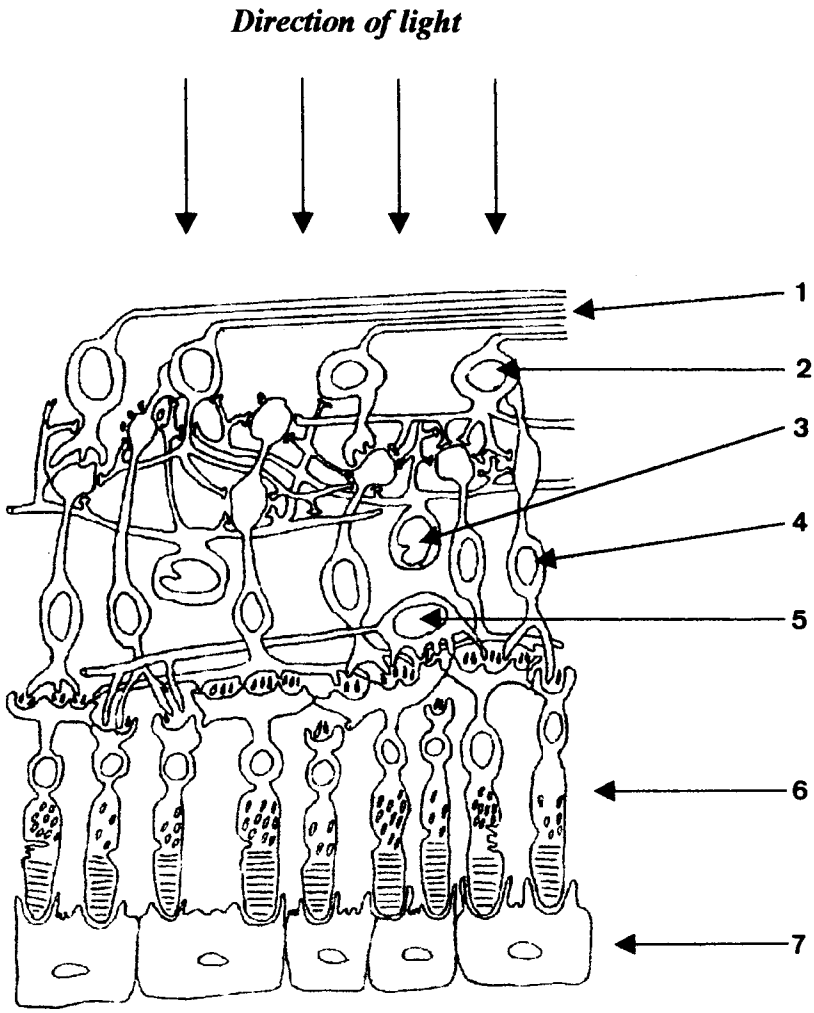
The photoreceptor cells contain light sensitive pigments. They are classified according to their shape as rod and cone cells. Rod cells are specialised for detection of low light levels (scotopic vision) and do not distinguish colour, whereas, cones function in bright light (photopic vision) and are responsible for colour vision. The human eye contains about a hundred million rods and about six million cones (Berman et al, 1991).

The photoreceptors have complex structure and consist of four distinct regions: the outer segment, the inner segment, the cell nucleus, and the synaptic pedicule. A schematic diagram of a rod cell is shown in Figure 5. Major structural differences are seen in rods and cones in that their outer segments have different photopigments incorporated into their membranes. In rod cells, this pigment is called rhodopin while cone cells have red, blue, and green pigments (Mathews and van Holde, 1990).

1.5.2. Rod outer segment

The rod outer segment (ROS) is the light-sensitive region of the receptor. Each ROS consists of a stack of membranous disc-like structures separated from a

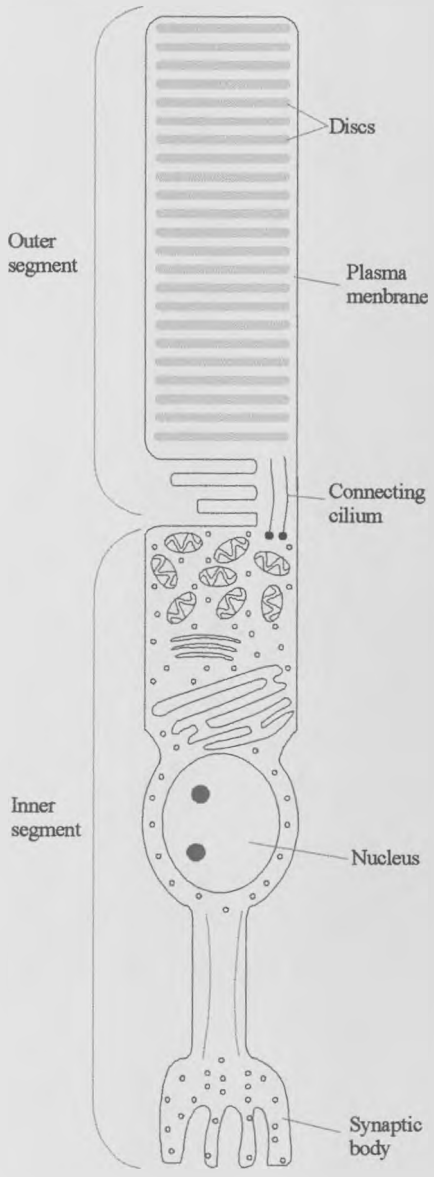
Figure 4: Schematic diagram of the layers of the retina



1 – Optic nerve fibers
2 – Ganglial neurons
3 – Amacrine cells
4 – Bipolar neurons
5 – Horizontal cell
6 – Photoreceptors (rods and cones)
7 – Pigmented epithelial cells

Adapted from Mathews and van Holde (1990)

Figure 5: Schematic diagram of the rod cell



Adapted from Stryer, 1995

surrounding plasma membrane. In cone outer segments, the lamellar structures are in continuity with the plasma membrane.

Outer segments disks in both rods and cones are formed from evagination of the plasma membrane of the cilium that joins the inner and outer segment. They are constantly removed by the phagocyte action of the pigment epithelium. This constant renewal of the ROS is essential for the maintenance of normal retinal light sensitivity (Roof and Heth, 1994).

1.5.3. Visual transduction

Visual transduction is the process by which light absorbed by the photoreceptors of the outer segment is converted to an electrical signal. This process involves various substances including vitamin A, rhodopsin, transducin, phosphodiesterase, cyclic GMP, arrestin or S-antigen, and interstitial retinal binding protein. These components interact to produce the photoresponse necessary for visual transduction.

Vitamin A is obtained in the diet in the form of a retinyl ester or β-carotene. It is then converted to retinaldehyde and reduced to retinol. In the intestinal mucosa it forms a complex with a specific retinol-binding protein, and is esterified to retinyl esters. It is then incorporated into chylomicrons and transported to the liver. After secretion it is transported to cells such as the retinal pigment epithelium (RPE), where it binds to cytosol retinal binding protein (Saari et al., 1982). The retinol undergoes further modifications until 11-retinaldehyde is formed. This is stored in the RPE until required to be utilised by the photoreceptors.

Rhodopsin is the visual pigment found in rod photoreceptor cells. It consists of a protein component, opsin, linked to a chromophore 11-cis retinal via a protonated Schiff base to a lysine side chain amino group. The absorption of a single photon of light by 11-cis retinal, results in its isomerisation to all-trans retinal. This initiates a series of conformational changes until metarhodopsin II is formed. Metarhodopsin II (R*) is an activated form of rhodopsin. A cascade of events is initiated, involving the proteins transducin, cyclic GMP phosphodiesterase and cyclic GMP. This cascade generates an impulse by closing ion channels in the rod cell membrane.

Metarhodopsin II activates a G-protein, transducin, by replacement of GDP with GTP in the transducin α-subunit (Tα). The complex Tα-GTP is dissociated from R* and activates phosphodiesterase, which hydrolyses the cGMP to 5GMP lowering the cGMP concentration in the rod cell. Depletion of cGMP leads to a subsequent closure of sodium channels, hyperpolarisation of the rod cell membrane, and electrical discharge (Mathews and Holde, 1990).

The termination of the cascade of events involves several mechanisms: sequential phosphorylation of R* by rhodopsin kinase followed by binding of another protein called arrestin (48K protein), and the Schiff base linkage to all-trans retinal becomes hydrolysed. Rhodopsin is regenerated by binding a new 11-cis retinal.

1.6. Retinitis pigmentosa research

Many studies have been carried out world-wide to determine the biochemical

and gene defects responsible of RP. This could be useful to the heterogeneity of RP and for an effective treatment to be developed.

1.6.1. Biochemical defects in retinitis pigmentosa

Biochemical studies have been undertaken in human RP and in the animal models for RP. These include studies of biochemical defects in rod outer segment renewal and in the various steps of the visual transduction cycle. Although much research has been done, the biochemical mechanisms responsible for the degeneration of the retina are still unknown.

1.6.1.1. Cyclic nucleotide defect

Cyclic guanosine 3', 5' monophosphate (cGMP) plays an important role in the visual process transduction. The concentration of cGMP in the rod outer segment is much higher than in other tissues. In addition, the cGMP metabolism is modulated by light. Evidence from animal models such as the retinal dystrophy (rd) mouse, Royal College of Surgeons (RCS) rats and dystrophic Irish setter dogs suggests that a decreased phosphodiesterase (PDE) activity results in increased levels of retinal cGMP, which is toxic to the photoreceptors (Chader et al, 1987). In addition, increased levels of retinal cGMP were observed in a study carried out on a human retinal tissue from a 17 year-old ADRP patient (Flannery et al, 1987). These studies suggest that there is evidence that cyclic nucleotide metabolism may play a role in the pathogenesis of some forms of retinal degeneration.

1.6.1.2. Rhodopsin metabolism

Rhodopsin is the visual pigment of the rod cells involved in the visual

transduction. The concentration of rhodopsin can be measured by psychophysical methods (Kemp and Faulkner, 1981). Abnormalities in rhodopsin levels may lead to the photoreceptor degeneration found in RP. In addition, reduced rhodopsin levels are related to the loss of the photoreceptor outer segments. In fact, rhodopsin levels and loss of the photereceptor outer segments have a linear relationship in some forms of RP (Kemp et al, 1984). In others forms of RP, the rhodopsin content may be normal, but any disruption in the rhodopsin molecule or its precursor may lead to the photoreceptor cell death.

1.6.1.3. Vitamin A

Vitamin A is the precursor of retinal, the light sensitive group in rhodopsin. It is essential for the visual system. In fact, the retina requires a continuous supply of this vitamin. A deficiency of vitamin A leads to the degeneration of the retina.

RP is associated with the systemic disorder abetalipoproteinemia (see section 1.4.4). This syndrome is caused by a deficiency of apo B, which results in the absence of low density lipoproteins and chylomicrons. The lack of chylomicrons may lead a disruption of the transport of vitamin A to the liver and subsequently to the eye, resulting in retinal degeneration. Treatment with this syndrome with large doses of vitamin A, vitamin E, and essential fatty acid supplementation reverse the progression of the visual problems in these patients (Schaefer, 1987).

Although various studies have been performed, there is no conclusive evidence that the deficiency of vitamin A is involved in RP. Clinical trials using vitamin A as treatment for RP have been reported to slow but not stop the progression of the disease (Berson et al, 1993) although there is some debate about

these results. Therefore, despite these results, no conclusive evidence has been suggested that defective metabolism or transportation of vitamin A contributes for the development of the disease, or that vitamin A therapy is effective in preventing the progression of the disease. However, recently a mutation in a retinaldehyde-binding protein has been found in autosomal recessive RP patients (Maw et al, 1997).

1.6.1.4. Taurine

Taurine is a free amino acid, which is found at high concentration in the retina of various species, including humans (Voaden et al, 1977). Specifically, taurine is found in high levels in the photoreceptors and is known to have a essential role in their maintenance and structure (Passantes-Moralles, 1986).

It has been demonstrated that cats maintained on a taurine-deficient diet present diminished ERG amplitude suggesting photoreceptor degeneration (Berson, 1982). The supplementation of the diet of these cats with taurine caused the ERG to revert to normal. This suggests that taurine plays a role in photoreceptor function.

Voaden and co-workers (1989) measured the uptake of taurine in the blood platelets of RP patients. Reduced taurine uptake was observed in ADRP type II patients, but not in type I patients (Hussain and Voaden, 1987). In addition, Airaksinen et al (1980) found the same results in a Finnish study, but the types of inheritance of the RP patients were unspecified. However, the taurine clearance in the blood of RP patients who were not genetically typed was compared to controls and no significant differences were detected (Benine et al, 1988). It could be suggested that taurine may play a role in some types of RP, however further studies with genetically classified RP are warranted.

1.6.2. Immunological studies in RP

Several reports have indicated alterations in cellular immunity in RP. Reactivity to retinal S-antigen and altered functions of T-cells have been described (Gailbraith and Fudenberg, 1984). In addition, monocytes from RP patients expressed diminished amounts of class II antigen (HLA-DR) and their mononuclear cells produced subnormal levels of interferon-gamma (Detrick and Hooks, 1987). Since both gamma-interferon and class II antigen are essential regulators of efficient immunoreactivity, these results may indicate an imbalance in the immune system in RP patients. This may suggest that some immune system activation may occur in association with the retinal degeneration. However, the contribution of immune factors to RP pathology remains unclear.

1.6.3. Molecular biology

RP is a group of inherited retinal degenerations that is genetically heterogeneous. The advances in the molecular biology field have facilitated the investigation of the gene defects responsible for RP. However, the heterogeneity of the disease may be the result of multiple mutant alleles at a single locus or multiple loci. This could be an obstacle for the identification of the defective gene, since any variation in a given candidate gene is likely to be present only in a small portion of the RP population.

Mutations have been described in RP in the rhodopsin gene (Dryja et al, 1990), the peripherin/rds gene (Farrar et al, 1991), the β-subunity of the rod cGMP

phosphodiesterase (McLaughlin et al, 1993), and the α-subunity of the cGMP-gated channels (Dryja et al,1995), among others.

McWilliam and coworkers (1989) were the first to detect linkage of ADRP to the polymorphic marker D3S47 on the long arm of chromosome 3. Since rhodopsin is located at 3q21-3 it was postulated that this may be the candidate gene for the ADRP form.

So far, at least 60 different rhodopsin mutations have been described (Rosenfeld and Dryja, 1995). About 25-30% of these mutations have been found in ADRP. Only a few, have been related to the autosomal recessive form of RP, and others have not been connected with particular types of RP. It seems that rhodopsin plays a role in the photoreceptor and any opsin mutation may interfere with normal photoreceptor function. However, it is not clear how these mutations lead to the photoreceptor degeneration (Rosenfeld and Dryja, 1995). There are many RP patients who apparently do not have any of the identified mutations (Bashir et al, 1992). In addition, McInnes and colleagues (1991), identified a new protein called ROM1, which has similarities to peripherin/RDS. These two membrane proteins are located in the outer segment disc rims. Mutation in ROM1 has been correlated with some forms of ADRP. Kajiwara et al (1994) described three families with mutations in the unlinked photoreceptor-specific genes ROM1 and peripherin/Rds, in which only double heterozygotes developed RP.

Humphries et al (1997) produced mice carrying a marked disruption of the rhodopsin gene. These animals may be provide a useful genetic background and could be used to express other mutant opsin transgenes and utilised as a model to

investigate the theraupetic potential of reintroducing functional rhodopsin genes into degenerating retinal tissues.

1.6.4. The importance of lipids in the retina

The retina is particularly rich in polyunsaturated fatty acids. The rod outer segment (ROS) disc membranes contain large amounts of docosahexaenoic acid (DHA) (22:6 n-3). It comprises approximately 50% of the esterified fatty acids of phospholipids. The ROS disc membranes are unusual for their high phospholipid content and low cholesterol content of the total lipid. The fatty acid composition of these phospholipids is exceptionally high in polyunsaturates. These factors contribute to the high degree of fluidity of the membrane (Fleisler and Anderson, 1983). DHA-rich phospholipids are tightly bound to rhodopsin. However, their function is still to be determined. It is likely that DHA plays a role in rhodopsin mobility and conformational changes (Wiedmann et al, 1988).

The biosynthesis and turnover of photoreceptor outer segments utilises a substantial amount of lipids. It seems that an adequate dietary supply of DHA is necessary for the maintenance of the photoreceptor structure and function. Any impairment of this process could affect ROS renewal and therefore affect retinal structure and function. It is known that DHA is not synthesised *de novo* by animals. Therefore, this fatty acid must be obtained from the diet or from dietary supply of its precursor α-linolenic acid (18:3n-3).

The retina is able to conserve DHA during n-3 fatty acid deficiency. In fact, the turnover of ROS is not affected by n-3 fatty acid deficiency (Wiegand et al, 1991), due to the ability of the retina to acquire n-3 fatty acids from the blood and

concentrate them into the photoreceptor membrane phospholipids. So the retina retains DHA despite a large amount being utilised by photoreceptors and phagocytosed by the pigment epithelium.

The possibility that DHA was recycled from RPE was proposed by Chen et al (1992). This suggests that DHA is conserved in the retina. However, this mechanism is not well understood. It was suggested that the transport of 22:6n-3 from the RPE to the retina may be mediated by specific carriers such as interphotoreceptor retinoid binding protein (Bazan et al, 1985), or lipoproteins (apo E) which have been found in the retina that affect intercellular transport of lipids (Bazan and Cai, 1990). The identification and characterisation of the carrier is necessary to elucidate the mechanism of conservation of DHA in the retina (Chen et al, 1992).

Several reports have been suggested that DHA plays a role in the structure and function of biological membranes specifically in the retina and the central nervous system. Neuringer et al (1986) reported that when pregnant rhesus monkeys and their infants were fed a low dietary intake of n-3 fatty acids, they showed about half the C22:6 n-3 content in the plasma phospholipid fraction of control animals. The retina of these animals also showed reduced levels of DHA. In addition, visual impairment with diminished ERG response were reported (Neuringer et al, 1984, 1986). It was also observed that the severe dietary deficiency of n-3 fatty acids during development of rhesus monkeys caused retinal damage which was irreversible, even though DHA was replaced (Neuringer et al, 1990). Decreased levels of C22:6 n-3 were also reported in animal models of retinitis pigmentosa such as miniature poodles when compared to controls (Wetzel et al, 1989).

These studies suggest that n-3 fatty acids play a role in retinal development and the visual process. However, it is still not clear whether deficiency of n-3 fatty acids causes retinal degeneration.

1.6.5. Lipids studies in retinitis pigmentosa

Retinitis pigmentosa has been associated with various abnormalities in lipid metabolism. Syndromes such as Refsum's disease and abetalipoproteinaemia are characterised by accumulation of plasma phytanic acid and lack of LDL, respectively. In addition, a deficiency in polyunsaturated fatty acid (PUFA) metabolism has been related to RP (Dawson and Newell, 1974). These authors observed elevated level of arachidonic acid (AA or 20:4n-6) in RP patients compared with controls.

Several groups have been reported abnormalities in the fatty acid composition of plasma lipids in RP and animals with inherited retinal degenerations. This phenomenon was first reported by Converse and coworkers (1983). In a plasma fatty acid survey in 69 RP patients, low levels of DHA were found in two X-linked families and one AD compared to unaffected members of their families. Additional studies of PUFA levels in five X-linked families showed that four of these families had low levels of DHA and two of them showed low level of arachidonic acid (20:4, n-6) as well (Converse et al, 1987). One of the X-linked families was reported to have consistently low levels of plasma and phospholipid DHA over a period of seven years (McLachlan et al, 1990).

Several studies have confirmed this finding (Gong et al, 1992 and Holman et

al, 1994). It seems that significant reduction in the blood levels of PUFA occur in all types of retinitis pigmentosa including Usher syndrome (Bazan et al, 1986).

It was also demonstrated that the miniature poodle and Abyssinian cat with progressive rod-cone degeneration also have low levels of DHA (Anderson et al, 1991).

Hoffman and coworkers (1993) determined the level of fatty acid in red cell membrane lipids and plasma lipids in ADRP patients. The plasma lipid samples showed significant increase in the n-6 PUFA whereas, the red cells had a decreased levels of n-3 and n-6 long chain PUFA. Subsequent studies of this group reported reduced levels of DHA in red blood cells from X-linked RP patients (Hoffman and Birch, 1994).

To investigate the possibility of a defect in the n-3 fatty acid pathway an oral supplementation of eicosapentaenoic (EPA or 20:5 n-3), a precursor of DHA, was administrated to a small group of ADRP patients with known rhodopsin mutation and to a control population (Hoffman et al, 1995). Raised levels of EPA and docosapentaenoic acid (22:5 n-3) were observed in both groups. However, the DHA level was found to be increased only in the control group suggesting a possibility of a metabolic defect in the final stages of DHA synthesis in the ADRP patients.

Althrough gene mutations for retina-specific proteins and enzymes are considered to be the principal factor for the development of many types of RP, all the above evidence of reduced DHA and alterations in arachidonic acid levels seem to be associated with the disease. Therefore any deficiency whether due to diet or defect in

the biosynthesis of C22:6 n-3 from its dietary precursor, or a defect in the blood lipoprotein transport system, could manifest in the retina causing degeneration.

Various studies have been reported that both hyperlipidaemia and hipolipidaemia are associated with RP. Converse and coworkers (1983) observed hyperlipidaemia in male RP patients over 35 years of age. Hypercholesterolaemia was also found in AD, AR and simplex RP in a German population (Jahn et al, 1987). Although hyperlipidaemia is more common in RP, hypolipidaemia was also observed in a few RP patients (Converse et al, 1983). All the above evidence indicates that abnormalities in lipid metabolism may play a role in the pathogenesis of the disease.

1.6.6. Apolipoprotein E in retinitis pigmentosa

Isoforms of apo E have been associated with abnormal plasma lipid level and poor uptake of lipids from the circulation (Assmann et al, 1984). Apo E is found in many organs including the brain and the retina, where it may play a role in lipid handling (Boyles et al, 1985; Mahley, 1988). It seems that a defective supply of the lipids to the retina may induce photoreceptor degeneration.

Jahn and coworkers (1987) reported a higher frequency of the unusual apo E2 isoforms in the German RP population. In addition, Huq and coworkers (1993) observed a four-fold increased in E2/E2 and an eight-fold increased in E4/E4 isoforms in the Scottish RP population. It seems that apo E plays a role in the disease.

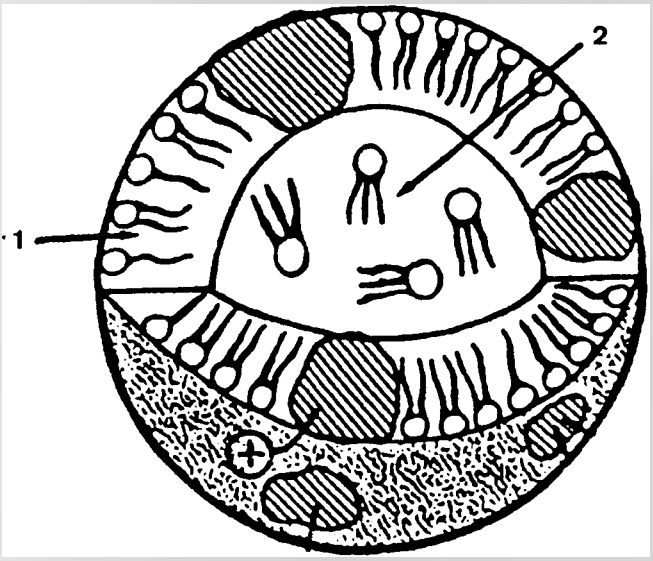
2. Lipoproteins

Lipoproteins are complex particles of protein and lipids held together by noncovalent bonds. They have various functions, a major one of which is to transport the water-insoluble lipids (triacylglycerols, cholesterol and cholesterol esters) through the body fluids. Lipoproteins have a wide range of components, and undergo complex metabolic pathways. The various types of lipoproteins share a globular structure (Figure 6). The core is made up of hydrophobic triglycerides and cholesterol esters surrounded by a hydrophilic surface layer of phospholipids, cholesterol and apolipoproteins. The phospholipid molecules are arranged on the surface of the lipoproteins so that their polar head groups face outward to the aqueous medium, while their fatty acid side chains penetrate and stabilise the interior of the particle (Tait and Shepherd, 1989). This kind of arrangement, with apolipoproteins on the surface, enables the lipoproteins to be recognised by plasma enzymes and cell receptors responsible for their catabolism.

2.1. Classification of lipoproteins

Plasma lipoproteins are classified on the basis of their densities on a salt gradient following ultracentrifugal separation. Each lipoprotein type shares the same lipid groups: cholesterol, phospholipids, cholesterol esters and triglycerides (Table 4). However each class has a different density due to varying amounts of lipid and protein. The higher the concentration of lipids, the less dense is the particle. According to increasing density, the lipoproteins are classified as chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low

Figure 6: Diagram showing the composition of a typical lipoprotein particle



1 – Polar lipid surface layer (phospholipid, choleterol, apoproteins)

2 – Non-polar lipid core (triglyceride, cholesterol ester)

Adapted from Tait and Shepherd (1989).

Table 4: Composition of lipoproteins

			PERCENTAGE OF TOTAL LIPID				
LIPOPROTEIN	**PROTEIN (%)**	**TOTAL LIPID (%)**	**TRI-GLYCERIDES**	**PHOSPHO-LIPIDS**	**CHOLESTERYL ESTERS**	**FREE CHOLESTEROL**	**FREE FATTY ACIDS**
CHYLOMICRONS	1-2	98-99	88	8	3	1	-
VLDL	7-10	90-93	56	20	15	8	1
IDL	11	89	29	26	34	9	1
LDL	21	79	13	28	48	10	1
HDL_2	33	67	16	43	31	10	-
HDL_3	57	43	13	46	29	6	6

Adapted from Mayes (1996).

density lipoprotein (LDL) and high density lipoprotein (HDL) (Table 5).

2.1.1.Chylomicrons

Chylomicrons are very large particles with a diameter of 75mm and a density range < 0.95g/ml. They consist of 98-99% lipid, of which triglyceride comprises 90%, and 2% proteins. The protein content of the chylomicrons in plasma is a higher proportion than that of those in lymph. In fact, the chylomicrons of lymph take up protein from other lipoproteins on going into the bloodstream. Chylomicrons are formed by the lymphatic system draining the intestine, their main function being to transport all dietary lipids into the circulation (Mayers, 1996). When secreted, they contain apo B-48, apo A-I and apo A-IV but after entering the plasma via the thoracic duct, they lose apo A-I and apo A-IV and acquire apo E and apo C. The apolipoprotein composition of the chylomicrons is shown in Table 6.

2.1.2. Very low density proteins (VLDL)

VLDL are large particles which float at a density of < 1.006g/ml. They are secreted into the bloodstream by hepatocytes and possibly by intestinal mucosa cells. Only 50-60% of their mass is triglyceride. Their surface contains free cholesterol, phospholipid and apo B-100. As chylomicrons, they obtain apo E and apo C in the plasma circulation from HDL. When triglyceride is removed, these additional surface components (apo C and apo E) are transferred back to HDL. The main function of VLDL is to transport the endogenous triglyceride to peripheral tissues.

Table 5: Classification and characteristics of lipoproteins

Fraction	Source	Density (g/ml)
Chylomicron	Intestine	< 0.96
VLDL	Liver and intestine	0.96 - 1.006
IDL	VLDL and chylomicrons	1.006 - 1.019
LDL	Catabolism of VLDL and IDL	1.019 - 1.063
HDL_2	Liver and intestine	1.063 - 1.125
HDL_3	Chylomicrons	1.125 - 1.210

Adapted from Marinetti (1990)

Diameter (nm)	Eletrophoretic mobility	Main function
100 - 1000	Remains at origin	Transport of exogenous triglycerides
30 -90	Pre-β	Transport of endogenous triglycerides
25 - 30	Broad-β	Precursor of LDL
20 - 25	β	Cholesterol transport
10 - 20	α	Reverse cholesterol transport
7.5 - 10	α	Reverse cholesterol transport

Table 6: The major apolipoproteins of the human plasma lipoproteins

Apolipoprotein	Lipoprotein distribution
ApoA-I	Chylomicrons, HDL
ApoA-II	Chylomicrons, HDL
ApoB-48	Chylomicrons
ApoB-100	VLDL, IDL, LDL
ApoC-I	Chylomicrons, VLDL, HDL
ApoC-II	Chylomicrons, VLDL, HDL
ApoC-III	Chylomicrons, VLDL, HDL
ApoE	Chylomicrons, VLDL, IDL, HDL

2.1.3. Intermediate density lipoprotein

In the early studies the low density lipoprotein was described as that floating at a density of 1.006-1.063g/ml. But later, it was realised that this fraction contains at least two types of lipoprotein particle. The minor subfraction is referred as LDL-1 or IDL and is found in the density range of 1.006-1.019g/ml. IDL are particles derived from the catabolism of VLDL. They are removed by liver or converted to LDL-2. Their lipid composition is intermediate between that of VLDL and LDL. The main apolipoproteins of IDL are apo C and apo E.

2.1.4. Low density lipoprotein

Low density lipoprotein (LDL-2) particles are the major sub fractions of LDL (density 1.006-1.063g/ml). They are called LDL-2 or simply LDL and are the main lipoprotein fractions of fasting plasma. LDL are formed as metabolic products of the enzymatic catabolism of VLDL via IDL and contain only apolipoprotein B. They contain up to 70% of total plasma cholesterol. Their main function is the supplying of cholesterol for various extrahepatic tissues and the transport of excess cholesterol from tissues to the liver where it can be excreted as biliary cholesterol (Tait, et al., 1989).

2.1.5. High density lipoprotein

High density lipoproteins (HDL) particles can be separated in the density range 1.063-1.21g/ml into three subfractions: HDL_1 (d = 1.055-1.085 g/ml), HDL_2 (d = 1.063-1.125) and HDL_3 (d = 1.125-1.21). They are secreted by liver and intestine

and may also be derived from surface remnants of VLDL and chylomicrons. Their main function is to take up cholesterol from cells and transport it back to the liver. This is called reverse cholesterol transport. The apolipoprotein composition of HDL is apo A-I, apo A-II, apo AIV, apo D and HDL_2 also contain apo E (Table 4).

Lipoproteins can also be classified by electrophoresis on the basis of electric charge. Electrophoresis can be carried out either on paper or on agarose gels. By this method, the lipoproteins can be classified as:

pre-β-lipoproteins (VLDL)

β-lipoproteins (LDL)

α-lipoproteins (HDL)

On electrophoresis, chylomicrons show no migration and remain at the origin (Havel and Kane, 1995).

2.2. Lipoprotein metabolism

Lipoprotein lipase (LPL) and hepatic lipase (HL) play a role in the metabolism of lipoproteins. The major function of LPL is to catalyse the hydrolysis of triglycerides of circulating chylomicrons, VLDL and IDL. However, hepatic lipase possesses a high affinity for HDL and it seems to be involved in the regulation of the metabolism of cholesterol-ester-rich lipoproteins (Marinetti, 1990). Apo C-II and apo A-I serve as cofactors for the enzymes lipoprotein lipase and hepatic lipase respectively.

There are two major pathways of lipoprotein transport: exogenous and endogenous. In addition, there is another indirect pathway called reverse cholesterol transport (Breslow, 1988).

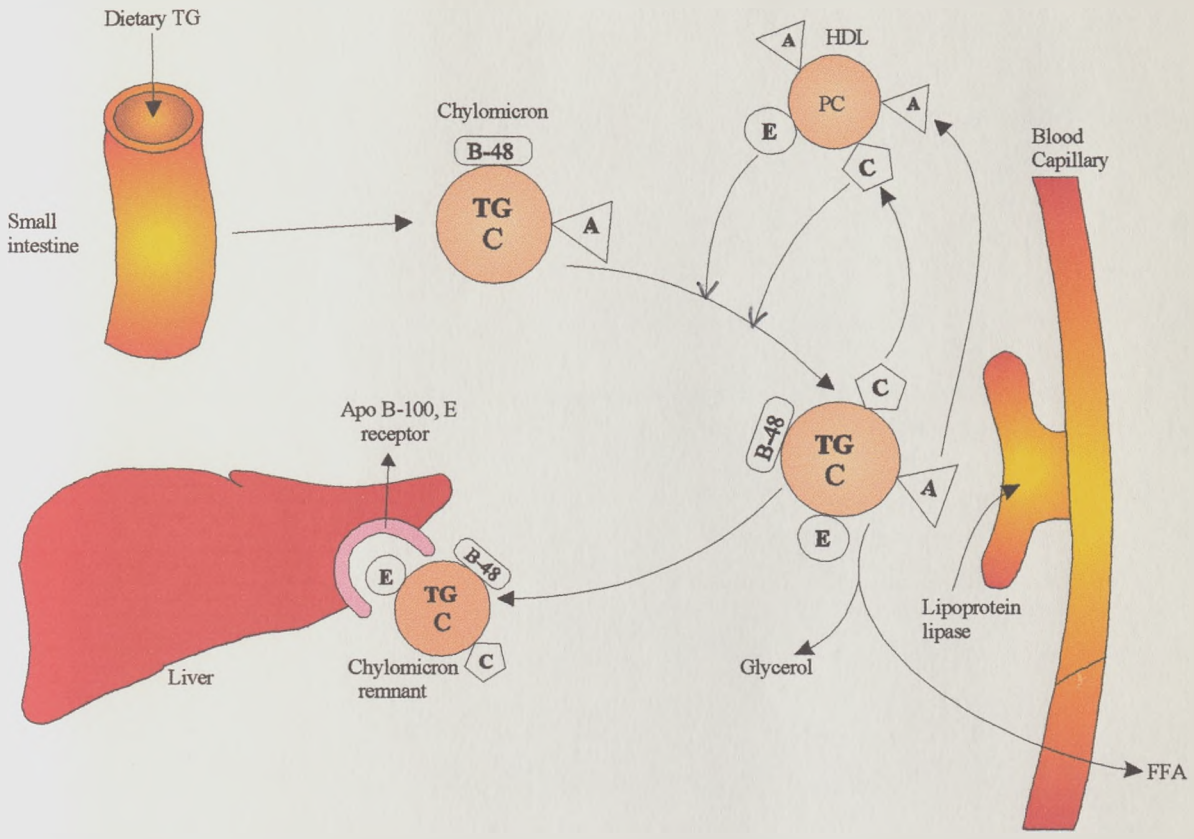
2.2.1. Exogenous pathway

Chylomicrons are formed from the exogenous fat. The lipids absorbed from the diet are hydrolysed by intestinal and pancreatic lipase. The free fatty acids and cholesterol resulting from this hydrolysis are absorbed and re-esterified in the intestinal epithelium to form triglycerides and cholesteryl esters (Fisher et al., 1989). The esterified lipids are then associated with phospholipids, free cholesterol and apolipoproteins and transported to the lymph where the chylomicrons are formed. After being synthesised, the chylomicrons enter the peripheral circulation via the thoracic duct. The newly synthesised chylomicrons contain only apo A and apo B-48. After entering the circulation, they obtain apo C and apo E from HDL (Figure 7).

Chylomicrons are degradated by the enzyme lipoprotein lipase that is located on the capillary endothelium of adipose tissues and skeletal muscles. The HPL hydrolyses the triglycerides to free fatty acid and glycerol. The free fatty acid is delivered to tissues such as adipose, for storage, and muscle, to be used as energy (Marinneti, 1990).

The resulting lipoprotein has lost most of its triglycerides and also apo C (which returns to HDL). It becomes smaller and denser and is called a chylomicron remnant. This particle is enriched in cholesterol and cholesteryl ester. It is taken up

Figure 7: Diagram of exogenous lipid transport pathway



Abbreviations:

TG – Triglycerides
C – Cholesterol and Cholesteryl ester
P – Phospholipids
FFA – Free fatty acids

Adapted from Mayes, P.A. (1996)

by the liver mediated by a specific hepatic membrane receptor that recognise apo E (Mahley at al., 1989).

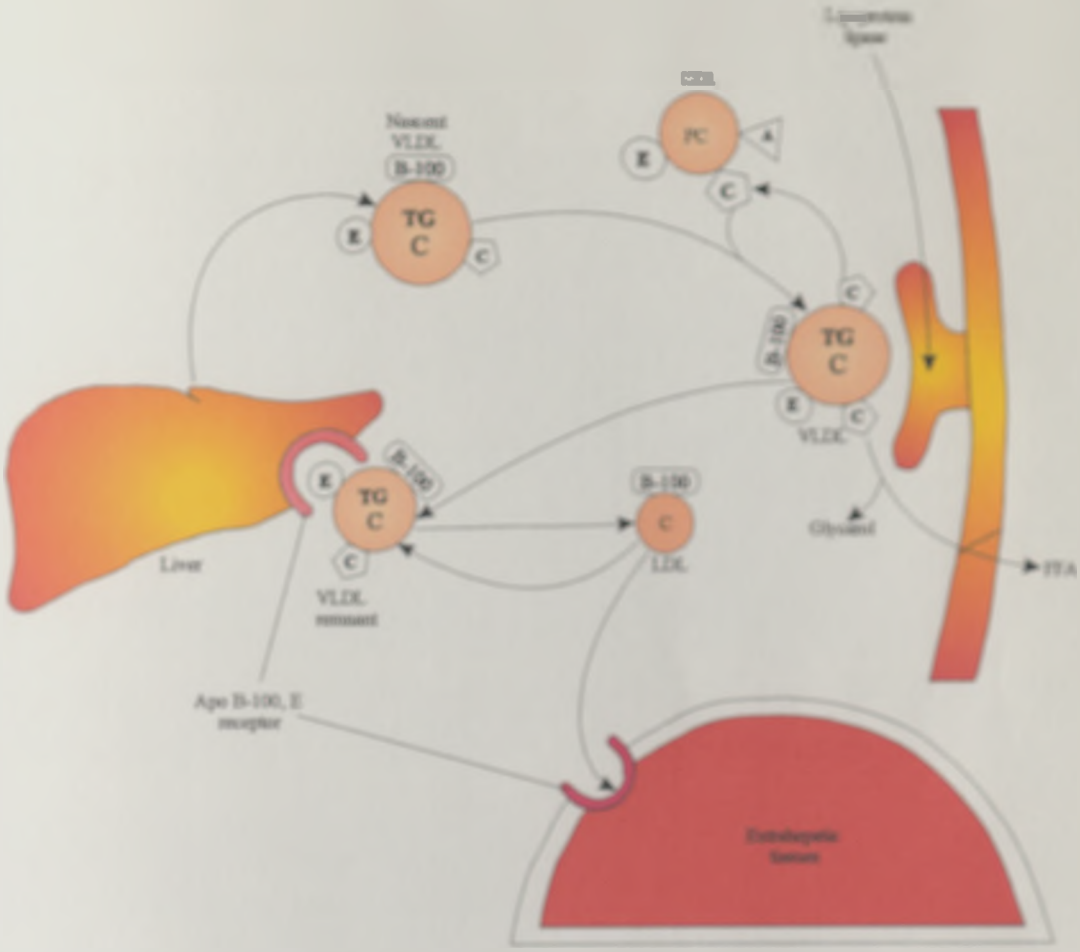
The chylomicron remnants are degraded by the enzyme hepatic lipase to smaller particles, which are enriched in cholesterol. The cholesterol is delivered to the liver, where it plays an important function in the regulation of hepatic cholesterol metabolism. The degradation products of the chylomicron remnant also are utilised for the synthesis of VLDL and HDL.

2.2.2. Endogenous pathway

Endogenous synthesis of triglycerides and cholesterol occurs in the liver where they are packaged with phospholipids and apolipoprotein and secreted as VLDL (Figure 8) (Marinetti, 1990). The nascent VLDL contain apo B-100 and apo E and as chylomicrons acquire apo C from the plasma or from HDL. Then they undergo lipolysis by the action of the enzymes lipoprotein lipase and hepatic lipoprotein lipase (Mahley et al., 1984; Lusis, 1988). The VLDL particle becomes smaller and the phospholipids, free cholesterol and apolipoprotein are released and taken up by HDL. This results in the formation of cholesterol-enriched particles called VLDL remnants or IDL (Tait et al., 1989).

The liver takes up some of the IDL via the LDL receptors, which recognise and bind apo E and apo B, and some are converted to LDL by the enzyme hepatic lipase. Therefore IDL normally are not accumulated in the plasma, because of their rapid removal or conversion to LDL.

Figure 8: Diagram of endogenous lipid transport pathway



Abbreviations: TG – Triglycerides
C – Cholesterol and Cholesteryl ester
P – Phospholipids
FFA – Free fatty acids

Adapted from Mayes, P. A. (1996)

LDL is the major cholesterol-carrying lipoprotein of plasma. It contains up to 70 per cent of the plasma cholesterol and its main function is to transport cholesterol from the liver to peripheral tissues. A specific LDL receptor (apo B/E receptor) recognises and binds LDL and thereby regulates the rate at which the cholesterol is transferred into the cells according to the cholesterol requirement for membranes,sterol hormones, or bile acid synthesis (Brown and Goldstein, 1976).

2.2.3. Reverse cholesterol transport

HDL is synthesised and secreted from both liver and intestine. Nascent HDL from intestine contains only apo A and acquires apo E and apo C from synthesised liver HDL in the circulation. A main function of HDL is to provide chylomicrons and VLDL with apo E and apo C required for their metabolism.

Nascent HDL synthesised in the liver is a discoidal particle containing phospholipid bilayers and apolipoproteins A-I, A-II, and E. In the circulation it acquires apo C and apo A from other lipoproteins and free cholesterol from extra-hepatic tissues and become enriched in cholesterol. The HDL particle acquires apo E in the interstitial fluid, which facilitates the acquisition of cholesterol by HDL. It seems that HDL-containing apo E has a special function in the delivery of cholesterol to the cells. Thus, apo E may be involved in the distribution of cholesterol from cells with excess cholesterol to other cells that requires cholesterol (Marinetti, 1990).

The free cholesterol is esterified by the action of the enzyme lecithin: cholesterol acyl transferase (LCAT), which is activated by apo A-I. Some of the cholesteryl esters are transferred to the core of the HDL particle to form mature

HDL, while some are transferred to chylomicron remnants, VLDL, IDL and LDL by cholesteryl ester transfer protein (CETP). At this stage the discoidal lipoproteins are transformed to spherical HDL particles (Eisenberg, 1984).

The HDL loses cholesterol by transferring cholesteryl esters to other lipoproteins. As result it can acquire additional cholesterol from peripheral tissues and transport them to the liver, where they are metabolised in bile acids. This process is known as reverse cholesterol transport (Figure 9).

2.3. Lipoprotein receptors

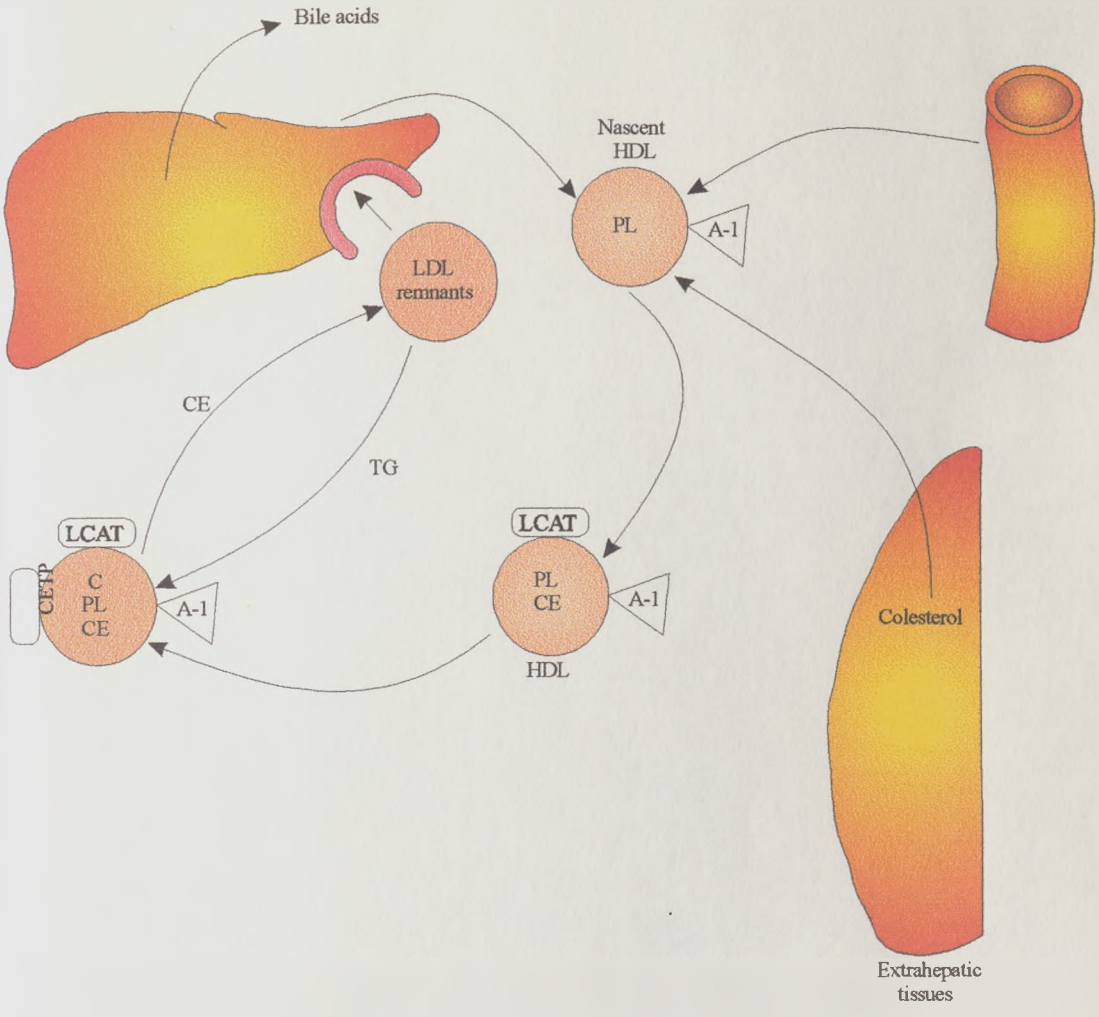
The major function of the lipoprotein receptors is to regulate plasma cholesterol homeostasis. These receptors have the ability to bind cholesterol-carrying lipoproteins and transfer them into cells by receptor mediated endocytosis. Therefore they are important in controlling the redistribution and catabolism of lipids in the body (Brown and Goldstein, 1983; Mahley et al., 1983).

2.3.1. The LDL receptor

The LDL receptor was first isolated on the surface of cultured human fibroblasts by Brown and Goldstein in 1976. Initially, it was thought that the LDL receptor bound only lipoproteins which contained apolipoprotein B-100, that is, LDL and VLDL. But then it was observed that lipoproteins which contain apo E such as HDL_c, also interact with high affinity with these receptors (Mahley et al., 1984).

The LDL receptor is also known as the apo B/E receptor. These receptors are present in both hepatic and extrahepatic tissues. The LDL receptor pathway is shown

Figure 9: Schematic diagram of reverse cholesterol transport



LCAT- Lecithin cholesterol acyltransferase

CE- Cholesterol ester

CETP - Cholesterol ester transfer protein

C-Cholesterol

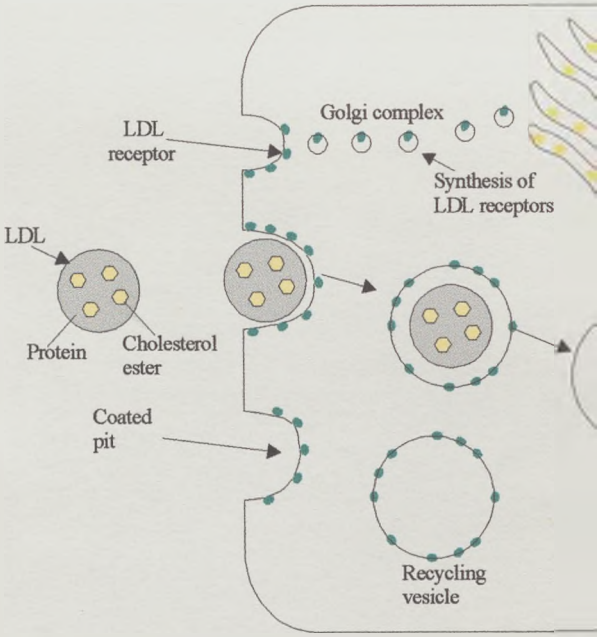
Adapted from Mayes , 1996.

in Figure 10. The LDL particle binds to the cell surface receptors (apo B/E receptor) which are located in regions called coated pits, containing the protein named clathrin. These coated pits invaginate forming coated vesicles and then fuse to form an endocytic vesicle. The LDL contained in these vesicles are delivered to lysosomes where the apo B and apo E are degraded to amino acids, and the cholesteryl esters are hydrolysed to free cholesterol by a lysosomal acid lipase (Stryer, 1995). The free cholesterol can be utilised for new membrane synthesis or can be re-esterified for storage inside the cell.

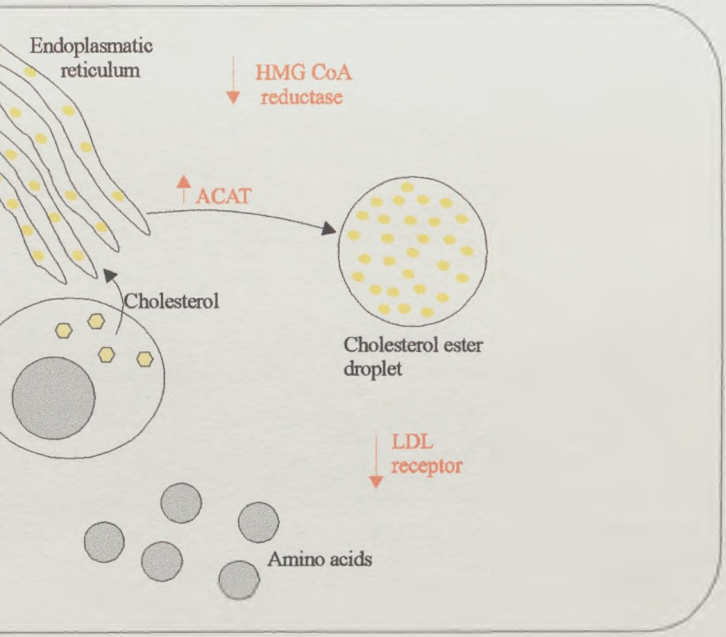
The uptake of LDL by cells is highly regulated by four mechanisms (Marinetti, 1990). First, as the cholesterol level increases in the cell, there is a suppression of the regulatory enzyme hydroxymethyl glutaryl-CoA reductase (HMGR). Second, the synthesis of the enzyme cholesterol esterase which hydrolyses cholesterol esters to free cholesterol and fatty acid is inhibited. Third, there is an activation of the enzyme acyl-CoA cholesterol acyl transferase (ACAT), which converts excess free cholesterol to cholesteryl esters, to be stored. Fourth, synthesis of the LDL receptors is inhibited. The regulation of the number of LDL receptors on the cell surface allows the cells to take up the right amount of cholesterol required for membranes, sterol hormones, or bile acid synthesis (Mayes, 1996).

Any malfunction of the LDL receptor can lead to a disease known as familial hypercholesterolaemia (FH) (Brown and Goldstein, 1976). In FH, affected individuals inherit defective genes encoding the LDL receptor protein, resulting in diminished or absent LDL receptor function. In consequence of this, the catabolism

Figure 10: The LDL-receptor pathway



Adapted from Gianturco and Bradley (1987)


Endoplasmatic reticulum
HMG CoA reductase
ACAT
Cholesterol
Cholesterol ester droplet
LDL receptor
Amino acids

of LDL is decreased resulting in decreased removal of the LDL particles from the plasma. This results in a high level of plasma LDL cholesterol, which can lead to atherosclerosis and premature death (Goldstein and Brown, 1983).

2.3.2. Chylomicron remnant receptor

The LDL receptor is the most thoroughly characterised receptor. Since its characterisation, an increasing number of related proteins have been isolated including LDL-related protein (LRP) (Herz et al., 1988), the VLDL receptor (Takahashi et al., 1992, and Sakai et al., 1993), LR8B (Novak et al., 1996), and apo E receptor 2 (apoE R2) (Kim et al., 1997). These receptors have similar functions to the LDL receptor.

The chylomicron remnant receptor, also known as the apo E receptor, was described in the liver with a high affinity for apo E-containing lipoproteins. The apo E receptor was said to be responsible for mediating receptor binding of chylomicron remnants, VLDL, and HDL-containing apo E (HDL_2 and HDL_c) (Mahley et al., 1984). In addition, it was demonstrated that in various systems including perfused liver, isolated liver membranes, and hepatocytes, there is a rapid clearance of chylomicron remnants from the plasma by apo E receptor-mediated endocytosis (Mahley and Innerarity, 1983). However, the apo E receptor itself was later shown not to exist (Beisiegel,et al 1987); the LRP performs some of its functions instead.

The LDL-related protein (LRP) is found in the liver and is located on the cell surface. It has high affinity for Ca^{2+}, which is required for binding to apolipoprotein (B, E). The structural and biochemical simularities with the LDL-receptor suggests

that LDL-related protein also recognises apo E. Therefore, it is involved in the uptake of apo E containing lipoprotein particles (Herz et al, 1988).

It has been demonstrated that the receptors and enzymes discussed in this section play special roles in lipid metabolism. Any defect, whether genetic or biochemical, may lead to profound effects on lipoprotein metabolism.

3. Apolipoproteins

Apolipoproteins are the protein constituents of plasma lipoproteins. They play an important role in plasma lipid transport and metabolism by acting as activators for enzymes or ligands for cell surface receptors (Mahley et al., 1984). Apolipoproteins have the ability to bind phospholipids located in specialised regions of the protein called amphiphatic helices. Because of this ability, apolipoproteins stabilise the pseudomicellar structure of lipoprotein particles. The amphiphatic helix has a spatial configuration of hydrophobic and hydrophilic amino acids (Patsch and Gotto, 1996). The hydrophobic face of the helix is associated with the fatty acyl chains of phospholipids, whereas the hydrophilic face is located close to the polar head groups of phospholipids. Therefore the amphipathic helix plays an important role in the structure and functions of the apolipoproteins (Segrest et al., 1992).

Most of the apolipoprotein genes consist of a multigene family (apo A-I, A-II, A-IV, C-I, C-II, C-III, and E) (Breslow, 1987; Li et al., 1988). The genes are functionally related and evolved from a common ancestor gene. They are characterised by the presence of repeated amphipathic helical regions, which are for the essential lipid binding properties. The chromosomal localisation of the

apolipoprotein genes are summarised in Table 7. The apolipoprotein genes present similar sequences in the coding region as demonstrated by both DNA and amino acid sequence. For instance, human apo B, E, and A-IV have up to 39% identity in their amino acid sequence (Li et al., 1988).

The apolipoproteins gene are also similar in genomic organisation, such as the intron/exon location, with the exception of apo B and various truncated forms such as apo B-48. In addition, the apo D gene is not a member of the apolipoprotein gene family as it differs in genomic organisation and primary structure (Drayana, 1987). The minor apolipoproteins such as apo H and apo J and I are classified as atypical apolipoproteins because they do not have structural homologies with either the apolipoproteins gene family or apo B (Patsch and Gotto, 1996). In addition, the amino acid sequence and lipid binding domains of the apolipoproteins F and G remain to be determined.

The properties of the apolipoproteins are discribed in Table 8. Only apo E will be discussed in detail in this thesis due to its relevance to this project.

3.1. Apolipoprotein E

Apolipoprotein E (apo E) is a common component of various classes of lipoproteins, including chylomicrons, VLDL and HDL. Initially, it was described in 1973 by Shore and Shore as a component of triglyceride-rich very low density protein (VLDL) and referred to as the arginine-rich protein, since it contained a high amount of arginine compared to other apolipoproteins (Weisgraber, 1994). Apo E serves various functions including mediating the cellular uptake and redistribution

Table 7: Characteristics of the human apolipoprotein genes

Apolipoprotein	Chromosomal location	Gene length (Kbp)	Exons/Introns
A-I	11	1.9	4/3
A-II	1	1.4	4/3
A-III	3	12.0	5/4
A-IV	11	2.6	3/2
B-100	2	44.0	29/28
B-48	2		
C-I	19	4.4	4/3
C-II	19	3.4	4/3
C-III	11	3.2	4/3
D	3	12.0	5/4
E	19	3.6	4/3

Adapted from Li et al., 1988; Fisher et al., 1989; Patsch and Gotto , 1996.

Table 8: Properties of the apolipoproteins

Apolipoprotein	Molecular weight – KDa	Number of amino acids	Tissue expression	Function
A-I	28	243	Liver and intestine	Structural, activator of LCAT, ligand for LDL binding, promotes cholesterol efflux.
A-II	17.4	77	Liver and intestine	Structural, activator for hepatic lipase.
A-III	20	169	?	Unknown
A-IV	46	376	Liver and intestine	Modulator of LPL activity, LCAT activator, function and in TG transport (?).
B-100	550	4563	Liver	Structural, secretion of VLDL, ligand for LDL receptor.
B-48	265	2152	Intestine	Structural, secretion of chylomicrons.

Table 8 continued.

Apolipoprotein	Molecular weight – KDa	Number of amino acids	Tissue expression	Function
C-I	6.6	57	Liver and intestine	Activator of LCAT, inhibits removal of TGRL by LDL receptor – related protein.
C-II	8.8	79	Liver and intestine	Activator of LPL.
C-III	9	79	Liver and intestine	Inhibitor of LPL, inhibits uptake of TGRL by liver.
D	20	169	Liver, intestine, spleen, pancreas, brain, adrenal glands and kidney	Binding of heme-related compounds (?), potential radical scavenger, reverse cholesterol transport (?).
E	39	299	Liver, intestine, spleen, pancreas, brain, adrenal glands and kidney	Ligand for LDL receptor and LDL receptor-related protein, reverse cholesterol transport, immunoregulatory properties, regulator of cell growth.

Adapted from Patsch and Gotto, 1996.

of cholesterol between peripheral tissues and the liver through the LDL receptor and the LDL receptor-related protein (Taylor et al., 1995). It is facilitated by the ability of apo E to be recognised by both apo E specific receptors on the liver and also LDL receptors on the liver and other peripheral tissues (Mahley et al., 1983).

Apolipoprotein E also appears to be involved in the repair response of tissue injury. In fact, increased amounts of apo E are found at sites of peripheral nerve injury and regeneration (Mahley, 1988). In addition, apo E has been identified upon autopsy in extracellular neurofibrillary tangles in the brains of individuals with Alzheimer's disease (Taylor et al., 1995).

3.1.1. Structure of apo E

Apolipoprotein E is a single polypeptide with a molecular mass of 34,200 Da as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Its primary structure was determined by amino acid sequencing of the purified protein and it was found to have 299 amino acids (Rall et al., 1982). This was confirmed by nucleotide sequencing of cDNA from apo E mRNA (McLean et al., 1984) (Figure 11).

The apo E molecule contains two structural regions, one located in the amino-terminal (residues 20-165), and another in the carboxyl-terminal (residues 225-299). These domains are joined by a central protease-susceptible region (residues 165-210) (Weisgraber, 1994). The amino-terminal region is highly stable and consists of two conserved sequences, one being the receptor binding region of apo E (residues 136-150), and another, the region between residues 29-61, whose function has not

Figure 11: Nucleotide and protein sequence of apo E mRNA

E4 DNA		ATG	AAG	GTT	CTG	TGG	GCT	GCG	TTG
E4 Protein		Met	Lys	Val	Leu	Trp	Ala	Ala	Leu
E4	CTG	GTC	ACA	TTC	CTG	GCA	GGA	TGC	CAG	GCC
E4	Leu	Val	Thr	Phe	Leu	Ala	Gly	Cys	Gln	Ala
	1									30
E4	AAG	GTG	GAG	CAA	GCG	GTG	GAG	ACA	GAG	CCG
E4	Lys	Val	Glu	Gln	Ala	Val	Glu	Thr	Glu	Pro
	*									
										60
E4	GAG	CCC	GAG	CTG	CGC	CAG	CAG	ACC	GAG	TGG
E4	Glu	Pro	Glu	Leu	Arg	Gln	Gln	Thr	Glu	Trp
										90
E4	CAG	AGC	GGC	CAG	CGC	TGG	GAA	CTG	GCA	CTG
E4	Gln	Ser	Gly	Gln	Arg	Trp	Glu	Leu	Ala	Leu
										120
E4	GGT	CGC	TTT	TGG	GAT	TAC	CTG	CGC	TGG	GTG
E4	Gly	Arg	Phe	Trp	Asp	Tyr	Leu	Arg	Trp	Val
										150
E4	CAG	ACA	CTG	TCT	GAG	CAG	CTG	CAG	GAG	GAG
E4	Gln	Thr	Leu	Ser	Glu	Gln	Val	Gln	Glu	Glu
										180
E4	CTG	CTC	AGC	TCC	CAG	GTC	ACC	CAG	GAA	CTG
E4	Leu	Leu	Ser	Ser	Gln	Val	Thr	Gln	Glu	Leu
										210
E4	AGG	GCG	CTG	ATG	GAC	GAG	ACC	ATG	AAG	GAG
E4	Arg	Ala	Leu	Met	Asp	Glu	Thr	Met	Lys	Glu
										240
E4	TTG	AAG	GCC	TAC	AAA	TCG	GAA	CTG	GAG	GAA
E4	Leu	Lys	Ala	Tyr	Lys	Ser	Glu	Leu	Glu	Glu
										270
E4	CAA	CTG	ACC	CCG	GTG	GCG	GAG	GAG	ACG	CGG
E4	Gln	Leu	Thr	Pro	Val	Ala	Glu	Glu	Thr	Arg

Figure 11: (contd)

										300
E4	GCA	CGG	CTG	TCC	AAG	GAG	CTG	CAG	GCG	GCG
E4	Ala	Arg	Leu	Ser	Lys	Glu	Leu	Gln	Ala	Ala
										330
E4	CAG	GCC	CGG	CTG	GGC	GCG	GAC	ATG	GAG	GAC
E4	Gln	Ala	Arg	Leu	Gly	Ala	Asp	Met	Glu	Asp
E2		**T**								
E3		**T**								360
E4	GTG	**CGC**	GGC	CGC	CTG	GTG	CAG	TAC	CGC	GGC
E4	Val	**Arg**	Gly	Arg	Leu	Val	Gln	Tyr	Arg	Gly
E3		**Cys**								
E2		**Cys**								
		112								
										390
E4	GAG	GTG	CAG	GCC	ATG	CTC	GGC	CAG	AGC	ACC
E4	Glu	Val	Gln	Ala	Met	Leu	Gly	Gln	Ser	Thr
										420
E4	GAG	GAG	CTG	CGG	GTG	CGC	CTC	GCC	TCC	CAC
E4	Glu	Glu	Leu	Arg	Val	Arg	Leu	Ala	Ser	His
										450
E4	CTG	CGC	AAG	CTG	CGT	AAG	CGG	CTC	CTC	CGC
E4	Leu	Arg	Lys	Leu	Arg	Lys	Arg	Leu	Leu	Arg
E2								**T**		
E3								**C**		480
E4	GAT	GCC	GAT	GAC	CTG	CAG	AAG	**CGC**	CTG	GCA
E4	Asp	Ala	Asp	Asp	Leu	Gln	Lys	**Arg**	Leu	Ala
E3								**Arg**		
E2								**Cys**		
								158		
										510
E4	GTG	TAC	CAG	GCC	GGG	GCC	CGC	GAG	GGC	GCC
E4	Val	Tyr	Gln	Ala	Gly	Ala	Arg	Glu	Gly	Ala
										540
E4	GAG	CGC	GGC	CTC	AGC	GCC	ATC	CGC	GAG	CGC
E4	Glu	Arg	Gly	Leu	Ser	Ala	Ile	Arg	Glu	Arg

Figure 11: (contd)

										570
E4	CTG	GGG	CCC	CTG	GTG	GAA	CAG	GGC	CGC	GTG
E4	Leu	Gly	Pro	Leu	Val	Glu	Gln	Gly	Arg	Val
										600
E4	CGG	GCC	GCC	ACT	GTG	GGC	TCC	CTG	GCC	GGC
E4	Arg	Ala	Ala	Thr	Val	Gly	Ser	Leu	Ala	Gly
										630
E4	CAG	CCG	CTA	CAG	GAG	CGG	GCC	CAG	GCC	TGG
E4	Gln	Pro	Leu	Gln	Glu	Arg	Ala	Gln	Ala	Trp
										660
E4	GGC	GAG	CGG	CTG	CGC	GCG	CGG	ATG	GAG	GAG
E4	Gly	Glu	Arg	Leu	Arg	Ala	Arg	Met	Glu	Glu
										690
E4	ATG	GGC	AGC	CGG	ACC	CGC	GAC	CGC	CTG	GAC
E4	Met	Gly	Ser	Arg	Thr	Arg	Asp	Arg	Leu	Asp
										720
E4	GAG	GTG	AAG	GAG	CAG	GTG	GCG	GAG	GTG	CGC
E4	Glu	Val	Lys	Glu	Gln	Val	Ala	Glu	Val	Arg
										750
E4	GCC	AAG	CTG	GAG	GAG	CAG	GCC	CAG	CAG	ATA
E4	Ala	Lys	Leu	Glu	Glu	Gln	Ala	Gln	Gln	Ile
										780
E4	CGC	CTG	CAG	GCC	GAG	GCC	TTC	CAG	GCC	CGC
E4	Arg	Leu	Gln	Ala	Glu	Ala	Phe	Gln	Ala	Arg
										810
E4	CTC	AAG	AGC	TGG	TTC	GAG	CCC	CTG	GTG	GAA
E4	Leu	Lys	Ser	Trp	Phe	Glu	Pro	Leu	Val	Glu
										840
E4	GAC	ATG	CAG	CGC	CAG	TGG	GCC	GGG	CTG	GTG
E4	Asp	Met	Gln	Arg	Gln	Trp	Ala	Gly	Leu	Val
										870
E4	GAG	AAG	GTG	CAG	GCT	GCC	GTG	GGC	ACC	AGC
E4	Glu	Lys	Val	Gln	Ala	Ala	Val	Gly	Thr	Ser

Figure 11: contd

									899
E4	GCC	GCC	CCT	GTG	CCC	AGC	GAC	AAT	CAC
E4	Ala	Ala	Pro	Val	Pro	Ser	Asp	Asn	His
									**

The position in bold indicates the cysteine-arginine interchanges at amino acid positions 112 and 158.

* NH_2-terminal

** COOH-terminal

Adapted from McLean, J.W. et al., (1984).

been determined (Krul and Cole, 1996). The carboxyl-terminal region contains the lipid binding activity of apo E, which consists of amphipathic helical domains (Weisgraber et al., 1994).

The predicted secondary structure of apo E consists of 62% α–helix, 9% β-sheet, 11% β-turn and 18% random coils (Rall et al.,1982; Mahley et al., 1988). The α-helical region, which is common to all apolipoproteins, corresponds to the lipid binding region.

Proteolysis of apo E by thrombin digestion generates a 22-KDa amino-terminal fragment (residues 1-119), and a 10KDa carboxyl-terminal fragment (residues 216-299) (Segrest et al., 1992). Wilson and coworkers (1991) determined the three dimensional structure of the 22KDa fragment of apo E by x-ray crystallography. It was observed that the amino-terminal domain of apo E contains five helices, four of which are arranged in an antiparallel four-helix bundle. The hydrophobic side chains are sequestered in the interior of the bundle and their basic and acid residues are involved in intra and inter-helical salt bridges. These salt bridges contribute to the stability of the structure. The hydrophilic side chains are exposed to solvent on the surface. The basic residues of the receptor binding region are not involved in salt bridges resulting in a formation of positive electrostatic potential in this region. In addition, the residues are free to interact with the LDL receptor (Weisgraber, 1994).

3.1.2. Heterogeneity of apo E

The heterogeneity of apo E was recognised by Shore and Shore (1973). Subsequently Utermann recognised that apo E polymorphism was genetically determined and a two allele model was proposed to explain the different patterns when subjected to isoelectric focusing (Utermann et al., 1977). Later, Zannis and Breslow (1981) proposed a three allele model for the multiple banded patterns based on two dimensional gel analysis. The apo E polymorphism can be explained by the presence of three alleles at a single gene locus in combination with the addition of one or more sialic acid residues to the product of each allele (Zannis and Breslow, 1981). The three alleles were designated as ε2, ε3 and ε4 and their gene products as E2, E3 and E4. Thus, there are three homozygous phenotypes, E2/E2, E3/E3 and E4/E4, and three heterozygous phenotypes, E4/E3, E4/E2 and E3/E2. The numeral designation corresponds to the relative pI of each protein on isoelectric focusing gels, with apo E2 being the most acidic isoform (pI ~ 5.7) and apo E4 the most basic (pI ~ 6.1).

3.1.3. Protein chemistry of apo E

Apo E is synthesised in the liver as a preprotein containing 317 amino acid residues, which includes an 18 residue signal peptide which directs the precursor to the plasma membrane. Initially it is secreted as the sialo form and then the preprotein undergoes intracellular proteolysis, glycosylation and extracellular desialylation, and the asialo apo E is released into the plasma (Zannis et al., 1984). The signal peptide is removed at this time. The mature apo E protein consists of 299 amino acid

residues (McLean et al., 1984; Zannis et al., 1984).

The molecular basis for apo E polymorphism was determined by analysis of the amino acid sequence of the three major isoforms (Rall et al., 1982). It was observed that the isoforms differed from each other by a single cysteine-arginine interchange at two positions: residue 112 and residue 158. These amino acid substitutions explain the charge differences among the major isoforms (Rall et al., 1982). The apo E3 isoform has a cysteine at residue 112 and contains arginine at residue 158. Apo E4 has arginine at both positions, whereas apo E2 has cysteine at both sites (Table 9). As a result, apo E4 has one unit of positive charge more than E3 which has one more positive charge than E2, as observed by isoelectric focusing polyacrylamide gel electrophoresis (Weisgraber et al., 1982). It was proposed that the amino acid substitutions are the result of mutations in the apo E gene, apo E3 (the most common phenotype) being the parent molecule, and apo E2 and E4 being mutants (Rall et al., 1982).

The minor isoforms are not due to polymorphism, but can be explained by post translational modification of the major isoforms (Zannis et al., 1984). About 80-85% of human plasma apo E is in the asialo form, whereas the remainder is present as monosialo and disialo isoforms (Zannis and Breslow, 1981). However, newly secreted apo E is highly sialylated (Zannis et al., 1984). The sialylated isoforms arise from the attachment of a carbohydrate moiety at a single site, threonine at residue 194 on the apo E molecule (Wernette-Hammond et al., 1989).

Table 9: The positions of the cysteine/ arginine interchanges in the three common apo E isoforms

	Residue number	
	112	**158**
E4	Arg	Arg
E3	Cys	Arg
E2	Cys	Cys

Abbreviations: Cys – Cysteine

Arg – Arginine

3.1.4. The apo E receptor binding region

Apo E plays an important role in lipid metabolism due to its ability to bind to the LDL receptor. Several studies have been done to determine the region of apo E that binds to the receptor. Initially, the region between residues 136-158 was studied because of its content of basic amino acid residues (Weisgraber et al., 1978). It was demonstrated that the binding activity of apo E was diminished by selective chemical modification of arginine and lysine residues, indicating the importance of these residues in the binding process (Mahley, 1988).

The three common isoforms were tested for receptor binding activity (Weisgraber et al., 1982). To determine the receptor binding activity, the apo E was complexed with phospholipids because in the lipid-free state the protein is inactive. The results showed that apo E3 and E4 competed effectively for binding to LDL receptors, suggesting that a arginine-cysteine interchange at position 112 does not alter the receptor binding activity of apo E. However, the apo E2 isoform was shown to be defective in binding to the receptor, indicating that the arginine (basic amino acid) at position 158 plays an important role in the receptor binding activity of the apo E molecule .

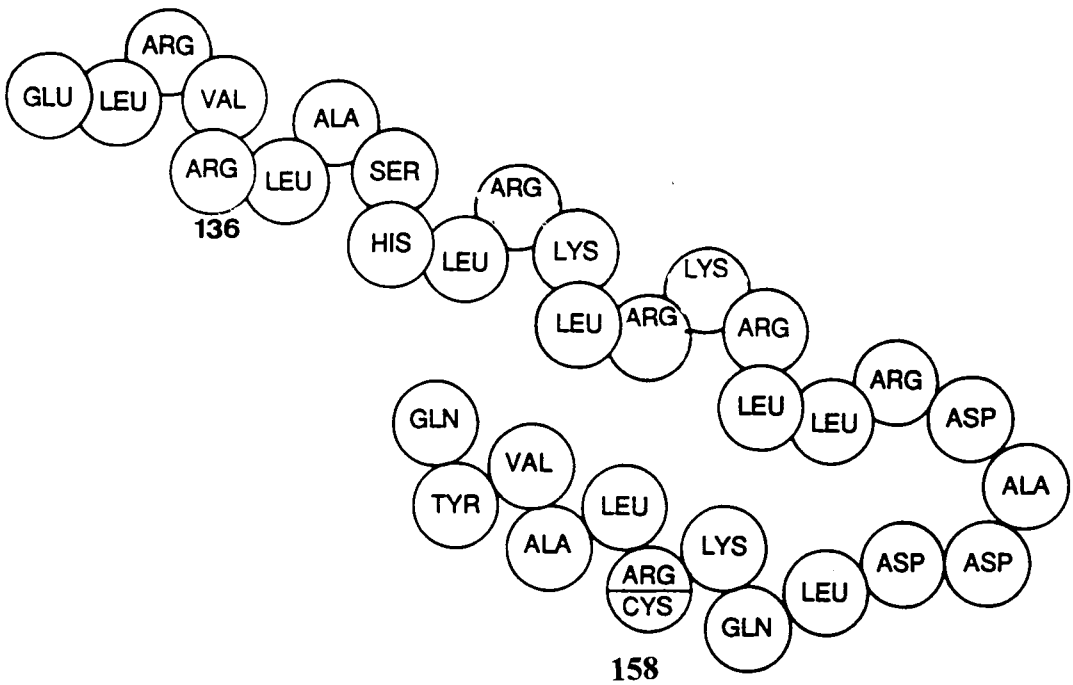
Another approach to determining the binding region was to cleave the apo E molecule into fragments by thrombin or cyanogen bromide and then to test for binding receptor activity (Innerarity et al, 1983). The fragments generated by thrombin digestion showed that only the 22 KDa fragment (residue 1-191) was active and the 10 KDa fragment had no activity (Innerarity, 1983). In addition, of the four

fragments produced by cyanogen bromide, only the fragment that contains residues 126-218 showed binding activity (Innerarity et al, 1983). This suggested that the receptor binding activity of apo E is located in the centre of the protein (Weisgraber, 1994). A further study involving monoclonal antibodies directed to the binding epitope, demonstrated that the receptor binding region was between residues 136-150 (Weisgraber et al, 1983). It was concluded that the residue arginine-158 does not interact directly with the receptor, but the basic amino acid in this position is important in maintaining the conformation of the binding region (Innerarity et al, 1984). The region of the apo E molecule that interacts with the LDL receptor is enriched in basic amino acids such as arginine and lysine (Figure 12). As a result, it was suggested that the basic receptor binding region of apo E binds to the LDL receptor via an ionic interaction between the basic residues in this region and the acid residues of the ligand binding domain of the LDL receptor (Brown and Goldstein, 1986). Thus, the basic amino acids in this region are essential for the conformation of apo E which bind the receptor. However, mutants outside this region may also affect the binding activity of the molecule (Innerarity et al, 1983; Weisgraber, 1983).

3.1.5. Methodology for apo E phenotyping

Apo E can be separated by isoelectric focusing (IEF) into three major isoforms E2, E3, E4 and a number of minor glycosylated isoforms. The major isoforms differ from each other in pI by a single unit charge. Apo E4 is the most basic and E2 the most acidic. Various methods for phenotyping have been developed. Previously, the most frequently used method was to isolate the VLDL from plasma

Figure 12: Receptor binding domain of apo E



Adapted from Mahley, 1988.

by ultracentrifugation followed by delipidation. After that, the proteins were submitted to IEF using a pH gradient. This method was laborious and required large amounts of plasma. It was also less suitable for large scale diagnosis and was expensive since ultracentrifugation was required to isolate VLDL. Because of these disadvantages, Menzel and Uterman (1986) developed a micromethod for phenotyping, which is based on IEF of delipidated serum, followed by immunoblotting using anti-apo E antibody as first antibody. This method utilises very small amounts of serum and allows mass screening for population studies. Due to the advantages of this method, we used it to develop this experimental work.

In addition to IEF, another method has been developed in which allele-specific oligonucleotide primers (ASP) are used to determine the apo E genotyping. Either hybridisation with oligonucleotides (Emi et al., 1988, Main et al., 1991) or restriction isotyping with allele-specific oligonucleotides (Hixson et al., 1990 and Dallinga-Thie et al., 1995) can be used to amplify the region including the polymorphic residues of the apo E gene. This method has become popular due to the facility of the polymerase chain reaction (PCR). It permits unambiguous phenotyping and can be used to identify unusual variants of apo E.

Isolelectric focusing, chemical modification and DNA sequencing techniques have allowed the characterisation of the rare apo E alleles. In addition to alleles occupying an unusual position on an IEF gel, mutants can be identified by migration on SDS-PAGE. It was suggested that the cysteine for arginine substitution at residue 158 in apo E modified the protein structure resulting in a different apparent

molecular weight on SDS-PAGE. In addition, chemical modification of the mutant isoforms can reveal the number of cysteine residues (Utermann et al., 1984). Treatment of the protein with cysteamine results in a reversible disulphide linkage with the cysteine residues, converting them into positively charged analogues of lysine. Consequently, E2 that has two cysteine residues will move up two places in an IEF gel to the E4 position, E3 will move up one and E4 will remain in the same position. Another method to identify mutants is to use allele-specific oligonucleotide probes and PCR.

3.1.6. Relative allele frequencies

The frequencies of the apo E phenotyping have been determined in several populations. It was observed that the allele E3 is the most common form in every population, whereas E2 and E4 phenotypes are the least common. The relative phenotypes and allele frequencies are summarised in Table 10.

It can be seen that there is similarity among the populations, with the exception of the Finnish and Japanese. Enholm and co-workers (1986), observed a 50% increase of the ε4 allele in the Finnish population. In addition, a significantly higher frequency of the ε3 allele and a significantly lower frequency of the ε2 and ε4 were reported in the Japanese population (Eto et al., 1986). This suggests that there may be racial differences.

3.1.7. Effect of apo E allele variation on plasma lipid abnormalities

There is evidence that the apo E polymorphism is associated with abnormal

Table 10: Apolipoprotein E phenotype and allele frequencies in various populations

Phenotype	Germany (Utermann et al., 1984)	U.S.A. (Ordoras et al., 1987)	New Zeland (Wardell et al., 1982)	Scotland (Cumming and Robertson, 1984)	Finland (Enholm et al., 1986)	Japan (Eto et al., 1986)
E4/E4	2.8	3.15	1.0	1.0	6.3	1.7
E3/E3	59.8	63.2	51.4	58.3	54.0	71.9
E2/E2	1.0	0.6	1.4	0.5	0.3	0.3
E4/E3	22.9	18.8	25.0	24.8	31.9	19.3
E4/E2	1.5	1.95	1.2	2.8	0.5	0.7
E3/E2	12.0	11.8	20.0	12.8	6.7	6.1
No of Subjects	1031	1204	426	400	615	576
Allele						
ε4	0.150	0.135	0.141	0.145	0.227	0.117
ε3	0.773	0.786	0.739	0.770	0.733	0.846
ε2	0.077	0.075	0.119	0.083	0.041	0.037

lipid and lipoprotein levels and atherosclerosis. Utermann and co-workers (1979) observed that in the normal population, individuals with the ε2 allele had lower levels of plasma cholesterol and LDL cholesterol levels than those with ε3 allele. In addition, Davignon et al (1988) found that individuals with the ε4 allele had an average higher levels of cholesterol and LDL cholesterol than individuals with the ε3 allele.

In several population studies, it was observed that the three isoforms of apo E have significantly different effects on plasma cholesterol and LDL levels (Hallman et al., 1991). In fact, it has been demonstrated that 60% of the variation in plasma cholesterol levels is genetically determined and that 14% of that variation is the result of apo E heterogeneity (Davignon et al., 1988).

In normal individuals, chylomicron remnants and VLDL remnants are rapidly cleared from the circulation by receptor-mediated endocytosis in the liver. In familial dysbetalipoproteinaemia or type III hyperlipoproteinaemia, there is an impairment of clearance of chylomicron and VLDL remnants due to a defect in apo E, often resulting in high levels of cholesterol and triglycerides in the plasma.

The apo E polymorphism has significant effects on lipid metabolism. It has been demonstrated that apo E2 is defective in binding to LDL receptors on skin fibroblasts, while apo E3 and E4 bind with high affinity (Weisgraber et al.,1982; Rall et al., 1982).

Several studies have reported that individuals with the ε2 allele have lower plasma cholesterol than ε3 subjects, and ε4 allele is associated with the highest levels

(Utermann et al., 1979). In several populations, including those of Germany (Utermann et al., 1987; Menzel et al., 1983), New Zealand (Wardell et al., 1982), Canada (Sing and Davignon, 1985), Scotland (Robertson and Cumming, 1985), Japan (Eto et al., 1988) and France (Boerwinkle et al., 1987), surveys have confirmed the effect of ε2 allele and ε4 allele in lowering and raising the plasma cholesterol levels respectively.

The primary effect of apo E polymorphism is likely to be on the metabolism of apo E-containing lipoproteins. The binding defect of apo E2 results in reduced catabolism of E2. Greg et al. (1986), using radiolabeled apolipoproteins demonstrated that E2 was catabolised more slowly than E3 which in turn was slower than E4. In addition, it was demonstrated that the E2 allele raises average apo E levels in the general population due to the relative accumulation of apo E containing lipoprotein. Conversely, the average effect of the E4 allele is to lower plasma apo E levels due to faster turnover rates of apo E containing lipoprotein (Boerwinkle and Utermann, 1988).

A model was proposed to describe the effect of apo E allele in plasma LDL levels (Boerwinkle and Utermann, 1988). In E2 homozygotes, fewer lipoprotein particles are delivered to the liver to provide cholesterol as a result of a defect in binding to LDL receptor. This results in an up-regulation of the LDL receptors to deliver more cholesterol into cells and therefore the level of cholesterol in the plasma is reduced. The opposite occurs with E4 homozygotes. The E4 individuals are readily taken up by receptors, consequently more cholesterol is delivered to the cell. This

results in a down-regulation of the LDL-receptor on the surface of the liver cells, resulting in an elevation of plasma cholesterol levels (Greg et al., 1986; Davignon et al., 1988; Boerwinkle and Utermann, 1988). In E3/E3 individuals, the particles are taken up normally by the receptors, resulting in an intermediate of cholesterol production.

Apo E2 and apo E4 are associated with hypertriglyceridaemia and hypercholesterolaemia respectively. In fact, apo E4 is related in type V hyperlipoproteinemia (Kuusi et al., 1988). In addition, apo E2 homozygotes are associated with type III hyperlipidaemia (HPL) (Utermann et al., 1975). This disorder is characterised by elevated plasma triglyceride and cholesterol levels (Table 11) as a consequence of impaired clearance of chylomicron remnants and VLDL due to a defect in apo E. The accumulation of these particles can result in xanthomatosis and premature coronary artery disease and peripheral vascular disease (Brown et al., 1983). Although almost every type III HPL individual has the E2/E2 phenotype (Utermann, 1987), only 1-2% of persons with this phenotype develop the disease. This suggests that the disease is polygenic and multifactorial. It is believed that the type III HPL is a result of two gene defects: one in the apo E structural gene and the other in another gene that influences chylomicron remnant synthesis or catabolism, but yet has not been identified (Breslow, 1987). A variety of factors such as age, sex, hormonal status, nutrition and alcohol consumption may also combine with the genetic background and exacerbate the disease (Davignon et al., 1988). It has been demonstrated that the E2 allele frequency is also increased in 1984). In fact, in type IV hyperlipidemia, there is an increased prevalence of the E2/E2 phenotype as

Table 11: Summary of human hyperlipoproteinaemias

Type	Major elevated lipoprotein	Plasma cholesterol	Plasma triglyceride	Defect or deficiency	Fasting plasma appearance
Ia	Chylomicrons	Normal	Grossly elevated	Lipoprotein lipase	Milky
Ib	Chylomicrons	Normal	Grossly elevated	Apo C-II	Milky
IIa	LDL	Elevated	Normal	LDL receptor	Clear
IIb	LDL,VLDL	Elevated	Elevated	LDL receptor, Apo B-100, VLDL overproduction Decreased VLDL degradation	Turbid or clear
III	IDL, VLDL	Grossly elevated	Grossly elevated	Apo E2 VLDL overproduction	Turbid
IV	VLDL	Slightly elevated	Elevated	VLDL overproduction Decreased VLDL degradation	Turbid or milky
V	Chylomicrons, VLDL	Slightly elevated	Grossly elevated	VLDL overproduction Decreased VLDL degradation Partial LPL deficiency Apo E4	Turbid or milky

Types are classified by Beaumont et al, 1970

Adapted from Marinetti, 1990

well as the E2/E3 and E2/E4 phenotypes (Davignon et al, 1988). In addition the E4 is associated with hypercholesterolemia (type II hyperlipidemia) and type V hyperlipoproteinemia (Ghiselli et al., 1982; Greg et al., 1983; Kuusi et al, 1988).

In addition, Eto and co-workers (1988) showed that in Japan both the E2 allele and E4 allele are associated with an increase risk of ischaemic heart disease as compared with the E3 allele.

3.1.8. Effect of the apo E variants in Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterised by the presence of senile plaques and neurofibrillary tangles in the brain. Recently apo E polymorphism has been implicated with this syndrome. The primarily synthesis of the apo E occurs in the liver, but it is also found in significant amounts in the brain. The presence of apo E was observed in amyloid plaques associated with Alzheimer's and Creutzfeldt-Jakob diseases (Namba et al., 1991). Also, it was found that apo E mRNA is increased several-fold in the brain of patients with AD (Diedrich et al., 1991).

Several studies have shown that a high frequency of apo ε4 alleles is associated with the late-onset familial and sporadic forms of AD (Corder et al., 1993; Roses, 1994). In addition, the gene from patients with the late-onset form of AD has been mapped and was found to be located in the same region of chromosome 19 as the apo E gene (Corder et al., 1993).

In Alzheimer's disease, apo E is bound to extracellular senile plaques, which contain the β-amyloid peptide 4. It was observed that the apo E4 binds more rapidly

to amyloid β-protein (minutes) than does the purified apo E3, which require hours (Strittmatter et al., 1993). Thus, this may provide insight into the functional role of apo E4 in AD.

Apo E genotypes were determined in 3 ethnic groups in a New York community, and it was found that the relative risk for AD associated with apo E4 homozygosity was increased in all ethnic groups (Tang et al., 1996). In a Scottish population study, with early onset AD was found an increased frequency of both homozygous and heterozygous apo E4 (St Clair et al., 1995). Several other population studies have been confirmed the increased risk for AD associated with the apo E4 allele (Poirier et al., 1993; Bickeboller et al., 1997).

Head injury is an epidemiological risk factor for Alzheimer's disease. It was observed that one third of individuals dying after a severe head injury present deposition of β-amyloid. It was reported that the frequency of apo E4 in those individuals with amyloid beta deposition following head injury was higher than in AD (Nicoll et al., 1995). However, it was also observed that there is a similar apo E4 frequency in individuals head injured without amyloid beta deposition compared to controls without AD. This confirm that apo E4 plays a role in amyloid beta deposition and other environmental or genetic factors for AD may act in an additive fashion (Nicoll et al., 1995).

3.1.9. Apolipoprotein E variants

Several apo E variants have been described, including the most common variant, apo E3. Several of those were found to be associated with familial

dysbetalipoproteinaemia and other types of hyperlipoproteinaemia and some of them are not associated with diseases. At the present 45 apo E variants have been described. Table 12 summarises the various types of apo E variants.

3.1.9.1. Common apo E variants

The three common apo E variants apo E2, E3, and E4, can be identified by IEF and by the polymerase chain reaction (PCR). Some apo E variants were characterised by isoelectric focusing. The apo E was designated as E1 when presented a single charge unit more acid compared with apo E2. In addition, variants which have an intermediate position between two isoforms were called $E3^{+}$ when it is slightly more more basic than apo E3 and designated $E4^{-}$ when it is slightly more acid than apo E4 and so on (de Knijff et al, 1994).

4. Apolipoprotein E and its relevance to RP

Apo E is synthesised primarily by the liver. It is also found in the nervous system where it seems to play a role in the central nervous system lipid homeostasis. Significants levels of apo E mRNA were observed in human brain tissues (Elshourbagy et al, 1985). In the CNS, all astrocyte cells, including specialised astrocytes (Bergmann glial, tanycytes, pituicytes and Muller cells) were found to contain a significant amounts of apo E (Boyles et al, 1985).

As discussed earlier, apo E is a polymorphic protein which has three isoforms: E2, E3 and E4. Apo E2 is associated with hyperlipidaemia type III. Apo E4 is associated with elevated cholesterol, increased risk for coronary heart disease and late onset Alzheimer disease. Apolipopoproteins have also been implicated in the

Table 12: Apo E variants

Variant name	Codon	Nucleotide change	Codon change	Disease associated
Apo E3 (Cys 112;Arg 158)	-	-	-	None
Apo E4 (Cys112→Arg)	112	TGC→CGC	Cys→Arg	N/HC
Apo E2 (Arg158→Cys)	158	CGC→TGC	Arg→Cys	N/FD-r
Apo E0	3598	A→G	Splice	FD-r
Apo E4-Philadelphia (Glu13→Lys; Arg145→Cys)	13 145	GAG→AAG CGT→TGT	Glu→Lys Arg→Cys	FD-d
Apo E0 (ΔG2919/2920/2921→ Stop60)	31-60	ΔG2919 or 2920 or 2921	Gly→FS→ stop at codon 60	FD-r
Apo E3-Leiden (Cys112→Arg; 7aa ins)	112 120-126 or 121-127	TGC→CGC Duplication of 21bp	Cys→Arg 7aa tandem duplication	FD-d
Apo E1 (Gly127→Asp; Arg158→Cys)	127 158	GGC→GAC CGC→TGC	Gly→Asp Arg→Cys	FD-r
Apo E2-Christchurch (Arg136→Ser)	136	CGC→AGC	Arg→Ser	FD-u
Apo E3 (Cys112→Arg; Arg142→Cys)	112 142	TGC→CGC CGC→TGC	Cys→Arg Arg→Cys	FD-d
Apo E3—Kochi (Arg145→His)	145	CGT→CAT	Arg→His	FD-u
Apo E2 (Arg145→Cys)	145	CGT→TGT	Arg→Cys	FD-u

Tabela 12: Contd

Variant name	Codon	Nucleotide change	Codon change	Disease associated
Apo E1-Harrisburg (Lys146→Glu)	146	AAG→GAG	Lys→Glu	FD-d
Apo E2 (Lys146→Gln)	146	AAG→CAG	Lys→Gln	FD-d
Apo E3-Washington (Trp210→Stop)	210	TGG→TAG	Trp→Stop	FD-r
Apo E2-Fukuoka (Arg224→Gln)	224	CGG→CAG	Arg→Gln	FD-u
Apo E5 (Glu3→Lys)	3	GAG→AAG	Glu→Lys	HC
Apo E5 (Glu13→Lys)	13	GAG→AAG	Glu→Lys	HC/HTG
Apo E2-Dunedin (Arg228→Cys)	228	CGC→TGC	Arg→Cys	HTG
Apo E2 (Val236→Glu)	236	GTG→GAG	Val→Glu	HTG
Apo E7-Suita (Glu244→Lys; Glu245→Lys)	244 245	GAG→AAG GAG→AAG	Glu→Lys Glu→Lys	HC/HTG
Apo E3 (Cys112→Arg; Arg251→Gly)	112 251	TGC→CGC CGC→GGC	Cys→Arg Arg→Gly	HTG
Apo E1 (Arg158→Cys; Leu252→Glu)	158 252	CGC→TGC CTG→GAG	Arg→Cys Leu→Glu	HC
Apo E4- -Freiburg (Leu28→Pro; Cys112→Arg)	28 112	CTG→CCG TGC→CGC	Leu→Pro Cys→Arg	N
Apo E3- -Freiburg (Thr42→Ala)	42	ACA→GCA	Thr→Ala	N

Tabela 12: Contd

Variant name	Codon	Nucleotide change	Codon Change	Disease association
Apo E5 (Pro84→Arg; Cys112→Arg)	84 112	CCG→CGG TGC→CGC	Pro→Arg; Cys→Arg	N
Apo E3 (Ala99→Thr; Ala152→Pro)	99 152	GCG→ACC GCC→CCC	Ala→Thr Ala→Pro	N
Apo E2 (Arg134→Gln)	134	CGG→CAG	Arg→Gln	N
Apo E4 (Cys112→Arg; Arg274→His)	112 274	TGC→CGC CGC→CAG	Cys→Arg Arg→His	N
Apo E4+ (Ser296→Arg)	296	AGC→CGC	Ser→Arg	N
Apo E5-Frankfurt (Gln81→Lys; Cys112→Arg)	81 112	-	Gln→Lys Cys→Arg	HC
Apo E1 (Arg142→Leu; Arg 158→Cys)	142 158	-	Arg→Leu Arg→Cys	FD-u
Apo E2 (Arg136→Cys)	136	-	ArgCys	FD-r
*Apo E2 (Δ209-212)	209-212	Δ209-212	TGA→FS→ stop at codon 209	HLP
**Apo E5 (Glu212→Lys)	212	GAG→AAG	Glu→Lys	HC/HTG
***Apo E3 (Arg145→Pro)	145	-	Arg→Pro	LPG
****Apo E3 (Cys112→Arg; Arg251→Gly)	112 251	-	Cys→Arg Arg→Gly	HTG

Table 12: Contd

Variant name	Codon	Nucleotide Change	Codon Change	Disease associated
*****Apo E3 (Arg136→His)	136	-	Arg→His	FD-r
******Apo E2 (Arg142→Leu)	142	-	Arg→Leu	HLP

Adapted from de Knijff, P. et al. (1994).

*Feussner, G., et al, 1996a.

** Feussner, G., et al, 1996b.

***Oikawa, S., et al, 1997.

****Zhao, S. P., et al, 1994.

*****Minnich, A., et al, 1995.

******Richard, P., et al, 1995.

Abbreviations: HLP - hyperlipidaemia;

HTG - hypertriglyceridaemia

HC – hypercholesterolaemia

N – normal

FD-r – familial disbetalipoproteinaemia recessive

FD-d - familial disbetalipoproteinaemia dominant

FD-u - familial disbetalipoproteinaemia unknown

LPG – lipoprotein glomerulopathy

Δ - deletion

pathogenesis of some types of RP. Apolipoprotein E and apolipoprotein B are important in the transport of lipids. Abnormalities in these apolipoproteins may prevent the eye from receiving the lipids required for the maintenance of the visual process.

Previous studies in the apo E in RP population have suggested that it may play a role in the disease. Jahn and coworkers (1987) determined apo E phenotyping in 139 German RP patients (Table 13). A ten increased-fold in the unusual apo E2/E2 phenotype was reported. This is significant since only 1% of the German control population are classified as E2/E2. In addition, Huq (1993) conducted a survey of apo E phenotype in 100 Scottish RP patients. A four-fold increase of E2/E2 and an eight-fold increase of E4/E4 were observed when compared to the Scottish control population (Table 14). The findings were statistically significant at the $P<0.05$ and $P<0.001$ levels respectively. The possibility of a new apo E variant was investigated by using sodium dodecyl sulphate and cysteamine modification. The results showed that two of the RP patients classified as E2/E2 behaved abnormally suggesting the possibility of a new apo E variant.

Souied and coworkers (1996) determined the apo E gene coding sequencing in 51 ADRP by SSCP analyses. No abnormal band that could indicate a new mutation was detected. In addition, there was no significant difference between the ADRP and control populations for allele frequency and apo E genotyping.

A survey of the apo E allele frequency in patients with age-related macular degeneration was carried out by La Paz and coworkers (1997). This disease is the

Table 13: Prevalence of apo E phenotypes in the German RP population

Apo E phenotypes	Patients	Prevalence in RP (%)	Prevalence in controls (%)
E2/E2	14	10.1*	1.0
E2/E3	27	19.4**	12.0
E2/E4	8	5.8	1.5
E3/E3	68	48.9	59.8
E3/E4	19	13.7**	22.9
E4/E4	3	2.2	2.8
Allele			
ε2	**ε3**	**ε4**	
0.227	0.654	0.119	

Levels of significance:

*p <0.05 **p <0.001

Taken from Jahn et al, (1987).

Table 14: Incidence of apo E phenotypes in the Scottish RP population

Apo E Phenotypes	Patients	Prevalence in RP (%)	Prevalence in controls(%)
E2/E2	4	4*	1
E2/E3	20	20	12
E2/E4	1	1	2
E3/E3	44	44	59
E3/E4	23	23	25
E4/E4	8	8**	1

Levels of significance

* $P < 0.05$ ** $P < 0.001$

Taken from Huq et al, 1993.

main cause of blindness among the elderly and is associated with cardiovascular diseases. The results showed no significant differences in the apo E allele frequency in the patients compared to control population.

In view of these results a new screening of apo E phenotyping in the Scottish RP population will be carried out in this project in order to confirm the results found by Huq and determine whether apo E is involved in the course of the disease.

5. Aims of the Project

5.1. To carry out a new screening of the common apo E phenotypes, using isoelectric focusing in the Scottish RP population, in order to confirm the results found by Huq (1993) and determine whether apo E is involved in the course of the disease.

5.2. To characterise apo E variants by using IEF, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and cysteamine modification.

5.3. To determine the apo E DNA sequences by using the polymerase chain reaction (PCR) in one of those patients whose apo E was found to behave abnormally by Huq (1990) and in those patients whose apo Es, during the course of this project, have been characterised as abnormal by IEF, SDS-PAGE or cysteamine modification.

5.4. To determine the level of the phytanic acid in patients with retinitis pigmentosa and whether they are related to the apo E phenotyping.

5.5. To measure phytanic acid in two suspected Refsum's disease patients, and study the variation with time (age), diet and weight.

Materials

This section describes the materials and equipment used in all the experiments carried out in the present work.

1. Apo E phenotyping

1.1. Chemicals

Glycine, Trizma base (Tris), urea, 2-mercaptoethylamine (cysteamine), 4-chloro-1-naphthol, amido black, ammonium persulphate, dithiothreitol, Coomassie Brilliant Blue R-250, N,N,N',N'-tetramethylethylamine (Temed), mixed bed resin, Tween 20, and low and high molecular weight marker proteins were purchased from Sigma-Aldrich Company Ltd.. Sodium decyl sulphate was supplied by Kodak Ltd. Ampholine pH 4-6 (manufactured by LKB, Sweden) was purchased from Pharmacia Ltd. Servalyte pH 4-6 and Servalyte pH 3-10 were obtained from Serva. "Marvel" dried skimmed milk was manufactured by Cadbury Ltd., Birmingham, England. Sodium hydroxide and sodium chloride were supplied by BDH. Protogel (30%) acrylamide/bisacrylamide solution (37.5:1) was obtained from Boehringer Mannheim. Immobilon-P transfer membrane was supplied by Millipore Ltd. Anti-apo E monoclonal antibody was kindly donated by the Lipid Research Laboratory, Glasgow Royal Infirmary, Scotland. Horseradish peroxidase anti-mouse IgG (sheep) was donated by the Scottish Antibody Production Unit (SAPU), Carluke, Scotland. The solvents used were analytical reagent grade: ethanol, anhydrous diethyl ether, methanol, acetic acid and perchloric acid 80% were purchased from the university chemical stores.

1.2. Equipment

The Protean II electrophoresis system and the Transblot cell were purchased from Bio-Rad Laboratories Ltd., Watford, England. The IEC Centra-4R centrifuge was purchased from Damon/IEC UK Ltd., Bedfordshire, England.

2. Apo E DNA sequencing

2.1.Chemicals

The chemicals used in this section were molecular biology grade. Trizma base, sucrose, magnesium chloride, Triton X-100, ethylenediamine tetracetic acid (EDTA), sodium dodecyl sulphate (SDS), sodium chloride, agarose gel and boric acid were supplied by Sigma-Aldrich Company Ltd, as were phenol:chloroform:isoamyl alcohol 25:24:1, and gel loading solution type 1. MicroAmp thin-walled reaction tubes with flat caps were obtained from Perkin Elmer-Applied Biosystems, Kelvin Close, Birchwood Park North, Warrington. The QIAquick Gel extraction Kit and QIAquick spin PCR purification Kit were supplied by QIAGen Ltd., Boundary Court, Gatwick Road, Crawley, West Sussex. A 100 bp DNA ladder was supplied by Gibco BRL, Paisley, Scotland. Taq-polymerase-Dynazyme II DNA polymerase was supplied by Flowgen, Lynn Lane, Shenstone, Lichfield, England. The solvents ethanol, chloroform, isopropanol, and glacial acetic acid were analytical reagent grade (Analar) obtained from standard laboratory grade.

2.2 Equipment

The Gyrovap GT centrifugal evaporator was supplied by V.A. Howe Company Ltd., Oxford, UK. The Biofuge 13 Microcentrifuge was obtained from

Heraeus Equipment Ltd., Brentwood, UK. The Applied Biosystems (ABI) 392 DNA/RNA synthesiser, ABI 373A Automated DNA Sequencer and PE DNA Thermal Cycler 480 were supplied by Perkin Elmer-Applied Biosystems, Warrington, UK. The GeneQuant II DNA/RNA calculator was supplied by Amersham Pharmacia Biotech, Little Chalfont, UK. The BioVision Gel Documentation System was obtained from Biogene Ltd. Kimbolton, UK.

3. Gas chromatography

3.1. Chemicals

All the solvents used in this work were HPLC grade. Methanol, chloroform, hexane and 14% boron trifluoride in methanol were supplied by Sigma-Aldrich Company Ltd, Poole, Dorset. Potassium chloride, sodium hydroxide and sodium chloride were all Aristar grade chemicals from Merck Ltd. (BDH), Lutterworth, Leicestershire. 'Tuftainer' silicone vials were obtained from Pierce and Warriner, Chester, Cheshire. Phytanic acid, phytanic acid methyl ester, pentadecanoic acid, pentadecanoic acid methyl ester, and lipid standard methyl esters were supplied by Sigma-Aldrich Company Ltd. Butylated hydroxy toluene was purchased from BDH Lab Supplies, Poole, England.

3.2. Equipment

The gas chromatography analysis was carried out on a Hewlett Packard 5890 series II gas chromatographic system connected to a Hewlett Packard HP3395 recorder/integrator system. A flame ionisation detector was used. The column was a 30m SP^{TM} 2330 fused silica capillary column with 0.32mm ID and 0.20μm film, and screw top clear vials were supplied by Supelco. The mixer Model A Whirli mixer

BP931263 was purchased from Fisions Scientific Apparatus, Loughborough. The hot plate was purchased from Rodwell Scientific Instruments Ltd., and the slow rotating mixer made by the Department of Pharmaceutical Sciences workshop. A 10µl micro syringe was obtained from Burke Analytical, Glasgow.

Methods

1. Selection of the patients

The patients involved in this work attended the RP clinic at the Western Infirmary, Glasgow. The diagnosis of the disease was based on the clinical examination. Several tests such as visual acuity, visual fields and electroretinograms (ERG) were carried out to confirm the diagnosis. The family history was taken from each patient to determine the mode of inheritance.

2. Blood collection

Venous blood (30ml) was taken from each patient. The blood samples were collected into 10 ml vacutainer tubes containing KEDTA (potassium ethylene diamine tetracetate) as an anticoagulant. The plasma was separated from the cells by centrifuging at 3000 rpm for 15 minutes in the IEC Centra-4R centrifuge. After that the plasma was pipetted off from the top using a Pasteur pipette. It was aliquoted in Eppendorf tubes and stored at -20^0C until assay. The cells were stored at -80^0C for the molecular biology study.

3. Selection of the control population

The control population used in this study was analysed in the Glasgow Royal Infirmary by Dr. Muriel Carlake and her associates in the Lipid Research Group

(unpublished). These data consisted of apo E phenotypes from a total of 3429 patients. This included various groups of studies performed from 1990 to 1997. A selection of these patients was carefully separated out in order to obtain a good representation of the normal population, eliminating any special age disease groups. In total 671 patients were selected and designated as the normal control population.

4. Determination of apo E phenotyping

4.1. Delipidation of the samples

Plasma (10μl) was mixed with 2.5 ml of a cold mixture of ethanol-ether (3:1). The mixture was incubated overnight at -20°C. Next morning it was centrifuged at 3000 rpm at 0°C for 30 minutes. The solvent was poured off carefully. The precipitate was washed with cold ether and allowed to stand at -20°C for a minimum of one hour. The samples were then centrifuged in the same conditions as above. The diethyl ether was discarded carefully and the precipitate was dried under nitrogen.

The precipitate was dissolved in 200μl of solubilising buffer (0.1 M tris base, pH10, 6 M urea, 1% sodium decyl sulphate) and 10μl of β-mercaptoethanol was added to the solution which was then incubated at 4^0C for 1 hour. Finally 60μl of the sample was applied to the isoelectric focusing gel (IEF).

4.2. Isoelectric focusing gel

This technique is used world-wide for the separation of amphoteric substances such as proteins. It is based on the separation of proteins according to their isoelectric point (pI) in a pH gradient. When a protein solution is subjected to a

pH gradient, the molecules will move until they reach a point at which they are uncharged.

The composition of the isoelectric focusing gel is given in Table 15 and is based on the methods of Menzel and Utermann (1986). The volume was prepared always in excess of requirements. The 8 M urea solution was prepared fresh each time and mixed with bed resin for 15 minutes. Then it was filtered into a conical flask. After that the required amounts of acrylamide, urea solution and ampholine mixture were mixed and degassed for 5 minutes using a water pump. Next the ammonium persulphate solution was added. Finally, just before gel casting, the Temed solution was added and the solution mixed by gentle swirling. The solution was poured into the plates and the comb was inserted to form the wells. It was allowed to polymerise for at least 2 hours at room temperature.

Each well in the gel was loaded with 60µl of sample. The sample was carefully overlayered with layering buffer (Table 16). Then the apparatus containing the gel sandwich was placed in the Protean II electrophoresis system. To prevent increase in the temperature during the run, it was cooled by circulating water. The anode (lower chamber) was filled with 10mM phosphoric acid buffer and the cathode(upper chamber) with 20mM sodium hydroxide buffer. The gel was allowed to run for 18 hours at a constant voltage of 250V with continuous cooling. Then the voltage was increased to 500V for 1 hour.

4.3. Immunoblotting of isoelectric focusing gels

The electroblotting was performed by using the method of Towbin et al. (1979). After running overnight the gel was carefully removed from the apparatus

Table 15: Composition of isoelectric focusing gels (Menzel and Utermann, 1986)

PAGE mix	12ml
8M urea	49ml
Ampholine mixture pH 3-10	2ml
Ammonium persulphate	0.115g in 4ml of 8M urea
Temed solution	5ml

Stock solutions:

1. PAGE-mix (Acrylamide/Bisacrylamide 37.5:1 solution)

2. Urea solution

48g of urea was dissolved in 80ml of distilled water and the volume made up to 100ml. This solution was freshly made and mixed with 3g of bed resin. It was filtered and degassed.

3. Ampholine mixture:

Ampholine pH 4-6	5.0 ml
Servalyte pH 4-6	5.0ml
Servalyte pH 3-10	2.0ml

4. TEMED solution:

Temed	0.2ml
8M urea	15ml
Distilled water up to	20ml

Table 16: Composition of solutions for IEF gels (Menzel and Utermann,1986)

1. Layering buffer

80% sucrose	2.0ml
Ampholine mixture pH 3-10	1.0ml
Distilled water up to	20 ml

2. Solubilizing buffer

Tris base	1.2g
Sodium decyl sulphate	1.0g
8M urea	75ml
Distilled water up to	100ml
Adjust pH to 10 with 1M HCl	

3. Cathode buffer (20mM sodium hydroxide)

Sodium hydroxide	1.6g
Distilled water up to	2 Litres

4. Anode buffer (10mM phosphoric acid)

Phosphoric acid 88%	5.6ml
Distilled water up to	5 Litres

and allowed to equilibrate in transfer buffer (Table 17) for 30 minutes with continuous shaking. While the gel was being equilibrated, a piece of the Immobilon-P transfer membrane was cut to the dimension of the gel. It was allowed to soak in 100% methanol for 15 seconds. Then it was washed with distilled water for 2 minutes and the membrane was equilibrated in the transfer buffer for at least 5 minutes. Finally the gel was assembled in the blotting apparatus. A "Scotchbrite" pad (Bio-Rad) was placed on one side of the cassette holder. Then the Immobilon membrane was placed on top of the gel and they were sandwiched between two sheets of 3MM filter paper and placed on top of the pad. To ensure a good transfer care was taken to remove all air bubbles between the membrane and the gel. Finally, a foam pad was placed on top of the filter and the cassette holder was placed in the tank of the blotting apparatus (Bio-Rad). The gel faced the cathode and the Immobilon membrane faced the anode. Then the tank was filled with transfer buffer. It was allowed to blot at 100V for 4 hours at 4°C.

After blotting the membrane was removed from the gel. It was equilibrated in blocking buffer for 1 hour at room temperature with continuous shaking. Then the blot was washed in buffer A for 20 minutes with constant shaking, changing the buffer five times. Next it was incubated with the first antibody (anti-apo E) overnight at room temperature with constant shaking. It was again washed in buffer A in the same conditions as above and incubated with the second antibody, HRP (horseradish peroxidase) anti sheep IgG for 2 hours at room temperature with shaking. It was again washed with buffer A as above. The blot was developed by incubation in substrate solution for 30 minutes in the dark. The reaction was stopped by washing the blot in distilled water. Finally the membrane was allowed to air-dry and stored

Table 17: Composition of immunoblotting solutions for IEF gels (Towbin et al., 1979, Menzel and Utermann, 1986).

1. Blotting buffer (25mM tris base, 192mM glycine, 20% methanol, pH 8.3

Tris base	9.0g
Glycine	43.2g
Methanol	600ml
Distilled water up to	3 litres

Adjust to pH 8.3 with Tris base or Glycine

2. Buffer A

Tris base	2.5g
Sodium chloride	18g
HCl (conc)	1.8ml
Tween 20	1.0ml
Distilled water up to	2 litres

Adjust to pH 7.4 with 1M HCl

3- Blocking buffer

Marvel	5.0g
Buffer A	100ml

Table 17 (contd.)

4. Substrate solution

4-chloro-1-naphthol	60mg
Methanol	10ml
PBS	60ml
H_2O_2	30µl

5- Phosphate buffered saline (PBS)

NaCl	0.8g
$Na_2H_2PO_4$	0.115g
KH_2PO_4	0.020g
Distilled water to	100ml

Adjust pH to 7.3 with 1M HCl

in a dark place.

5. Characterization of apo E

5.1. Cysteamine modification

Cysteamine modification was performed using the method of Weisgraber et al. (1981). Plasma (10μl) was added to 90μl of freshly prepared 0.4M cysteamine solution. It was then incubated at 37°C for 18 hours. After this time the samples were delipidated as previously described in section 4.1. The pellet protein was dissolved in sample buffer (see Table 16) in the absence of β-mercaptoethanol. It was allowed to stand at 4°C for 30 minutes. Then 60μl of each sample was loaded into the gel and submitted to IEF followed by immunoblotting as in section 4.3.

5.2. Determination of the molecular weight of apo E by SDS-Gel

5.2.1. Laemmli gels

This technique is extensively used to analysing protein mixtures qualitatively. It is based on the separation of protein according to their molecular size. Therefore, it can be used to determine the relative molecular weight of the proteins and it is also useful to monitor protein purification. The composition of Laemmli gels is shown in Table 18.

The delipidation of the sample was carried out as described in section 4.1. It was dissolved in Laemmli sample buffer and boiled for 5 minutes prior to loading. The Laemmli gel system consists of a separating gel and a stacking gel. After preparation, the resolving gel was poured into the gap between two plates and care

Table 18: Composition of Laemmli gels (Laemmli, 1970)

1. Resolving gel

Acrylamide(29.2%) / Bis(0.8%)	26.6ml
x4 Resolving gel buffer	20.0ml
Distilled water	31.8ml
10% SDS	0.8ml
10% Ammonium persulphate	0.8ml
TEMED	0.08ml

2. Stacking gel

Acrylamide(29.2%) / Bis(0.8%)	1.7ml
x4 Stacking gel buffer	2.5ml
Distilled water	5.8ml
10% SDS	0.1ml
10% Ammonium persulphate	0.1ml
TEMED	0.01ml

Table 18: contd

3. Acrylamide(29.2%) / Bisacrylamide(0.8%)

Acrylamide	29.2g
Bisacrylamide	0.8g
Distilled water up to	100ml

4. 4x Resolving gel buffer

Tris	18.2g
Distilled water up to	100ml

Adjust the pH to 8.8 with 1M HCl

5. 4x stacking gel buffer

Tris	6.1g
Distilled water up to	100ml

Adjust the pH to 6.8 with 1M HCl

6. Running buffer

Tris	3.1g
Glycine	14.4g
SDS	1.0g
Distilled water up to	1000ml

Table 18: contd

7. Laemmli sample buffer

Tris	0.75g
Glycerol	10ml
SDS	2.0g
Bromophenol blue	0.001g
Distilled water up to	100ml

Adjust pH to 6.7 with 1M HCl

8. 10% Ammonium persulphate (to be prepared immediately before use)

Ammonium persulphate	0.1g
Distilled water	1.0ml

was taken to avoid any bubbles. It was then overlaid with isobutanol and allowed to polymerise. After polymerisation, the isobutanol was poured off and the gel surface washed with tap water and stacking buffer. The stacking gel was poured on the resolving gel and the comb inserted to form the sample wells. After polymerising the comb was carefully removed and the sample wells filled with buffer. The samples (30µl) were carefully loaded into the wells. A low molecular weight marker was run in each gel. The gel was run at 120V until the samples had passed through the stacking gel. After that a constant voltage (220V) was applied until the samples had reached the bottom of the gel.

5.2.2. Neville gels

The discontinuous system of Neville (1971) was used with some modifications as described by Utermann et al. (1984). The method was modified by including 0.1% SDS in the upper and lower gel solutions. The composition of the gel is given in Table 19. The plasma was delipidated as described before (section 4.1.). The dried protein was dissolved in 200µl of Neville sample buffer and 10µl of β-mercaptoethanol was added. The solution was boiled for 3 minutes and allowed to cool.

The gel system (1mm thick gel) consists of a 13% resolving gel and a 3% stacking gel. After preparing the resolving gel, the mixture was poured between two glass plates (12x12) until it reached a height of 8 cm. Then a few drops of isobutanol were added to form a straight surface. It was polymerised for 30 minutes. After polymerisation the isobutanol was poured off and the plates washed with a large amount of water. The stacking gel was poured on top of the resolving gel and the

Table 19: Composition of Neville gels (Neville, 1971, Utermann et al., 1984)

1. Running gel

Acrylamide	11.0g
Bisacrylamide	0.10g
Running gel buffer	100ml
Ammonium persulphate	0.05g
Temed	150µl

2. Stacking gel

Acrylamide	3.0g
Bisacrylamide	0.2g
Stacking gel buffer	100ml
Ammonium persulphate	0.05g
Temed	150µl

3. Running gel buffer

Tris base	51.7g
2M HCl	15.4ml
Distilled water up to	1000ml

Adjust to pH 9.18 with 2M HCl

Table 19:(Contd)

4. Stacking gel buffer

Tris base	6.55g
1M H_2SO_4	10.7ml
Distilled water up to	1000ml

Adjust pH to 6.14 with 1M H_2SO_4

5. Upper reservoir buffer (cathode)

Boric acid	2.47g
Tris base	4.92g
SDS	1.0g
Distilled water up to	1000ml

Adjust pH to 8.64 with Tris

6. Lower reservoir buffer (anode)

As running gel buffer.

7. Sample buffer

SDS	0.9g
Cathode buffer	100ml
Bromophenol blue	0.001g

comb inserted to form the wells. It was allowed to polymerise for 1 hour. After that the comb was removed and the 30µl of each sample applied into each well. A low molecular weight marker mixture (10µl) was run in each gel. The gel was run at 50V overnight until the dye front was 1cm from the bottom of the gel.

5.2.3. Immunoblotting of SDS-Gels

After electrophoresis, the gel was submitted to immunoblotting as described in section 4.3. with some modifications. The composition of the immunoblotting solutions is given in Table 20. A piece of the gel corresponding to the molecular weight markers was cut off and stained with a solution of amido black (Table 20) for 1 minute. It was destained with acetic acid (12%). The gel containing the proteins was washed in the blotting buffer for 30 minutes followed by immunoblotting on Immobilon-P membrane in the same blotting buffer at 100V for 3 hours at 4°C. After immunoblotting, the blot was immersed in the blocking buffer and incubated for 1 hour at room temperature with shaking. Then the blot was washed with tris saline buffer for 20 minutes, changing the buffer every five minutes. It was followed by incubation with anti apo E antibody at room temperature overnight with continuous shaking. The blot was then washed again as above and incubated with the second antibody horseradish peroxidase anti sheep IgG, for 2 hours at room temperature with shaking. It was again washed in tris saline buffer and developed by incubation in a substrate solution for 30 minutes protected from the light. Washing the blot with water stopped the enzyme reaction. It was allowed to dry and stored in the dark.

Table 20: Composition of immunoblotting solutions for SDS-PAGE (Towbin et al., 1979).

1. Blotting buffer

Glycine	43.2g
Tris base	9.0g
Methanol	600ml
Distilled water up to	3 litres

Adjust to pH 8.3 with Tris or Glycine

2. Tris saline buffer

Tris saline	4.8g
Sodium chloride	16.3g
Distilled water up to	2 litres

Adjust to pH 7.4 with 1M HCl

3. Phosphate buffered saline (PBS)

NaCl	0.8g
$Na_2H_2PO_4$	0.115g
KH_2PO_4	0.020g
Distilled water up to	100ml

Adjust pH to7.3 with 1M HCl

4. Blocking buffer

Bovine serum albumin	3.0g
Normal goat serum	5ml
Tris saline buffer up to	100ml

Table 20: (contd)

5. Substrate solution

4-chloro-1-naphthol	18mg
Methanol	3.0ml
PBS	18ml
H_2O_2	60µl

6. Amido black

0.1 % w/v in methanol: water: acetic acid (45:45:1)

6. Apo E DNA sequencing

6.1. The apo E gene

The apo E gene is located on chromosome 19 (Das et al, 1985). It is 3,597 nucleotides in length and contains four exons and three introns (Paik, et al, 1985), producing a mRNA of 1163 nucleotides. Initially, the apo E is synthesized as a prepeptide of 317 amino acid residues which includes an 18 residue signal peptide which directs the precusor to the plasma membrane. The signal peptide is removed at this time. The mature apo E protein consists of 299 amino acid residues (McLean et al.,1984; Zannis et al., 1984). The mature protein is coded mostly by exon 3, with the amino acid N terminal sequence arising from exon 2.

6.2. Polymerase chain reaction

The polymerase chain reaction (PCR) is a powerful method in which a short strand of a DNA molecule is amplified many times. The amplification is carried out by a thermostable DNA polymerase (Taq) isolated from Thermus aquaticus.

The DNA is denatured by heating and separates into two single strands of DNA. Then a pair of a short oligonucleotide primers are attached on to each single stranded molecule (template). The primers act as the starting point for synthesis of new complementary strands. One primer is complementary to a sequence at the beginning of the target segment on the DNA and the second is complementary to a sequence at the end of the target segment on the antiparallel strand. This reaction is catalysed by a DNA polymerase enzyme and requires the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) which acts as substrates.

The PCR reaction is taken through 20-30 cycles of replication. Each cycle doubles the copy number of the target sequence with high specifity. The amplified DNA segment can be purified and characterised by direct sequencing.

6.3. Genomic DNA isolation

DNA was isolated from whole blood of subjects who had been previously phenotyped. 200µl of blood was lysed with 800µl of blood lysis buffer (10mM tris, 320mM sucrose, 5mM $MgCl_2$, 1% Triton x100, pH 7.5), mixed with a micropipette and then centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and the nuclear pellet washed with a further 200 µl of lysis buffer, re-centrifuged and the supernatant removed as before. The pellet was resuspended in 15µl of 10X TNE (500mM tris, 1M NaCl, 10mM EDTA, pH 7.0) and 20µl of 10% SDS. After the pellet was dissolved, 135µl of Milli-Q water (ultrapure 18 mega oms water) was added and phenol:chloroform extraction carried out according to Sambrook et al, 1989. Cold phenol:chloroform:isoamyl alcohol (25:24:1), was added (200µl) and mixed carefully until an emulsion was formed. Then it was centrifuged at 13,000 rpm for two minutes. The upper, aqueous, phase was transferred to a fresh tube and re-extracted with phenol:chloroform:isoamyl alcohol (25:24:1). Next, the aqueous supernatant was extracted with 200µl of chloroform to remove residual phenol and centrifuged for 1 minute at 13,000 rpm. The upper phase was transferred to a clean tube and two and half volumes of cold absolute ethanol were added and incubated at -20°C overnight. It was then centrifuged at 13,000 rpm for 10 minutes and the supernatant discarded. The pellet was washed with 300µl of cold 70% ethanol, re-centrifuged, the solvent discarded and the pellet dried. Finally the pellet was

dissolved in 50µl of Milli-Q water and the concentration determined by reading the absorbance at 260nm.

6.4. PCR amplification of the apo E gene

6.4.1. Amplification of exon 3

Exon 3 comprises most of the apo E sequence. The apo E receptor binding activity is located in this region in the vicinity of residues 136-158. This region is enriched in basic amino acid residues (Rall et al., 1982) which play an important role in this interaction.

Exon 3 of the apo E gene was amplified by PCR in a thermal cycler using a 21-mer sense primer (5' GAC GAG ACC ATG AAG GAG TTG 3') and a 19-mer antisense primer (5' GCG TGA AAC TTG GTG AAT C 3'). The sense primer was synthesised complementary to a sequence at the beginning of the exon 3 coding region, while the antisense primer was synthesised using a sequence from the intron region following the exon 3 coding sequence. These primers were used to amplify an 800 bp fragment. The reaction mixture was prepared by adding 100 pmol of each primer, 10µl of 10X Taq buffer, 2µl of the mixture of four dNTPs, each at a concentration of 1.25mM, 400ng of DNA template and 2.5 Units of Taq DNA polymerase in a final volume of 100µl. The mixture was transferred to a 0.5 ml thin-walled GeneAmp Reaction tube below two drops of mineral oil.

The mixture was heated at 96°C for 10 minutes to denature the DNA and then subjected to 20 cycles of amplification: denaturation (96°C for 2 minutes), primer annealing (57°C for 1 minute and 30 seconds) and extension (65°C for 4 minutes).

After completion of the cycles there was a final extension at 65° for 10 minutes. After 20 cycles, the reaction was temporarily stopped by cooling to 4°C, an extra 2.5 Units of Taq Dynazyme was added and the amplification continued for a futher 15 minutes.

6.4.1.1. Visualisation of the PCR product

The PCR product was analysed by electrophoresis on a 0.7% agarose gel in TBE buffer 1X (10.8 g tris, 5.5g boric acid, 0.83g EDTA) and 2μl of ethidium bromide solution (10mg/ml). A 100 bp ladder marker was co-electrophoresed as a size marker. It was run for 30 minutes at 75V. After electrophoresis, the gel was illuminated with ultra violet light and photographed to visualise the DNA fragment.

6.4.2. Amplification of exon 2

Exon 2 of the apo E gene was amplified by using an 18-mer sense primer (5' CCT AGG TAG CTA GAT GCC 3') and an 18-mer antisense primer (5' CCA AGA CTT AGC GAC AGG 3'). Both the sense and antisense primers were synthesised to be complementary to sequences in the intron regions, before and after the exon 2 coding region respectively. These primers were used to amplify a 353 bp fragment. The PCR mixture was prepared as in section 5.4.1. The mixture was denatured at 96°C for 10 minutes and subjected to 35 cycles of amplification consisting of denaturation (96°C for 2 minutes); annealing (45°C for 1 minute and 30 seconds) and extension (50°C for 4 minutes). After 20 cycles an extra 2.5 Units of Taq Dynazyme was added and the reaction continued for further 15 cycles.

6.4.2.1. Visualisation of the PCR product

The amplified fragment corresponding to exon 2 of the apo E gene was visualised using the same conditions as in section 6.4.1.1.

6.5. Purification of the DNA fragment

The amplified fragment was purified to remove the primers, DNA polymerase and deoxynucleotide triphosphates prior to sequencing. The fragment was purified either from the gel or from the PCR product.

6.5.1 Purification of the DNA fragment from the gel

The DNA fragment was purified from 0.7% agarose gels electrophoresed in TAE buffer 1X (4.84g tris, 1.14 ml glacial acetic acid, 2ml EDTA solution 0.5M, pH 8.0) using a QIAquick gel extraction kit. The required fragment was excised from the gel and placed in a tube. It was weighed and 3 volumes of buffer QX1 was then added and incubated at 50°C for 10 minutes to solubilize the gel. One gel volume of isopropanol was added and the pH of the mixture was checked and adjusted to a pH of less than 7.5 with 2M sodium acetate (pH 4.5) if necessary. The mixture was then loaded on a QIAquick spin column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and 750µl of buffer PE was added to the column. It was again centrifuged as above and the flow-through discarded. The column was then centrifuged again to remove residual wash buffer. Next, the column was placed in a clean microcentrifuge tube and 50µl of Milli-Q water was added to the center of the column to elute the DNA. Finally the concentration of the solution was measured at 260 nm. Then the purified PCR product was sequenced.

6.5.2. Purification of the PCR product

The PCR product was purified using a QIAquick PCR purification kit. One volume of the PCR product was placed in the clean microcentrifuge tube and five volumes of the PB buffer was added and mixed. Care was taken to avoid any mineral oil. The mixture was loaded into a QIAquick spin column connected to a collection tube. The column was centrifuged for 60seconds at 13,000 rpm. The flowthrough was discarded and the column was washed with 750µl of PE buffer and centrifuged for a further 60 seconds at 13,000rpm. The flowthrough was discarded and the column re-centrifuged to remove residual buffer. The QIAquick column was placed in a clean 1.5ml micro centrifuge tube and 50µl of Milli-Q water was applied to the centre of the column. The column was centrifuged for 30 seconds to elute the DNA and the concentration of the purified amplified product measured at 260 nm. The purified DNA was placed in the freezer until required for sequencing

6.6. Apo E DNA sequencing

Sequencing was performed by the Molecular Biology Laboratory in the Faculty of Science, Strathclyde University. Mr. Rothwell Tate carried out the sequencing reaction with my assistance.

The direct sequencing method of Sanger-Coulson was used to sequence the apo E PCR product from selected patients. DNA was sequenced with fluorescent dye-labelled dideoxynucleotides (PRISM kit, PE-Applied Biosystems Division, Warrington, Cheshire, UK) and the sequence determined using an Applied Biosystems 373A DNA sequencer.

7. Determination of phytanic acid by gas chromatography

The amount of phytanic acid can be estimated by using chromatography and spectroscopic approaches (Christie, W. W., 1982). However gas chromatography is used extensively in the analysis of all the major lipid classes. The technique requires the vaporisation of the sample prior to passing through a column (stationary phase) at a suitable temperature using a stream of carrier gas (mobile phase). The separation of the compounds occurs by adsorption effects if the prepared column consists of particles of adsorbent or by partition effects if the particles of adsorbent are coated with a liquid which forms a stationary phase.

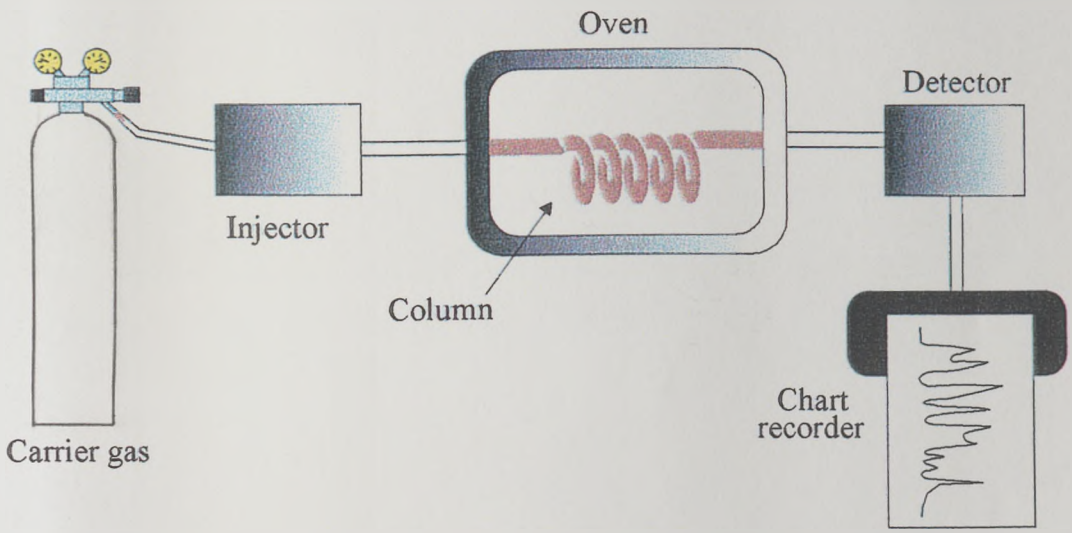
The most useful application of gas chromatography in lipid analysis is in the determination of the fatty acid composition of lipids. Volatile derivatives (such as methyl esters) of the fatty acids can be analysed without any decomposition.

The essential components of a gas chromatographic system consist of a carrier gas supply, a flow regulator to monitor the flow of the carrier gas, a sample inlet system for injecting the sample and a thermostated oven to subject the column to the required temperature. The analytical column contains the stationary phase. A detector and a chart recorder were connected to the equipment. Figure 13 shows a schematic representation of a gas chromatographic system.

7.1. Sample preparation

All lipids when left for a time exposed in air and in the daylight go rancid; this is caused either by hydrolysis or by oxidation. Therefore, care is necessary in handling the lipid samples to avoid oxidation. If this is not prevented the lipids

Figure 13: Diagrammatic representation of a gas chromatographic system



Adapted from Zubrick, J.W. (1997)

extracted would contain oxidative products which may lead to ambiguous results. In order to prevent lipid oxidation, antioxidants should be added to the sample to be analysed.

The lipids extracted from tissues contain non-lipid contaminants. They must be removed before the lipids are analysed. Care should be taken to avoid the loss of specific components. A mixture of two solvents is usually used since a single solvent can extract only few lipids. The solvent used should be sufficiently polar and have hydrogen bonding properties to remove all lipids from their association with cell membranes or with lipoproteins. It is important that the solvent does not react chemically with the lipids. The non-polar contaminants must be eliminated from the extract by washing.

7.2. Extraction of lipids

Lipid extraction was carried out using the method of Folch et al (1957). Plasma (0.5 ml) was placed in a 25 ml quick-fit stoppered tube and 2µl of butylated hydroxytoluene (BHT) (1% w/v BHT in 2:1 (v/v) chloroform:methanol) (Table 21) was added as antioxidant. Then 0.1 ml of pentadecanoic acid (450 µg/ml), used as internal standard, was added. To this mixture, 5 ml methanol and 10 ml chloroform were added in order to free the lipid from protein/lipid interactions, especially the acid minor components such as phosphatidyl inositol and phosphatidyl serine. The mixture was flushed with nitrogen (to remove any air and prevent oxidation of the sample), stoppered and placed on a rotary mixer for 30 minutes at 4°C and then filtered.

Table 21: Composition of the solutions for lipid extraction

Reagents	**Solution preparation**
Chloroform/methanol 2:1 v/v	50ml chloroform was added to 50ml methanol
Butylated hydroxy toluene (BHT)	BHT(0.1g) was dissolved in 10ml of chloroform/methanol (2:1 v/v) solution
Potassium chloride 0.88% w/v	Potassium chloride 0.88g was dissolved in 100ml distilled water
Methanol/water 1:1 v/v	Equal volumes of methanol and water were mixed
Methanolic sodium hydroxide	Sodium hydroxide (1.0g) was dissolved in 50ml methanol and allowed to stand overnight
Saturated sodium chloride	Sodium chloride (16.0g) was dissolved in 50ml distilled water

The crude lipid extract contains any molecule that has the property to be dissolved in organic solvents. Therefore, at this stage, the filtrate contains both lipid and non-lipid contaminants. The following steps involved the purification of the lipid extract. A volume of 0.88% (w/v) potassium chloride, corresponding to a quarter of the total volume of the filtrate was added and flushed with nitrogen, shaken vigorously for 15 seconds and allowed to stand for the two layers to be formed. Of the two layers formed, the top layer was discarded using a pasteur pipette. The remaining solution was further washed with methanol:water (1:1) (v/v), equal to one quarter of the total volume, flushed with nitrogen and shaken for 15 seconds and allowed to stand for the separation of two layers. The top one was discarded, and the remaining solution was washed further with 0.88% potassium chloride equal to a quarter of the total volume, followed by the same procedure as described above. Finally, the solution containing the pure lipid layer was filtered and dried under nitrogen with the tube maintained in a water bath at 60°C. This dried lipid was taken up in 0.5 ml of hexane to be methylated. At this stage, the sample can be stored at -20°C until derivatisation is carried out.

7.3. Preparation of fatty acid methyl esters

To the lipid stored in 0.5 ml of hexane, 1 ml of methanol and 1 ml of methanolic sodium hydroxide (1g NaOH in 50 ml methanol) were added. The mixture was mixed and transferred to a 'Tuftainer' silicone vial and flushed with nitrogen. The container was placed in a boiling water bath for 3 minutes. After returning to room temperature, 1 ml of 14% boron trifluoride (BF_3) in methanol was added to the solution, it was flushed with nitrogen, and the container replaced in the

boiling water bath for further 3 minutes. Finally, the sample was transferred back to a clean tube to which 8 ml hexane and 5 ml saturated sodium chloride solution were added. Then it was flushed with nitrogen, vortex mixed, and left for 30 minutes at 4°C until the two layers separated. The upper layer containing the methyl esters was transferred to a clean tube, blown dry under nitrogen, and re-dissolved in 0.1 ml of hexane for analysis by gas chromatography.

7.4. Analysis of phytanic acid methyl ester by gas chromatography

To quantify the amount of phytanic acid in the plasma the use of an internal standard was necessary. Pentadecanoic acid is a compound which is of similar chemical nature and also elutes close to the phytanic acid. It was used as internal standard because it does not occur naturally in plasma, does not interfere with any other fatty acid and therefore elutes individually. To determine its retention time and the position of the peak, a pure sample of the internal standard was subjected to the same analytical conditions. As a result a single peak was obtained on the chromatogram. A similar retention time should be obtained every time a sample containing pentadecanoic acid is run. As only methylated samples could be analysed by GC, for this experiment pentadecanoic methyl ester was used instead of pentadecanoic acid. This meant that a sample could be injected straight in the chromatograph, avoiding having to carry out the extraction process.

It was also necessary to determine the retention time of the phytanic acid in order to identify its peak position on the chromatogram. This fatty acid did not elute as a single peak from the plasma sample but was followed by various peaks corresponding to other fatty acids. A sample of phytanic acid methyl ester was run by

itself and its retention time and peak position were obtained, In addition, a sample containing five different fatty acids was injected in order to determine their retention times (Table 22) and so identify other peaks present on the chromatogram. After determining the exact retention time of the phytanic acid and the internal standard, the amount of phytanic acid could be determined in the plasma sample of the RP patients. A known amount of the internal standard was introduced into the sample in the early stage of the extract procedure. Therefore, any loss of standard during the analysis will be identical with the loss of the compound to be analysed.

7.5. Calibration curve

The analysis of fatty acids by GC can be either qualitative or quantitative. In this project, a quantitative approach was taken to quantify the level of phytanic acid in each of the RP patients.

In order to determine the concentration of phytanic acid in those samples, a calibration curve of concentration of phytanic acid versus peak area ratio was constructed. This was achieved by analyzing five samples of the control human plasma, each containing a known amount of phytanic acid. A stock solution of phytanic acid of 100μg/ml was prepared and the desired amount of phytanic acid over the concentration of 20-120μg/ml was introduced to the plasma control. In addition, pentadecanoic acid (45μg/ml) was added to each sample control in a fixed concentration (Table 23). All the solutions were prepared in chloroform. Lipid extraction following methylation was carried out as section 7.2. The sample was then injected in the GC equipment always using the conditions shown in Table 24. The corresponding peaks were identified by comparing with their retention time

Table 22: The retention time of the fatty acid methyl ester standard

Fatty acid	Retention time (min)
Pentadecanoic acid methyl ester (45μg/ml)	13.480
Phytanic acid methyl ester (50μg/ml)	16.530
Palmitic acid methyl ester (50μg/ml)	15.205
Stearic acid methyl ester (50μg/ml)	18.182
Oleic acid methyl ester (50μg/ml)	18.735
Linoleic acid methyl ester (50μg/ml)	19.836

Table 23: Solutions for the preparation of the calibration curve

Concentration	Phytanic acid (100μg/ml)	Pentadecanoic acid (450μg/ml)
20μg/ml	0.2ml	0.1ml
40μg/ml	0.4ml	0.1ml
60μg/ml	0.6ml	0.1ml
80μg/ml	0.8ml	0.1ml
100μg/ml	1.0ml	0.1ml
120μg/ml	1.2ml	0.1ml

Table 24: Gas chromatography conditions

Column	SP™ 2330 fused silica capillary column
Injector temperature	220^0C
Initial temperature	100^0C
Initial time	1 minute
Rate	5^0C/min
Final temperature	205^0C
Detector temperature	235^0C
Final time	7 min
Detector	Flame ionisation
Carrier gas	Helium
Run time	27 min

previously determined as shown in Table 22. The peak area associated with the fixed amount of internal standard is used to calculate the relative retention time ratios and relative peak area ratios for each peak in the calibration curve as well as in the sample to be analysed. Therefore, a calibration curve consists of a plot of relative peak area ratio against the known concentration of the analyte. Thus, the amount of the analyte in the test sample can be calculated.

Results

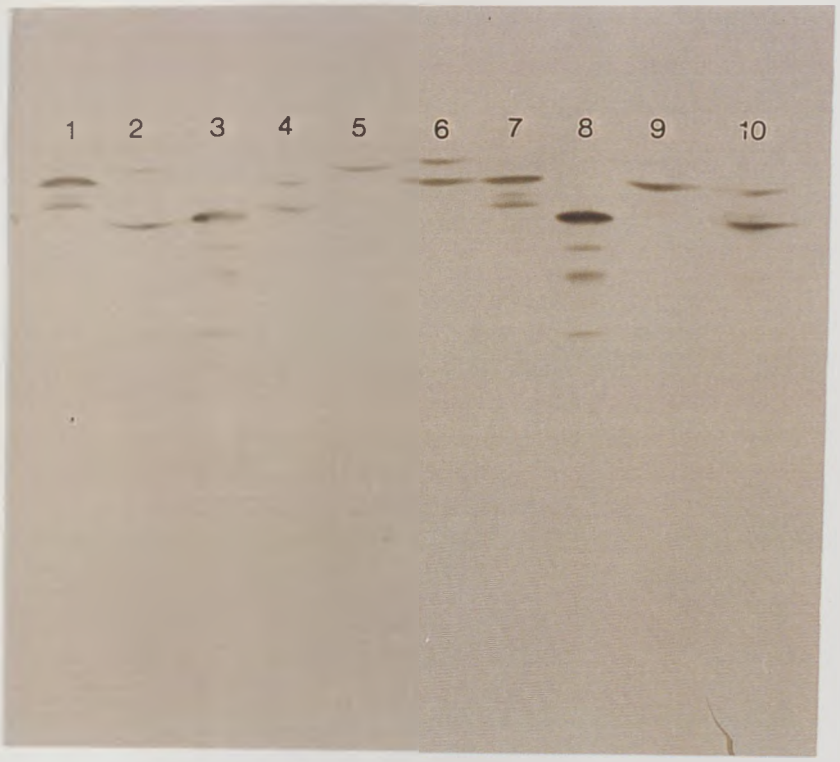
The first part of this project was to determine the apo E phenotyping in the Scottish RP patients including some of those already done by Huq. This study was carried out with the collaboration of the RP clinic in the Western Infirmary, Glasgow. The suspected RP patients were reported by their GPs. In the RP clinic some specialised tests were carried out in order to confirm the diagnosis. In addition, family history was taken to classify the type of inheritance.

1. ApoE phenotyping

The apo E phenotype was determined by isoelectric focusing by using the method of Menzel and Utermann (1986). Delipidated plasma samples were applied to an isoelectric focusing vertical slab gel system followed by blotting to nitrocellulose membrane and immunoblotting with monoclonal anti-apo E antibody as the first antibody.

In this method plasma or serum can be used instead of VLDL. The advantage is that only a small volume (10μl) is needed. However the success of this method depends on the antibody used: a specific antibody was essential. The monoclonal anti-apo E antibody used was kindly donated by the Royal Infirmary where it was routinely used for apo E phenotyping. As shown in Figure 14 the apo E antibody was specific and reacts with the apo E bands. It was also observed that the reactivity of the anti-apo E antibody depends on the concentration of the apo E itself. This could explain by the different intensities of the apo E bands observed between the samples.

Figure 14: Immunoblot illustrating various apo E phenotypes



Lane

1. DW E4/E4
2. KB E4/E2
3. HF E2/E2
4. HW E3/E2
5. LG E4/E4
6. PA E4/E3
7. MK E3/E3
8. IC E2/E2
9. RG E3/E3
10. SC E3/E2

Storage and handling precautions for the plasma sample should be taken in account in order to get true apo E bands. The plasma should be separated from the cells as soon as collected and stored frozen at -20^{o} C in small aliquots. The plasma should be defrosted only once to avoid deterioration of apo E caused by the many defrostings. When apo E decomposition occurs it can present bands that are one or more charge units more acid than the starting material. This can also occur if the plasma is stored for extended periods of time. Thus, it is very important to be aware of this problem when IEF is used for apo E phenotyping.

IEF is a gradient gel in which the proteins migrate according to their charge. By this method apo E can be designated as E2, E3 and E4. The apo E2 isoform is the most acid band with an isoelectric point (pI) of 5.5 and apo E4 the most basic with a pI of 5.75. Apo E3 is the intermediate one which shows a band in the position between apo E2 and E4 and has a pI of 5.6 (Brown et al, 1983). There are three homozygous phenotypes: E2/E2, E3/E3 and E4/E4 and three heterozygous phenotypes: E2/E3, E2/E4 and E3/E4. The phenotypes were determined by visual observation of the bands on the blotting membrane.

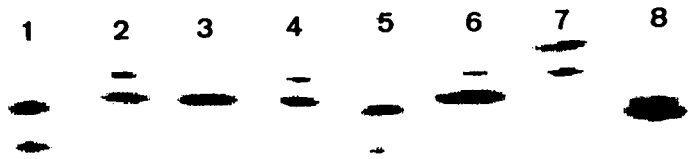
In addition to the major bands, some minor bands also appear, confusing the phenotype classification. These minor bands represent sialylated derivatives of the major isoforms that are formed as a result of post translational modification. In some cases the presence of these minor bands results in uncertain phenotypes. To overcome this problem, the samples can be treated with neuraminidase before focusing, in order to remove the sialic acid which interferes in the phenotypes. In the present work only the samples that presented uncertain results were treated with

neuraminidase, due to the additional expense.

The following strategy was adopted for the interpretation of the phenotypes. When two major bands were present on the blotting membrane, the relative proportions of them were considered. The concentration of the more acidic band must be greater than the basic one. Therefore, the presence of two major bands corresponding to E2 and E3 position can be classified as heterozygous E2/E3 if the E2 band is more intense than E3 band. However when the E3 band appeared more intense than the E2 band the sample was classified as homozygous E3/E3. When the phenotype was not clear, the sample was sent to the Royal Infirmary to be repeated in order to confirm or not the result. This was done because the method used in this study was the same method used in that laboratory.

Figure 15 shows apo E phenotyping from selected subjects. It can be seen that the samples SL (lane 2), HH (lane 4) and KG (lane 6) showed two distinct bands, one in the position corresponding to the E3 position and the other higher than the E2 position. These subjects were classified as $E3/E2^{+}$. A sample JR (lane3) presented a single band whose position was higher than E2. It was designated as $E3^{-}/E3^{-}$. Those samples were sent to the Royal Infirmary to be repeated and found to have the same results. Further work was carried out to investigate the abnormal behavior of these samples on IEF.

Figure 15: Blot showing apo E phenotyping from selected subjects



Lane

1. WA E2/E2

2. SL E3/E2^{+}

3. JR E3^{-}/E3^{-}

4. HH E3/E2^{+}

5. HF E2/E2

6. KG E3/E2^{+}

7. DW E4/E4

8. IC E2/E2

2. Population study

2.1. Control population

The control population data from the Royal Infirmary were used in the present study. The results obtained in this control population were similar to those found by Cumming and Robertson (1984) (Table 25). It was decided to use these data because the methods used for apo E phenotype in both this study and at the Royal Infirmary were the same. In addition, this is more recent data, and it relates specifically to Glasgow, whereas Cumming and Robertson were based in Aberdeen.

2.2. RP population

In the present study, the apo E phenotypes of 104 RP patients were determined. In total 101 different RP families were studied. However, some of these families had been already analysed by Huq (1990). Therefore, only 77 families (Table 26) done in this work were considered in the statistical analyses. In addition only one member of each family was considered. The common families were included just once when both studies were combined in the statistical analyses.

All genetic types of RP were represented in this sampled RP Scottish population. It can be seen in Table 27 that almost half of the families were simplex. This was similar to the fraction presented in other population studies shown in Table 1. Thus, this sampled Scottish RP population corresponds to the widespread distribution of the disease. The distribution of the apo E phenotypes according to the mode of inheritance is represented in Table 28.

Table 25: Comparation of two Scottish control populations

Phenotype	This study	This study (%) (n=671)	Cumming and Robertson, (1984) (n=400)	Cumming and Robertson, (1984) (%)
E2/E2	7	1.0	2	0.5
E3/E2	73	10.9	51	12.8
E4/E2	16	2.4	11	2.8
E3/E3	399	59.5	233	58.3
E4/E3	164	24.4	99	24.8
E4/E4	12	1.8	4	1.0

Table 26: The apo E phenotypes of the 77 RP patients

Number	Initials	Type of RP	Apo E phenotyping
1	AB	AD	E2/E3
2	AC	AD	E3/E3
3	AH	AD	E3/E3
4	AL	AD	E3/E3
5	EC	AD	E3/E3
6	KB	AD	E2/E4
7	GB	AD	E3/E4
8	HW	AD	E2/E3
9	JS	AD	E2/E3
10	JG	AD	E3/E3
11	JH	AD	E4/E4
12	JC	AD	E2/E3
13	JD	AD	E3/E3
14	MC	AD	E3/E3
15	MF	AD	E3/E3
16	ML	AD	E3/E3
17	MW	AD	E3/E3
18	RF	AD	E2/E4
19	RK	AD	E3/E3

Table 26 contd

Number	Initials	Type of RP	Apo E phenotyping
20	WM	AD	E2/E3
21	CMC	AR	E3/E4
22	DC	AR	E3/E3
23	DM2	Simplex	E4/E4
24	HM	AR	E2/E3
25	JR	AR	E2/E3
26	MK	AR	E3/E3
27	MW	AR	E3/E3
28	MP	AR	E4/E4
29	PA	AR	E3/E4
30	PW	AR	E3/E3
31	SJ	AR	E3/E3
32	RW	AR	E2/E3
33	JC	Multiplex	E2/E3
34	GB	Refsum	E3/E3
35	AW	Simplex	E3/E3
36	AM	Simplex	E3/E3
37	AH2	Simplex	E3/E3
38	AB2	Simplex	E3/E3
39	CA	Simplex	E3/E3
40	CU	Simplex	E3/E4

Table 26 contd

Number	Initials	Type of RP	Apo E phenotyping
41	CC	Simplex	E3/E4
42	CD	Simplex	E3/E3
43	CH	Simplex	E3/E3
44	DM	Simplex	E2/E4
45	EC	Simplex	E3/E4
46	HC	Simplex	E3/E3
47	HR	Simplex	E3/E3
48	HF	Simplex	E2/E2
49	JM	Simplex	E3/E3
50	JW	Simplex	E3/E4
51	JM	Simplex	E3/E3
52	JB	Simplex	E3/E3
53	KG	Simplex	E2/E3
54	LT	Simplex	E3/E4
55	MJW	Simplex	E3/E3
56	MY	Simplex	E3/E3
57	NA	Simplex	E3/E3
58	PG	Simplex	E3/E3
59	RW	Simplex	E3/E4
60	RG	Simplex	E3/E3
61	RM	Simplex	E2/E3

Table 26 contd

Number	Initials	Type of RP	Apo E phenotyping
62	SH	Simplex	E2/E3
63	SS	Simplex	E2/E3
64	SA	Simplex	E3/E3
65	SL	Simplex	E3/E3
66	AS	Ushers	E3/E3
67	EB	Ushers	E3/E4
68	IM	Ushers	E3/E3
69	ID	Ushers	E3/E3
70	MW	Ushers	E2/E3
71	SH	Ushers	E3/E3
72	SC	Ushers	E2/E3
73	WD	Ushers	E3/E4
74	DW	X-linked	E4/E4
75	IC	X-linked	E4/E4
76	MM	X-linked	E3/E3
77	PD	X-linked	E3/E3

Abbreviations:

AD: Autosomal Dominant

AR: Autosomal Recessive

Table 27: Distribution of the genetic types in the sampled Scottish RP population

Type of RP	No. of families	% of Total
Simplex	31	40.5
Multiplex	1	1.3
X-linked	4	5.2
Autosomal Dominant	21	27.3
Autosomal Recessive	11	14
Autosomal Recessive Syndrome*	9	11.7

* This includes 8 cases of Usher syndrome and 1 cases (two sisters) of Refsum's disease.

Table 28: Distribution of the apo E phenotypes according to the mode of inheritance of RP

	Genetic type of RP						
Apo E phenotype	**Simplex**	**Multiplex**	**X-linked**	**Autosomal Dominant**	**Autosomal Recessive**	**Autosomal Recessive Syndrome**	**Total (n=77)**
E2/E2	1	-	-	-	-	-	1
E3/E2	4	1	-	6	3	2	16
E4/E2	-	-	-	2	-	-	2
E3/E3	19	-	2	11	5	5	42
E4/E3	6	-	-	1	2	2	11
E4/E4	1	-	2	1	1	-	5

In this study the frequency of the E2/E2 isoform was only slightly above normal compared to the Scottish control population (Table 29). However, a four to six-fold increase in E4/E4 was observed as compared with the controls. The incidence of the apo E phenotypes in the Scottish RP population found in this work was also compared with the results presented by Huq (1990) (Table 29). It was observed that the incidence of E2/E2 was greater than controls in the Huq study while in the present study it appeared to be normal. On the other hand, the incidences of E3/E2 and E4/E4 were in accordance with her results in that in both studies they were increased compared to the control population. In addition, the incidence of E4/E2 was normal in both Scottish RP populations, while the incidence of E4/E3 was decreased in this study and normal in Huq's study.

Table 30 shows the incidence of apo E phenotypes and allele frequencies in the RP populations (both studies) compared to the controls. It can be observed that the incidences of E2/E2 and E4/E4 were increased. In addition, the allele frequency of ε2 was increased in both the Scottish RP population (Table 30) as well as in the German RP population (Table 13)

This work was performed using the same methodology as Huq did. Therefore it was decided to do the statistical analysis on the combined studies. This is shown in Table 30. A 2x2 Chi-squared Test was performed on each phenotype to check whether the levels were statistically significant.

The contigency table for the incidence of E2/E2 in this study was constructed; the increase in E2/E2 was not statistically significant. In addition, when the contigency table of E2/E2 phenotype was examined in the combined studies, it

Table 29: Incidence of apo E phenotypes in the Scottish RP population compared to Huq's study and control population

Apo E phenotype	This study (n=77)	This study (%)	Huq's study (n=100)	Huq's study (%)	Control population (n= 671)	Control population (%)
E2/E2	1	1.3	4	4	7	1.0
E3/E2	16	20.8	20	20	73	10.9
E4/E2	2	2.6	1	1	16	2.4
E3/E3	43	55.8	44	44	399	59.5
E4/E3	10	13.0	23	23	164	24.4
E4/E4	5	6.5	8	8	12	1.8

Table 30: Incidence of apo E phenotype and allele frequency in the Scottish RP patients compared to controls

	E2/E2	E3/E2	E4/E2	E3/E3	E4/E3	E4/E4
Both stydies (n=177)	5	38	3	86	34	13
(%)	2.8	20.3	1.8	48.6	19.2	7.3
Control population (n=671)	7	73	16	399	164	12
(%)	1	10.9	2.4	59.5	24.4	1.8

Allele		
ε2	**ε3**	**ε4**
*0.139	0.686	0.175
**0.077	0.771	0.152

* Both studies

**Control population

was not found to be significant at the $p<0.05$ level (Table 31). However, the contingency table of E4/E4 in the present work showed that the increase was significant at the $p<0.01$ level (Table 32) and at the $p<0.001$ level when the E4/E4 contingency table was analysed in both studies (Table 33). These results suggest that E4/E4 may be implicated in RP.

3. Characterisation of the selected apo E phenotypes

The increase in the apo E2/E2 phenotypes found by Huq and the confirmation that apo E4/E4 was increased in RP patients led us to investigate the possibility of apo E variants. By the conventional IEF method the patients classified as E2/E2 and E4/E4 showed bands that focused at the normal positions. There is, however, the possibility that when there is a mutation it does not cause a charge difference. This, would not be observed by the IEF technique since this method separates proteins according to their charge. Therefore, chemical modifications were carried out to investigate this possibility. The selected E2/E2 and E4/E4 individuals are listed in Table 34. In addition, several heterozygous patients with unusual band patterns were examined (MB, MF, JR, SH in Figure 16a, b).

3.1. Cysteamine modification

The three common apo E isoforms (E2, E3 and E4) differ from each other by their cysteine content. Apo E2 and E3 contain two and one cysteine residues respectively, while apo E4 lacks cysteine residues.

Cysteamine modification of cysteine residues can be used to characterise apo E variants. When apo E is treated with cysteamine the disulphide combines with

Table 31: Contingency table comparing the incidence of E2/E2 in the Scottish RP population (both studies) to Scottish controls

	E2/E2	**Other Phenotypes**
RP (n = 177)	5	172
Control (n = 671)	7	664

Chi-squared test: $T = 3.19$ $p < 0.10$

Table 32: Contingency table comparing the incidence of E4/E4 in the Scottish RP population (this study) to Scottish controls

	E4/E4	**Other Phenotypes**
RP (n = 77)	5	72
Control (n = 671)	12	659

Chi-squared test: $T = 4.94$ $p < 0.01$

Table 33: Contingency table comparing the incidence of E4/E4 in the Scottish RP population (both studies) to Scottish controls

	E4/E4	**Other Phenotypes**
RP (n = 177)	13	164
Control (n = 671)	12	659

Chi-squared test: T = 15.2 $p < 0.001$

Table 34: Selected RP subjects for cysteamine modification

Initials of patients	Genetic type of RP	Apo E phenotypes
IC	Simplex	E2/E2
WA	Simplex	E2/E2
HF	Simplex	E2/E2
DW	X-linked	E4/E4
JH	AD	E4/E4
DM	Simplex	E4/E4
MP	AR	E4/E4
LG	AD	E4/E4

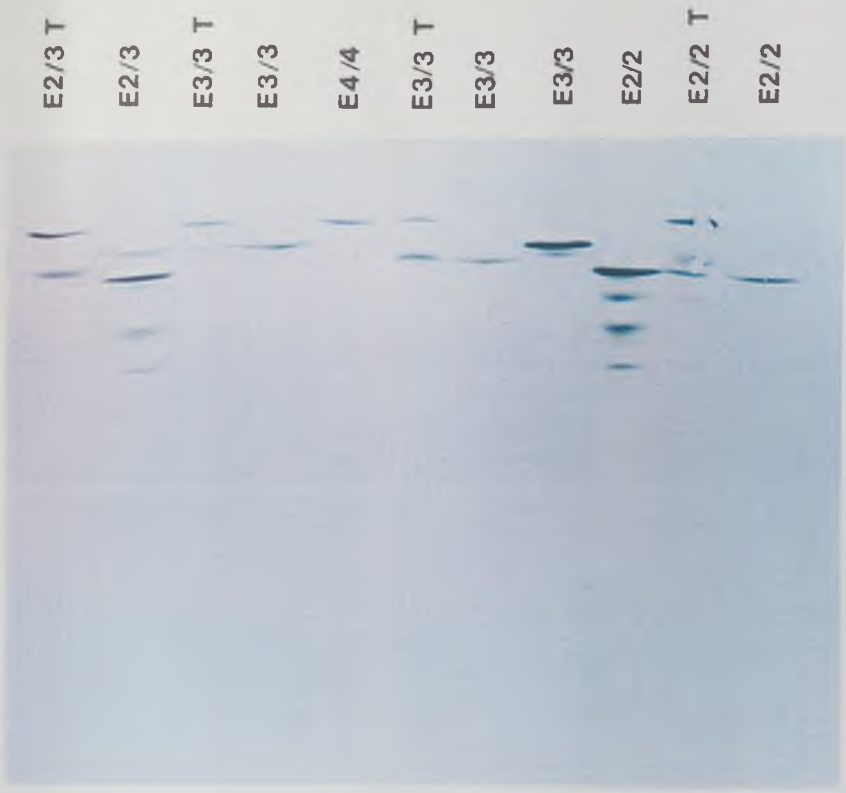
cysteine residue resulting in a lysine analogue side chain which adds a positive charge on the protein for each cysteine present. Therefore when apo E2, which has two cysteines, is treated with cysteamine it is expected to move up two positive charge units, while apo E3, with one cysteine, should move up one positive unit. Apo E4 remains in the same position due to the absence of cysteine residues. Thus, after cysteamine treatment apo E2 and apo E3 bands will focus at the position corresponding to E4. This charge modification can be monitored by IEF.

Figure 16a and 16b illustrate a typical blot of a cysteamine modification experiment in which the samples moved to the positions according to their cysteine content. Cysteamine modification of selected subjects is shown in Figure 16b. The E2/E2 subjects: IC (lane 1), WA (lane 2) and HF (lane 3) behaved as expected, that is, the E2 band moved up two positive charges. This indicates the presence of two cysteine residues in these individuals. This result was unexpected for the IC patient who was found by Huq to behave abnormally. In her work, when treated with cysteamine this patient's apo E moved up only one positive charge suggesting the presence of one cysteine residue instead of two. Further investigation was carried out on this subject (see below). In addition, all the E4 samples remained in the same position after cysteamine treatment as expected. This suggests that these samples do not have cysteine.

3.2. Characterisation of apo E isoforms by SDS-PAGE

The apo E isoforms E2, E3 and E4 have different mobility on SDS-PAGE (Utermann et al, 1979). Apo E2 can be distinguished from E3 and E4 by its slower mobility on SDS-PAGE. This suggests that E2 has a higher apparent molecular

Figure 16a: Blot showing cysteamine modification of selected subjects

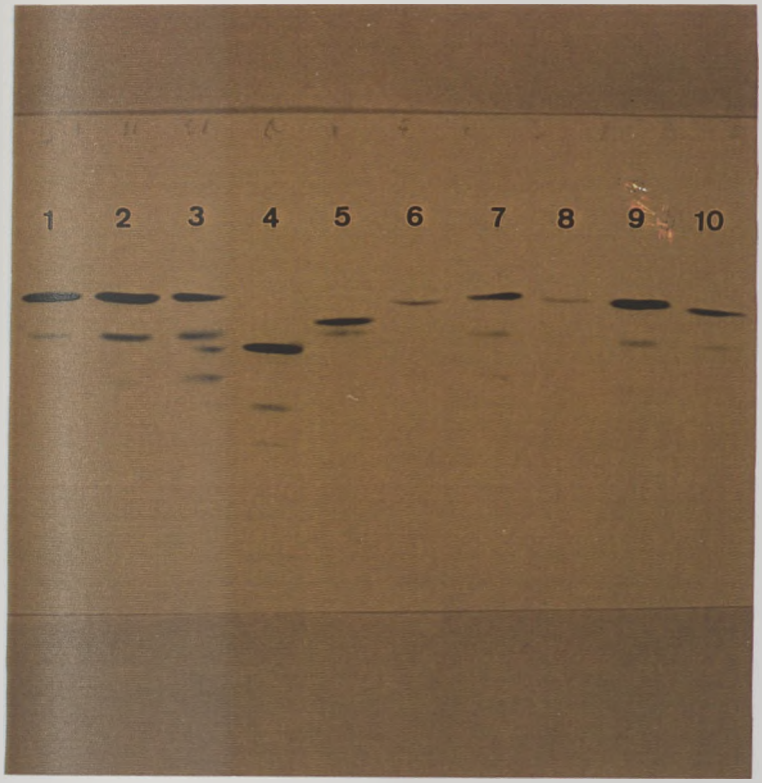


Lane

1. MB E3/E2 T
2. MB E3/E2
3. MF E3/E3 T
4. MF E3/E3
5. LG E4/E4
6. JR E3/E3 T
7. JR E3/E3
8. MK E3/E3
9. IC E2/E2
10. IC E2/E2 T
11. HF E2/E2

T – treated with cysteamine

Figure 16b: Blot showing cysteamine modification of selected samples



Lane

1. IC E2/E2 T
2. WA E2/E2 T
3. HF E2/E2 T
4. IC E2/E2
5. SH E3/E3
6. LG E4/E4 T
7. SH E3/E3 T
8. LG E4/E4
9. DM E4/E4 T
10. DW E4/E4 T

T – treated with cysteamine

weight than E3 and E4. The difference is calculated to be 1500 Da (Utermann et al, 1979). However, apo E3 and E4 bands appear in the same position indicating that the presence of the cysteine residue in apo E3 (position 112) does not interfere in its electrophoretic mobility in relation to E4. This method is useful to characterise apo E variants. Utermann and coworkers (1984) described the apo E2 variants E2 ($Arg_{145}\rightarrow Cys$) and E2 ($Lys_{146}\rightarrow Gln$) by this method. Therefore, it was used to investigate the mobility of the selected patients listed on Table 35.

3.2.1. Laemmli SDS-PAGE

Laemmli SDS-PAGE was used to investigate the mobility of the apo E isoforms in some subjects who behaved abnormally on IEF (Figure 15). These selected individuals are shown in Figure 17.

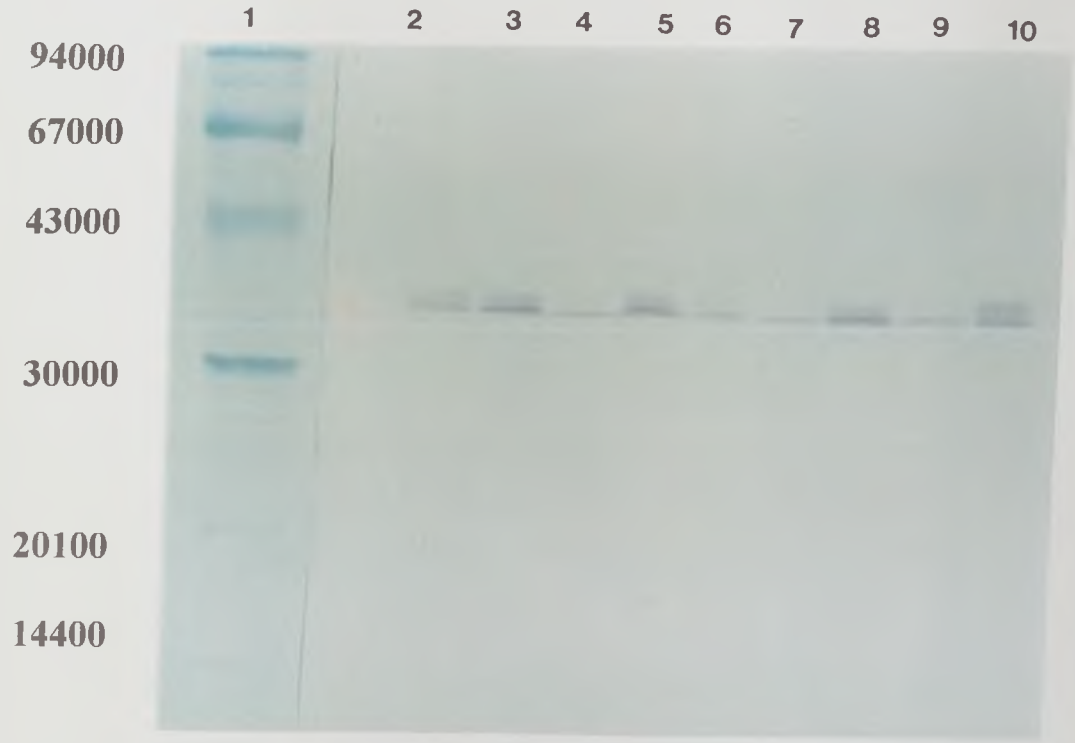
Delipidated plasma from these subjects was dissolved in the Laemmli sample buffer and 20μl was loaded into each well. A high marker protein was also used in each gel to monitor the molecular weight of the apo E bands on the SDS-PAGE. Subjects SL (lane 4), HH (lane 6) and KG (lane 7) who were previously classified as $E3/E2^{+}$ were expected to present two bands on this method. However, only one band showed up.

In addition, the subject JR (lane 9) was classified as $E3^{-}/E3^{-}$ on the basis of IEF. This sample showed a single band whose position was higher than the normal E2/E2. On the SDS-PAGE, it was shown to be in the same position as the E3 band, suggesting the presence of only one cysteine residue. The possibility of these individuals having an apo E variant was further investigated by sequencing their

Table 35: Selected subjects characterised by SDS-PAGE

Initials	Apo E phenotype
HH	$E3/E2^{+}$
KG	$E3/E2^{+}$
SL	$E3/E2^{+}$
JR	$E3^{-}/E3^{-}$
HF	E2/E2
WA	E2/E2
IC	E2/E2
DM	E4/E4
LG	E4/E4
PM	E4/E4
DW	E4/E4
IC2	E4/E4
JH	E4/E4

Figure 17: Laemmli SDS-PAGE to determine the apparent molecular weight of apo E from selected RP subjects



Lane

1. Marker protein
2. MK E3/E3
3. IC E2/E2
4. SL E3/E2^{+}
5. WA E2/E2
6. HH E3/E2^{+}
7. KG E3/E2^{+}
8. HF E2/E2
9. JR E3^{-}/E3^{-}
10. IC E2/E2

apo E DNA genes.

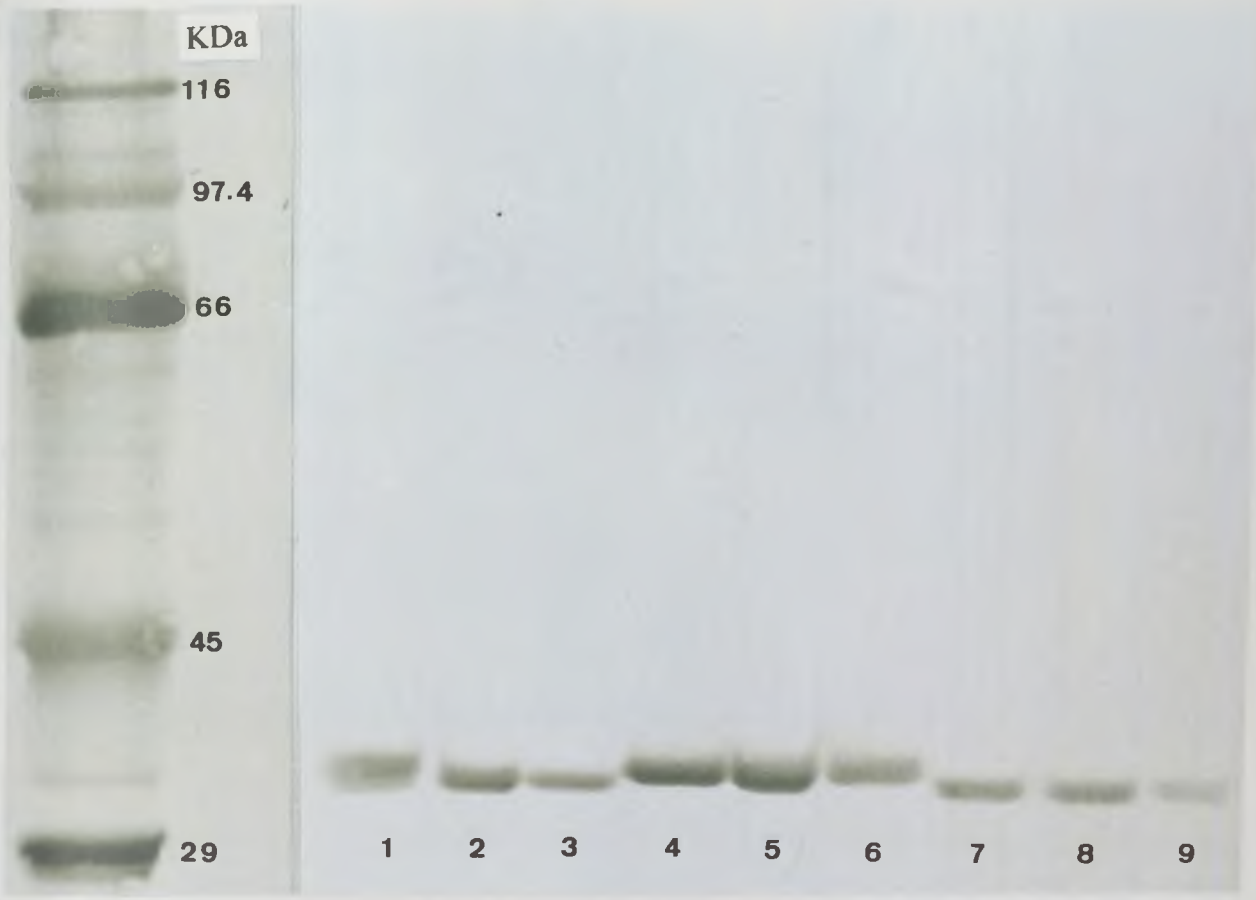
3.2.2. Neville SDS-PAGE

To investigate the different mobilities of E2 and E4 bands from selected subjects, the technique of SDS gel electrophoresis in discontinuous buffer systems introduced by Neville (1971) was used with some modifications as described by Utermann and co-workers (1984). The advantage of this method is to fractionate plasma proteins and provide high resolution patterns.

It can be seen in Figure 18 that apo E from the subjects phenotyped as E2/E2, IC (line 4), WA (lane 5) and HF (lane 6) had a slightly retarded mobility compared to E3/E3 and E4/E4 subjects HC (lane 3), JH (lane 7), LG (lane 8) and DW (lane 9). This result suggests that these patients have normal apo E2 since on SDS-PAGE the mobility of apo E2 is expected to be slightly retarded compared to E3 and E4. This change in mobility is attributed to the presence of a cysteine residue at position 158. Therefore, these patients classified as E2/E2 probably have apo E with two cysteine residues.

The result presented by subjects IC, WA and HF on Neville gels was consistent with that found in the cysteamine modification experiments. However, it is not possible to predict whether these cysteine residues are located at the normal position from these results. Therefore, these subjects were further characterised by having their apo E genes sequenced.

Figure 18: Neville SDS-PAGE from selected samples



Lane

1. DM E4/E4
2. MK E3/E3
3. HC E3/E3
4. IC E2/E2
5. WA E2/E2
6. HF E2/E2
7. JH E4/E4
8. LG E4/E4
9. DW E4/E4

4. Apo E DNA sequencing

Huq (1990) reported that the apo E from a RP subject who was classified as E2/E2 on the basis of IEF behaved abnormally after chemical modification. When IC's plasma was treated with cysteamine, the change in the isoelectric point of the apo E was not consistent with the phenotype. Cysteamine affects the mobility of apo E according to the number of cysteine residues. This patient's apo E was expected to move to the E4 position, since he was classified as E2/E2 on IEF and so his apo E should contain two cysteine residues at position 112 and 158. Instead, the E2 band moved up only one charge unit, indicating that this isoform only had one cysteine residue. This result suggests that this patient may have a substitution in the sequence of the amino acids causing the difference in charge as shown by IEF. This result leads us to investigate the possibility of a new apo E variant by using DNA sequencing. In addition, these subjects who had abnormal behaviour on IEF in the present work were also investigated by this method. These selected subjects are shown in Table 36.

4.1. Polymerase chain reaction

The polymerase chain reaction (PCR) is a powerful method in which a short strand of a DNA molecule is amplified many times. The amplification is carried out by a thermostable DNA polymerase (Taq) isolated from *Thermus aquaticus*. Figure 19 illustrates a PCR reaction.

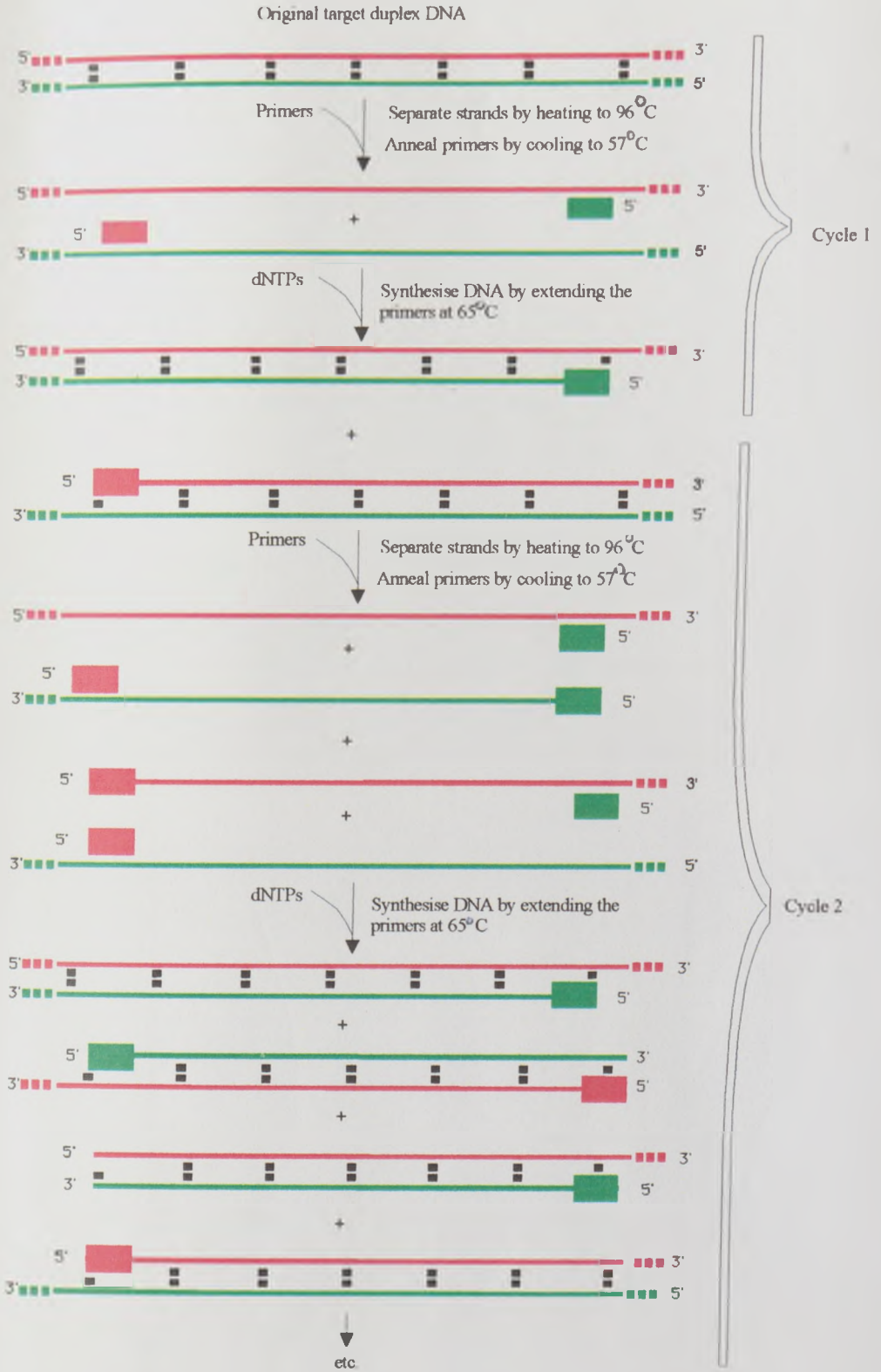
The DNA is denatured by heating into two single strands DNA. Then a pair of a short oligonucleotide primers are attached on to each single stranded molecule

Table 36: Selected subjects characterised by DNA sequencing

Initial	Apo E phenotype (by IEF)	Cysteamine modification
IC	E2/E2	Abnormal (Huq, 1990) *
HF	E2/E2	Normal
WN	E2/E2	Normal
WM	E2/E2	Normal
HH	E3/E2$^+$	Normal
SL	E3/E2$^+$	Normal
SJ	E3/E2$^+$	Normal
KG	E3/E2$^+$	Normal
DW	E4/E4	Normal

*Cysteamine modification was normal in the present work

Figure 19: Schematic representation of a PCR reaction



Adapted from Voet and Voet (1995)

(template). The primers act as the starting point for synthesis of a new complementary strand. One primer is complementary to a sequence at the beginning of the target segment on the DNA and the second is complementary to a sequence at the end of the target segment on the antiparallel strand. This reaction is catalysed by a DNA polymerase enzyme and requires the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) which act as substrates.

The PCR reaction is taken through 20-30 cycles of replication. Each cycle doubles the copy number of the target sequence with high specifity. The amplified DNA segment can be purified and characterised by direct sequencing.

4.2. PCR amplification of the apo E gene from selected patients

The apo E gene comprises four exons and three introns making an mRNA of 1,163 nucleotides (Paik et al, 1985). The nucleotide and protein sequence is illustrated in Figure 11. Initially, the apo E is synthesized as a prepeptide of 317 amino acid residues which includes an 18 residue signal peptide which directs the precursor to the plasma membrane. The signal peptide is removed at this time. The mature apo E protein consists of 299 amino acid residues (McLean et al., 1984; Zannis et al., 1984).

The polymerase chain reaction was used to amplify the apo E gene. This method allows the detection of common and rare apo E variants not detected by IEF. The strategy was first to design primers to amplify exon 3 (Figure 20). This region includes the LDL-receptor binding activity which is located between residues 136 and 158. It also contains the residues 112 and 158 which define the three major apo E isoforms. The visualization of the exon 3 PCR fragment is shown in Figure 21.

Figure 20: The apo E coding sequence

Exon 1

ATGAAGGTTCTGTGGGCTGCGTTGCTGGTCACATTCCTGGCAG

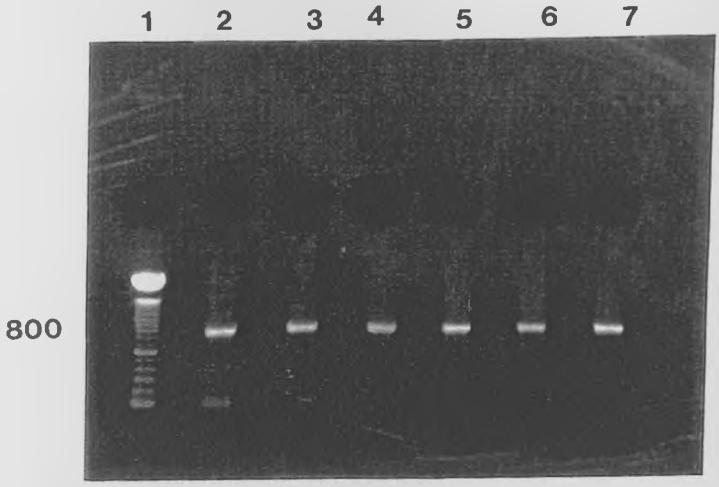
Exon 2

GATGCCAGGCCAAGGTGGAGCAAGCGGTGGAGACAGAGCCGGAGCCCG
AGCTGCGCCAGCAGACCGAGTGGCAGAGCGGCCAGCGCTGGGAACTGGC
ACTGGGTCGCTTTTGGGATTACCTGCGCTGGGTGCAGACACTGTCTGAGC
AGGTGCAGGAGGAGCTGCTCAGCTCCCAGGTCACCCAGGAACTGAG

Exon 3

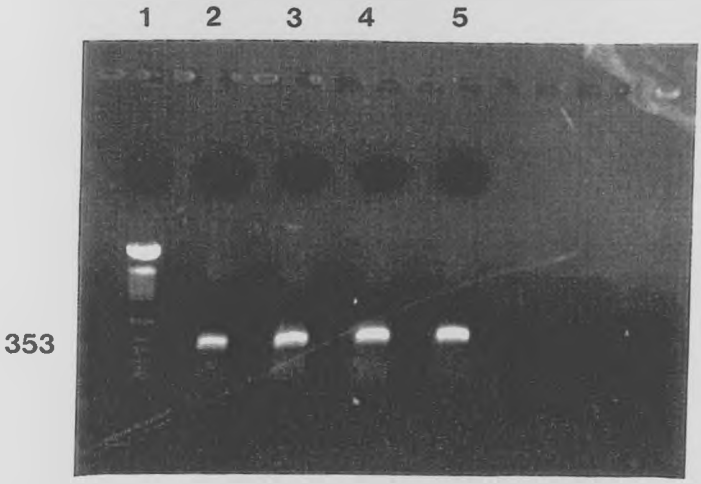
GGCGCTGATGGACGAGACCATGAAGGAGTTGAAGGCCTACAAATCGGAA
CTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGT
CCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGG
ACCTGCGCGGCCGCCTGGTGGAGTACCGCGGCGAGGTGCAGGCCATGCT
GGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGC
AAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGCGCC
TGGCAGTGTACCAGGCCGGGGCCCGCGAGGGCGCCGAGCGCGGCCTCAG
CGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGGGCCGCGTGCGG
GCCGCCACTGTGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCC
AGGCCTGGGGCGAGCGGCTGCGCGCGCGGATGGAGGAGATGGGGAGCC
GGACCCGCGACCGCCTGGACGAGGTGAAGGAGCAGGTGGCGGAGGTGC
GCGCCAAGCTGGAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCGAGC
CTTCCAGGCCCGCCTCAAGAGCTGGTTCGAGCCCCTGGTGGAAGACATGC
AGCGCCAGTGGGCCGGGCTGGTGGAGAAGGTGCAGGCTGCCGTGGGCAC
CAGCGCCGCCCCTGTGCCCAGCGACAATCACTGA

Figure 21: Visualisation of the PCR product



Exon 3

1- 100 bp DNA ladder
2- IC
3- DW
4- WM
5- WN
6 - KG
7 - SL



Exon 2

1 – 100 bp DNA ladder
2 – IC
3 - KG
4 – DW
5 – WN

The PCR product obtained from subject IC was purified and sequenced completely in both directions on the Applied Biosystems 373A DNA Sequencer. Using a computer program, the sequences were compared and aligned. The results demonstrated that IC had the normal sequence for E2 with cysteine residues at positions 112 and 158. This result was not in accordance with the cysteine modification result found by Huq (1990) that suggested the presence of only one cysteine residue. It was thought that maybe the expected mutation could be present in the other allele. Again an oligonucleotide sense primer was designed to amplify it. The fragment was purified and sequenced. The results showed a normal sequence in exon 3 (Figure 22). Therefore, no mutation was found in exon 3 for both alleles. To investigate further, it was decided to sequence the remaining expressed exon. Sense and antisense primers were synthesised to amplify exon 2. The amplified segment is shown in Figure 21. The PCR product was purified and sequenced. The sequence was compared and aligned and found to have a normal sequence. Unfortunately no mutation was found in exon 2 (Figure 23) either.

The apo E DNA sequencing from IC was in accordance with the results found in this work. In fact, when IC's apo E was treated with cysteamine it moved to the E4 position indicating the presence of two cysteine residues. The apo E DNA sequence showed that the cysteine residues were located in the normal positions 112 and 158 (Table 37). Therefore, IC was found to have a normal apo E2 sequence, by two techniques.

Exon 1 from the apo E gene has eighteen nucleotides which corresponding to the signal peptide. It does not contain any of the protein coding region, therefore

Figure 22: Chromatogram showing the exon 3 sequence from IC subject

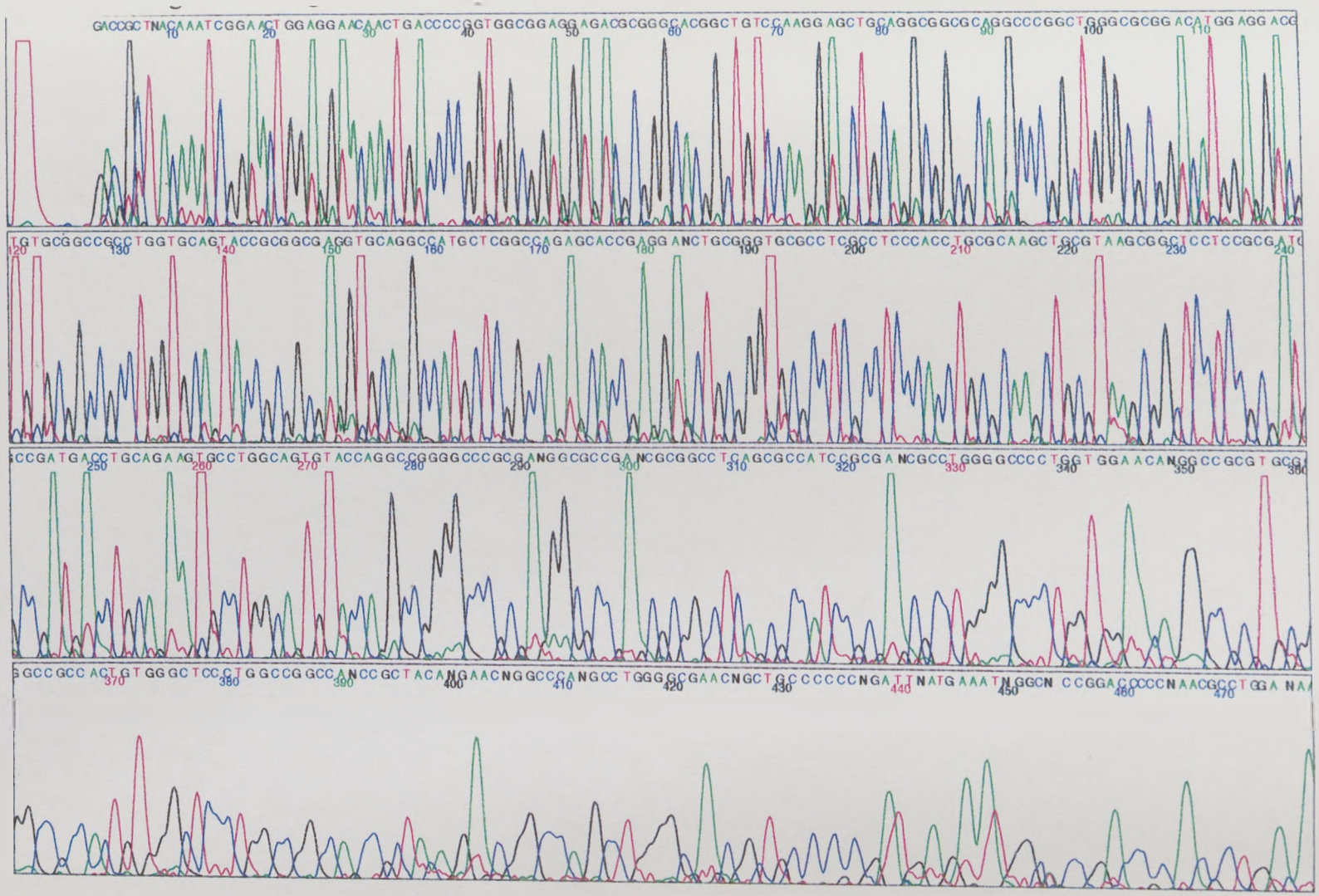


Figure 23: Chromatogram showing the exon 2 sequence from IC subject

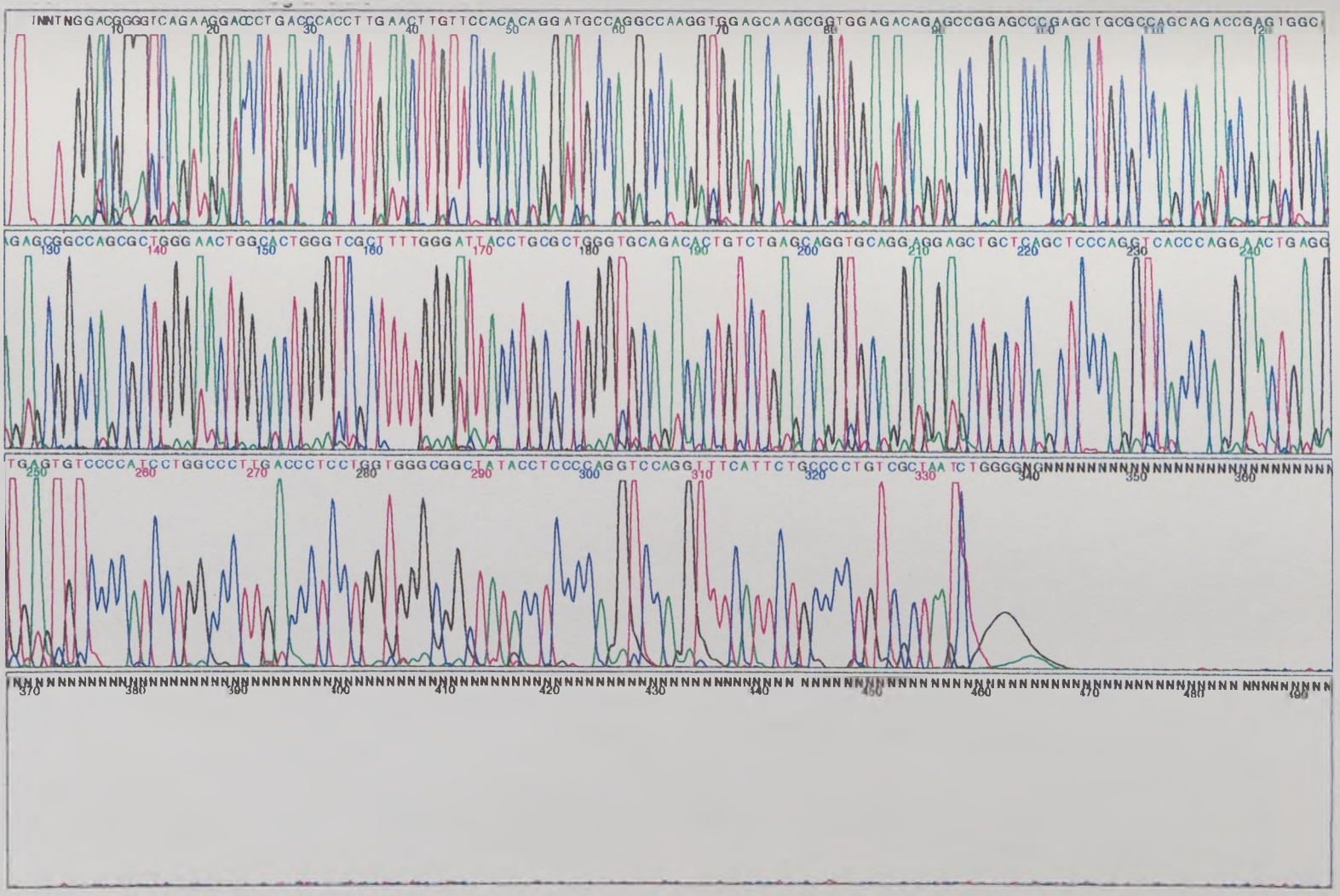


Table 37: DNA sequence of exon 3 (residues 112 and 158) from selected subjects

Initial	Residue 112	Residue 158	Genotyping
IC*	TCG (Cys) TCG (Cys)	TCG (Cys) TCG (Cys)	E2/E2
HF	TCG (Cys) TCG (Cys)	TCG (Cys) TCG (Cys)	E2/E2
DW	CGC (Arg) CGC (Arg)	CGC (Arg) CGC (Arg)	E4/E4
HH	TCG (Cys) TCG (Cys)	CGC (Arg) CGC (Arg)	E3/E3
SL	TCG (Cys) TCG (Cys)	CGC (Arg) CGC (Arg)	E3/E3
KG	TCG (Cys) TCG (Cys)	CGC (Arg) CGC (Arg)	E3/E3
WN	TCG (Cys) TCG (Cys)	TCG (Cys) CGC (Arg)	E2/E3
WM	TCG (Cys) CGC (Arg)	TCG (Cys) CGC (Arg)	E2/E4
SJ	TCG (Cys) TCG (Cys)	TCG (Cys) CGC (Arg)	E2/E3

* In the case of IC, the two alleles were determined separately using different primers. For the others, the two alleles were determined together from a single PCR experiment.

it was not sequenced.

The subject DW was phenotyped as E4/E4 on the basis of IEF (Table 36). When treated with cysteamine the E4 band remained in the same position, indicating the lack of cysteine residues. However, the presence of two bands on SDS-PAGE leads us to investigate further. It was decided to amplify exon 3. The amplified segment was purified and directly sequenced. The sequence was aligned and compared with normal apo E sequences. The results showed a normal sequence for exon 3 as well as confirming the E4/E4 phenotype found on IEF. It was decided to amplify the exon 2. This segment was purified and sequenced. It was found to have a normal sequence for exon 2 of apo E. Since this sample has a normal apo E sequence, the presence of an extra band on SDS-PAGE could be due to over loading of the sample.

Subjects HF, WM and WN were classified as E2/E2 on IEF. This result was confirmed by the presence of two cysteine residues as demonstrated by cysteamine modification. Since the E2/E2 phenotype was found at a higher SDS-PAGE position in RP patients than in the normal population (Huq, 1990), it was decided to amplify exon 3 to investigate the possibility of a new apo E variant. Subject HF's apo E was confirmed by DNA sequence to be homozygous E2/E2 and it was also demonstrated to have a normal sequence. Subject WN was found to have heterozygous E2/E3 by DNA sequencing. This result was not in accordance with that found on IEF. The presence of the band corresponding to E3 was absent on IEF. In addition, the subject WM, previously phenotyped as homozygous E2/E2 on IEF, was also found to be heterozygous E2/E4 by DNA sequence analysis and to contain a normal DNA

sequence. This suggests that IEF is not always a very reliable method for apo E phenotyping, since some bands may not be detected by this method resulting in misinterpreted results.

The subjects KG, HH, SL and SJ were classified as E2/E2^{+}, due to the band being slighty more basic than the normal E2 band on IEF. These results led us to determine the apo E DNA sequence from those individuals. Exon 3 was amplified from each of these patients. It was purified and sequenced. The subjects KG, HH and SL were found to have a cysteine residue at position 112 and an arginine at residue 158 (Table 37). Therefore these subjects were classified as E3/E3 by DNA sequence. These samples also were demonstrated to contain a normal DNA sequence. The subject SJ was found to be heterozygous E2/E3. In this case the apo E3 band was not picked up by IEF. The reason that these bands did not show in the normal position corresponding to the E3 band could be that the samples were stored for a long time before analysis.

5. Phytanic acid

Phytanic acid is a 20 carbon saturated branched-chain fatty acid. Its origin is exogenous, phytanic acid being a normal constituent of food and dairy products and ruminant meat. Unlike others fatty acids, phytanic acid cannot be metabolised by β-oxidation due to the presence of the methyl side chain on the β carbon which blocks the enzymatic action. An initial α-oxidation is needed. However, alpha oxidation of phytanic acid is defective in patients with Refsum's disease and the disorders of peroxisome biogenesis (Zellweger syndrome, infantile Refsum's disease). This defect results in phytanic acid accumulation in the plasma and the tissues.

Refsum's disease is a rare autosomal recessive neurologic disorder associated with an elevated concentration of phytanic acid in the serum and the tissues. The clinical features include atypical retinitis pigmentosa, peripheral neuropathy, ataxia and elevated cerebrospinal fluid protein concentration. The diagnosis is confirmed by plasma phytanic acid measurement.

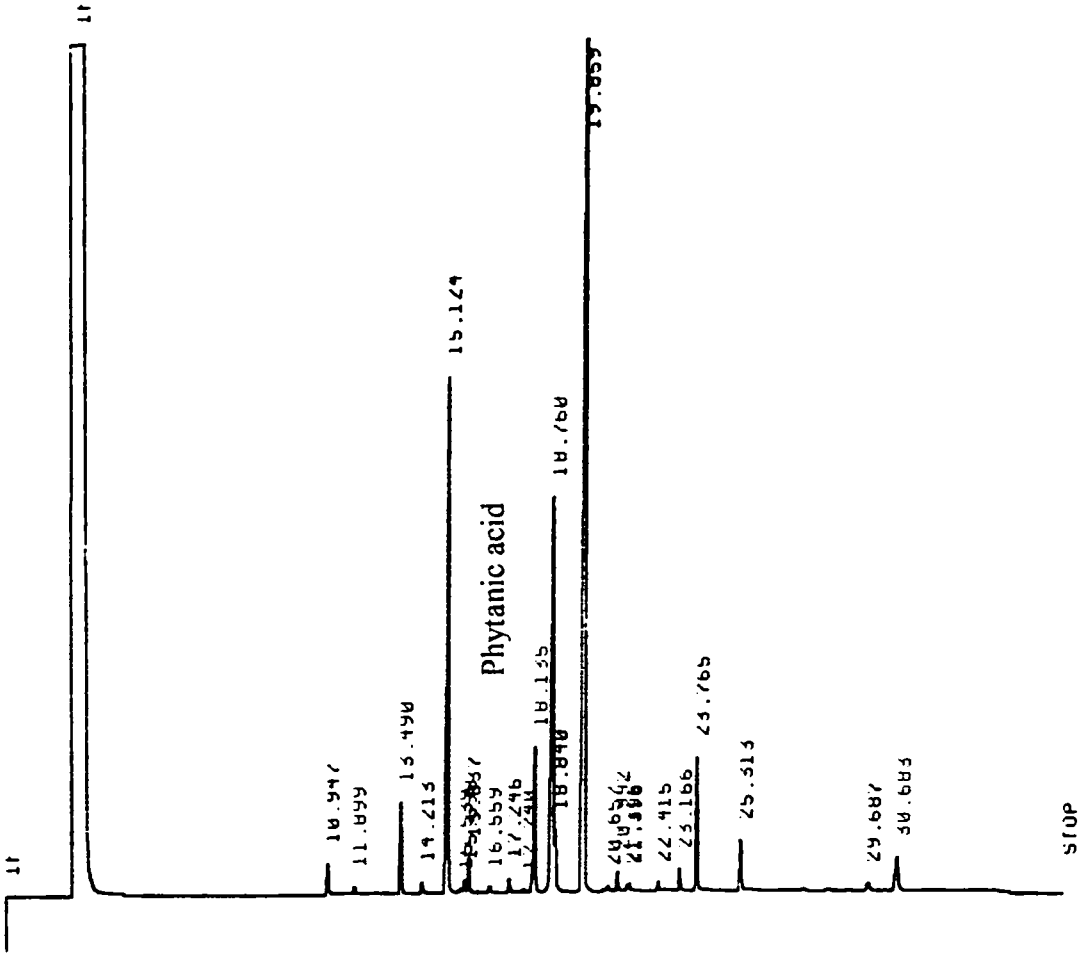
A method for the identification of phytanic acid in the plasma by gas chromatography was developed in order to quantify the phytanic acid level (Manerikar, 1996).

5.1. Calibration curve

The analysis of plasma sample by GC shows various peaks corresponding to diverse fatty acid plasma constituents. Thus, the determination of the retention time of phytanic acid and the internal standard were necessary in order to identify their peaks. A chromatogram from a normal subject is shown in Figure 24. Others peaks were also identified by eluting a standard containing five different fatty acids.

To quantify phytanic acid in the plasma, a calibration curve was determined. This was achieved by analysing five standard samples of healthy human plasma, each lacking phytanic acid, containing an added known amount of phytanic acid. The desired amount of phytanic acid was taken from a stock solution of 100μg/ml and added to the plasma sample. In addition, a fixed amount of internal standard (pentadecanoic acid) (45μg/ml) was also added to each sample. The amount of phytanic acid in each of the standards is shown in Table 23. Lipid extraction as. previously described was followed for each sample, prior to analysis by GC.

Figure 24: Chromatogram of fatty acid methyl ester extracted from normal plasma, showing very little phytanic acid (16.559 min)



Each sample was eluted and its relative peak area ratio calculated by dividing the area of the phytanic acid peak by the area of the internal standard peak for each sample. A calibration curve was prepared by plotting relative peak area ratio versus the known concentration of the phytanic acid. Therefore, the concentration of phytanic acid in the test sample could be calculated.

5.2. Determination of phytanic acid in two suspected patients

Two Scottish sisters were examined in the RP clinic at the Western Infirmary Glasgow, and reported to have clinical features suggestive of Refsum's disease. They were further examined by a specialist Refsum's disease clinic in London.

It was reported that "the examination of the 23 year old female patient (GB) revealed poor night vision for many years and loss of peripheral pigmentary retinopathy. Her sense of smell was very poor and she was able to distinguish smells only with difficulty. Radiological examination of the hands and feet revealed symmetrically short distal phalanxes of the thumb, index and middle fingers. The fifth metacarpals were also short. In the feet, the great toes and the fourth and fifth toes were short". These skeletal abnormalities are commonly and easily observed on patients suffering from Refsum's disease.

"The second patient, (GM) a 25 year old female, was found to have, as her sister, poor night vision and loss of peripheral vision. Poor sense of smell was also observed and hearing loss. She has gait ataxia with a tendency to fall to the left. Skeletal signs in the hands and feet were shown to be similar to those found in her sister" (Gibberd, personal communication).

Bood samples from these patients were collected in KEDTA vacutainer tubes. The plasma was separated from the cells by centrifugation. It was stored in small aliquots at -20^0C until the extraction procedure was carried out. The phytanic acid analysis by GC was raised in both subjects GM (Figure 25) and GB (Figure 26) at the levels 161 μmol/L and 200 μmol/L respectively (normal <33μmol/L). In the case of these patients, however, the level of phytanic acid was not severely elevated. In fact, the plasma level of phytanic acid is much higher at the diagnosis of classic Refsum's disease (more than 800 μmol/L). It must be taken in account that an initial phytanic acid analysis may be normal in Refsum's disease as this depends on age, body fat mass and diet. These levels might rise with age if they are untreated. Therefore, the cardinal features of pigmentary retinopathy, RP, raised phytanic acid and shortened fingers led the consultants to conclude that it is quite likely that the patients have Refsum's disease.

Meanwhile, a second phytanic acid analysis was carried out six months after the first one. Phytanic acid was found to be increased for both patients at levels 229 μmol/L for GM and 234 μmol/L for GB. Figure 27 shows that the level of phytanic acid in both subjects has been increased with the passage of time.

5.3. Treatment

By the time that the patients were examined by the specialist, the level of phytanic acid was higher for both GM and GB. The raised phytanic acid levels and the cardinal features led the specialist to confirm that the sisters have Refsum's disease.

Figure 25: Chromatogram of fatty acids from subject GM

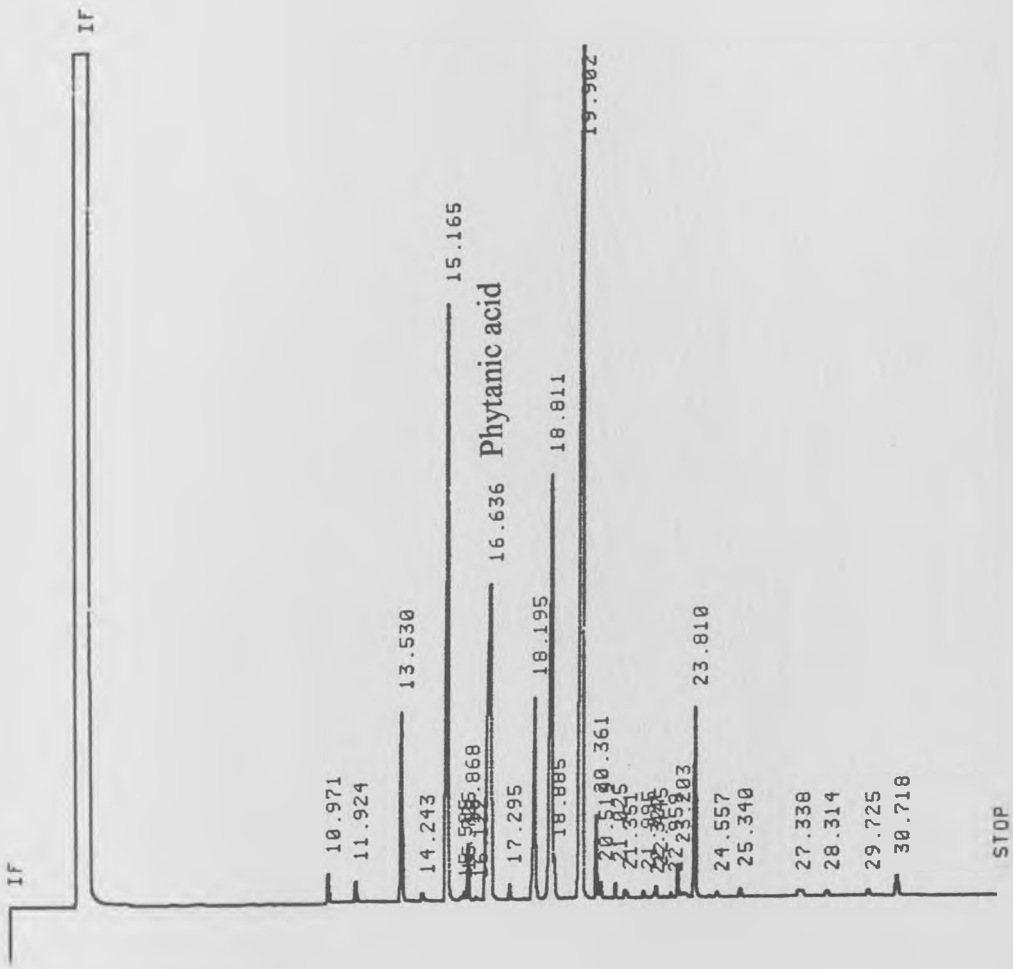


Figure 26: Chromatogram of fatty acids from subject GB

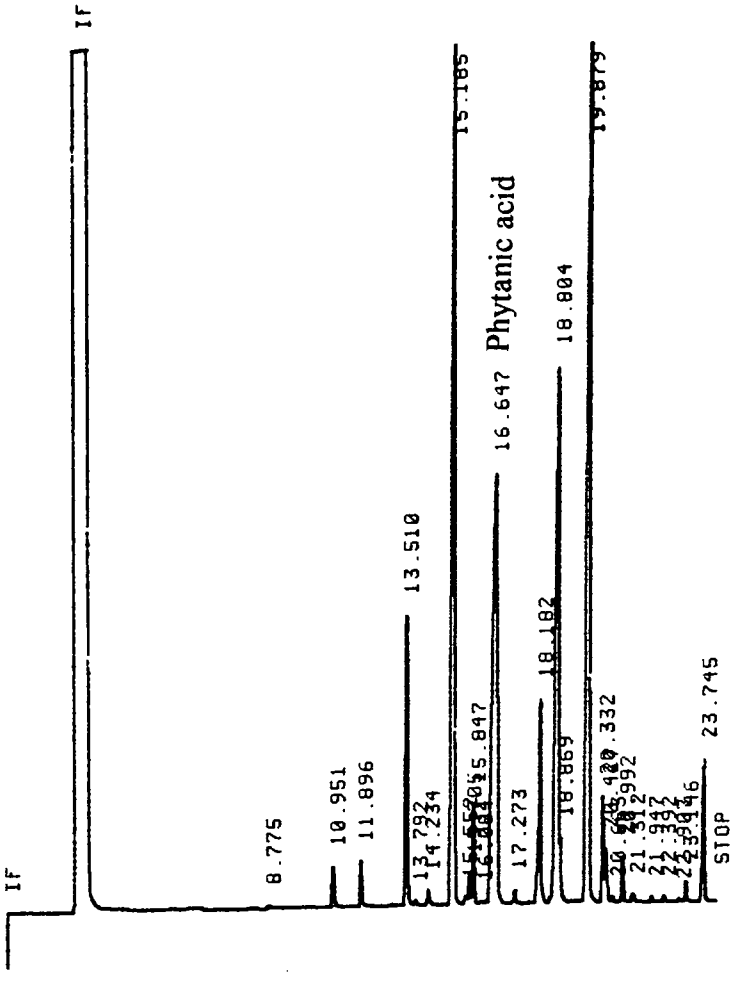
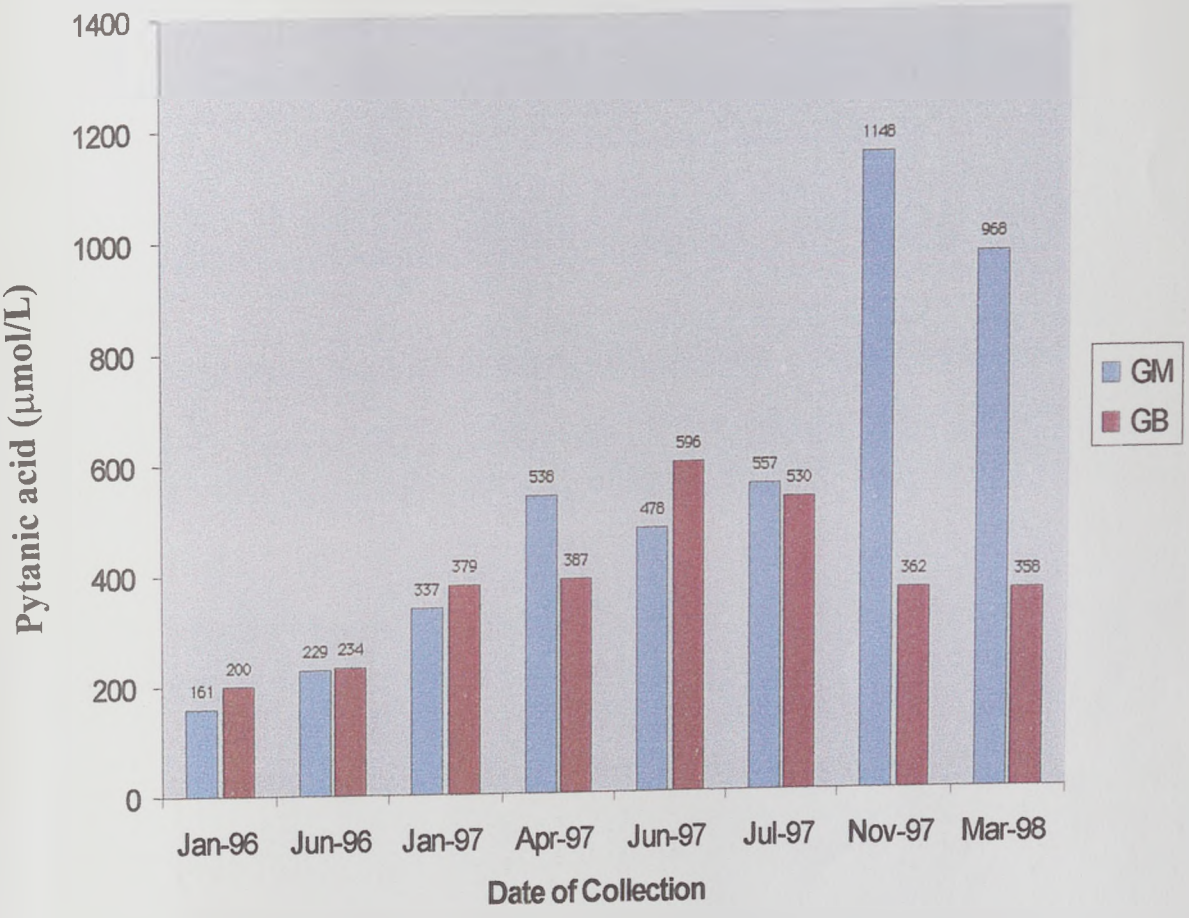


Figure 27 - Phytanic acid concentration in the plasma of the patients GM and GB



Once diagnosed these patients were started on an appropriate diet monitored by an experienced dietitian. The treatment consists of avoiding animal fats and milk products and also foods that contain phytanic acid. Unlike previous studies, leafy vegetables containing porphyrin-bound phytol in the form of chlorophyll can be safely included in the diet since phytanic acid is not formed from bound phytol (Coppack et al, 1988). It is advised that this treatment should be initiated as soon as possible and continued for life. This is essential to prevent extensive demyelination of nerves. As long as the phytanic acid intake levels remain low the plasma concentration will also remain low. Depending on the exacerbation of the disease, plasmapheresis may be used to lower the phytanic acid levels rapidly and prevent possible fatal sequelae (Claridge et al, 1992).

In the case of GM and GB, the phytanic acid levels were moderately raised. Therefore, the treatment has been mainly by low phytanic acid diet. As our patients were overweight, GM grade II obese and GB grade I, it was decided for both that they should start with a phytanic acid diet in which loss of weight would be achieved. They were advised to lose weight slowly. Although the success of the treatment depends on the patient maintaining body weight, this could safely done because the initial level of phytanic acid was only moderately elevated. In fact, when body weight falls the fats stored are utilised for energy. However the phytanic acid released is not used resulting in its accumulation. This can lead to the exacerbation of the symptoms. Table 38 shows the variation of phytanic acid analysis in these patients related to weight loss. As can be seen the phytanic acid levels increased with the weight loss in both subjects.

Table 38: Phytanic acid versus weight for subjects GM and GB

	Patient			
	GM		GB	
Date of collection	Phytanic acid µmol/L	Patient weight (Kg)	Phytanic acid µmol/L	Patient weight (Kg)
Jan – 1997	337	89.1	379	74.8
Apr – 1997	538	86.0	387	73.4
Jun – 1997	478	-	596	66.8
Jul – 1997	557	76.0	530	65.3
Nov – 1997	1148	74.7	362	67.5
Mar – 1998	968	70.4	358	71.3

5.4. Phytanic acid in RP patients previously phenotyped

Phytanic acid can account for 53% of total fatty acids in plasma and up to 50% of total fatty acids in the liver and kidneys of Refsum's disease patients. Phytanic acid replaces the long chain fatty acids in phospholipids (phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine) (Skrbic and Cummings, 1969) and in triglycerides (McBrinn and O'Brien, 1968). Steinberg and coworkers (1970) investigated the distribution of phytanic acid among the plasma lipid classes in Refsum's disease patients. It was observed that 60% of plasma phytanic acid was found in the triglycerides and about 25% in phospholipids. However, only 2.3% of phytanic acid was found in the cholesterol ester fraction.

Phytanic acid is transported in body fluids by lipoproteins. The distribution of phytanic acid in lipid subfractions was analysed in patients with Refsum's disease (Wierzbicki, et al, 1998). A significant proportion was found to be present in VLDL (16.2% ± 12.6%) and HDL (14.3% ± 12.2%). However, it was observed that the transport of phytanic acid occurs mostly in LDL (34.8% ± 12.6%).

Apolipoprotein E has been associated with RP. Apo E is a major constituent of chylomicrons, VLDL and HDL. It is recognised by the LDL receptor on the liver and other peripheral tissues. Apo E is a polymorphic protein whose major function is to transport lipids among tissues and organs. In the present work, an investigation of phytanic acid in RP patients with known apo E isoform was carried out. This was done in order to determine whether the different apo E isoforms are implicated in the accumulation of phytanic acid in these patients, and whether the two factors, abnormal apo E and raised phytanic acid, could work together in the pathogenesis of

RP. Alternatively, phytanic acid could be an indication of defects in the peroxisomes, and defective peroxisomes might not synthesise DHA as effectively: this could be a risk factor in multifactorial RP, along with certain apo E phenotypes.

Plasma samples were obtained from a total of 60 RP patients attending the RP clinic at the Western Infirmary, Glasgow. These patients had had their apo E phenotyped previously. The lipids were extracted and the fatty acids methylated and then analysed by capillary gas chromatography (Table 39). The phytanic acid level was plotted for each of the apo E phenotypes (Figure 28). It seemed that the patients with E2/E2 phenotype had elevated phytanic acid levels compared to the other groups. Statistical analysis was performed using the Student's t-test. It was found that phytanic acid in E2/E2 RP subjects was significantly raised compared to E3/E3, the largest group, at level $p<0.0001$. To investigate the relationship between phytanic acid level and apo E phenotype an analysis of phytanic acid levels in the control population with known apo E phenotype, particularly E2/E2 and E3/E3 is necessary. However, there was no time for analysing this in the present study. This was recently investigated by Resch (personal communication). It was observed that the E2/E2 control population has increased plasma phytanic acid levels compared to the E3/E3 control population. This results suggest that a subpopulation of apo E2/E2 individuals may transport phytanic acid less efficiently than the E3/E3 individuals.

5.5. The analyses of apo E phenotype and phytanic acid levels in the subject CM

Mutations in the rhodopsin gene are responsible for 25-30% of autosomal dominant form and some recessive forms of RP. The investigation of various RP

Table 39: Phytanic acid versus apo E phenotype in RP subjects

Initial	Apo E phenotype	Phytanic acid (μmol/l)
WG	E2/E3	14.11
IM	E3/E4	19.1
SJ	E2/E3	13.1
WA	E2/E2	23.3
CMC	E3/E4	9.5
IC	E2/E2	32.22
HF	E2/E2	16.8
MW	E2/E3	22.10
JW	E3/E4	10.7
SS	E2/E3	15.69
HW	E2/E3	12.3
HMcD	E2/E3	11.4
JH	E4/E4	12.98
RW	E2/E3	12.75
JR	E2/E3	10.9
MB	E2/E3	8.2
KG	E2/E3	9.0
JC	E2/E3	24.7
PR	E2/E3	7.01
DW	E4/E4	13.4

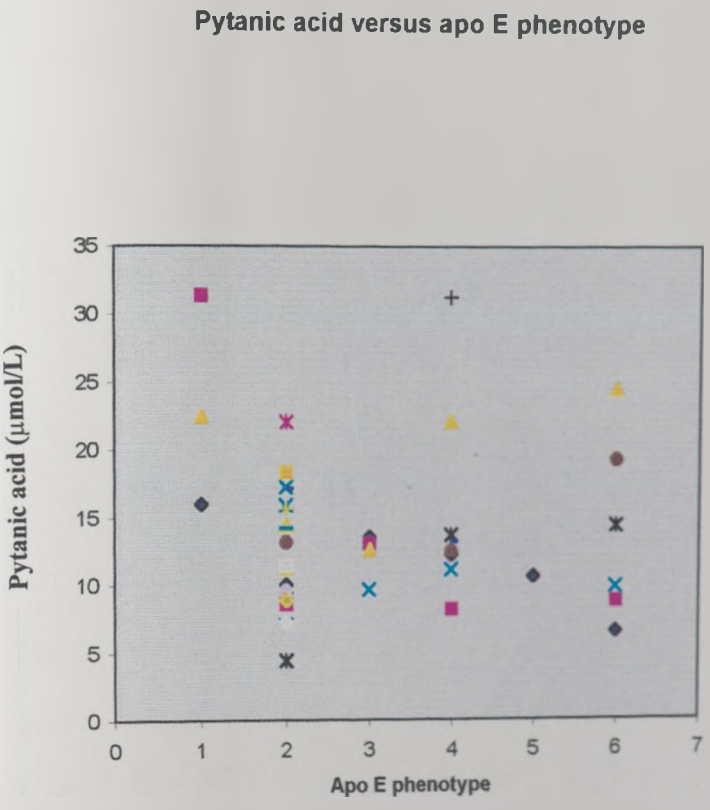
Table 39 (contd)

Initial	Apo E phenotype	Phytanic acid (μmol/L)
WN	E2/E3	13.1
WM	E2/E4	12.17
RF	E2/E4	11.05
KB	E2/E4	9.0
MF	E3/E3	7.4
IC	E4/E4	13.8
MY	E3/E3	10.71
MMcC	E3/E3	10.26
SL	E3/E3	14.5
AW	E3/E3	8.59
DC	E3/E3	19.3
CD	E3/E3	14.9
MC	E3/E3	7.1
MK	E3/E3	18.6
CN	E3/E3	10.9
AC	E3/E3	8.2
JG	E3/E3	11.5
RK	E3/E3	12.1
ML	E3/E3	18.1
JM	E3/E3	17.9
LG	E4/E4	10.5
DI	E3/E3	10.4

Table 39 (contd)

Initial	Apo E phenotype	Phytanic acid (μmol/L)
NA	E3/E3	9.3
AL	E3/E3	15.2
AS	E3/E3	8.1
WR	E3/E3	12.2
AH	E3/E3	8.1
JR	E3/E3	10.2
PW	E3/E3	14.5
HC	E3/E3	12.7
EC	E3/E3	9.5
HH	E3/E3	14.0
LT	E3/E3	25.3
GB	E3/E4	15.24
CU	E3/E4	18.3
JR	E3/E3	15.0
IS	E3/E3	10.8
SH	E3/E3	14.7
EB	E3/E4	7.3
WD	E3/E4	20.1

Figure 28: Plot showing apo E phenotype versus phytanic acid



Lane

1 – E2/E2

2 – E3/E3

3 – E4/E4

4 – E3/E2

5 – E4/E2

6 – E4/E3

patients for rhodopsin mutations by Whitehead and colleagues from the University of Aberdeen, in collaboration with our group, revealed a nucleotide change GCC→TCC at codon 299 (Ala299→Ser) in an isolated case of RP (CM). This rhodopsin sequence change destroys a BsoF1 recognation site in exon 4 of rhodopsin gene.

Further investigation in the family members of the patient showed that his mother and one brother also carried the mutation Ala299→Ser. However these members are not affected by RP. In addition, the investigation in unrelated controls revealed the absence of an Ala299→Ser change. The possibility that subject CM would have a polygenic inheritance form was investigated. It has been postulated that abnormalities in lipid handling or metabolism may contribute to the expression of the disease (McColl and Converse, 1995). This led us to investigate specific lipid groups. The apolipoprotein E phenotype from subject CM and his brother was determined and it was found that they have the same apo E phenotype, E4/E3. In addition, they presented normal phytanic acid plasma levels and the level of arachidonic acid and docosahexaenoic acid were not significantly different between the two brothers. Therefore, no evidence for abnormalities in lipid handling were found that could contribute to the expression of the disease in subject CM. The explanation could be that the Ala299→Ser change may be a rare benign rhodopsin variant.

Previous studies in this laboratory indicated the involvement of lipids in RP. Rod outer segment (ROS) disc membranes contain large amounts of polyunsaturated fatty acids (PUFA), specifically docosahexaenoic acid (DHA). This fatty acid comprises about 50% of the total ROS phospholipids.

It has been demonstrated that ROS disc membranes have a low cholesterol content and high polyunsaturated fatty acid-phospholipid content. These factors contribute to the high fluidity of the disc membranes. As a result, rhodopsin translational motion is very rapid; this may be necessary for rhodopsin activity (Berman, 1991). Therefore, the presence of DHA in ROS disc membranes may be essential to maintain rhodopsin activity.

Converse and coworkers (1983) first observed hyperlipidaemia in RP patients compared to spouses and siblings, as well as to unrelated controls. They found that male RP patients over 35 were hyperlipidemic. These results were confirmed by Jahn and colleagues (1987). They found that cholesterol levels were significantly increased in autosomal dominant, autosomal recessive and isolate RP. Another group also reported hypercholesterolemia in three ADRP families (Voaden, et al, 1989). These results suggest that lipid abnormalities are present in different types of RP. Therefore, it may contribute to the expression of the disease.

In addition to these studies, Converse and coworkers (1983, 1989), also reported hypocholesterolaemia in a large number of autosomal dominant families. To investigate whether lipid abnormalities are implicated with RP, specific lipids and lipoproteins, including apolipoprotein E, were analysed. Apolipoprotein E is

important in the transport of lipids. It has been reported that the E2/E2 isoform is associated with type III hyperlipoproteinaemia.

The association of apolipoprotein E with retinitis pigmentosa was first observed by Jahn and colleagues (1987). In a survey in a German RP population, they observed a 10-fold increase in the uncommon E2/E2 isoform. In addition Huq and coworkers (1993) found a four-fold increase in the incidence of E2/E2 and eight-fold increase in E4/E4 in the Scottish RP population. They also reported two suspected apo E variants in Scottish RP patients.

In view of these results, it was decided to identify and characterise these suspected apo E variants. In addition, a new apo E phenotype survey in the Scottish RP population was initiated in order to identify further possible apo E variants and characterise them also.

In the present work phytanic acid levels were determined in two RP patient sisters suspected of having Refsum's disease, as a function of age and dietary history. In addition, a survey of phytanic acid levels in the RP population was undertaken as part of a study of the relationship between phytanic acid and apo E phenotype in these patients.

1. Apo E phenotype

The isoelectric focusing gel technique, followed by immunoblotting was carried out in the present work. This conventional method allowed a rapid screening of a large number of samples. The utilisation of a monoclonal anti apo E antibody was necessary in order to achieve good results. When the interpretation of the

phenotype was not clear, a second opinion was obtained from D. Bedford, an expert on apo E phenotyping in the Royal Infirmary. Storage and handling precautions were taken in order to avoid deterioration of the samples. In addition, each sample was defrosted only once.

2. Population study

In total 101 RP families were investigated. However, only 77 of these families were considered in the statistical analyses because the others had been used in Huq's (1990) statistical analysis. With only a few exceptions, the phenotyping of the patients by Huq was confirmed in the present study.

In our survey the increase in the E2/E2 phenotype in RP was shown to be not significant. This finding does not agree either with the results presented in the German RP population, nor with Huq's results. On the other hand, there was confirmation of the increased frequency of apo E4/E4 in the Scottish RP population. The statistical analysis showed this increase to be significant at the $p<0.01$ level. The prevalence of the E3/E2 phenotype was significantly higher than in the control population. However, the prevalence of E4/E2 was not increased and the prevalences of E3/E3 and E4/E3 were diminished. In addition, there was an increase in the allele frequency of $\varepsilon 2$ and $\varepsilon 4$ when compared to the control population.

In the present work, the methodology used previously by Huq (1990) was followed. Therefore, it was decided to do the statistical analysis in both studies. The increase in the E2/E2 phenotype in RP was not found to be significant at the $p<0.05$ level. However, the prevalence of E4/E4 was significant at the $p<0.001$ level.

Both E2 and E4 isoforms are known to be involved in lipid abnormalities. It seems that abnormalities of plasma lipoprotein metabolism may contribute to the pathogenesis of the disease. It is known that the E2 isoform is involved in hyperlipoproteinaemia and E4 is related to hypercholesterolaemia. Recently the E4 isoform has also been related to Alzheimer's disease. The presence of apo E in the retina suggests that the increased prevalence in both E2 and E4 isoforms may have some significance to the expression of RP. However, it is unlikely that apo E2/E2 and E4/E4 by themselves cause some type of RP, since the number of people who have these isoforms is greater than those who have RP.

Another possibility is that RP is polygenic or multifactorial. In this case the patient would inherit a gene that has the potential to cause RP and the presence of another factor like apo E2/E2 and E4/E4, which contributes to the expression of the disease. However the possibility of an affected sibling inheriting both genes would be small. Therefore E2/E2 and E4/E4 are expected to occur more in isolated cases of RP. In fact, this was observed in the German population where 13 out of 14 of the E2/E2 were simplex cases, as well as in Huq's study, where 7 out of 12 of the E2/E2 and E4/E4 were simplex. In the present study the E2/E2 subject was also found to be a simplex case, however the various types of RP were represented in E4/E4 phenotypes.

The possibility of a genetic linkage between apo E and RP genes would be supported, though not proven, if there was an increase in the heterozygote phenotypes of E2 and E4 and the ε2 and ε4 allele frequencies. In fact, only the E3/E2 heterozygote was significantly increased. The others were found to be normal or

diminished compared to the control population. The allele frequencies of ε2 and ε4 were increased compared to the control population. The ε3 allele frequency was diminished. Therefore, there is evidence for some genetic linkage between RP and apo E. This is hard to reconcile with the fact that the apo E4/E4 patients had a variety of RP types, and RP has been mapped to many chromosomal locations, of which only one, a type of ADRP, is on chromosome 19 (Maghtheh, et al., 1994). It may be that a few of the E2/E2 and E4/E4 cases can be attributed to linkage while the others represent a multifactorial form of the disease, with E2/E2 and E4/E4 being risk factors.

There is a possibility of a new mutation in the apo E gene in those individuals phenotyped as E2/E2 and E4/E4, which could be directly involved either alone or in combination with another gene or factor in the pathogenesis of the disease in these subjects. This hypothesis was investigated in the present work.

3. Characterisation of selected apo E phenotypes

The increased prevalence of the E2/E2 phenotype found by Jahn and coworkers (1987) and Huq (1990), and the confirmation of the increased prevalence of E4/E4 in the Scottish RP population lead us to further investigate these subjects as well as those whose apo E had an abnormal behaviour on IEF.

3.1. Cysteamine modification

This technique modifies the cysteine residues by adding a positive charge to each cysteine protein content. Thus, it reveals whether an isoform contains the predicted number of cysteine residues.

The cysteamine modification of the five E4 homozygote subjects and also the E2 band of the patient HF behaved as expected. It seems that these subjects have a normal apo E4.

Huq (1990) reported that the E2/E2 RP subject (IC) behaved abnormally when treated with cysteamine. It was suggested that this patient may have an apo E variant. This finding lead us to further investigate this subject. The cysteamine modification was repeated and the result contradicted the earlier findings. The E2 band moved up two positive charges and focused at the E4 position. This indicated that subject IC has two cysteine residues. Therefore, further investigation was needed to characterise this patient.

3.2. Characterisation of apo E isoforms by SDS-PAGE

This method is very useful to characterise apo E variants. Apo E2 can be distinguished from E3 and E4 by its slower mobility on SDS-PAGE. This occurs due to the presence of a cysteine residues at position 158. Some apo E variants were identified by this method (Utermann and coworkers, 1984).

The Laemmli SDS-PAGE technique was first used to characterise the E2E2 subjects IC, WA and HF. However, in this method the higher apparent molecular weight of E2 could not be distinguished from E3. The band of the E3/E3$^-$ subject (JR) appeared in the same position as the normal E3 isoform. In addition, the RP subjects HH, KG and SL, classified as E3/E2$^+$ due to the presence of an E2 band focusing in a higher position than normal E2, were analysed by Laemmli SDS-PAGE. Two distinct bands were expected, one band corresponding to the E2 position and another one corresponding to the E3 position, since E2 has a higher apparent

molecular weight than E3. However, this was not observed and only a single band showed up. One possibility for this apparent molecular weight could be that the bands corresponding to the $E2^+$ position on IEF are variants of apo E which contain just one cysteine residue. Therefore, on SDS-PAGE these bands appeared to have the same mobility as apo E3.

The results obtained in Laemmli-SDS were not satisfactory since it is unlikely that all three E2/E2 samples analysed by this method have just one cysteine each. Thus, the alternate Neville SDS-PAGE technique was used. The E2 band from subjects IC, WA and HF now appeared to have a slightly lower mobility than the E3 and E4 bands. These results were in accordance with the cysteamine modification, which suggested the presence of two cysteine residues. However, the position of these cysteine residues can not be predicted by this method. Therefore, these subjects were further characterised by DNA sequencing.

4. Apo E DNA sequencing

One of the aims of this study was to determine the apo E DNA sequence of the RP subject IC. This patient was classified by Huq (1990) and confirmed in the present study as having E2/E2 phenotype. Cysteamine modification of this patient suggested the presence of only one cysteine residue (Huq, 1990). However, it was not confirmed in this work since this subject's apo E behaved as expected when treated with cysteamine.

The apo E DNA sequence from subject IC was found to be normal. In fact, this result was expected since the apo E from this patient behaved normally on cysteamine treatment and also on SDS-PAGE. Therefore, a mutation in the apo E2

and also on SDS-PAGE. Therefore, a mutation in the apo E2 gene was not found that could explain the involvement of this particular phenotype in RP.

The subjects DW and HF, classified as E4/E4and E2/E2 respectively on the basis of IEF, were also found to contain a normal apo E DNA sequence and had their phenotypes confirmed by this method. However, the subjects WN and WM classified as E2/E2 on IEF was found to be heterozygous by DNA sequencing: WN was E3/E2 and WM was E4/E2. In addition, the subjects KG, HH, SL were phenotyped as E3/E2^{+} on IEF and they were found to be homozygous E3/E3 by DNA sequencing. However the subject SJ (E3/E2^{+}) had her phenotype confirmed by DNA sequencing. Therefore, there was a lack of concordance between these two methods. Civieira and coworkers (1996) also reported a lack of concordance when they examined apo E phenotypes by IEF and genotypes by restriction enzyme analysis of the polymerase chain reaction. One reason for those missed bands on IEF could be that these bands were so faint that they did not show up on IEF. On the other hand, all these patients had normal apo E sequences. Therefore, in the present study, the presence of suspected apo E mutations in these RP patients was not confirmed.

5. Analysis of phytanic acid in two suspected Refsum's disease subjects

The analysis of phytanic acid levels in the two RP subjects GM and GB were 161μmol/L and 200μmol/L respectively. These initial levels were not particularly elevated since Refsum's disease patients at the time of the diagnosis have plasma phytanic acid levels above 800μmol/L (Britton et al,1989). This could be explained by the early diagnosis of these patients. However, the presence of clinical features of

Refsum's disease led the specialists to confirm the diagnosis. Therefore, in January 1977 they were put on low phytanic acid diet as advised by the dietition.

At this time the plasma phytanic acid level from the patient GM was 337μmol/L and her weight was 89.1Kg. As she was overweight, it was decided to aim for a low phytanic acid diet in which loss of weight would be achieved. On this regime, in April 1997, her weight dropped to 86.0 Kg. In consequence of this her plasma phytanic acid had risen to 538μmol/L. In June 1977, her phytanic acid level dropped to 478μmol/L. However it was not possible to get her weight at this time. In July 1997, her plasma phytanic acid level had risen to 557μmol/L and she had lost a significant amount of weight. At this time she was 76Kg. In November 1997, the level of phytanic acid was at a peak 1148μmol/L and her weight 74.7 Kg. She was not very well at this time due to the exacerbation of the disease. In March 1988, her phytanic acid level had dropped to 968μmol/L in spite of lost of weight (70.4 Kg). In April 1988, her phytanic acid level continued to drop and it was 744μmol/L. Again her weight was not available. From these data, it can be concluded that the plasma phytanic acid level can increase with loss of weight in spite of low phytanic acid intake. This is due to the mobilisation of phytanic acid from adipose tissue. However, in March 1998, both phytanic acid level and weight had dropped. It could be that since this patient had been on a low phytanic acid diet for a long period of time, all the stored phytanic acid had been released from the adipose tissues. Therefore, if she were to continue to the low phytanic acid intake, the level of phytanic acid would probably continue to drop in spite of further loss of weight.

The second patient GB had plasma phytanic acid levels of 379μmol/ in January 1997. At this time her weight was 74.8Kg. She also started in a low phytanic acid diet. This patient was successful in losing weight slowly. In April 1997, her phytanic acid level had slightly increased in 387μmol/L, and her weight had fallen to 73.4 Kg. In June 1997, she managed to drop her weight to 66.8 Kg. In consequence of this her phytanic acid level increased to 596μmol. In July 1997, despite her losing weight (65.3 Kg), her phytanic acid had slightly decreased to 530μmol/L. At this time, the patient was feeling well and in November 1977 she was putting on some weight (67.5Kg). Her plasma phytanic acid level dropped to 362μmol/L. The patient had regained weight and in March 1998 she was 71.3Kg and had phytanic acid level of 358μmol/L. It seems that at this stage both patients are able to keep to the low phytanic acid diet. However, it is important to avoid rapid loss of weight.

5.1. Phytanic acid in RP patients previously phenotyped

Since phytanic acid is transported by lipoproteins, an investigation was carried out to determine whether the different apo E phenotypes are involved differently in the transport of this fatty acid. The results revealed that the E2/E2 RP patients have increased phytanic acid levels compared to other groups. These results were shown to be statistically significant when compared to the E3/E3 RP subjects. The relationship between plasma phytanic acid and apo E is unknown. It is possible that both abnormal apo E2/E2 and phytanic acid are risk factors for RP and the disease may be expressed with the combination of these factors. Alternatively, raised phytanic acid may be an indicator of peroxisome dysfunction, which could be a risk factor. Another possibility is that E2/E2 individuals transport phytanic acid poorly.

resulting in its accumulation compared to other apo E phenotypes. In this case E2/E2 control subjects would contain also high phytanic acid levels compared to the E3/E3 controls.

This possibility was recently investigated by Resch in our laboratory. Phytanic acid levels were determined in five E2/E2 non-RP (control) subjects and five E3/E3 controls. An increase in the phytanic acid levels in E2/E2 was also observed compared to the E3/E3 controls. It seems that apo E2/E2, which is known to be defective in binding to the apo B-E receptor, may transport phytanic acid less efficiently.

The control population, both E2/E2 and E3/E3, surprisingly had higher phytanic acid levels than the RP population. This could be due to the fact that the two populations were analysed by different people at different time. However, it should be noted that the E2/E2 and E3/E3 control samples were obtained from hospital routine laboratories and the increase in the phytanic acid concentration could be explained by the fact that apo E phenotype is usually determined in hyperlipidaemic patents and in individuals suspected of having type III hyperlipoproteinaemia. These individuals have increased levels of triglycerides and cholesterol. Since 60% of the total phytanic acid is present in triglyceride fractions (Steinberg et al. 1970) and more recently was found to be mostly in LDL (rich in cholesterol). It is possible that these E2/E2 and E3/E3 controls are hyperlipidaemic and have higher phytanic acid as a result. To resolve this question, the control and RP populations should have had their cholesterol and triglyceride levels determined in order to determine whether they are normolipidaemic subjects.

Conclusion

Retinitis pigmentosa is a group of inherited progressive retinal degenerations. The association of lipid abnormalities in some forms of RP indicates that they may be involved in the pathogenesis of the disease. It is postulated that the presence of specific lipids in the retina is fundamental for the normal function of the photoreceptors. Therefore, defects in the metabolism or in the transport of these lipids may contribute to the retinal degeneration found in RP. In the present study, the involvements of apolipoprotein E and phytanic acid were investigated in RP patients. Apo E transports lipids in the plasma and in the retina while phytanic acid may indicate a defect in peroxisome function.

The incidence of apo E2/E2 was found to be not significant in this study, when compared to a Scottish control population. When the statistical analysis was performed in the combined studies, it was not found to be significant at the $p<0.05$ level, although with $p<0.10$, there appeared to be a trend towards increased E2/E2. The frequency of the ε2 allele was significantly increased. In addition, the incidence of E4/E4 was statistically significant in both this study and in the combined study at the $p<0.01$ and $p<0.001$ levels respectively. Therefore, the increased incidence of E4/E4 in the RP population was confirmed in this study. However, the increased incidences in E2/E2 observed by both Jahn and colleagues in the German RP population and Huq's study in the Scottish RP population were not confirmed in this study.

The possibility of a genetic linkage between apo E and RP would be strengthened if an increase in the heterozygote phenotypes of E2 and E4 is observed.

However, only the heterozygote E3/E2 was increased. Unfortunately it was not possible to study large families by linkage analysis to confirm this possibility. In addition, the fact that both ε2 and ε4 alleles were increased could indicate a linkage in some types of RP but so far only one common type of ADRP has been mapped to chromosome location 19q13.4, close to the apo E gene (19q13.2). Therefore, it may be possible that there could be a dysequilibrium of apo E in this type of ADRP as result of linkage. It is more likely that in the majority of RP type, the dysfunctional apo E2 and E4 contribute to the pathogenesis in combination with some non-linked RP genes.

The hypothesis of a new mutation in E2/E2 and E4/E4 RP subjects was not confirmed. However, since the apo E DNA sequence was investigated in only a few patients this possibility may not be entirely excluded.

In this study, two suspected Refsum's patients were confirmed by analysing their phytanic acid levels. These patients were put on a low phytanic acid diet and their plasma phytanic acid was monitored during this study. It was observed that the phytanic acid level increased when there was loss of weight. This presumably occurs due to the mobilisation of the lipids to be used as energy source, resulting in the accumulation of phytanic acid in the plasma.

The relationship between apo E phenotypes and phytanic acid revealed that the E2/E2 RP subjects had higher phytanic acid levels than the other groups. However, a recent investigation in this laboratory showed that the E2/E2 controls also had a tendency towards higher phytanic acid levels than the E3/E3 controls. This may suggest that apo E2/E2 transports phytanic acid to target tissues poorly resulting

in its accumulation. Another possibility is that the E2/E2 subjects could be hyperlipidemic and thus have increased triglycerides and cholesterol levels.

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