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DETAILED CLINICAL PHENOTYPING IN AGE-RELATED MACULAR DEGENERATION

MD THESIS
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Dr Samantha Sujata Dandekar

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Age-related Macular Degeneration (AMD) is a degenerative disorder that accounts for about 50% of blindness in England and Wales. At present there is no effective treatment. It occurs in genetically susceptible individuals exposed to environmental factors such as smoking but so far specific factors remain to be identified.

For this descriptive study, 879 patients with Age-Related Maculopathy (ARM) and AMD and 44 spouses with normal maculae, to act as a comparison group, were recruited from a tertiary referral centre. The clinical phenotypes were analysed from fundus photographs, fluorescein angiography and autofluorescence (AF) images. Fundus features were characterised as they are thought to reflect the genes conferring risk in an individual and may allow greater understanding of disease mechanisms.

These data demonstrated (1) A revised grading system shown to be reproducible for use with digital images (2) A moderate concordance rate for phenotype between eyes with end-stage AMD (kappa statistic=0.48; 95% CI = 0.38-0.57, $p<0.001$). (3) Distinct characteristics, including a larger area and higher counts of soft drusen with focal areas of increased AF, in fellow eyes of those with unilateral visual loss due to geographic atrophy (GA). (4) An increased susceptibility of the inferotemporal macula to GA. (5) Preserved integrity of the retinal pigment epithelium (RPE) in the initial stages of CNV development as identified from AF images. (6) Loss of scotopic rather than photopic function over areas of increased AF, as determined by fine matrix mapping, indicating the preferential vulnerability of rods. (7) No difference in smoking history between those with neovascular compared to non-neovascular AMD.

Although AMD has been extensively investigated, this study extends our knowledge of retinal AF, the relative susceptibility of rods compared to cones at the macula and suggests both eyes of an individual are more discordant for late stages of AMD compared to drusen.

CONTENTS

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List of abbreviations

A2E	N-retinylidene-N-retinylethanolamine
ABCA4	Gene encoding an ATP-binding cassette (ABC) transporter protein (ABCR) that is defective in Stargardt disease
ACE	Angiotensin Converting Enzyme
AIF	Apoptosis inducing factor
AF	Autofluorescence
AMD	Age-related macular degeneration
APOE	Apolipoprotein E
AREDS	Age-related eye disease study
ARM	Age-related maculopathy
CNV	Choroidal neovascularisation
DD	Disc diameters
DNA	Deoxyribose nucleic acid
DZ	Dizygotic
EDCCS	Eye disease case control study
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1 gene
ELOVL4	Elongation of very long chain fatty acids gene associated with autosomal dominant Stargardt-like macular dystrophy
FFA	Fluorescein angiography
FMM	Fine matrix mapping
GA	Geographic atrophy
ICG	Indocyanine Green Angiography
IPCV	Idiopathic polypoidal choroidal vasculopathy
JPEG	'Joint photographic experts group' that create the standards for still image compression; file interchange format
MIDD	Maternally inherited diabetes and deafness
MPS	Macular photocoagulation study
MZ	Monozygotic
PAS	Periodic acid Schiff stain

PDT	Photodynamic therapy
PED	Pigment epithelial detachment
PEDF	Pigment epithelial derived factor
Peripherin/RDS	Gene coding for integral membrane glycoprotein found in the rim regions of photoreceptor cell discs
POLA	Pathologies Oculaires Liees a l'Age Study
RAP	Retinal angiomatous proliferation
RCO	Royal College of Ophthalmologists
RDS	'Retinal degeneration slow' phenotype, observed in mice as a model for peripherin/rds-mediated retinitis pigmentosa (RP)
RNA	Ribonucleic acid
ROS	Rod outer segments
RPE	Retinal pigment epithelium
SER	Smooth endoplasmic reticulum
SLO	Scanning laser ophthalmoscope
TAU	Microtubule associated protein which is the basic component of intraneuronal and glial inclusions observed in many neurological disorders
TIFF	Tagged Image File Format
TIMP-3	Tissue inhibitor of metalloproteinases-3 gene
VA	Visual acuity
VEGF	Vascular endothelial growth factor
VMD2	This vitelliform macular dystrophy gene encodes bestrophin, a transmembrane protein at the basolateral portion of the retinal pigment epithelium

1 Introduction & Background

1.1 What is age-related macular degeneration?

Age-related macular degeneration (AMD) has been divided into early disease termed age-related maculopathy (ARM) that has little effect on vision, and late disease termed age-related macular disease (AMD) which is a degenerative disorder causing significant visual impairment, which is more frequent after the age of 65 years.

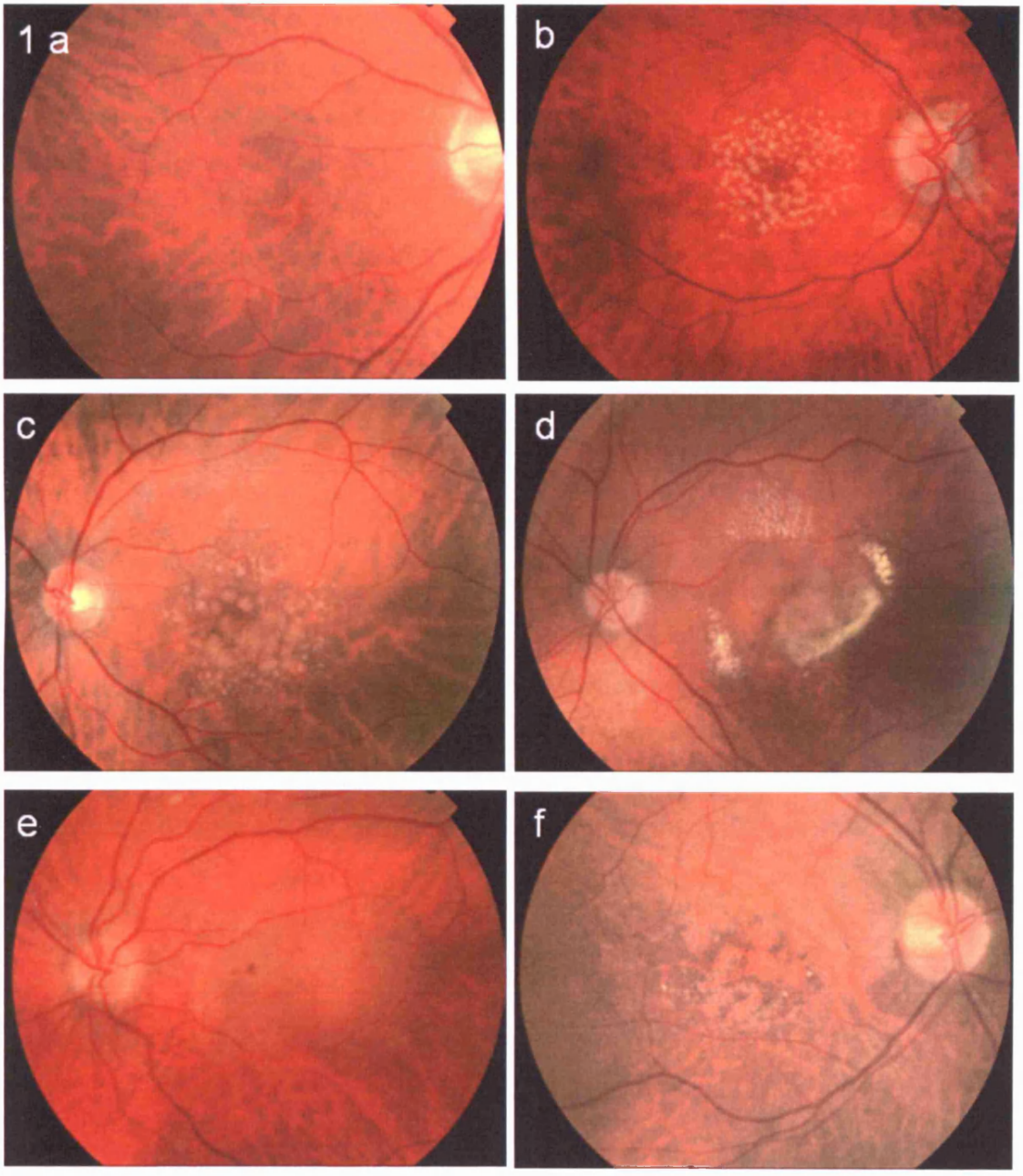
ARM is defined clinically by areas of drusen deposit that can either be hard and small, soft and intermediate or large (see figures 1a-c [page 11]) and / or areas of hyperpigmentation (in the outer retina or choroid) or hypopigmentation of the retinal pigment epithelium (RPE),). The presence of soft drusen alone is sufficient for the diagnosis of ARM whereas small hard drusen do not themselves characterize the disorder (Bird, A. C. *et al*; 1995).

In the later stages, AMD affects several layers of the central retina including the RPE, the photoreceptor cell layer and the choriocapillaris (Green, W. R.; 1999),(Bird, A. C. *et al*; 1995) causing visual impairment. The lesions causing visual loss, namely choroidal neovascularisation (CNV) (see figure 1d), pigment epithelial detachment (PED) (see figure 1e) and geographic atrophy (GA) (see figure 1f) appear to occur as a reaction to ARM. AMD causes loss of central vision in patients but peripheral visual field is predominantly left intact.

More recently, additional phenotypes of macular disease in the elderly have been characterised, including retinal angiomatous proliferation (RAP) and idiopathic polypoidal choroidal vasculopathy (IPCV). RAP occurs on a background of ARM and involves invasion of the outer retina by retinal blood vessels and may precede or occur simultaneously with CNV (Yannuzzi, L. A. *et al*; 2001). Idiopathic PCV was a term coined in 1990 by Yannuzzi, but the condition was originally described as lesions occurring in the peripapillary region in African women (Stern, R. M. *et al*; 1985).

Figure 1

Fundus photographs demonstrating (a) small hard drusen only (<63μm)- classified as the comparison group(b) intermediate sized soft drusen (125-250μm) (c) intermediate and large soft drusen (125-500μm) (d) a choroidal neovascular lesion with subretinal scar and exudates (e) a pigment epithelial detachment with dome like elevation of the RPE (f) multiple areas of geographic atrophy



Since then, a macular variant of IPCV has been described (Yannuzzi, L. A. *et al*; 1990) involving polypoidal dilatations arising from the choroidal vascular network but its relevance to ARM is unknown.

The importance of AMD is underlined by the fact that it accounts for about 50% of blind or partially sighted registrations in England & Wales (Evans, J.; 1995) and with the population over the age of 60 set to increase by 45% in the next 20-25 years, this will represent a huge burden on the National Health and Social Services (RCO; 2000)(Royal College guidelines). In addition the prevalence of AMD appears to be increasing at a rate that is not fully explained by the ageing population (Evans, J. *et al*; 1996) and occurring at an earlier age than before causing further increases to public health costs.

There is currently no treatment that will have an influence on blindness from AMD in the community, and the results of treatments (i.e. submacular surgery, argon laser, radiotherapy, photodynamic therapy) over the years have been disappointing. Although the prognosis will be affected in some cases, treatment serves only to reduce the magnitude of visual loss; gain of visual acuity rarely occurs. This has stimulated research into the fundamental causes of the disorder in the hope that new therapeutic approaches will emerge. It is believed that AMD occurs in those with a genetic predisposition and becomes evident with appropriate environmental pressures. Environmental factors have been sought and attempts have been made to identify those genes involved. Alongside this, efforts have been made to determine the intermediate disease mechanisms.

1.2 Physiology of the tissues involved and mechanisms of disease

1.2.1 Normal macula

The macula is anatomically defined as the area within the arcades centred on the foveola in which the ganglion cell layer is more than one cell in thickness. It has an approximate diameter of 5.5mm (Hogan, M. J. *et al*; 1971).

1.2.2 The ageing macula

Ageing is the single most important risk factor for retinal disease in the Western World (Ganley and Roberts 1983). The mechanisms underlying these retinopathies are not understood, but it is believed that changes in the retinal pigment epithelium (RPE) and Bruch's membrane play a vital role in degenerative changes in the adjacent photoreceptors, neural retina and the supporting choroid (Hogan, M. J.; 1972).

1.2.2.1 Ageing of the photoreceptors

The outer segments of the photoreceptors are constantly being renewed with the distal disc membranes being shed each day. Thirty to fifty photoreceptors are apposed to each RPE cell, and it is within the RPE cell that phagocytosis, fusion of lysosomes to form phagolysosomes and digestion of the outer segments occurs (Feeney L 1976 Exp Eye Res). Much of the degraded material is then recycled to provide the substrate for formation of new outer segment membranes. There is good evidence to suggest that the RPE discharges cytoplasmic material into the inner portion of Bruch's membrane (Feeney-Burns, L. *et al*; 1980). This material is believed to consist of degradation products from rod outer segment (ROS) renewal however, neither rhodopsin sequences nor phagosomal enzyme activity have been shown in Bruch's membrane using monoclonal antibodies (Feeney-Burns, L. *et al*; 1988). An explanation for this was that superficial hydrophilic antigen binding sites on the molecules for which the antibodies were specific had been altered or destroyed by

lysosomal enzyme digestion within the phagolysosomal system of the RPE, prior to the formation of definitive lipofuscin granules. The authors concluded that these monoclonal antibodies are of limited value in studying degradation products from rod outer segment renewal (Feeney-Burns, L. *et al*; 1988).

With age, human rods and cones appear to undergo degeneration and distortion of the outer segments (Curcio, C. A. *et al*; 1996; Grindle, C. F. *et al*; 1978). Histological studies have shown a selective vulnerability of rods compared to cones. In maculae of older adults lacking visible drusen and pigmentary change (i.e., they do not have ARM), the number of cones appears to remain stable, but the number of rods decreases by up to 30% and the greatest loss occurs in the parafoveal area (Curcio, C. A. *et al*; 1993).

There is also suggestive evidence that rod and cone photopigments regenerate by different mechanisms. In frog retinas separated from the RPE, cone opsin, but not rhodopsin, regenerates spontaneously (Goldstein, E. B. *et al*; 1973; Hood, D. C. *et al*; 1973). Mata and co-workers very recently found several catalytic steps of an alternate visual cycle that mediates pigment regeneration in cones. This alternate pigment regeneration is independent of the RPE and may involve Müller cells (Mata, N. L. *et al*; 2002). This implies that visual-pigment regeneration in cones is not exclusively dependent on RPE function as it is in rods.

Throughout life, oxidative damage to the photoreceptor outer segments is thought to occur as a result of light energy allowing the formation of singlet oxygen free radicals. The outer segments are particularly vulnerable to oxidative damage as they are rich in polyunsaturated fatty acids (Kennedy et al 1995, Katz 1978) and dissolved oxygen is concentrated in the fatty acid layer. These oxidative changes may make the outer segments more resistant to degradation by the RPE cell.

1.2.2.2 Ageing of the retinal pigment epithelium (RPE)

The adult RPE is a non-dividing system that sustains a number of functions essential for the preservation of photoreceptor cells (Boulton 2001). It is composed of a single layer of hexano-cuboidal epithelial cells positioned between the neural retina and the choroid. Its predominant roles include:

- Regulation of the transport of nutrients and waste products to and from the retina through the expression and activity of specific proteins.
- Phagocytosis and degradation of photoreceptor outer segments (approx a million rod discs per RPE cell per year (Grindle, C. F. *et al*; 1978).
- Protection against free radical damage induced by high oxygen levels and light irradiation and absorption of unused optical radiation that has passed through the neural retina.
- Storage of Vitamin A and its conversion to a form that can be utilised by the photoreceptors for the synthesis of rhodopsin (Hogan, M. J.; 1972).
- Production of growth factors that influence the function of neighbouring tissues.
- Ionic homeostasis of the outer retinal extracellular space

Given the high metabolic load placed on the RPE, it is not surprising that this tissue consistently undergoes ageing changes.

It is the phagocytosis and degradation of photoreceptor outer segments that is responsible for the formation of the compound, lipofuscin. The accumulation of this autofluorescent compound is one of the most pronounced changes to occur with ageing in the RPE (Wing, G. L. *et al*; 1978) and is at least partly responsible for the subsequent pleomorphic changes, loss of intact melanin granules, significant metabolic changes and cell loss that occurs (Boulton, M. *et al*; 2001).

It is lipofuscin accumulation that is believed to contribute to the pathogenesis of age-related macular degeneration as photoreceptor loss has been directly correlated with lipofuscin concentration in the opposing RPE (Dorey, C. K. *et al*; 1989).

1.2.2.2.1 Lipofuscin

Lipofuscin is a heterogeneous material made up of lipids, proteins and fluorescent compounds that accumulate within the human RPE as yellow-brown refractile granules (Feeney-Burns 1980, Kennedy C 1995, Eldred 1988 Exp Eye Res). It characteristically fluoresces maximally with short wavelength light excitation (Feeney, L.; 1978) increasing in intensity with age (Boulton, M. *et al*; 1990; Okubo, A. *et al*; 1999). When age is plotted against RPE residual body content or RPE autofluorescence, a quadratic relationship is obtained. The greatest accumulation of RPE autofluorescence occurs in the first few decades of life and reaches a plateau by the eighth to ninth decade. This can be explained by the fact that lipofuscin has a finite half-life (Delori, F. C. *et al*; 1995a) and photoreceptor population decreases after the sixth decade (Curcio, C. A. *et al*; 1993).

1.2.2.2.2 Origin of lipofuscin

Substantial evidence now supports the proposal that lipofuscin is derived from photoreceptor outer segment contents. After phagocytosis and digestion by lysosomal enzymes including Cathepsin D (Feeney-Burns 1984 IOVS), the resulting compounds are able to diffuse out of the phagolysosome into the cytoplasm (Feeney-Burns L 1983). The degradative process however is incomplete (see figure 2-[page17]), resulting in residual bodies seen on electron microscopy as electron dense inclusions containing lipofuscin granules within the RPE (Katz, M. L. *et al*; 1982).

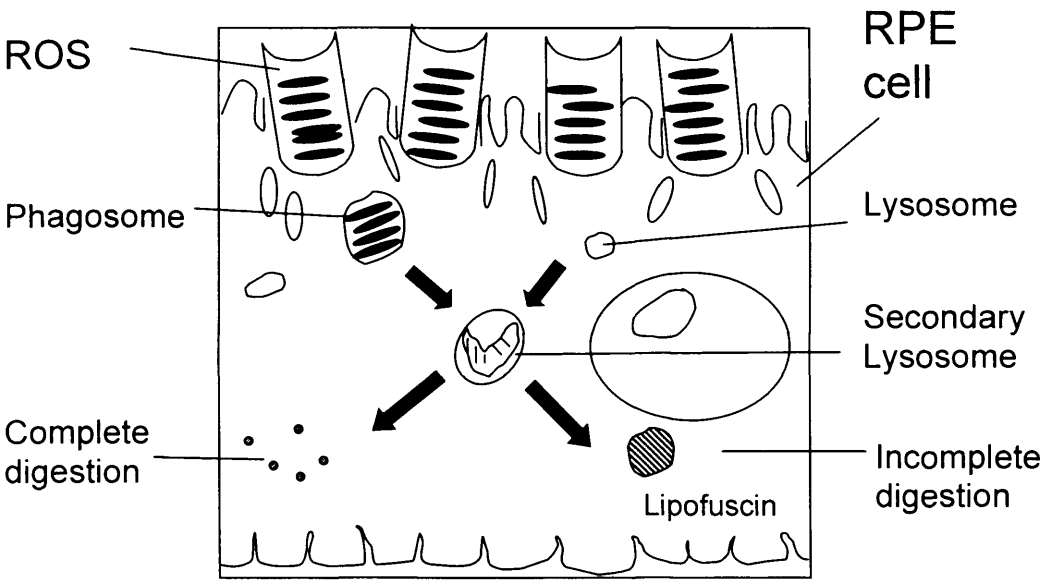


Figure 2: Diagram showing origin of lipofuscin in RPE cell. Modified from Kennedy et al 1995.

Further evidence comes from experimental rats with retinae lacking photoreceptors due to light damage or a genetic defect that show less residual body accumulation (Katz et al 1986, 1989) (Boulton 1986). Additionally RPE cells cultured without exposure to photoreceptor outer segments accumulate very little lipofuscin (Boulton et al 1989), but if these cells are allowed to phagocytose exogenously added outer segments, they quickly accumulate large amounts of lipofuscin (Boulton et al 1989).

It was previously believed that the lipofuscin-containing residual bodies were undegradeable and therefore accumulated over time. Katz however, demonstrated that if albino rats were given intravitreal injections of the protease inhibitor leupeptin (which blocks the degradation of outer segment proteins), there was a rapid accumulation of lipofuscin-like inclusions within the RPE (Katz, M. L. *et al*; 1999). Twelve weeks following the injection, the amounts of inclusion material and fluorescence returned to normal levels. This suggested that the age-related increase in RPE lipofuscin results from an imbalance in the rates of lipofuscin formation and disposal rather than an absence of a disposal mechanism (Katz, M. L. *et al*; 1999).

1.2.2.2.3 Composition of lipofuscin

Lipofuscin is a mixture of several distinct fluorescent components and may contain up to ten different fluorophores (Eldred, G. E. *et al*; 1988). Among the likely candidates for conversion into these fluorophores are vitamin A compounds, which are present in the retina and RPE in high concentrations. Dietary deficiency of retinoid precursors (vitamin A palmitate) have been shown to eliminate the orange emitting fluorophore of lipofuscin and reduce the number of lipofuscin granules in rats (Katz, M. L. *et al*; 1986).

An important component of lipofuscin is the fluorophore known as A2E, (N-retinylidene-N-retinylethanolamine) a pyridinium bisretinoid, which is formed in the photoreceptor outer segment membrane from sequential reactions of 'phosphatidylethanolamine' and 'retinal' to produce the phosphatidylethanolamine-bisretinoid A2-PE; subsequent hydrolysis releases the fluorophore A2E (Liu, J. *et al*; 2000).

1.2.2.2.4 Toxicity of lipofuscin

The concept that excessive lipofuscin can be harmful is supported by observations in Stargardt macular dystrophy (Allikmets, R. *et al*; 1997) and other inherited dystrophies such as Best vitelliform macular dystrophy (Kramer, F. *et al*; 2000). Lipofuscin levels in these patients were found to be significantly higher than those in normal subjects of the same age (Delori, F. C. *et al*; 1995b),(von Ruckmann, A. *et al*; 1997b). Histological studies have also shown that lipofuscin concentration directly correlates with photoreceptor cell loss in humans (Dorey, C. K. *et al*; 1989).

Excessive levels of lipofuscin have been postulated to compromise essential RPE functions by various mechanisms (Feeney-Burns, L. *et al*; 1984; Schutt, F. *et al*; 2000). Firstly it acts as a photosensitiser in blue light, generating free radicals within the RPE cell (Boulton,M;1990). Secondly the fluorophore, A2E, has surfactant like properties causing disintegration of the lysosomal membrane resulting in a rise in the pH which would disable the lysosomal enzymes (Schutt, F. *et al*; 2002). Thirdly, A2E it is believed to be toxic to mitochondria by releasing cytochrome C and AIF (apoptosis-inducing factor), which initiates apoptosis (Sparrow, J. R. *et al*; 2000; Suter, M. *et al*; 2000). Fourthly, there may be

a mechanical effect caused by the inclusion bodies engorging the cells with non-functional waste that may damage cellular architecture particularly the smooth endoplasmic reticulum (SER) and finally, there may be a reduction in the recycled material needed for synthesis of photoreceptor outer segments (Okubo, A. *et al*; 2000).

Okubo et al also showed widespread structural changes in the outer retina following repeated injections of the lysosomal protease inhibitor E-64 into the vitreous of Sprague Dawley rats (Okubo, A. *et al*; 2000). Progressive accumulation of A2-E occurred within the RPE followed by loss of SER, shortening and loss of photoreceptor outer segments, alteration of choroidal capillaries and invasion of Bruch's membrane by fibroblasts and pericytes. She postulated that the high levels were witness to lack of avaliable catabolites that are recycled to provide the substrate for photoreceptor outer segment renewal.

It is hoped that increased knowledge of in vivo lipofuscin accumulation, which is now possible with the advent of autofluorescence imaging (Delori, F. C. *et al*; 1995a; von Ruckmann, A. *et al*; 1997a), may give important clues as to the pathogenesis and progress of ageing in the human eye, especially at the macula.

1.2.2.3 Ageing of Bruch's membrane

Bruch's membrane undergoes progressive thickening and hyalinisation with an increase in basophilia and periodic acid -Schiff (PAS) staining throughout life (Hogan, M. J. *et al*; 1967; Pauleikhoff, D. *et al*; 1990c; Sarks, S. H.; 1976). Electron microscopy has also shown changes in the collagen and elastic fibres of the membrane with increased calcification (Grindle, C. F. *et al*; 1978; Hogan, M. J. *et al*; 1967).

Unlike RPE changes, the thickening of Bruch's membrane with age is linear and is thought to be due to accumulation of waste material (Okubo, A. *et al*; 1999). These changes are recognised clinically as drusen when the deposits are focal. The thickness would be influenced by the rate of deposition and the rate of clearance. This material is thought to be cytoplasmic in origin. It is thought that the RPE discharges material into the inner portion

of Bruch's membrane to achieve cytoplasmic renewal, an attribute of non-dividing cells that are metabolically active. It is thought ultimately to be cleared by the choroid although this process is poorly understood (Feeney-Burns, L. *et al*; 1980; Sheraidah, G. *et al*; 1993). Since the thickening of Bruch's membrane cannot be fully explained by ageing alone, other factors must play a role (correlation between age and Bruch's membrane thickness; R^2 = 0.57) (Okubo, A. *et al*; 1999).

Lipid content in Bruch's membrane, especially the accumulation of neutral lipids and phospholipids (Holz, F. G. *et al*; 1994a; Sheraidah, G. *et al*; 1993) has been shown to increase with age. Experiments using material extracted from Bruch's membrane in donor eyes using lipid solvents and then analysed using thin-layer and gas chromatography have confirmed this (Sheraidah, G. *et al*; 1993). A second series of specimens, where the pigment epithelium was removed and then analysed, gave similar results confirming that the lipids were from Bruch's membrane (Sheraidah, G. *et al*; 1993). Subsequent studies then confirmed a reduction in the hydraulic conductivity of the layer primarily due to this accumulation of lipid (Fisher, R. F.; 1987; Moore, D. J. *et al*; 1995).

All the above changes are associated with progressive disruption of Bruch's membrane and believed to contribute to a decrease in the exchange efficiency between the choriocapillaris and pigment epithelium.

Okubo studied the relationship between ageing in Bruch's membrane and that of the retinal pigment epithelium. Changes in both layers increased with age and although there was a linear relationship between the two, giving strength to the belief that the deposits in Bruch's membrane are derived from the RPE, there was considerable variation within each age group and between specimens. The R^2 correlation was 0.18 to 0.37 using electron and light microscopy respectively implying that only a small proportion of thickness of Bruch's membrane can be explained by increase in RPE residual bodies or autofluorescence (Okubo, A. *et al*; 1999).

1.2.2.4 Ageing of the choriocapillaris

Thickening of the intercapillary pillars and dropout of the choriocapillaris are seen histologically with age and in AMD (Lutty, G. *et al*; 1999; Sarks, S. H.; 1976). The subsequent reduced blood flow to the choriocapillaris has been associated with the appearance of filling defects on ICG angiography (Lutty, G. *et al*; 1999), and has been implicated as a pathogenetic factor in choroidal neovascularisation via angiogenic factor release induced by hypoxia (Mori, F.; 2003). The structure of the blood vessels has also been described to change from a sinusoidal system to become more tubular with age (Olver, J. M.; 1990).

1.2.3 Pathological consequences of the ageing process

1.2.3.1 Mechanism of drusen formation

Drusen are thought to be the clinically recognisable discrete deposits seen histologically on the inner surface of Bruch's membrane. They can be focal and nodular or more diffuse in nature.

The origin of drusen has been disputed. Two theories referred to by Coats in 1905 as the 'transformation' and 'deposition' theory of drusen biogenesis may both play some role (Hageman, G. S. *et al*; 2001). The former involved the transformation of degenerating RPE cells into drusen and the latter proposed that drusen result from the secretion of abnormal material derived from RPE or photoreceptors into Bruch's membrane (Hageman, G. S. *et al*; 2001). It is also thought that inflammatory components are important in the development of drusen (Hageman, G. S. *et al*; 2001). Complement components and fibrinogen, thrombin and acute phase proteins such as vitronectin have all been found to be contained within drusen but their role in the pathogenesis of AMD is unclear. Degenerating RPE cells and debris trapped between the RPE monolayer and Bruch's membrane may act as a chronic inflammatory stimulus and target for complement attack (Hageman, G. S. *et al*; 2001). Recent evidence suggests that a polymorphism in the CFH (complement factor H) gene may be an important factor in AMD susceptibility (Klein, R. J. *et al*; 2005)(see page 51).

Various studies have confirmed the presence of lipids and proteins in drusen. Pauleikhoff and colleagues showed a correlation between the lipid biochemical composition and fluorescein binding properties of drusen. Other studies have identified several proteins that are believed to undergo oxidative modifications (i.e. TIMP 3, clusterin and vitronectin) and may play a role in drusen formation (Crabb, J. W. *et al*; 2002).

While focal thickening of Bruch's membrane can be seen clinically, diffuse thickening is not visible on biomicroscopy. It has been postulated that "prolonged choroidal filling" on

fluorescein angiography (Pauleikhoff, D. *et al*; 1990b) may be a consequence of this diffuse thickening. Similar changes in Bruch's membrane are known to occur in Sorsby fundus dystrophy due to mutations in TIMP-3 and are also associated with prolonged choroidal filling (Capon, M. R. *et al*; 1989; Polkinghorne, P. J. *et al*; 1989). In AMD, it is eyes with patchy choroidal fluorescence in the transit phase of the angiogram that tend to have fewer, central drusen and are hypothesised to have diffuse changes in Bruch's membrane (Pauleikhoff, D. *et al*; 1990c; Piguet, B. *et al*; 1992). This may hamper diffusion between the choriocapillaris and retinal pigment epithelium and cause secondary changes to occur in the choroidal capillaries. Further, scotopic threshold studies using the Humphrey automated perimeter and fine matrix mapping showed up to 3.4 log units of scotopic threshold elevation in areas of choroidal perfusion abnormality. It has been postulated that diffuse deposits in Bruch's membrane might account for both the perfusion abnormality and the functional loss seen (Chen, J. C. *et al*; 1992). Slow choroidal filling, caused by diffuse deposits therefore represent a risk factor for pigment epithelial detachment or choroidal neovascularisation in AMD.

1.2.3.2 Mechanism of pigment epithelial detachments (PED)

The theory that the sub-retinal pigment epithelial fluid in a PED is derived from the active outward movement of water from the RPE rather than from the choroid was introduced in 1986 (Bird, A. C. *et al*; 1986; Holz, F. G. *et al*; 2004). It was proposed that a reduction in the hydraulic conductivity of Bruch's membrane would cause increased resistance to water flow towards the choroid, thus causing it to accumulate in the sub-retinal pigment epithelial space. As stated above, Bruch's membrane increases in thickness with age (Green, W. R. *et al*; 1977) and subsequent studies have confirmed a reduction in the hydraulic conductivity of the layer (Fisher, R. F.; 1987; Moore, D. J. *et al*; 1995) primarily due to the progressive accumulation of neutral lipids (Holz, F. G. *et al*; 1994a; Sheraidah, G. *et al*; 1993). This would subsequently increase the hydrophobicity of Bruch's membrane causing the development of a pigment epithelial detachment (Bird, A. C. *et al*; 1986). Clinical observations by Pauleikhoff and colleagues are in accordance with this hypothesis as they have shown that drusen in the fellow eyes of those with PED are more densely packed,

larger and less fluorescent on fluorescein angiography implying that they are hydrophobic due to the presence of neutral lipid (Pauleikhoff, D. *et al*; 1990a).

1.2.3.3 Mechanism of choroidal neovascularisation and angiogenesis

In choroidal neovascularisation (CNV), blood vessels derived from the choroid extend through Bruch's membrane and into the sub-RPE space (Green, W. R. *et al*; 1993) usually in association with diffuse or focal drusen. This process may also be associated with a cellular breakdown of Bruch's membrane with the presence of multinucleated giant cells (Penfold, P. L. *et al*; 1987) or the stimulation of blood vessel growth by macrophages on the inner surface of Bruch's membrane (Sarks 1976). The current theory however is that CNV is likely to occur in response to the release of an angiogenic stimulus. Evidence that the RPE may produce this stimulus comes from work by Uyama and colleagues (Yamagishi, K. *et al*; 1988) where sodium iodate ($NaIO_3$) was used to damage RPE cells in primates while inducing CNV with photocoagulation. Viable RPE was required at least initially to allow induction of new vessel growth.

Vascular endothelial growth factor (VEGF) is believed to be the predominant stimulus to new vessel growth in retinal disorders including diabetes, retinopathy of prematurity and central retinal vein occlusion. It has been proposed that it is produced in response to hypoxia or ischaemia. Although other growth factors such as basic fibroblast growth factor (bFGF)(Ogata, N. *et al*; 1996) and transforming growth factor – beta (TGF-β) (Ogata, N. *et al*; 1997) have been suggested to promote the development of CNV, increasing evidence suggests that VEGF is the most likely candidate (Schwesinger, C. *et al*; 2001; Wada, M. *et al*; 1999; Wells, J. A. *et al*; 1996) even though CNV is not obviously an ischaemic-driven process. Wells et al demonstrated that vitreous levels of VEGF are significantly elevated in those eyes with subretinal neovascularisation (Wells, J. A. *et al*; 1996). Intrachoroidal neovascularisation has been shown to occur in transgenic mice over-expressing VEGF in the RPE (Schwesinger, C. *et al*; 2001). Similarly, CNV has been induced in rats when an adeno-associated viral vector encoding human VEGF 165 has been injected into the subretinal space (Wang, F. *et al*; 2003). Additionally, both VEGF and VEGF receptor

mRNA expression has been localised to retinal pigment epithelial cells and endothelial cells by in situ hybridisation techniques within experimentally induced CNV (Wada, M. *et al*; 1999).

Further studies have shown that there is likely to be an imbalance of growth factors operating in the development of CNV, namely VEGF stimulating growth and PEDF (pigment epithelium –derived factor) suppressing growth (Ohno-Matsui, K. *et al*; 2001). PEDF is a dual function protein which acts as a neurotrophic factor promoting the survival of photoreceptors (Cayouette, M. *et al*; 1999), and is also a potent inhibitor of angiogenesis (Dawson, D. W. *et al*; 1999). It is thought to act directly on endothelial cells, halting the formation of new vessels by inducing the apoptotic death of endothelial cells that have been stimulated to form new vessels (Stellmach 2001} (Mori, K. *et al*; 2002).Work on vitreous samples of patients with CNV due to AMD has shown deficient levels of PEDF (Holekamp, N. M. *et al*; 2002). Additionally in the presence of oxidative stress in vitro, PEDF levels were shown to decrease and disrupt the balance, promoting angiogenesis (Ohno-Matsui, K. *et al*; 2001). Others have also shown that increased expression of PEDF in the eye reduces the amount of ocular neovascularisation in mice and in a model of laser induced CNV (Mori, K. *et al*; 2001).

In addition to VEGF and PEDF, recent results have implicated angiopoietins 1 and 2 (ang 1&2) to play a role in CNV formation. Ang 1 promotes vascular integrity and maturation and inhibits apoptosis and ang 2 is an antagonist of ang 1 and promotes VEGF-induced angiogenesis. These factors may also be modulated by VEGF (Hangai, M. *et al*; 2001; Holz, F. G. *et al*; 2004).

Observations that up-regulation and suppression of growth factors are able to influence the behaviour of endothelial cells in vitro and inhibit new vessels in vivo support the belief that these may lead to alternative and more selective treatment strategies in the coming years.

1.2.3.4 Mechanisms of geographic atrophy

It was Blair in 1975 who described geographic atrophy (GA) of the RPE as a separate disease entity secondary to macular degeneration (Blair, C. J.; 1975). It is defined as a discrete area of retinal depigmentation >175 μm, characterised by a sharp border and the presence of visible choroidal vessels at the posterior pole (Bird, A. C. *et al*; 1995). Histologically, this represents RPE cell degeneration with accumulating lipofuscin granules which aggregate as clumps. The overlying photoreceptors also degenerate becoming grossly abnormal with shortened inner segments and fragmented outer segments. Bruch's membrane becomes hyalinised and there is a variable degree of choroidal atrophy and partial obliteration of capillaries by fibrous tissue (Sarks, J. P. *et al*; 1988; Sarks, S. H.; 1976).

One of the explanations for the focal nature of the atrophic process, has been ascribed to the lobular microvascular anatomy of the choriocapillaris. It was thought that closure or sclerosis of the underlying choriocapillaris lobule could result in focal atrophic areas of the RPE. Both the lobules and areas of atrophy are of similar size (Maguire, P. *et al*; 1986) but the fact that the choriocapillaris changes could occur secondary to changes in the RPE cannot be discounted.

With the knowledge of lipofuscin accumulation during ageing and the advent of autofluorescence (AF) imaging, attention has switched to the RPE cell being the initial site of disease. Evidence suggests that lipofuscin reflects the inability of the RPE to recycle phagosomal contents and as it accumulates, its toxicity (see section 1.2.2.2.4) causes a number of changes within the RPE, photoreceptors, Bruch's membrane and choroidal capillaries resulting in areas of atrophy (Okubo, A. *et al*; 2000). Focal increases in autofluorescence, seen on scanning laser ophthalmoscopy, have been shown to represent increased levels of lipofuscin within the RPE (Delori, F. C. *et al*; 1995a) and are thought to predict the development of atrophy (Holz, F. G. *et al*; 2004). Additionally, areas with high levels of autofluorescence seen at the perimeter of established geographic atrophy have been shown to become atrophic within 1 year (Holz, F. G. *et al*; 2001). If the presence of increased levels of lipofuscin or A2-E is important in the pathogenesis of GA, this would

argue against the use of vitamin A supplements. Instead a dietary restriction of vitamin A or ways of slowing down retinoid recycling would be advocated for possible treatment strategies (Holz, F. G. *et al*; 2004).

1.2.4 Why the macula?

Attempts have been made to explain why lesions associated with ageing are more frequently seen in the macular and perimacular areas compared to more peripheral areas of the eye.

One explanation from Gass is the organisation of the rich vascular system at the macular region. Here the blood passes from large arterial trunks in the choroid to large sinusoidal spaces of the choriocapillaris. The intracapillary pressure and choroidal blood flow are likely to be very high and it is thought that age changes may cause decompensation in an area of greatest intracapillary stress (Gass, J. D.; 1967). No further evidence however suggests that macular degeneration is due to capillary disease (Hogan, M. J. *et al*; 1967).

Bruch's membrane has also been shown to differ structurally at the macula, with a decreased thickness (3-6 times) of the elastic lamina at the macular region compared to extramacular regions (Chong, N. H. *et al*; 2005). In cases with active CNV and disciform scars the integrity of the layer was significantly reduced becoming more porous compared to controls (Chong, N. H. *et al*; 2005). This may help to explain why the macula is more susceptible to AMD allowing choroidal vessels to breach Bruch's membrane and grow into the retina.

The distribution of lipofuscin and cytoplasmic volume within the RPE increases from the periphery to the posterior pole (Feeney-Burns, L. *et al*; 1984; Weiter, J. J. *et al*; 1986). One explanation is that the higher ratio of photoreceptors to RPE cells in the macula area results in increased phagocytic and metabolic load on the RPE and increased accumulation of lipofuscin (Dorey, C. K. *et al*; 1989). Marshall *et al* showed a similar profile of lipofuscin to the distribution of rod photoreceptor cells across the retina (Marshall, J.; 1987) and

Dorey demonstrated that the temporal equator had much higher ratios of rod photoreceptors than the nasal equator.

Other factors implicated include the high metabolic rate in the macular region and the long term effects of light (Gass, J. D.; 1967) although no further evidence has substantiated this.

1.3 Prevalence and incidence of ARM/AMD

1.3.1 Prevalence of ARM/AMD

The prevalence of AMD in Caucasian populations in the Western World is increasing. In Britain in 1950, only 14% of blind registrations were attributable to AMD and by 1990 this figure had increased to nearly 50% (Evans, J. *et al*; 1996). This figure is greater than would be expected when adjusted for age and so other factors must also be involved (Evans, J. *et al*; 1996). This increase may partly be explained by a change in the classification or coding of AMD over the years and partly by improvements in the treatment of the other blinding conditions assessed such as glaucoma and cataract.

Population based studies have shown variable estimates for the prevalence of ARM/AMD amongst different populations (see table 1 {page 29}), but collectively they all show that the frequency increases with age. Reasons for differences between studies include differences in definitions, methodology, socio-demographic factors and genetic differences among the groups being studied (Kahn, H. A. *et al*; 1977), (Bressler, N. M. *et al*; 1989; Klein, R. *et al*; 1992; Mitchell, P. *et al*; 1995; Vingerling, J. R. *et al*; 1995b).

Examples of differences in definitions include the Framingham Eye Study (Leibowitz, H. M. *et al*; 1980) and the National Health and Nutrition Eye Study (Goldberg, J. *et al*; 1988) which included visual acuity in the definition whereas other population-based studies (Beaver Dam, Rotterdam and Chesapeake Bay Study) did not. Drusen were also included in the definition of 'dry' or non-neovascular AMD in the Framingham study increasing its prevalence in relation to the neovascular form. The Rotterdam Study (Vingerling, J. R. *et al*; 1995b), the Blue Mountains Eye Study (Mitchell, P. *et al*; 1995) and the Beaver Dam

Eye study (Klein, R. *et al*; 1992) all used a grading system with standard protocols to grade fundus photographs objectively (based on the Wisconsin Age-related Maculopathy Grading System -WARMGS) (Klein, R. *et al*; 1991). Essentially a transparent grid, centred on the foveola was placed over colour 35mm slides to allow grading of the various features within 3000μm of the centre of the macula and the National Health and Nutrition Examination Survey (NHANES) (Klein, B. E. *et al*; 1982) was based on clinical examination only.

Table 1: Prevalence of ARM/AMD amongst different populations

Study	Time period	No of participants	Age range	Prevalence of ARM (%) (difficult to compare between studies)	Prevalence of late AMD (%)
Framingham, U.S. (Kahn 1977)	1973-1975	2477	52-85	8.8	1.5 % (exudative) (Leibowitz 1980 surv)
Beaver Dam Eye Study, U.S. (Klein 199)	1988-1990	4771	43-86	20% (any soft drusen) 13% (pigment abnormality)	1.6% exudative 1.2%, GA 0.6%
Blue Mountains Eye Study, Australia (Mitchell 1995)	1992	3654	49+	13.3% (any soft drusen) 12.6% (pigment abnormality)	1.9% total (exudative 1.3% atrophic 0.7%)
Rotterdam Study, Netherlands (Vingerling 1995)	1990-1993	6251	55-98	Higher rates of soft drusen compared to Beaver Dam and Blue mountains but not comparable. 7.2% (Pigment abnormalities)	1.7% total (exudative 7.4% > 85 yrs; atrophic 3.7% > 85 yrs)
Reykjavik Eye Study Iceland (Jonasson, F. *et al*; 2003)	1996-2001	1021	50+	Age 50-59; 4.8% intermediate soft drusen, 1.2% large soft, 0.6% large semisolid. Age > 80; 18.2%, 10.9% and 25.5% respectively	exudative 2.3% (≥ 70 years) atrophy 9.2% (≥ 70 years)

1.3.1.1 Prevalence of geographic atrophy

Geographic atrophy is a less common cause of visual loss in AMD in most surveys, but increases sharply in prevalence with age (Klein, R. *et al*; 1997; Mitchell, P. *et al*; 1995). In

the Beaver Dam Study the prevalence increased from 0% in those less than 54 years to 2.0% in those over 75 years and in the Rotterdam Study the prevalence increased from 0.1% (≤ 64 yrs) to 3.7% in those greater than 85 years. GA has different prevalences in different populations. In Rotterdam, North America and Australia the prevalence appears to be half that of neovascular AMD (Klein, R. *et al*; 1997; Mitchell, P. *et al*; 1995; Vingerling, J. R. *et al*; 1995b). In Greenland and Iceland however, geographic atrophy occurs more frequently than neovascular AMD. In the latter, the prevalence is 9.2% in those >70 years compared to 2.3% with exudative macular degeneration in the same age group (Jonasson, F. *et al*; 2003). Reasons for this are unclear but environmental factors such as diet, notably vitamin A intake, or genetic influences may play a role.

1.3.1.2 Prevalence of neovascular AMD / exudative AMD

Exudative AMD, which is responsible for much of the blindness caused by AMD, was twice as prevalent as GA in the Beaver Dam (Klein, R. *et al*; 1992), Blue Mountain Study (Mitchell, P. *et al*; 1995) and the Rotterdam Study (Vingerling, J. R. *et al*; 1995b) (see table 1 {page 29}). In the Framingham study however, drusen were included in the definition of non-exudative AMD thus lowering the proportion of exudative AMD (Leibowitz, H. M. *et al*; 1980). No differences existed between the sexes once the results were adjusted for age (Vingerling, J. R. *et al*; 1995b) (Mitchell, P. *et al*; 1995).

1.3.2 Incidence of AMD

According to the Rotterdam study, the incidence rate of AMD was 1.2 per 1000 persons per year for subjects aged 55 years and over, increasing to 8.8 per 1000 persons per year in those subjects aged 85 years and older (Klaver, C. C. *et al*; 2001). This rate is lower than that shown by the Beaver Dam Study where the incidence rate for those over the age of 85 was calculated as approximately 27 per 1000 persons per year (Klaver, C. C. *et al*; 2001) (Klein, R. *et al*; 1997). Although the follow up period in the Rotterdam study was shorter and only a small number of participants developed incident AMD, the difference appears considerable and is consistent over the age groups. This is also in accord with other reports suggesting that there are global differences in the occurrence of AMD (Klaver, C. C. *et al*; 2001). The incidence of AMD in the contralateral eye of subjects already affected by

unilateral AMD was markedly increased at 170.6 per 1000 persons per year suggesting that age-related macular degeneration in one eye considerably increases the risk to the second eye(Klaver, C. C. *et al*; 2001).

1.4 Risk factors for AMD

1.4.1 Demographic factors

1.4.1.1 Age profile

Age is known to be the strongest risk factor as all studies have shown a marked increase in prevalence with age. In the Framingham eye study, "senile macular degeneration" was infrequent below the age of 65 (1.2%) and increased rapidly with age to 6.4% for ages 65-74 and to 19.7% for ages 75 and older (Leibowitz, H. M. *et al*; 1980). The vast majority were classified as 'dry' AMD although drusen and pigment alterations were included in this definition making it difficult to compare with other studies. The Beaver Dam Study (1987-1990) reported on 4926 people between the ages of 43 and 86. People 75 years of age or older had significantly higher frequencies ($p<0.01$) of large drusen (≥125μm, 24% v 1.9%), soft indistinct drusen (23% v 2.1%), retinal pigment abnormalities (26.6% v 7.3%), exudative macular degeneration (5.2% v 0.1%) and geographic atrophy (2% v 0%) compared to those aged 43 to 54 years (Klein, R. *et al*; 1992).

1.4.1.2 Gender

Unlike the Rotterdam (Klaver, C. C. *et al*; 2001) or the Framingham Study, the Beaver Dam Study, after adjusting for age, found an increased incidence of early ARM (x2.2) and over seven times the incidence of late ARM in women over 75 years (Klein, R. *et al*; 1997). The Blue Mountain Study found higher frequencies for soft drusen in women but this did not reach statistical significance (Mitchell, P. *et al*; 1995). Arguments put forward, after age adjustment, as to the cause of this difference include the differences in health seeking behaviour between the sexes (Evans, J. R.; 2001). It has also been suggested that there may be a loss of a protective effect of oestrogens in post menopausal women. The Eye Disease Case Control Study showed that women taking replacement oestrogen were at

a reduced risk of neovascular AMD (Evans, J. R.; 2001). After further analysis of pooled data however, from Beaver Dam, Rotterdam and the Blue Mountains, it was concluded that there were no significant gender differences in the prevalence of neovascular or atrophic AMD (Smith et al 2001).

1.4.1.3 Race

AMD is believed to be a disease predominantly affecting Caucasian western populations. The prevalence, especially of neovascular disease, has been shown to vary between racial groups being lower in afro-Caribbean populations (Friedman, D. S. *et al*; 1999; Schachat, A. P. *et al*; 1995). With regard to studies of photoreceptor to RPE cell ratios in the macula, it was found that afro-Caribbeans had fewer photoreceptors to each RPE cell compared to whites older than 50, indicating less lipofuscin accumulation and subsequently less photoreceptor cell death (Dorey, C. K. *et al*; 1989).

Within the Western World, the age specific incidences of AMD appear to be lower in the Netherlands compared to the US (Klein, R. *et al*; 1997)and are consistent over the age groups. This difference is not explained by risk factors such as smoking or cardiovascular factors (Klaver, C. C. *et al*; 2001). It remains to be seen if other environmental or genetic factors are accountable.

In Japan, AMD was virtually unknown 30 years ago but the prevalence has dramatically increased in urban communities recently, most likely due to environmental factors (Yuzawa, M. *et al*; 1997). The Inuit of Greenland interestingly appear to have a particularly high incidence of AMD (Rosenberg, T.; 1987), the cause of which is still unknown. Genetically they are clearly distinct from western Caucasian societies and their lifestyle is quite different.

1.4.2 Genetic factors

1.4.2.1 Evidence for genetic involvement

Over the last three decades there has been increasing evidence of the role of genetic influences in the pathogenesis of age-related macular disease. It was Don Gass in 1973 who reported that 20% of 200 patients had 'a positive family history of loss of central vision'. Since then several twin and sibling studies have suggested that a genetic predisposition exists (Klein ML et al, 1994; Meyers SM et al,1995; Piguet B et al 1993; Hammond et al 2002) and that various environmental factors may trigger the disorder in susceptible individuals. In a study by Seddon et al (Seddon 1997) the prevalence of age-related maculopathy in the first-degree relatives of subjects with the disease was found to be greater than those without the disease. This was particularly the case with exudative rather than non-exudative disease. This led to the conclusion that the relative contributions of genetic and environmental factors may differ in the various phenotypes of AMD.

The genes conferring risk are as yet unidentified but it is recognised that AMD is a complex disorder in which several genes are likely to be involved.

1.4.2.1.1 Twin studies

Klein et al (1994) reported eight of nine monozygotic (MZ) twins concordant for AMD with a similar fundal appearance. In the ninth pair, one twin had advanced exudative ARMD with vision loss in one eye, while the other had large, confluent drusen and good visual function in both eyes. *Meyers et al* (1995) reported 100% concordance in MZ twins (n=25) compared to 42% concordance in dizygotic (DZ) twins (n=12). The twin pairs however were not necessarily concordant for individual phenotypes of AMD and showed variable clinical expression between twins and between eyes of the same twin. Concerns with this study included a recruitment bias towards women and monozygotic twins. There is also potential for discordant dizygotic twin pairs to become concordant with time. Hammond et al more recently (*Hammond 2002*) demonstrated that the concordance of ARM in MZ twins was 0.37 compared to 0.19 in DZ twins. The most heritable phenotypes were soft drusen $\geq$ 125 μm and $\geq$ 20 hard drusen. No case had evidence of geographic

atrophy or neovascular AMD. These studies represent good evidence of a genetic role in AMD.

1.4.2.1.2 Familial aggregation studies

Piguet et al (1993) compared the density, size and confluence of drusen between AMD patients and their spouses and siblings and found statistically significant concordance with the siblings, but not with the spouses, for number and density of drusen in the central macula. Other studies have also shown greater concordance between siblings for features of AMD than between spouses (Gorin, M. B. *et al*; 1999; Heiba, I. M. *et al*; 1994; Seddon, J. M. *et al*; 1997; Silvestri, G. *et al*; 1994).

Klaver (Klaver, C. C. *et al*; 1998b) carried out a familial aggregation study based on probands from the population- based Rotterdam Study. The prevalence of early and late ARM assessed from colour photographs was significantly higher in first-degree relatives of 87 patients with late ARM compared to first-degree relatives of 135 control subjects. A relative risk of 4.2 was obtained. It was also found that relatives of patients developed ARM at a younger age, which suggests genetic factors may play a role in determining age of onset.

Hyman (Hyman, L. G. *et al*; 1983) found that 20% of the siblings of index cases were affected compared to 8% of the siblings of controls, demonstrating a 2.9 fold increased risk of ARMD if one or more family members was affected. Silvestri (Silvestri, G. *et al*; 1994) observed a family history of ARMD in 58.3% of affected patients compared to none in the controls. One important limitation of the study was the inability to examine all patients and the reliance on reports from other ophthalmologists and optometrists. The late onset of disease and variable expressivity also makes identification of the disease difficult in family members who may not have reached the age of disease expression or had insufficient disease to experience symptoms.

The Beaver Dam Eye Study (Klein, B. E. *et al*; 2001) investigated the 5-year incidence of specific lesions of age-related maculopathy in the younger sibling of those with an affected

older sibling with the same lesion. The odds ratios for the younger sibling developing each specific lesion was 1.82 (95% CI 0.91- 3.66) for soft drusen, 3.59 (95% CI 1.71-7.57) for increased retinal pigment and 10.32 (95% CI 0.83-128.58) for exudative AMD. These results were consistent with genetic influences being important in specific phenotypes of AMD.

1.4.2.2 Approaches for identifying genetic risk factors

1.4.2.2.1 Linkage studies

Linkage analysis allows the mapping of genes within a pedigree using genetic markers (usually microsatellite markers such as (CA)n repeats of the DNA sequence) that are sufficiently polymorphic. This ensures a reasonable chance that family members will be heterozygous, having two different alleles, and therefore be informative for the marker. If the disease locus is on the same chromosomal segment as one of the markers they will tend to segregate together provided they are not separated by recombination during meiosis (Strachan, T. *et al*; 1999a).

A simple form of linkage analysis commonly used is sib-pair analysis. Here pairs of affected siblings, who share a proportion of their parental alleles, are typed for markers. The chromosomal regions, where there is greater than expected sharing, are studied allowing candidate susceptibility regions to be defined. However, these are often large making the subsequent determination of the susceptibility genes a real challenge. The next step is to seek associations in the general population between the disease and alleles of candidate genes or markers within the susceptibility region. (Strachan, T. *et al*; 1999b).

1.4.2.2.2 Association studies

An alternative strategy to linkage mapping is to look for statistical associations in the general population between the disease and some marker genotype. This approach can be used with case control studies to look for sequence variants or candidate genes enriched in one group compared to the other. With associations between an allele and a disease, the allele itself increases the susceptibility or risk of an individual developing the disorder but

does not necessarily cause the disease. This type of study is dependant upon the founder effect whereby a population is genetically homogeneous and descended from a small number of original founders. It relies on a base change which has occurred in a common ancestor where a significant proportion of those affected have inherited the mutation (Strachan, T. *et al*; 1999a). The choice of controls is also crucial to ensure that study patients and control groups are drawn from genetically similar populations (Strachan, T. *et al*; 1999b).

1.4.2.3 Genetics of complex disorders

Attempting to map genes in complex disorders is complicated by the fact that multiple genes are likely to either interact or act independently with environmental variables to cause susceptibility to disease. Even when using large numbers of families, initial detection of loci by linkage analysis may be difficult, either because their predisposing effects are mild, are masked by interaction with other loci or are strong but relevant to only a small subset of cases (Field, L. L. *et al*; 1994). Despite this, there is currently great optimism for the discovery of genes in complex disorders.

Research in other complex diseases has benefited from the identification of biochemical pathways involved and likely candidate genes. Alzheimer disease, an adult onset neurodegenerative disease is an example of this (Sisodia, S. S. *et al*; 2002). The past decade has seen the identification of 3 causative genes (β-amyloid precursor protein, presenilin 1, presenilin 2) and one susceptibility gene (apolipoprotein E $\in$4) for Alzheimer's disease. Disappointingly these genes although involved in transmitting familial Alzheimer disease, have not been shown to be important in simplex cases.

Potential advantages of studying a disorder such as AMD include the existence of several inherited monogenic retinal dystrophies with phenotypes similar to that of AMD. The genes responsible for these disorders have been previously characterised and will serve as invaluable candidate genes for subsequent association studies. The subdivision of AMD into its various phenotypes will allow purer samples of disease to be investigated with molecular genetic techniques in the future in the hope of finding gene associations.

Additionally as our knowledge increases of the underlying mechanisms involved in AMD, these pathways can be investigated for further candidate genes.

1.4.2.4 What is known to date?

1.4.2.4.1 Linkage studies

A large pedigree of 10/21 members with AMD was identified in the US allowing linkage to be carried out. Those affected had predominantly large soft drusen (n=7, with 2 cases with early GA) or extensive geographic atrophy (n=3).The disorder was found to segregate as an autosomal dominant trait with the disease locus mapping to chromosome 1q25-q31, between markers D1S466 and D1S413. The multipoint lod score was found to be 3.00 giving evidence for linkage (Klein, M. L. *et al*; 1998). The LOD (logarithm of the odds) score is a statistical estimate of whether 2 loci lie near each other and therefore are likely to be linked.

More recently, the 1q31 locus was independently shown to map to other families with age-related macular degeneration using an expanded genome-wide scan (Weeks, D. E. *et al*; 2001).

1.4.2.4.2 Association studies and candidate genes

Several hereditary retinal dystrophies have some phenotypic similarity to AMD. Stargardt disease (*ABCA4* gene) (Allikmets, R. *et al*; 1997) commonly results in macular atrophy (see figure 3a [page 40]) and mutations in the *peripherin /RDS 172* gene give rise to a phenotype very similar to geographic atrophy (see figure 3b) (Downes, S. M. *et al*; 1999). *ELOVL 4* is associated with dominant Stargardt disease and has been evaluated in AMD (Ayyagari, R. *et al*; 2002). Drusen nasal to the disc is a characteristic feature in Doyne honeycomb retinal dystrophy (see figure 3c) or Malattia Leventinese (see figure 3d) (*EFEMP1* [EGF-containing fibulin-like extracellular matrix protein 1] gene) (Stone, E. M.

et al; 1999). A gene similar to *EFEMP1*, namely a member of the fibulin gene family, has recently been implicated in AMD(Stone, E. M. *et al*; 2004).

Choroidal neovascularisation, which is a prominent feature in Sorsby fundus dystrophy (see figure 3e) (*TIMP3* gene) (Weber, B. H. *et al*; 1994) is also a common finding in AMD. *VMD 2* is the gene known to cause Best disease which is also associated with neovascularisation in the later stages (Petrukhin, K. *et al*; 1998) and adult onset vitelliform macular dystrophy (see figure 4a [page 41]) can be mistaken for drusen or RPE pigmentation without the assistance of autofluorescence imaging (see figure 4a&b). The genes responsible for these conditions are therefore attractive candidate susceptibility genes for AMD. Various association studies have been carried out to investigate the role of these genes in AMD.

- ABCA4 gene

The *ABCA4* gene, which encodes an ATP-binding cassette (ABC) transporter protein ABCR, corresponds to a previously identified rod outer segment protein called rim protein (RmP), is known to be defective in Stargardt disease (Allikmets 1997 Nat Genet). The protein, ABCR, is believed to be involved in the transport of vitamin A across membranes between the rod and RPE cell. This is disrupted in Stargardt disease resulting in an abnormal accumulation of a rhodopsin by-product, N-RPE (N-retinylidene phosphatydal ethanolamine) which accumulates in the rod outer segment. This then results in lipofuscin accumulating within the RPE (Eagle, R. C., Jr. *et al*; 1980; Glazer, L. C. *et al*; 2002; Mata, N. L. *et al*; 2001; Weng, J. *et al*; 1999) leading to RPE cell and rod cell death. These changes are characterised by early adult onset of progressive atrophy of the macular RPE and photoreceptors not dissimilar to the appearance of geographic atrophy in AMD. Stargardt disease patients also demonstrate peripheral yellow flecks at the posterior pole which fluoresce brightly on AF imaging (von Ruckmann, A. *et al*; 1997b) (see figure 4c&d).

In an association study, 167 unrelated AMD patients were screened for alterations in all 51 exons of the ABCA4 (ABCR) gene (Allikmets, R. *et al*; 1997). The results showed 13

different AMD-associated alterations, mostly missense mutations in conserved amino acid positions, in 26 AMD patients (16%). These alterations were concluded to be associated with AMD on the basis that they were significantly less frequent in controls (0.45%). Critics have since expressed their concern that sequence variations which occurred in controls were ignored and DNA from controls was not completely screened. In a subsequent study, 215 individuals with Stargardt disease, 182 cases of AMD and 96 controls were equally screened for sequence variations (Stone, E. M. *et al*; 1998). A large degree of sequence variation was seen in all three groups with no significant difference between the proportion of AMD and control subjects with non-conservative variants or single DNA changes.

Further investigation of the two most frequent AMD associated variants in *ABCA4*, by the international ABCR screening consortium (Allikmets, R.; 2000) have shown that G1961E was found in 1.56% patients with AMD compared to 0.32% control subjects (OR: 5.0, 95%CI 1.6-20) and D2177N was present in 1.77% AMD cases compared to 0.64% controls (OR: 2.8, 95%CI 1.2-7.4). The patients in this study were segregated by phenotype into "dry" and "wet" forms of AMD but the prevalence of both the G1961E and D2177N variants were not significantly different between the 2 groups (OR:1.77 95% CI 0.9-3.4). Critics have argued that the phenotyping was not precise and this remains a major weakness of the study. In addition the original series was included in the second study.

The role of ABCR in AMD remains as yet unresolved. Although various studies have conflicting results, no study has been large enough to exclude the gene. Bernstein (Bernstein *et al* 2002) concluded that there is mounting evidence to suggest that heterozygous variants in ABCR may well contribute to AMD susceptibility.

Figure 3

Fundus photographs demonstrating (a) Stargardt macular dystrophy (b) *peripherin /RDS 172* gene mutation phenotype (c) Doyne honeycomb retinal dystrophy (d) Malattia Leventinese (e) Sorsby fundus dystrophy with CNV like phenotype (f) variant of Sorsby fundus dystrophy with appearance similar to GA

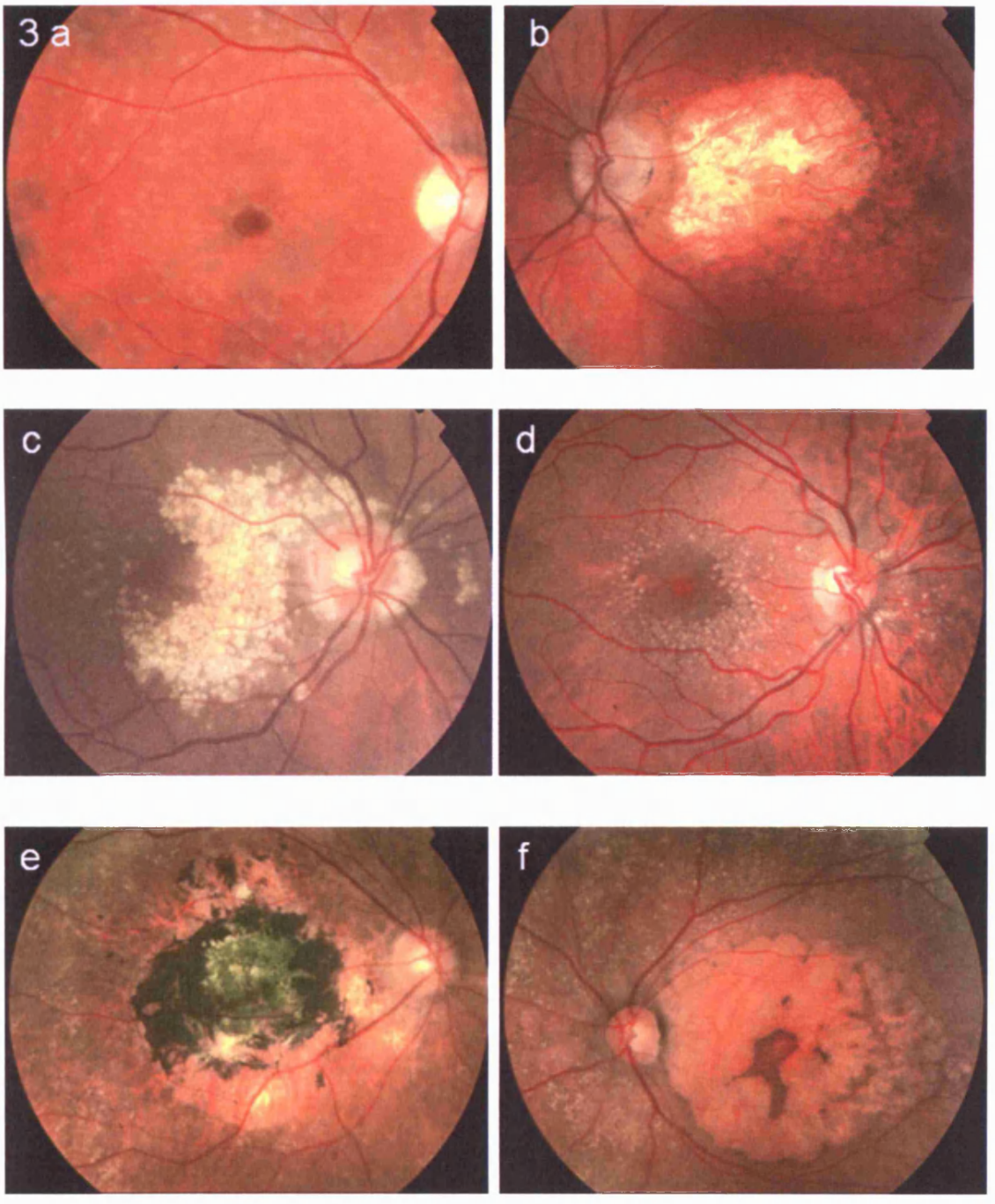


Figure 4 (a to h)

Fundus photographs with corresponding AF images demonstrating (a&b) Adult vitelliform macular dystrophy (c&d) Stargardt macular dystrophy with lipofuscin deposits (e&f) Best macular dystrophy (g&h) pattern dystrophy (butterfly phenotype)

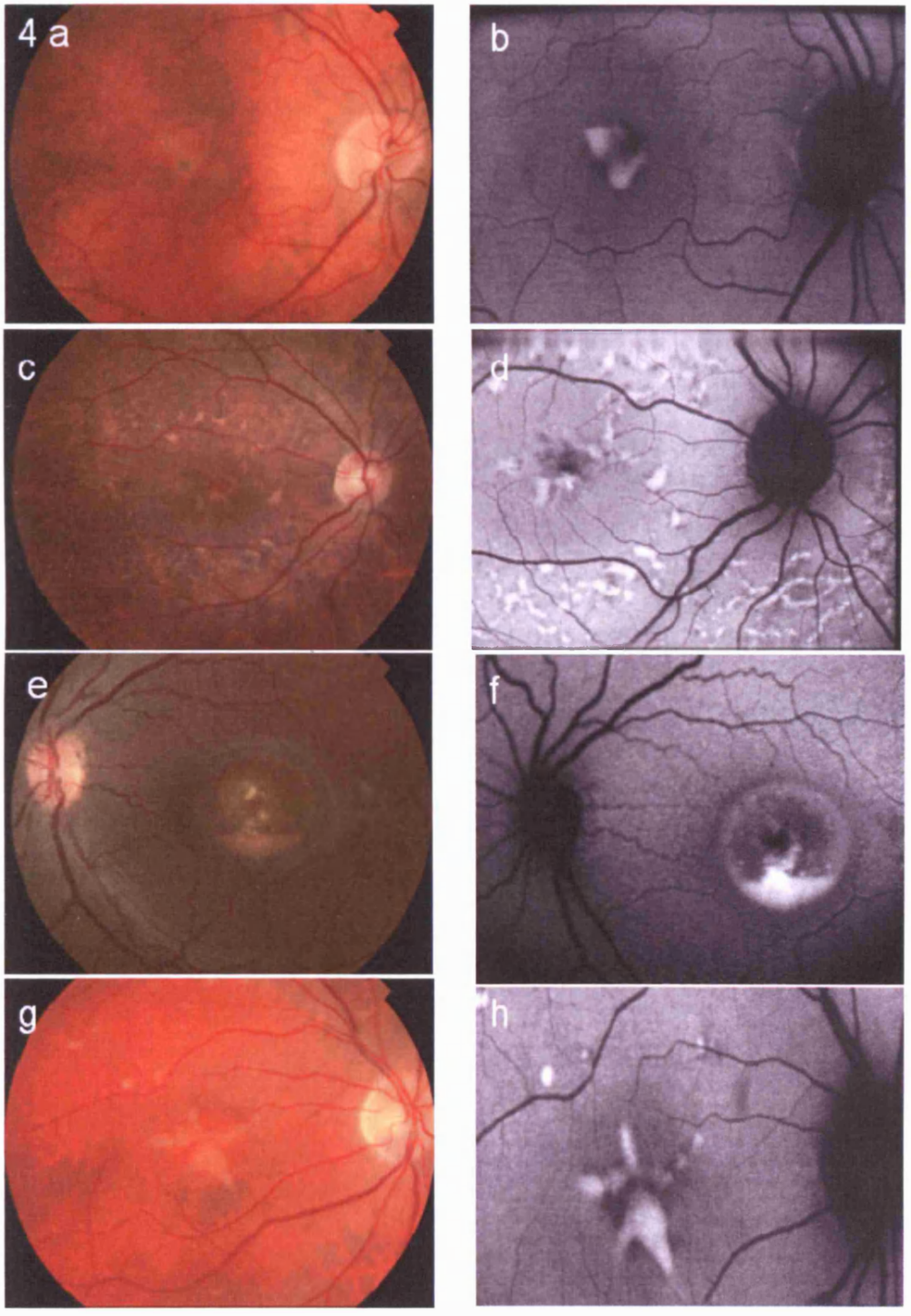
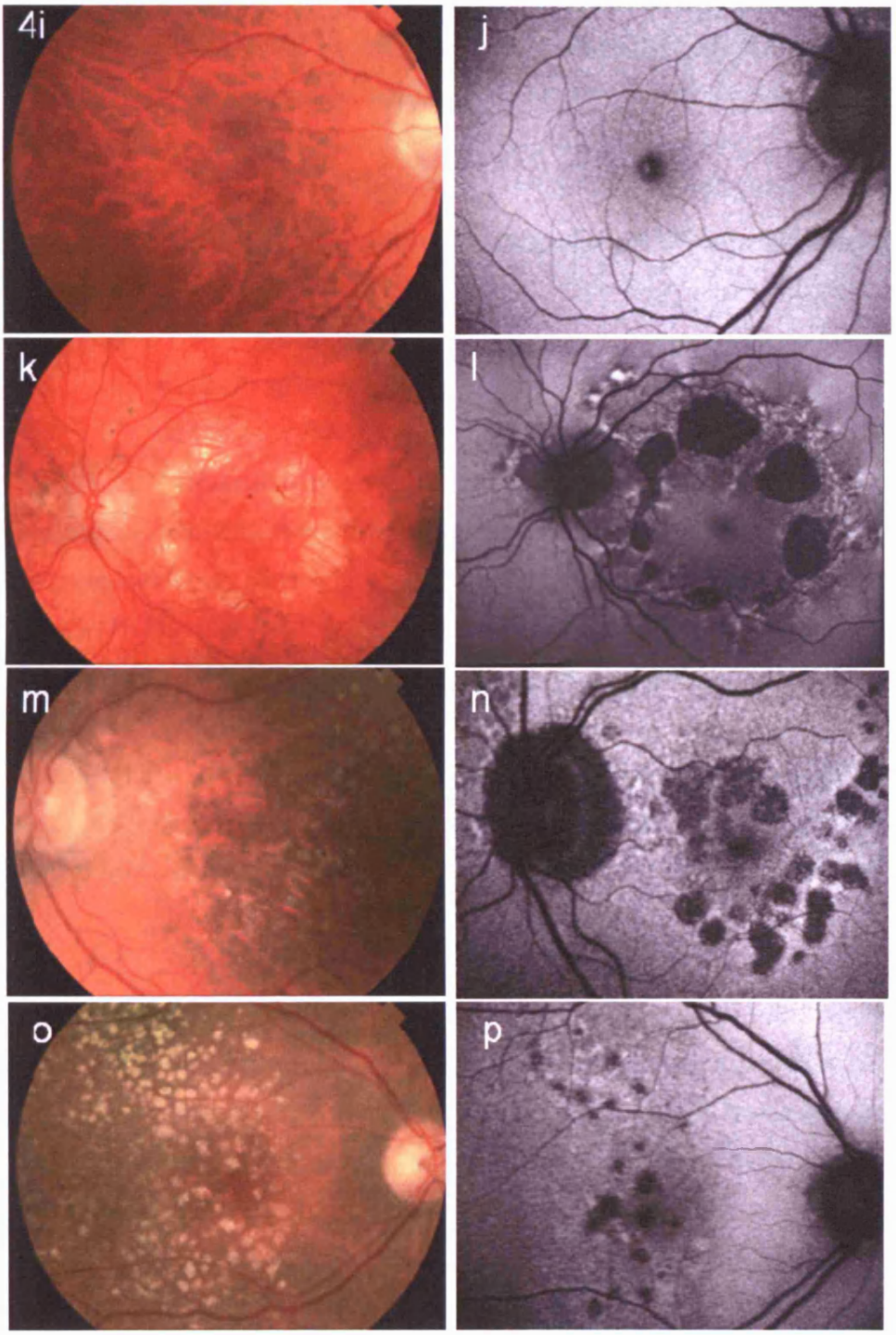


Figure 4 (i to p)

Fundus photographs with corresponding AF images demonstrating (i&j) a 'comparison group' eye (k&l) maternally inherited diabetes and deafness (MIDD) with perimacular areas of atrophy (m&n) geographic atrophy which is more readily seen with AF imaging, especially where extensive drusen is present (o&p)



- Peripherin / RDS gene

Mutations in this gene can present with a variety of phenotypes including a dystrophy looking very similar to geographic atrophy (RDS mutation 172) (see figure 3b [page 40]) (Downes *et al* 1999). Other dystrophies include pattern dystrophy (see figure 4g&h [page 41]) which can be mistaken for bilateral drusen to the untrained eye (see figure 1b [page 11]).

Mutation screening in 50 AMD patients however did not identify significant sequence changes (Silvestri *et al* 1997).

- EFEMP1 gene

A single non-conservative mutation (Arg345Trp) in the gene *EFEMP1*(EGF-containing fibrillin-like extracellular matrix protein 1) or also known as fibulin 3 has been found to be associated with both Doyne honeycomb retinal dystrophy and Malattia Leventinese (Stone et al 1999). This autosomal dominant disease is characterised by drusen deposits similar in appearance to that in AMD. The distinguishing feature described in Malattia is radial drusen (see figure 3d [page 40]) and in Doyne honeycomb dystrophy, is drusen nasal to the optic nerve head (see figure 3c). Although *EFEMP1* is an attractive candidate gene for AMD and is expressed in tissues close to the site of drusen formation (RPE and retina), the mutation was not found in a panel of 494 patients with AMD (Stone, E. M. *et al*; 1999).

- Fibulin Genes

There has been a lot of interest in the fibulin gene family recently (closely related to EFEMP 1) which is thought to play an important role in AMD. It was a recent study from Iowa City, that showed amino acid-altering sequence variations in the fibulin 5 gene (now published as fibulin 6) in seven of 402 patients with AMD, whereas none were observed among the 429 control subjects ($P<0.01$). Several of the altered residues have been

conserved in evolution. All seven patients had small circular drusen as the clinical phenotype (Stone, E. M. *et al*; 2004).

- TIMP-3 gene

Sorsby fundus dystrophy, an autosomal dominant disorder caused by mutations in the tissue inhibitor of metalloproteinases-3 gene on chr 22 (*TIMP-3*) (Weber, B. H. *et al*; 1994), has a phenotypic appearance not dissimilar to choroidal neovascularisation in AMD (Capon, M. R. *et al*; 1988) (see figure 3e). A further mutation in the gene has also been associated with a phenotype more like geographic atrophy (see figure 3f). The gene is thought to be involved in extracellular matrix modelling (controlled by matrix metalloproteinases) and the normal turnover of proteins. Histologically, mutations in this gene causes widespread marked thickening of Bruch's membrane and subretinal deposits (Capon, M. R. *et al*; 1989) which is seen clinically by choroidal perfusion abnormalities (Pauleikhoff, D. *et al*; 1990b) (Polkinghorne, P. J. *et al*; 1989). Linkage studies, association studies, sib pair analysis and mutation screening looking for a role in AMD have not yet yielded any positive results to date (De La Paz, M. A. *et al*; 1997; Felbor, U. *et al*; 1997).

Studies of protein content of drusen have interestingly shown the presence of TIMP-3 and oxidative modifications of TIMP-3 and are thought to be important in drusen formation (Crabb, J. W. *et al*; 2002).

- Bestrophin gene

VMD2 has been identified as the gene causing Best disease, which is also an autosomal dominant macular dystrophy mapping to 11q13 (Petrukhin, K. *et al*; 1998). The disorder is associated with an accumulation of autofluorescent material in the RPE thought to represent lipofuscin (see figure 4e&f [page 41]) (von Ruckmann, A. *et al*; 1997b) but as yet, the gene has not been found to play a major role in AMD (Allikmets, R. *et al*; 1999).

The following three candidate genes have no recognised specific phenotype but may still play a role in increasing susceptibility to AMD.

- APOE gene

Apolipoprotein E (ApoE) is a polymorphic protein that plays an important role in lipid metabolism in the central nervous system and in neurodegeneration. It exists in three forms, E2, E3 and E4 coded by three co-dominant alleles ε2, ε3 and ε4 each associated with different biochemical properties (Mahley, R. W.; 1988). The ε4 allele is associated with elevated cholesterol, increased risk of coronary heart disease (Davignon, J. *et al*; 1988) and the earlier onset of Alzheimer disease (van Duijn, C. M. *et al*; 1994). It was the lipid composition of drusen that prompted analysis of the Apo E gene.

Two studies have shown a protective effect of the ApoE ε4 allele in AMD. Souied et al (Souied, E. H. *et al*; 1998) demonstrated a lower frequency of ε4 allele in 116 unrelated patients with exudative AMD in one eye and soft drusen in the other compared to 168 age and sex matched controls ($p<0.002$). The risk for an individual with the ε4 allele to be affected was 4.8 times lower than that for an ε4 noncarrier. This finding was also found by Klaver et al (Klaver, C. C. *et al*; 1998a) who reported a reduced risk of AMD in association with the ε4 allele (OR 0.43; 95%CI 0.21-0.88). This group also confirmed an increased risk of AMD with the ε2 allele (OR 1.5; 95%CI 0.8-2.82) and showed ApoE immunoreactivity in drusen and basal laminar deposit.

One hypothesis that explains the protective effect of ApoE4 protein in contrast to E2 and E3 is that it lacks cysteine residues at certain positions preventing the formation of disulphide bridges and dimers. This would favour easier transport of smaller lipid particles and in turn could prevent the formation of soft drusen by allowing better elimination of lipids through Bruch's membrane (Souied, E. H. *et al*; 1998).

Although none of the candidate genes above have been implicated to have a major role in AMD as yet (Schultz, D. W. *et al*; 2003), it is felt that there is insufficient evidence at present to exclude them.

- Angiotensin Converting Enzyme (ACE) gene (Alu polymorphism)

The Alu insert, responsible for the polymorphism, occurs in intron 16 of the ACE gene (Rigat, B. *et al*; 1992). It is thought to regulate circulating levels of ACE in the bloodstream (Rigat, B. *et al*; 1990) and ACE itself has been localised to the retinal pigment epithelial cells (RPE), choroid and neural retina (Wagner, J. *et al*; 1996). Recent studies have shown that this Alu +/+ polymorphism has a protective effect for ARMD in that it occurred 4.5 times more frequently in an age-matched controls compared to those with atrophic AMD (Hamdi, H. K. *et al*; 2002) but there was no statistically significant difference in the frequency of the polymorphism between controls and those with neovascular AMD. Although ACE is an enzyme known to be involved in regulating blood pressure and electrolyte balance, one potential mechanism is that the renin-angiotensin system has been shown to regulate cell proliferation and cell death (Fernandez, L. A. *et al*; 1985). Interestingly, in likeness to the Apoe ε2 allele, the Alu polymorphism has also been shown to be a risk factor for Alzheimer disease (Kehoe, P. G. *et al*; 2003).

- Complement Factor H Polymorphism (CFH)

One of the most exciting new developments in AMD genetics has been the identification of polymorphisms within the complement factor H gene (on chromosome 1q32) which are thought to be strongly associated with AMD(Haines, J. L. *et al*; 2005). These polymorphisms have been proposed to explain up to 40-60% of AMD susceptibility (Haines, J. L. *et al*; 2005; Klein, R. J. *et al*; 2005). The most common variant which represents a tyrosine –histidine change at amino acid 402 significantly increases the risk of AMD with odds ratios between 2.45 and 5.57 (Haines, J. L. *et al*; 2005).

Several lines of evidence make the CFH polymorphism an attractive candidate for AMD. Firstly, the gene is located on chr 1q31 which has been repeatedly implicated in AMD(Weeks, D. E. *et al*; 2001). Secondly, CFH is a key regulator of the alternative complement system, protecting against infection and attacking diseased and dysplastic cells. Various components of the complement cascade have previously been identified within drusen(Hageman, G. S. *et al*; 2001). Thirdly, both age and smoking, two significant risk factors in AMD, influence plasma levels of complement factor H(Klein, R. J. *et al*; 2005). Additionally, drusen of a similar composition to that in AMD are found in the kidney disease, membranoproliferative glomerulonephritis type II, which is known to be caused by CFH deficiency(Mullins, R. F. *et al*; 2001).

1.4.3 Environmental risk factors

It is believed that environmental factors play an important role in AMD in that they may trigger the disease in genetically susceptible individuals. Studies of AMD in communities such as those from Japanese fishing villages who have migrated from rural to urban areas and adapted a more westernised lifestyle as well as others in Europe, Greenland and North America, would contribute greatly to our knowledge but these have yet to be carried out.

Various environmental factors identified as a result of the larger epidemiological studies are given below. The extent to which these factors are involved remains controversial.

1.4.3.1 Cardio-vascular factors

1.4.3.1.1 Smoking

There has been increasing interest in the link between smoking and AMD. Oxidative stress (Beatty, S. *et al*; 2000) and atherosclerosis (Vingerling, J. R. *et al*; 1995a) have been proposed as potential mechanisms to explain the causal association. It is known that tobacco smoke lowers plasma antioxidant levels (Sanders, T. A. *et al*; 1993),(Stryker, W. S. *et al*; 1988) and reduces macular pigment density (Beatty, S. *et al*; 2000) thus allowing more oxidative damage to occur at the retina.

A recent article in the BMJ has highlighted that more than a quarter of all cases of AMD with blindness or visual impairment in the UK are attributable to current or past exposure to smoking (Kelly, S. P. *et al*; 2004). Other studies have shown a three to four-fold increased age adjusted risk of age-related macular degeneration in current smokers compared to never smokers from a pooled analysis of 3 cross sectional studies from Australia, North America and Europe(Smith, W. *et al*; 2001).

It has also been demonstrated that the association is stronger between smoking and late AMD (CNV or GA) compared to early ARM (Drusen and pigment abnormalities) (Smith, W. *et al*; 1996),(Delcourt, C. *et al*; 1998). Some studies have reported that smoking is associated with only neovascular AMD (Klein, R. *et al*; 1993),(Vingerling, J. R. *et al*; 1995a), others with only atrophic AMD (Vinding, T. *et al*; 1992) and in a study of women by Seddon and colleagues, there was an association with both types of AMD (Seddon, J. M. *et al*; 1996). The Blue Mountain study however did not show an association between smoking and type of late AMD but this could be attributed to small numbers of patients in the study with CNV(n=50) and GA (n=22).

1.4.3.1.2 Hypertension

Hypertension is believed to damage choroidal vasculature. Although the Macular Photocoagulation Study Group did find a relationship between hypertension and an increased risk of AMD (MPS; 1997) none of the major population-based studies (Beaver Dam, Rotterdam, Blue Mountains or the POLA [Pathologies Oculaires Liees a l'Age] study) confirmed this (Smith, W. *et al*; 2001) (Delcourt, C. *et al*; 2001). It has been suggested that if a moderate increase in risk existed with hypertension, a large number of cases of disease would be required to detect this, and hence this may be the reason why certain studies have failed to find a statistically significant association (Evans, J. R.; 2001). It is unlikely however that hypertension has a strong direct effect on the development of AMD.

1.4.3.1.3 Atherosclerosis

The association of atherosclerosis and AMD was investigated by the Rotterdam study (Vingerling, J. R. *et al*; 1995a). Using carotid ultrasonography, people with plaques in the carotid bifurcation were at increased risk of ARMD (OR 4.5; 95%CI 1.9-10.7). A subsequent Finnish study looked at people with retinal arteriosclerosis (marked generalised narrowing of the arterioles) and found a higher prevalence of age-related macular degeneration but only 2 people in the age group studied did not have arteriosclerotic changes (Evans, J. R.; 2001).

1.4.3.2 Dietary factors

1.4.3.2.1 Dietary fat / cholesterol

The Beaver Dam study demonstrated that a high intake of saturated fat and cholesterol was associated with an increased risk of early rather than late ARM (Mares-Perlman, J. A. *et al*; 1995). Serum lipids and dietary fat have been hypothesised to increase the risk of AMD by promoting arteriosclerosis in the choroidal vasculature (Bird, A. C. *et al*; 1986). Alternatively, increases in serum fatty acids and triglycerides have been proposed to increase deposition of neutral fats in Bruch's membrane that would adversely affect the supply of nutrients and removal of waste products from the RPE (Mares-Perlman, J. A. *et al*; 1995). This deposition of fats is also thought to decrease hydraulic conductivity of Bruch's membrane resulting in the development of RPE detachments (Moore, D. J. *et al*; 1995).

In contrast to the Beaver Dam study, dietary data from the eye disease case control study (EDCCS) did not show any association with dietary cholesterol or saturated fats but rather an increase in risk for AMD with a higher intake of monounsaturated and polyunsaturated fats and linoleic acid (an omega 6 fatty acid). Omega 3 fatty acids and a higher frequency of fish intake with low levels of linoleic acid were however found to be protective (Seddon, J. M. *et al*; 2001).

1.4.3.2.2 Dietary antioxidant micronutrients

Dietary antioxidants are increasingly thought to have a protective role in reducing oxidative damage to the retina caused by incoming blue light and formation of oxygen free radicals. The retina is perceived as being particularly susceptible due to its high consumption of oxygen, its high proportion of polyunsaturated fatty acids and its exposure to visible light throughout life (Beatty, S. *et al*; 2000). It is thought that age related macular damage may occur in those patients with reduced amounts of antioxidant micronutrients in the serum or retina. High levels of macular pigments, primarily lutein and zeaxanthin, may also limit retinal damage (Beatty, S. *et al*; 2000).

Further evidence supporting this comes from observations by the EDCCS group. They found that those with higher serum levels of the carotenoids lutein, zeaxanthin and β-carotene, had a markedly reduced risk of neovascular AMD (OR =0.4; 95% CI 0.2-0.6)(EDCCS; 1993). Additionally, those with a higher dietary intake of carotenoids had a 43% lower risk for neovascular AMD compared to those with the lowest intake (odds ratio, 0.57; 95% confidence interval, 0.35 to 0.92; P for trend = .02) (Seddon, J. M. *et al*; 1994).

Since serum and retinal antioxidants are potentially modifiable risk factors, there has been much interest in the role of dietary supplements in reducing the risk of AMD (Beatty, S. *et al*; 1999).

The most promising supplementation clinical trial to date is the age-related eye disease study (AREDS 2001, report no. 8). This studied the effect of antioxidants (beta-carotene, Vit C, E) and zinc supplements on the progression of AMD and visual loss from AMD in 3640 participants aged between 55-80 years over 6.3 years. Patients were divided into 4 categories according to the severity of AMD and all cases recruited had a best corrected visual acuity of at least 20/32 in the study eye. Category 1 consisted of all those essentially free of AMD with no more than 5 small drusen in each eye (<63μm). Category 2 consisted of those with multiple small drusen or non-extensive intermediate drusen (63-124 μm) or pigment abnormalities. Category 3 participants required at least one large druse (125μm), extensive intermediate drusen or an area of extrafoveal geographic atrophy. Finally

Category 4 included those with advanced AMD (central geographic atrophy or choroidal neovascularisation) in one eye and no advanced AMD in the study eye. Interestingly eccentric GA was not included in the definition of advanced AMD.

All categories were then randomised to 4 treatment groups (A) antioxidants only (500mg vitamin C, 400 IU vitamin E and 15mg beta-carotene) (B) zinc (80mg as zinc oxide with copper to prevent anaemia) (C) antioxidants plus zinc (D) placebo. The results showed that participants in category 3& 4 did benefit, albeit modestly, from supplementation with antioxidants and zinc in that the risk of progressing to advanced AMD was reduced by 25% and the risk of visual loss (15 letters) was reduced by 19%. In both groups the combination of antioxidants plus zinc was more effective than each supplement alone. When analysed individually, the effect was however only statistically significant in category 4 and only in those with CNV in the affected eye. Why it did not protect in group 3 or in those with geographic atrophy in the first eye is not explained.

As a result of other studies during the course of the trial, beta- carotene was found to be associated with an increased risk of lung cancer in smokers. Treatment regimens were therefore altered and beta- carotene is now not recommended for smokers (AREDS; 2001a)

Criticisms of this study include the fact that since category 3 was the largest group, it was statistically more likely to show an effect if one existed. Additionally, there was evidence from the trial to suggest that antioxidants and zinc may be harmful in those with moderate geographic atrophy. Other concerns have been raised as to the dose of zinc which may have toxic side effects including a link with Alzheimer disease (Koh, J. Y.; 2001; Newsome, D. A. *et al*; 1988).

Further studies of Vitamin E supplements have failed to show a protective effect in AMD (Taylor, H. R. *et al*; 2002) and the results of various lutein supplement trials are awaited.

1.4.3.3 Other factors

Body Mass Index greater than 30 (Delcourt, C. *et al*; 2001), sunlight exposure (Cruickshanks, K. J. *et al*; 2001; Darzins, P. *et al*; 1997), blue iris colour (Mitchell, P. *et al*; 1998), early menopause (EDCCS; 1992);(Klein, M. L. *et al*; 1994; Vingerling, J. R. *et al*; 1995c) and raised fibrinogen levels (Smith, W. *et al*; 1998) have all been linked to an increased prevalence of AMD. Conflicting evidence however exists for most of these factors (EDCCS; 1992) making interpretation of their importance difficult. One of the difficulties, especially with measuring light exposure, is the accuracy of data obtained and the considerable recall bias amongst patients.

1.5 Phenotyping of AMD / classification

1.5.1 Why is phenotyping important?

There is evidence that certain attributes of the phenotype of ARM reflect the influence of the genes conferring risk (Piguet, B. *et al*; 1993). Certain phenotypes such as large soft drusen and multiple hard drusen show stronger genetic influences than phenotypes with other features such as pigmentary changes (Hammond, C. J. *et al*; 2002). To date, little attempt has been made to separate out distinct phenotypes in ARM or AMD and no association has been found with any specific candidate gene. It may be that subdivision according to phenotype will allow generation of purer samples of disease, which may in turn help to identify the genes contributing to the pathogenesis of the disease. It may be that once various phenotypic characteristics are segregated, certain genetic variants may be significantly more prevalent in the specific phenotypic groups compared to controls.

At present, it is not clear which features of ARM and AMD will show the strongest correlation to genetic risk. Any attempt to phenotype ARM and AMD will depend on a widely accepted classification system for grading of lesions. Such a grading system has been established by the International ARM Epidemiological Study Group (Bird, A. C. *et al*; 1995). In this study a revised version of the grading system is introduced and validated for use with digital images (see results section).

Another benefit of accurate phenotyping is that it allows the discarding those cases that do not have AMD. With careful assessment of drusen distribution, autofluorescence patterns and distribution of atrophy, conditions such as Doyne honeycomb dystrophy (see figure 3c [page 40]), pattern dystrophy (see figure 4g&h [page 41]), adult onset Stargardt disease and Maternally Inherited Diabetes and Deafness (MIDD) can be identified and excluded from the analyses. The latter is a relatively recently discovered mitochondrial disorder which is seen in 0.5% to 2.8% of patients with type II diabetes related to a point mutation at position 3243 of mitochondrial DNA (Guillausseau, P. J. *et al*; 2001). It is associated with younger onset of diabetes, neurosensory hearing loss with a normal or low body mass index and 46% of non insulin dependent patients progressed to insulin dependence after approximately 10 years. In the eyes, patients have been found to have patches of atrophy at the posterior pole sparing the fovea (see figure 4k&l [page 42]).

Lastly, once patients have been categorised phenotypically and once genetic variants have been identified, this will allow further investigation of various environmental factors which are thought to play a role in AMD aetiology.

1.5.2 The international classification and grading system (colour images)

In 1995 the International ARM epidemiological Study Group proposed a classification system for ARM and AMD to try to standardise the definitions and the grading of colour fundus photographs (Bird, A. C. *et al*; 1995). They defined the disorder as affecting the macular area (approx 5.5mm in diameter centred on the foveola) in those ≥ 50 years of age. The presence of soft drusen (≥63μm) and RPE pigmentary hyperpigmentation and/or RPE hypopigmentation constituted early ARM whereas the presence of geographic atrophy of the RPE or neovascular AMD (including RPE detachment, subretinal or sub RPE neovascular membranes, subretinal haemorrhages, fibrous scarring or exudate in the absence of other retinal vascular disorders) was defined as late ARM or AMD. Visual acuity was not required to make the diagnosis.

In order to perform the grading, a standard grid on a transparent background made up of three concentric circles of radii 500μm, 1500μm and 3000μm is used to define the central circle, the inner and outer macula. Four further radial lines subdivide the areas into subfields. The central circle is then centred on the fovea of the 30° or 35° stereoscopic colour fundus image. The number of drusen, drusen morphology (hard, soft, distinct and indistinct), drusen size, area covered by drusen, presence or absence of hypo and hyper pigmentation are then graded separately in each zone. Additionally, the presence, location and area covered by geographic atrophy (GA) (min 175μm) and the presence, location, and area covered by the typifying features of neovascular AMD (see earlier) are also graded separately. Wherever, neovascular AMD develops in an eye with previous GA this is reclassified as neovascular AMD and where atrophy develops around or replaces previous neovascular disease, the classification remains as neovascular AMD. All studies utilising the Wisconsin grading system (including the International classification) focus predominantly on the posterior pole (up to 6000μm diameter), largely disregarding the retinal periphery. Others studies (Lewis, H. *et al*; 1985) have previously demonstrated a correlation between peripheral reticular degeneration and AMD that can occur before the onset of macular changes. A further study however (Sunness, J. S. *et al*; 1985), did not show any difference in peripheral retinal function between eyes with AMD and the fellow eye with only drusen as assessed by electrophysiology. Static perimetry also showed sensitivity loss in the central 20 degrees, but normal thresholds peripheral to this. These results therefore suggest that retinal function abnormalities are confined to the central retina in AMD.

1.5.3 Digital Versus Film photography

Film-based fundus photography is currently acknowledged as the gold-standard in both clinical practice and research, but is often complicated with difficulties in archiving photographs and limited transfer of images amongst clinicians. With the advent of advanced digital technology, electronic storage, software manipulation and improvements in image quality (Lee, S. C. *et al*; 2001) digital imaging is fast becoming a realistic alternative.

A recent study investigated the feasibility of using digitised colour fundus transparencies for grading diabetic retinopathy and showed good agreement between the digitised images and 35 mm colour transparencies viewed as slides (George, L. D. *et al*; 1999). Regarding AMD, one recent study has semi-quantitatively compared standard fundus photographs to digitised images but the study population, was not restricted to ARM and AMD patients, the grading only included four categories (drusen characteristics), and kappa statistics were not performed (Lee, M. S. *et al*; 2000).

Potential disadvantages of digital photography include the loss of quality and resolution when images are compressed to allow for storage requirements and poorer stereopsis.

1.5.4 Autofluorescence (AF) imaging

As lipofuscin is now believed to play a central role in the pathogenesis of age-related macular degeneration (AMD), the technique of autofluorescence (AF) imaging using a confocal scanning laser ophthalmoscope (SLO) has become an invaluable tool. Observations by Delori and co-workers (Delori, F. C. *et al*; 1995a) provided evidence that autofluorescence is largely derived from lipofuscin in the RPE. He demonstrated that the spectral characteristics of the dominant fundus fluorophore and the retinal location between choriocapillaris and the photoreceptors correlated with RPE lipofuscin. Further evidence supporting the origin of autofluorescence arising posterior to the neurosensory retina comes from the increased autofluorescence seen in stage IV macular holes where there is a full thickness neurosensory retinal defect (von Ruckmann, A. *et al*; 1997a).

Von Rückmann and colleagues were the first to publish images of the spatial distribution of fundal autofluorescence in normal subjects and those with age-related macular degeneration and macular dystrophies (von Ruckmann, A. *et al*; 1995; von Ruckmann, A. *et al*; 1997a; von Ruckmann, A. *et al*; 1997b).

1.5.4.1 Normal subjects

The characteristics and the rate of accumulation of lipofuscin at different sites in the posterior pole and the changes with age have been documented in a population of normal subjects (Delori, F. C. *et al*; 2001; von Ruckmann, A. *et al*; 1997b).

Fundus AF intensity is highest at the posterior pole (between 5°and 15° from the fovea) and dips at the fovea and toward the periphery. The optic disc and retinal blood vessels are shown as dark structures and these features remain consistent regardless of age. This indicates that the image is not caused by reflected light as the disc is the most reflectant feature of the fundus (von Ruckmann, A. *et al*; 1997a). Delori and colleagues found the spatial distribution of fluorescence to be 40% lower in the fovea than at 7 degrees eccentricity and asymmetrically distributed around the fovea. The fluorescence was maximal at 11 degrees temporally, 7 degrees nasally, 13 degrees superiorly and 9 degrees inferiorly. This distribution of lipofuscin generally matches that of rods and reflects the pattern of age- related loss of rod photoreceptors (Delori, F. C. *et al*; 2001).

1.5.4.2 AF in age-related macular degeneration

The spatial distribution of AF of the various phenotypes in age-related macular degeneration described to date is given below.

1.5.4.2.1 Drusen

In patients with early age-related macular degeneration with non-crystalline drusen deposits at the level of Bruch's membrane (inner collagenous layer), the intensity of autofluorescence has been shown to be no greater than background levels when compared to age-matched controls. This was true for both hard and soft drusen (von Ruckmann, A. *et al*; 1997a). Lois subsequently described patterns of AF in eyes with drusen, where large foveal drusen corresponded to areas of increased AF, but drusen outside the foveal area did not show changes in AF (Lois, N. *et al*; 2002). A reticular pattern of AF can be seen outside the foveal area but does not always correspond to drusen. It is thought that these areas represent varying quantities of lipofuscin within the RPE.

Delori has looked at AF associated with drusen using a different imaging device (Delori, F. C. *et al*; 2000). In contrast he describes a distinct pattern associated with individual hard (>60 μm) and soft drusen with a centre of low autofluorescence surrounded by an annulus of high autofluorescence. A possible explanation for this appearance is the peripheral displacement of the RPE cells over the drusen causing central thinning of RPE and a peripheral increase in autofluorescence. This pattern however has not been reported with SLO autofluorescence imaging and is likely to be due to the different methodology used.

1.5.4.2.2 Hyperpigmentation

In eyes with focal hyperpigmentation or pigment figures at the level of the retinal pigment epithelium there was intense increased AF corresponding to these regions (von Ruckmann, A. *et al*; 1997a) (Solbach, U. *et al*; 1997). This could be explained by the presence of melanolipofuscin (von Ruckmann, A. *et al*; 1997a) or possibly an increased metabolic activity of the RPE in areas with hyperpigmentation (Solbach, U. *et al*; 1997).

1.5.4.2.3 Geographic atrophy

In geographic atrophy, there is consistently decreased autofluorescence corresponding to the area of atrophy and photoreceptor loss (von Ruckmann, A. *et al*; 1997a). Various patterns in the junctional zone of geographic atrophy have been described (Holz, F. G. *et al*; 1999a). These include a continuous band of increased autofluorescence surrounding the atrophy, a diffusely increased autofluorescence at the entire posterior pole and finally small focal spots of increased autofluorescence at the junctional zone. These differing patterns in geographic atrophy may represent varying reactive changes in the surrounding RPE cells and are thought to precede cell death (Holz, F. G. *et al*; 1999a). Follow up of these patients with areas of increased autofluorescence surrounding geographic atrophy has shown the development of new atrophic patches within these regions within a year. These observations strengthen the theory that excessive RPE lipofuscin accumulation is involved in the pathogenesis of geographic atrophy associated with age-related macular degeneration.

1.5.4.2.4 Choroidal neovascularisation

In those eyes with choroidal neovascularisation, the autofluorescence has been described as irregular, with regions of increased and decreased autofluorescence compared to background levels. The intensity is less than background over disciform scars (von Ruckmann, A. *et al*; 1997b). Increased AF around the edge of the lesion has also been described often corresponding to areas of irregular pigmentation (von Ruckmann, A. *et al*; 1997a)

1.5.4.2.5 Pigment epithelial detachments

Retinal pigment epithelial detachments older than 6 months showed a mild, diffusely increased autofluorescence corresponding exactly with the detached area and this persisted for 2 months after the detachment had flattened.

1.6 Natural history and risk of visual loss with ARM

1.6.1 Drusen

The presence of drusen is the ophthalmoscopic hall-mark of age-related maculopathy (ARM). Various studies have shown that increased drusen number (Holz, F. G. *et al*; 1994b), larger size (Gregor, Z. *et al*; 1977; MPS; 1997)(MPS), increased confluence (Klein, R. *et al*; 1997; Smiddy, W. E. *et al*; 1984)and decreased fluorescence on fluorescein angiography (Pauleikhoff, D. *et al*; 1990a)are important factors in conferring risk for visual loss and AMD (Strahlman, E. R. *et al*; 1983) (Bressler, S. B. *et al*; 1990; Gass, J. D.; 1973; Gregor, Z. *et al*; 1977). In particular, the macular photocoagulation study (MPS; 1997) identified that the presence of 5 or more drusen (RR=2.1;95%CI 1.3-3.5) and 1 or more large drusen (RR=1.5; 95%CI 1.0-2.2) were 2 important risk factors for the development of CNV in the fellow eye of those with CNV in the first eye. Pauleikhoff also demonstrated that fellow eyes of those with PED's were more likely to have larger, more densely packed and less fluorescent drusen (i.e. more hydrophobic) than fellow eyes of CNV (Pauleikhoff,

D. *et al*; 1990a). This is consistent with the proposed pathogenesis of PED's and the neutral lipid theory (Bird, A. C. *et al*; 1986; Pauleikhoff, D. *et al*; 2002; Sheraidah, G. *et al*; 1993).

The Beaver Dam study (Klein, R. *et al*; 1997) and the Chesapeake Bay Waterman Study (Bressler, N. M. *et al*; 1989) demonstrated that small hard drusen are common and not necessarily correlated with a risk of advanced AMD. This was also confirmed by the results of the AREDS study (AREDS; 2001a) where patients in category 1 (i.e. no more than 5 small drusen (<63μm) in each eye) had a very low risk of progression to advanced AMD over 5 years (0.005%). Those in category 2 (extensive small drusen or non-extensive intermediate drusen) had a 1.3% 5yr risk of progression and those in category 3 ,with extensive intermediate sized drusen or at least one large druse (125μm) had a significant risk of progression of 27%. Category 4, although they had the highest risk of progression were not classified according to drusen size.

Smiddy and colleagues (Smiddy, W. E. *et al*; 1984) identified drusen associated with focal hyperpigmentation and more than a minimal degree of confluence as significant risk factors for exudative AMD in those with bilateral drusen but interestingly drusen size was not considered important. In Holz's prospective study of 126 patients with bilateral drusen (Holz, F. G. *et al*; 1994b) again drusen size did not appear to increase the risk of AMD but drusen number was found to be important as none of the eyes with less than 10 central drusen developed AMD.

The more diffuse linear deposits, that cause thickening of Bruch's membrane, do not appear to have a clinical correlate but they do produce changes on fluorescein angiography and indocyanine green (ICG) angiography causing slowing of choroidal perfusion and may be an important factor in the development of geographic atrophy (Pauleikhoff, D. *et al*; 1999). Further study of these patients using the Humphrey automated perimeter and fine matrix mapping revealed discrete areas of scotopic threshold elevation implying functional loss in the regions with choroidal perfusion abnormality (Chen, J. C. *et al*; 1992)consistent with the proposal that continuous thickening of Bruch's membrane may act as a barrier to diffusion between the RPE and choriocapillaris (Pauleikhoff, D. *et al*; 1990b).

1.6.2 RPE pigmentation

Focal RPE hyperpigmentation has been shown to be an important risk factor. One study (Smiddy, W. E. *et al*; 1984) found an increased risk of 23 times of developing exudative maculopathy with focal pigmentation associated with drusen (95%CI ;4-259) The MPS, Beaver Dam and Rotterdam study also found focal RPE hyperpigmentation to be a major risk factor for developing CNV(MPS; 1997);(Klein, R. *et al*; 1997) (Klaver, C. C. *et al*; 2001).

Gregor et al (Gregor, Z. *et al*; 1977) however found no association between pigment clumping, with or without drusen and the development of AMD in his study of 104 patients.

1.6.3 Natural history of pigment epithelial detachments

Historically pigment epithelial detachments (PED's) have been reported to have a good prognosis. In 1979, the natural history of 50 eyes with avascular serous PED's was reported on (Meredith, T. A. *et al*; 1979). Subretinal new vessels did not develop in any patient under the age of 56 years or if the lesion was less than 1 disc diameter in size. The risk of visual loss increased with age with 9.1% of those less than 55 yrs losing greater than 1 line of Snellen which increased to 51.3 % in those older than 55. Patients were also more likely to develop vascular complications if the lesion was subfoveal (39.4 % v 16.7%). Unfortunately the study involved limited numbers and follow up. A further study by Elman and colleagues (Elman, M. J. *et al*; 1986) described the natural history of PED in 110 patients. Thirty-two percent developed a choroidal neovascular complex within an average of 20 months and this was associated with an acuity of 6/60 or worse. Risk factors for CNV development and poor visual acuity were (1) sensory retinal detachment (2) increased size of the PED (3) a hot spot on fluorescein angiography (4) late or irregular filling and (5) notching. Thirty-nine percent of eyes retained an acuity of 6/9 or better.

In a further study of 101 patients with different types of PED (Pauleikhoff, D. *et al*; 2002) namely polypoidal choroidal vasculopathy (PCV), vascular PED and avascular PED, all

types were found to have a similar clinical course with respect to visual loss, enlargement and regression. The mean final visual acuity was 1/18 in avascular PED, 1/24 in vascular PED and 1/30 in the PCV group, but these were not statistically different. The size of the lesions however, did show statistically significant differences, with avascular lesions being the smallest, vascular PED's being intermediate and PCV associated with the largest PED's. Interestingly the avascular PED's subsequently developed geographic atrophy whereas the other two groups more frequently resulted in RPE tears and disciform scaring.

A further group, which are often referred to as 'drusenoid' PED or 'serogranular PED'(see figures 11 d&e [page 133]) represents an area of approximately ½ disc diameter of confluent soft drusen under the centre of the macula. These patients are at risk of developing GA or CNV. In a study by Roquet of 31 patients(Roquet, W. *et al*; 2004), 50% were shown to develop GA after 7 years and those with a lesion larger than 2DD or with metamorphopsia as a symptom were at an increased risk of progressing to atrophy or CNV after 2 years ($p<0.01$). Over 10 years, 75% progressed to atrophy and 25% to CNV with a poor visual prognosis.

1.6.3.1 RPE rips

Schoeppner et al reported a high incidence of visual loss (37%, 59% & 80% in the 1st, 2nd & 3rd yrs respectively) in the fellow eye of those patients with RPE tears in the first eye (Schoeppner, G. *et al*; 1989). The causes of visual loss included retinal pigment epithelial detachments with choroidal neovascularisation and RPE tears. If the characteristics of drusen truly determine the subsequent lesion that develops, identifying eyes at high risk of developing PED's may allow prophylactic measures to prevent their development and subsequent visual loss. Pauleikhoff also noted that RPE tears were only seen in larger volume PED's (Pauleikhoff, D. *et al*; 2002) where a further increase in volume could cause sufficient tangential stress to rupture the RPE. If the size of PED's could in some way be limited, this complication may be avoided with its inherent loss of vision.

1.6.4 Natural history of geographic atrophy

It was Sarks who described the clinic-pathological correlation of RPE atrophy and then subsequently described its evolution and rate of progression (Sarks, J. P. *et al*; 1988; Sarks, S. H.; 1976).The process commonly occurs in eyes with drusen and pigmentary alterations although it may also follow flattening of RPE detachments (Bird, A. C. *et al*; 1986; Elman, M. J. *et al*; 1986; Meredith, T. A. *et al*; 1979). The progression has been described to occur in three phases (Maguire, P. *et al*; 1986). In the initial phase, eyes show focal discrete areas of RPE atrophy in the parafoveal area, mostly skirting fixation producing a horse-shoe-shaped lesion with no thinning of the RPE. This stage is usually associated with numerous drusen and retention of good visual acuity (Sarks, J. P. *et al*; 1988) (Maguire, P. *et al*; 1986). Following this, the second phase occurs in which there is foveal involvement, a precipitous loss in acuity often down to count fingers, coarse foveal granularity, thinning of the RPE and a regression of macular drusen (Schatz, H. *et al*; 1989). Histologically, regressing drusen are associated with macrophages and material not removed becomes crystalline which can be seen clinically (Sarks, J. P. *et al*; 1988). The final phase results in multifocal areas of atrophy becoming confluent to produce circular areas of atrophy involving the entire central macula.

Sarks showed that the size of the atrophy and the percentage of foveal involvement increased annually by more than 100% at first, slowing rapidly once the GA had involved the previous areas of atrophy (Sarks 1988). It was also observed that significant visual loss occurred at a rate of 8% per year and that the atrophy tended to expand faster in those under 75 compared to patients over 75 (Schatz, H. *et al*; 1989).

Sunness et al also reported a significant decline in visual acuity over time as areas of GA continued to enlarge, even when already large at baseline (Sunness, J. S. *et al*; 1999a). Those with VA better than 20/50 had the highest rate of acuity loss. 27% of these eyes had acuities of 20/200 or worse at 4 years. Maguire and Vine also reported that the average interval from onset to legal blindness was nine years (Maguire, P. *et al*; 1986). The development of CNV in those eyes with GA is another reason for further loss in vision. Sunness reported rates of CNV development of 2% by 2 years in those patients with

bilateral GA and no CNV at baseline and 11% by 4 years (Sunness, J. S.; 1999). In those with GA in the study eye and CNV in the fellow eye at baseline, these figures rose to 18% by 2 years and 34% by 4 years. CNV did not occur at sites within the atrophy, but rather at the peripheral border or in the spared foveal region. The size of central atrophy also appeared to be important whereby an area of central atrophy less than 2 disc areas was associated with an increased risk of developing CNV. Sarks also found that the risk of CNV declined once the GA had involved all areas of incipient atrophy (Sarks, J. P. *et al*; 1988).

Improvement of visual acuity over time in patients with geographic atrophy has also been reported (Sunness, J. S. *et al*; 2000). Six out of 36 patients with bilateral GA experienced a two or more line improvement with better fixation in the worse seeing eye, which occurred at the same time as vision deteriorated in the better seeing eye. None of the patients however noted the change themselves. Unlike CNV, where the lesion can involute causing improvements in VA (Singerman, L. J. *et al*; 1985), regression does not occur in GA. Any improvement in vision therefore is presumably due to eccentric fixation to an area of surviving retina (Sunness, J. S. *et al*; 2000).

1.6.5 Natural history of CNV.

CNV secondary to AMD is usually associated with profound visual loss especially when new vessel formation results in serous and/or haemorrhagic detachment of the RPE and the formation of a disciform scar. This term 'disciform degeneration of the macula' was initially coined by Oeller in 1905 (Gass, J. D.; 1967) and was subsequently used to describe disc shaped lesions occurring in the macular region of elderly individuals. Today we use the term to indicate the formation of a scar following exudation and haemorrhage at the macula.

Clinically CNV is classified according to the position of the lesion in relation to the fovea as seen during the transit phase of the fluorescein angiography (FFA). Lesions are described as **extrafoveal** when the entire complex is located 200μm or farther from the

centre of the foveal avascular zone (FAZ) and probably account for no more than one quarter of those cases that threaten central vision (Bressler, N. M. *et al*; 1987). **Juxtafoveal** complexes are required to have their posterior edge or associated blood/blocked fluorescence between 1 and 199μm from the centre of the FAZ and **subfoveal** lesions have evidence of neovascularisation under the centre of the FAZ (Chamberlin, J. A. *et al*; 1989).

1.6.5.1 Classic and occult CNV

Subfoveal CNV is then further classified as either 'classic' or 'occult' according to MPS guidelines again as defined by the appearances on FFA (MPS; 1991).

Classic CNV is characterised by an area of choroidal hyperfluorescence with well demarcated boundaries that can be discerned in the early phase of the angiogram. In later phases, progressive pooling of dye leakage occurs in the overlying subsensory retinal space and usually obscures the boundaries of the CNV.

Occult CNV however is represented by two patterns of hyperfluorescence. Stereoscopic angiography is advised to aid in the diagnosis. The two forms are known as 'fibrovascular pigment epithelial detachment (PED)' and 'late-phase leakage of an undetermined source'. The former describes an area of irregular elevation of the RPE which are neither discrete nor as bright as classic CNV in the transit phase. They then develop an area of stippled hyperfluorescence within 1-2 minutes after the injection and by 10 minutes there is persistent staining or leakage within a sensory retinal detachment. These PED's should not be confused with the typical serous detachments of the RPE also seen in AMD. The second type of occult lesion often appears as speckled hyperfluorescence with leakage and pooling of dye in the overlying subsensory retinal space occurring at between 2 and 5 minutes after dye injection. The source of this leakage cannot be discerned in the earlier phases of the FFA and the boundaries of the lesion are never well demarcated (MPS; 1991).

Gass initially suggested that choroidal neovascularisation in AMD grew primarily under the RPE, hence explaining the poor prognosis following surgery with damage to the RPE

(Gass, J. D.; 1994). In a subsequent clinicopathological study by Lafaut and colleagues, an attempt was made to correlate histology with classic and occult complexes. Although the classic CNV's had a major subretinal fibrovascular component and the occult membranes predominantly contained a fibrovascular component beneath the RPE, 10/18 classic membranes also contained a sub RPE component and 2/10 occult membranes contained a subretinal component making it difficult to make any definite correlations. Fibrin was also noted to occur at the lateral edges of classic membranes and to occur on the inner surface of occult membranes (Lafaut, B. A. *et al*; 2000).

The above definitions have become increasingly important since the advent of Photodynamic therapy (PDT) but unfortunately they do not have any direct histological correlation. Additionally, since most CNV's associated with AMD have been described as occult and subfoveal (Bressler, N. M. *et al*; 1987), treatment options are severely limited at present.

Natural history studies have shown that visual improvement can occur in those with CNV. Singerman (Singerman, L. J. *et al*; 1985)showed that 4 out of 20 patients with macular disciform degeneration in the first eye, spontaneously improved their acuity by 5 lines or more when the second eye underwent a decrease in vision. In a further study of those with occult CNV however, the outcome was more variable. 32% cases more than doubled their original size and median visual acuity loss was 2.5 lines after 9-12 months of follow up. Classic CNV was said to develop in 52% of those with occult CNVand in those with a classic component at baseline, more subretinal fibrosis developed causing further visual loss (Stevens, T. S. *et al*; 1997).

1.6.5.2 Risk to second eye

Studies have shown that AMD usually affects both eyes over time (Gass 1973, Gregor 1977). The Framingham eye study showed that the proportion of cases that were unilateral decreased with age from 90% (15/16) in those less than 65 to only 40% (34/78) in those older than 75. In patients with a unilateral advanced form of the disease it is of interest to

be able to estimate the risk to the second eye. This risk has been calculated by several groups including Gregor et al (Gregor, Z. *et al*; 1977) who reported a 12-15% annual risk of developing a disciform lesion in the second eye of 104 patients with a unilateral disciform in the first eye. This agrees with the incidence of 12% found by Teeters and Bird (Teeters, V. W. *et al*; 1973). A further study (Bressler, S. B. *et al*; 1982) found a cumulative risk of 29% of developing a sub-retinal CNV in a fellow eye with macular drusen over 3 years. Strahlman (Strahlman, E. R. *et al*; 1983) and Holz (Holz, F. G. *et al*; 1994b) reported much lower risks to the second eye of between 3 to 10% per year. These studies however had various follow up times and differences in the severity of macular changes which could account for the diversity of risk.

The Rotterdam study provided information on incidence of AMD in the contralateral eye of subjects already affected by unilateral AMD (170.6 per 1000 persons per year). The most predictive stage for development of AMD was ARM stage 3, which comprises the presence of either soft, indistinct drusen or soft drusen with pigmentary irregularities (Klaver 2001 IOVS). The most important predictors of progression were more than 10% of macular area covered by drusen, presence of depigmentation, and presence of hyperpigmentation.

The AREDS study found the risk of progression to advanced AMD in those with central GA or CNV in the first eye (category 4) to be 43% over 5 years. Those with non-central GA had a risk of 27% over 5yrs (category 3). Both groups were found to benefit from vitamin supplements.

1.6.6 Symmetry between eyes

Marked symmetry of drusen between eyes has been demonstrated by various groups (Gass 1973, Coffey AJH 1986, Lewis H 1986, Barondes 1990). Barondes showed close concordance (kappa 0.7 -0.9) with respect to drusen size, number, density and fluorescence with fluorescein angiography which was greater than would be expected by chance alone. This led to the conclusion that drusen may result from metabolic malfunction specific to the

patient rather than the non specific result of ageing. In addition it is likely that both eyes have similar risk of visual loss at least in terms of drusen characteristics.

Geographic atrophy has been quoted to be bilateral in 48 –65 % of cases (Sarks, J. P. *et al*; 1988), (Green, W. R. *et al*; 1985), (Bellmann, C. *et al*; 2002), (Wang et al BJO1998) and it was Sunness who described the high correlation in size and progression of GA between eyes. More recently, Bellman and colleagues have used autofluorescence imaging to record the symmetry of lesions in patients with geographic atrophy. Autofluorescence imaging allows an accurate assessment of the area of RPE loss. Seventy-two patients were assessed for number of atrophic areas, size of atrophy and the size of the convex hull around all the lesions within an eye. There was no statistically significant difference when these variables were compared between eyes. These findings are in accordance with the view that AMD is not merely the result of a non-specific process and implies that both eyes are under similar genetic and/or environmental influences.

A further study of symmetry of disciform scars in 81 patients observed a significant correlation between eyes in terms of final scar size ($r=0.5$, $p<0.01$) (Lavin, M. J. *et al*; 1991). It was suggested that the similarity in scar sizes between eyes may reflect the effect of some underlying inherited or systemic factor. In a study looking at subtypes of neovascular complexes, Chang et al concluded that 48/55(87%) patients with a vascularised PED occult lesion in the first eye developed a similar type of occult lesion in the second as did 49/58(84%) of the group with occult CNV without a serous PED (Chang, B. *et al*; 1995).

Concerning patients with Pigment epithelial detachments, Chuang and Bird reviewed the prevalence of bilateral tears. They found that 10/22 patients with loss of vision in their second eye as a result of an RPE tear had a similar lesion in the first eye (Chuang, E. L. *et al*; 1988). Schoeppner et al (Schoeppner, G. *et al*; 1989) also reported a high incidence of visual loss (80% after 3 years) in the fellow eye of 43 patients with RPE tears in the first eye. Visual loss in the fellow eye was most frequently precipitated by pigment epithelial detachment, which occurred in 23 patients and 15 of these developed a tear.

Regarding people with bilateral involvement of AMD, the Beaver Dam Study identified 34/4926 individuals. 15% had pure GA in both eyes, 56% had exudative macular degeneration in both eyes and 29% had GA in one eye and exudative changes in the fellow eye (i.e. a discordant phenotype). In the Blue Mountain Eye Study 80% of the 230 gradable cases of AMD or early ARM were found to be bilateral, 57% for AMD and 77% for early ARM. GA was bilateral in 56%, neovascular AMD in 40% and distinct soft drusen in 47%, with hyperpigmentation and hypopigmentation being bilateral in only 38% and 28% of cases respectively (Wang, J. J. *et al*; 1998).

Unfortunately, as in the Beaver Dam and Blue Mountain Study, many population studies have small numbers with late stage maculopathy making it difficult to assess overall symmetry between eyes in late stage disease. Larger cohorts of patients with AMD in both eyes will allow a more accurate assessment of symmetry.

1.7 Current treatments in AMD

1.7.1 Laser photocoagulation Therapy

Until relatively recently, laser photocoagulation therapy was the only effective treatment available for CNV. Between 1979 and 1994, the Macular Photocoagulation Study Group conducted a number of clinical trials on patients with neovascular lesions in one or both eyes(MPS; 1986; MPS; 1990; MPS; 1991). CNV lesions were classified into those that were extrafoveal (200 μm or more from the centre of the macula), juxtafoveal (more than 1μm but less than 200μm from the foveal centre) and sub-foveal (lesion under the foveal centre). The study group set out indications and guidelines for Argon and Krypton laser treatment of the various neovascular lesions and defined the terms 'classic' and 'occult lesions'(MPS; 1991). Although still used in predominantly extrafoveal lesions there are limitations of this treatment. Firstly, only 10-15% of all neovascular lesions are considered

small enough and sufficiently delineated by fluorescein angiography to be eligible for treatment. Secondly, even if the laser treatment is successful – that is, the new blood vessels appear closed on fluorescein angiography, at least 50% have a recurrence of leakage within 2 years. A further problem is that in the treatment of subfoveal lesions, laser causes an immediate reduction in central vision, even though in the long term these patients suffer less visual loss(MPS; 1991). These problems have sparked the need to find alternative, less destructive treatments for AMD.

1.7.2 Photodynamic Therapy

Photodynamic therapy has now been introduced as the mainstay of treatment for subfoveal, predominantly classic neovascular lesions following the treatment of AMD with photodynamic therapy (TAP) and verteporfin in photodynamic therapy (VIP) studies (Barbazetto, I. *et al*; 2003; TAP study Group; 1999; VIP Study Group; 2001; VIP Study Group; 2002). It involves the intravenous administration of a photosensitiser dye called verteporfin and then laser irradiation of the macular region. The destruction of the CNV lesion is confined to the new, abnormal blood vessels since it is a nonthermal process leading to the localised production of reactive oxygen free radicals which mediate cellular, vascular and immunological injury. Typically the leaking vessels remain closed for several weeks but often multiple treatments are necessary. The trials showed that treatment delayed or prevented loss of vision after two years of follow up in patients with predominantly classic lesions. Some of the limitations of this treatment however are the limited number of patients with predominantly classic lesions, the high cost of the photosensitiser dye and the need for recurrent treatments after 3 month intervals.

1.7.3 Transpupillary Thermotherapy

Transpupillary thermotherapy (TTT) is a laser photocoagulation treatment characterised by large spot, long-pulse and low-irradiance infrared diode (810nm) laser exposure. It has been used successfully to treat occult subfoveal choroidal neovascularisation (CNV). The

treatment is administered as a subthreshold photocoagulation with no visible endpoint and no ophthalmoscopically apparent chorioretinal change. This may make it difficult to assess whether adequate treatment has been given. It has however been observed to result in closure of CNV membranes and resorption of intraretinal and subretinal fluid with little or no damage to the overlying retina (Reichel, E. *et al*; 1999). The mechanism of action, although currently unknown, is thought to involve the alteration of choroidal blood flow(Ciulla, T. A. *et al*; 2001).

1.7.4 Anti VEGF Treatments

Since VEGF has been shown to be an important cytokine stimulus for the growth of new vessels in the eye in both AMD and diabetic retinopathy, anti-VEGF therapy is thus a potential treatment for neovascular AMD. Two anti-VEGF agents are now clinically available for the treatment of neovascular AMD: pegaptanib sodium (macugen), which is an oligonucleotide aptamer which has an ability to bind and inactivate VEGF and bevacizumab (Avastin), which is an antibody for metastatic colorectal cancer given systemically(Michels, S. *et al*; 2005). Unlike bevacizumab, which binds all VEGF isoforms, pegaptanib targets only VEGF165, the isoform responsible for pathological ocular neovascularization and thus an ideal target for treatment of AMD(Ng, E. W. *et al*; 2005). The aptamer (pegaptanib sodium) has also been shown to significantly reduce vascular permeability in animals and in human studies, 3 months after intravitreal administration, 80% of eyes showed stabilised or improved vision with 27% showing an increase in threee or more lines on the ETDRS chart(Eyetech Study Group; 2002). While these agents offer promising therapies, there is potential for local and systemic adverse sequelae since VEGF serves numerous essential functions in extraocular tissues(van Wijngaarden, P. *et al*; 2005).

1.7.5 Anecortave Acetate

Anecortave acetate is an angiostatic synthetic derivative of cortisol that is being currently evaluated for the treatment of exudative age-related macular degeneration (ARMD). It is delivered as a posterior juxtascleral depot injection (15mg) and has been shown to stabilise

vision compared to placebo and inhibit neovascular lesion growth when given at 6 monthly intervals(Schmidt-Erfurth, U. *et al*; 2005). Reported side-effects have included cataract formation, decreased visual acuity, ptosis, ocular pain and subconjunctival hemorrhage(Schmidt-Erfurth, U. *et al*; 2005) and there is also a risk of raised intraocular pressure.

1.7.6 Retinal Translocation

The development of increasingly refined vitreoretinal surgical techniques has resulted in a variety of surgical procedures for age-related macular degeneration (AMD). These have included submacular surgery with removal of choroidal neovascular membranes and subretinal blood and macular translocation surgery(Conti, S. M. *et al*; 2005). The latter involves translocating the macula with respect to the retinal pigment epithelium and choroid and performing a 360 degree retinectomy. Problems with this treatment include the need for concurrent squint surgery to reposition the fovea with the risk of anterior segment ischaemia and the risk of recurrence of subfoveal RPE atrophy following the procedure(Cahill, M. T. *et al*; 2005).

To date most of the treatments available have had minimal impact in reducing blindness from AMD. It may be that in the future, combination treatments will need to be given to provide the best chances of success in reducing visual loss.

2 Aims

The aims of this descriptive study were:

1) To collect a large cohort of ARM and AMD patients and carry out a more detailed phenotype analysis than has previously been performed. Techniques for phenotyping are to include colour photography, autofluorescence imaging and fluorescein angiography where clinically indicated.
2) To validate grading techniques used for the assessment of 35mm colour transparencies and digital colour images for phenotyping of ARM and AMD.
3) To investigate whether phenotype analysis of patients with ARM and AMD using colour fundus photography, autofluorescence imaging and fluorescein angiography, where available, can increase our knowledge base regarding:
    - The concordance and symmetry between eyes.
    - The fellow eye characteristics in those with unilateral visual loss and whether they relate to the type of AMD in the affected eye.
    - The regional susceptibility of the macula to geographic atrophy and its pattern of progression.
    - The integrity of the retinal pigment epithelium (RPE) in patients with choroidal neovascularisation at different stages of development.
    - The regional variation in photoreceptor function in differing lesions in AMD
4) To investigate the relationship between smoking and the different phenotypes of AMD.

3 Clinical Methods

3.1 Patient / Spouse recruitment

Patients and spouses (acting as a comparison group) presenting to medical retina clinics at Moorfields Eye Hospital & King's College Hospital were invited to enter the study. Ninety percent of patients were recruited by myself. The remaining 10% were recruited with the help of Tunde Peto (Reading Centre co-ordinator) Sharon Jenkins (genetic study co-ordinator) and Hendrik Scholl (visiting retinal fellow).

3.1.1 Inclusion criteria:

- Patients over the age of 50 with West European family-origin with early or late age-related macular degeneration in one or both eyes.
- Spouses of patients, over the age of 50, if they have no signs of age-related macular disease on biomicroscopy examination (Comparison group).
- Patients and spouses willing to take part in the study after receiving a full explanation of the nature of the study.

3.1.2 Exclusion criteria:

- Patients and spouses not wishing to participate.
- Eyes with opaque media that did not allow gradeable colour photos.
- Presence of retinal disease or other cause of visual loss other than due to age-related macular degeneration.

Informed consent was obtained in all patients and spouses entering the study after an explanation of the purpose and requirements of the study. This study was conducted in accordance with the tenets of the Declaration of Helsinki, and with the approval of the institutional ethical committee (see appendix 3).

Each participant was asked about a family history of retinal disease, past or current smoking, number of cigarettes smoked per day and for how long. Visual acuity was tested using the Snellen chart at 6 metres rather than logMar acuity since the phenotyping of AMD according to the International Classification does not include visual acuity in the definition and it was felt that Snellen acuity would be quicker to assess since these were available in all patient cubicles and could be converted to logMar if needed (see chart below). Additionally, visions could also be compared to previous visual acuities which would also have been measured with the Snellen chart. A full dilated ophthalmological examination was also carried out.

LogMar is an acronym for Log_{10} of the Minimum Angle of Resolution (MAR). The MAR is taken as the stroke width of the letters, which is $1/5^{th}$ of the vertical angle it subtends. Thus a 6/6 letter which subtends 5 minutes of arc, equates to a MAR of 1 minute and a logMar of 0 ($\text{Log}_{10}(1)=0$)(Thompson, D; 2005). One disadvantage of the logMar notation is that for letter sizes smaller than 6/6, the logMar score is negative.

Tables have therefore been calculated to allow Snellen acuity to be converted to logMar acuity. An example is shown below (Edited from (Thompson, D; 2005)).

Snellen Acuity	LogMar
6/4	-0.2
6/6	0
6/9	+0.2
6/12	+0.3
6/18	+0.5
6/24	+0.6
6/36	+0.8
6/60	+1.0

3.2 Data Collection

3.2.1 Microsoft access database

All data were entered into an access database designed for the purpose of the study (see figure 5 for entry data sheet).

For the purposes of this MD by July 2003, data from 1300 patients had been collected. The individual studies however were carried out at different time periods and therefore the total number of patients differs between studies.

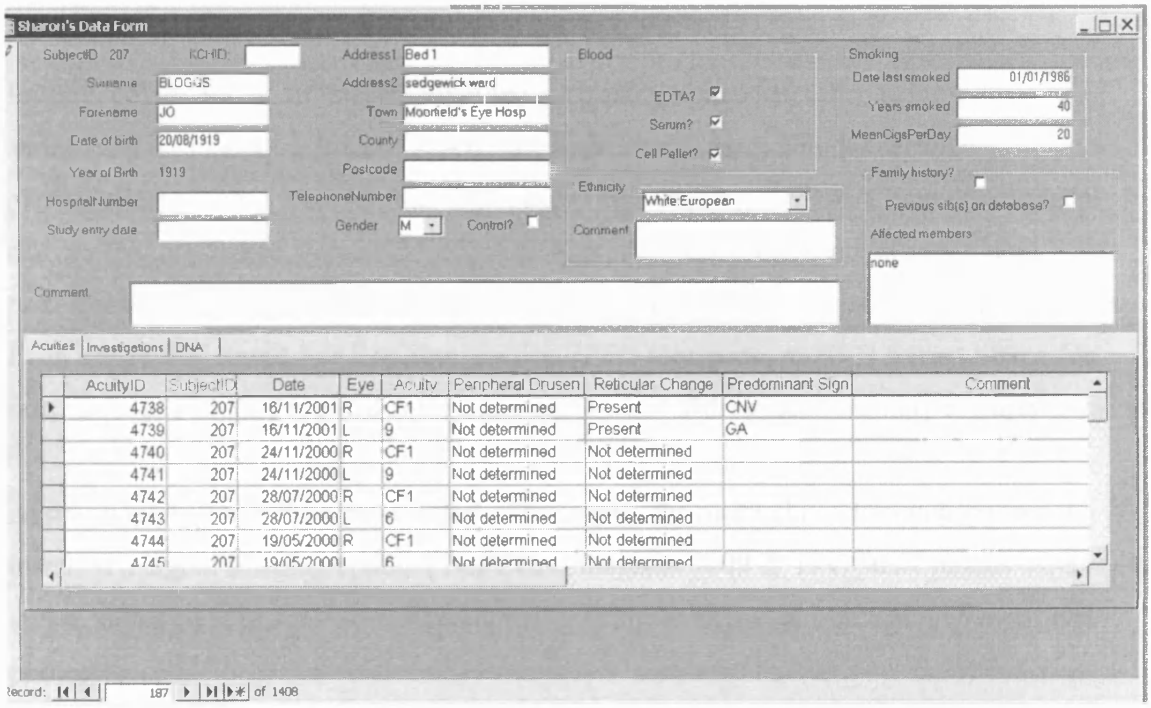


Figure 5: Microsoft Access data entry sheet

3.2.2 Queries

Once all data were entered into the access database, 'select queries' were devised to analyse and sort the data. Specifically patients with different phenotypes could be subdivided, i.e. those with GA in 1 or both eyes, those with CNV in 1 or both eyes and those with a discordant phenotype (those with GA in 1 eye and CNV or PED in the other eye). Cross tab

and select queries were also used to subdivide patients into those with unilateral and bilateral visual loss based on visual acuities at recruitment.

3.3 Imaging:

3.3.1 Colour photography:

Digital imaging has become progressively more sophisticated over previous years and, with the advantages of ease of electronic storage, image transfer, digital analysis and software manipulation, this has become the imaging technique of choice.

Initially colour 30° & 50° stereoscopic fundus photographs were taken using a Zeiss FF-series fundus camera on 35mm Ektachrome slide transparency film by certified photographers. These were all then digitised by myself using a Nikon Coolpix 990 digital camera (Nikon, Tokyo, Japan), 35mm slide film adapter and commercially available light box. The settings were chosen to optimise image quality at a resolution of 1024x768 pixels. Each image was stored as a low-compression JPEG (compression (8:1) format with a file size of about 170 KB) to allow reasonable quality but with reduced memory requirements.

Approximately 4 months after the start of the study, the medical illustration department installed a digital imaging system (TOPCON Imagenet 2000 © TRC 501X fundus camera) and so subsequent images were taken digitally using standard settings again by certified photographers.

3.3.2 Autofluorescence imaging:

Autofluorescence images were taken of all eyes by myself (providing there was no significant lens opacity) using either:

(1) A prototype confocal scanning laser ophthalmoscope (cSLO) donated by Zeiss (Zeiss, Jena, Germany). This takes a 40° field of view using a confocal aperture. The confocal detection unit employs a pinhole aperture (400μm) to suppress light originating from outside the focal plane. This enhances the image contrast compared with non-confocal images and also ensures that the autofluorescence recorded is derived from the ocular fundus provided that the focus is on the retinal surface. The ametropic corrector is employed to correct for refractive error and to focus on the structure of interest (the macula). An argon blue laser (488nm) is used for excitation and directed into the dilated or undilated pupil, delivered from an external source. A wide-pass filter with short-wavelength cut-off of 521nm is inserted in front of the detector to record fundus autofluorescence. Emitted light is then detected above the barrier filter wavelength and recorded as a series of images. Several images can be recorded and digitised at 768 x 576 resolution with an in-house programmeme written in C programmeming language utilising functions from the Matrox© Image Library (Matrox Imaging Products Group, Quebec, Canada). Thirty-two individual images were automatically aligned and averaged to reduce noise. As a result, a single image was obtained for each study eye. A recent study has shown good repeatability with moderate inter-observer reproducibility using the same Zeiss system for AF imaging (Lois, N. *et al*; 1999).

Or

(2) The Heidelberg Retinal Angiograph scanning laser ophthalmoscope (SLO) camera (Heidelberg Engineering, Heidelberg, Germany) – The optical and technical principles have been previously published (Holz, F. G. *et al*; 1999a) but in brief, two scanning mirrors provide the horizontal and vertical scanning directions. The illumination beam has a diameter of 3mm and the pupil of the eye under investigation is either dilated or undilated. The field of view can vary between 10°x10° to 30°x30°. The latter field of view was used in this study. Once again, an argon blue laser (488nm) is again used for illumination and the barrier filter has a short wavelength cut-off of 500nm to suppress reflected blue argon excitation light by a factor of 10^{-6}. The optics allow ametropia compensation between -12 and +12 D. To amplify the autofluorescence signal, 9 images are aligned and a mean image calculated using image analysis software. Images are then digitised and processed using a

flexible frame processor (up to 20 frames/second) provided by Heidelberg Engineering (Heidelberg Eye explorer, Version 2.1.0, 11/2002 for windows 98/NT/2000/XP) and displayed on a computer screen. Each frame contains 512 X 512 pixels and is saved as a 256 KB image file (Holz, F. G. *et al*; 2001). All autofluorescence images were taken either by myself (70%) or Sharon Jenkins (genetic study co-ordinator) (30%).

3.3.3 Fluorescein angiography:

Fluorescein images were taken according to standard protocol for the department by certified photographers only when clinically indicated for suspected choroidal neovascularisation. Five millilitres of 20% sodium fluorescein was injected into the antecubital vein and images were taken using:

- A Zeiss 35mm camera (Zeiss, Oberkochen Germany) or
- Topcon IMAGEnet 2000 © digital camera

The digital images were stored as JPEG files to reduce file storage requirements.

Fundus images (35mm slide transparencies) taken prior to the recruitment date were also digitised by myself using a Nikon Coolpix 995 digital camera (Nikon, Tokyo, Japan), slide film adapter and commercially available light box.

All images were stored digitally in an image database in folders named according to their unique study number. Each folder contained images divided with respect to type of image ('c' for colour, 'f' for fluorescein and 's' for SLO AF) with the date taken.

3.4 Grading techniques (colour, AF)

All images were evaluated at a reading centre at Moorfields Eye Hospital by specially trained ophthalmologists or certified graders. Two to three readers graded each image independently, and in the case of disagreement a further reader was asked to mediate. For the initial studies all grading was carried out equally by myself, Tunde Peto and Hendrik

Scholl and subsequently all grading was carried out by myself and Irene Leung (certified grader).

3.4.1 Colour photographs

The grading system used for colour images was a modified version of that established by the International ARM Epidemiological Study Group (Bird, A. C. *et al*; 1995). This system was used to assess and quantify abnormalities of age-related maculopathy (ARM) and degeneration (AMD) by zone, in each eye from digital images. In order to assess its reliability and validate its use with digital images, the inter- and intra-observer variability was calculated as part of the study (see Chapter 4.1.1).

Since the grading system had been developed for use with 35mm film images and not for digitised fundal photographs (Nikon 995 Coolpix) or digital images (Topcon IMAGE net 2000 ©), it was important to evaluate the effect of the digitisation process by comparing kappa values for the grading of ARM and AMD. This analysis was performed as part of the study and gave high levels of agreement between the two methods (see chapter 4.1).

3.4.1.1 35mm film grading

For grading purposes, all 35mm images were viewed and graded using standard stereoscopic viewing spectacles (Pocket Stereoscopes, Cartographic Engineering Limited, Hampshire, UK; magnification of x2.0) on an x-ray viewing light box (CABIN CL-5000 Series Light Panel; colour temperature of 5000 K). The grading process used a standard acetate grid template with three circular zones (diameter 1000, 3000 and 6000μm). Spokes help to centre the grid on the macula and a set of graduated circles with diameters of 63 μm, 125 μm, 250 μm, and 500 μm were used to estimate drusen size, pigment abnormality size, and total area covered by geographic atrophy and/or neovascular AMD. Data were collected using grading forms and entered into a database.

3.4.1.2 Digital grading

All digital images were viewed using the commercial image processing package Photoshop version 6.0 at a resolution of 1024 x 768 pixels (Adobe Systems Inc., San Jose, CA). Images were saved as low compression JPEG (compression (8:1) formats with a file size of about 170 KB to allow for maximum storage. Grading was then performed on a DELL 17 inch monitor with contrast and brightness standardized. Prior to grading, stereo pairs of images could be viewed using a stereoviewer programme developed in collaboration with scientists at the visual science laboratory at the Institute of Ophthalmology (London) in conjunction with a 'prism viewer.'

One image from each stereo pair was then chosen for grading. The posterior pole was arbitrarily divided into sectors by overlaying a standard digital grid template [produced in Adobe photoshop version 6.0 (Adobe Systems Inc., San Jose, CA) and sized according to the International ARM Epidemiological Study Group and Wisconsin ARM grading system (designed for 30° images)] onto the 30° photograph and centred on the fovea (see figure 6). This grid size was chosen as this is the standard grid size used in macular studies (Bird, A. C. *et al*; 1995). The three circles making up the grid represented the central, middle and outer subfield with diameters of 1000, 3000 and 6000 μm respectively. The adjacent circles represented drusen sizes ranging from 63-500μm.

When a 50° photograph was used, the grid size was scaled down proportionately (approx by 0.6) and centred on the macula to allow the same area within the arcades to be graded. It is acknowledged that there may have been some discrepancy between the exact size of grading area between images but every attempt was made to keep the sizing of the grid standardised between images.

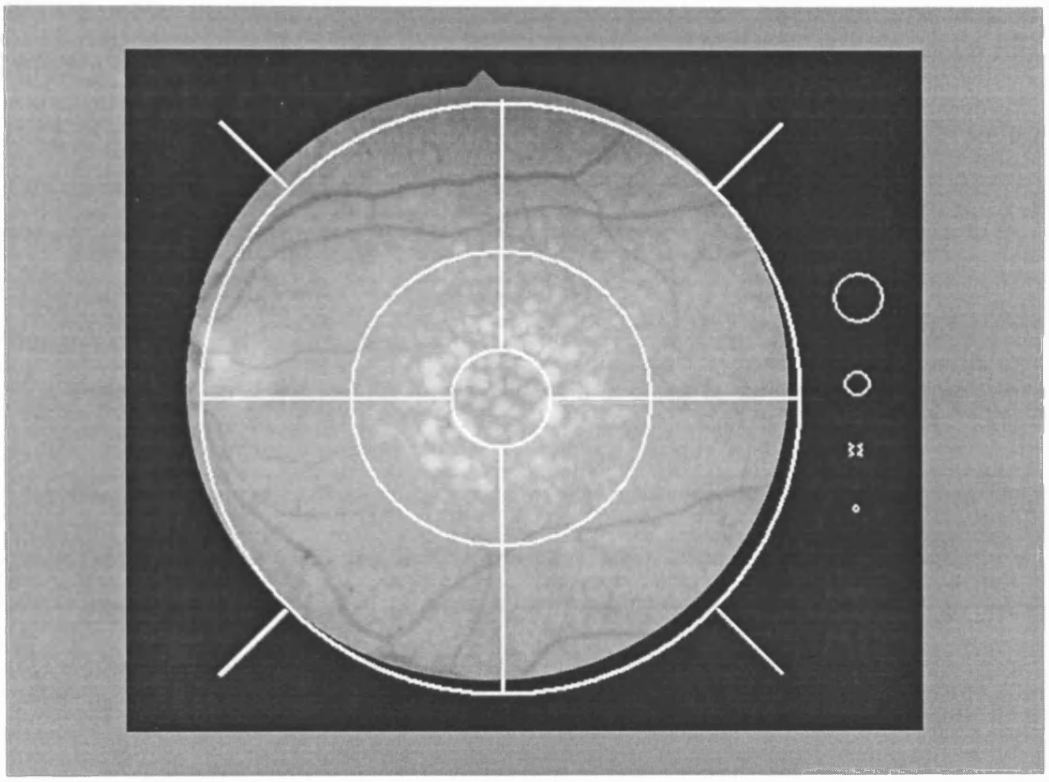


Figure 6: Digital grid template overlying digital image adapted from the International ARM Epidemiological Study Group and Wisconsin ARM grading system

3.4.1.2.1 Software manipulation

In the present study, digitised images were not manipulated by any image processing software. Optimisation of contrast, sharpness and brightness and the use of digital zoom are widely available in commercial image processing software (such as Photoshop, Adobe). It has been shown that software manipulation of images can improve the agreement in grading performance of diabetic retinopathy (George 1999). It is however known that this process can accentuate artefacts and result in pixilation or "blockiness" of the images. For the purposes of the study it was presumed that software manipulation would confound the data in an uncontrolled manner and so was not performed, although a potential to further improve grading performance cannot be excluded.

3.4.1.3 Grading categories

Both 35mm film and digital images were then graded using a coding manual (see appendix 1) with individual scores for:

Photo quality [ranging from perfect image (grade 1) to very poor quality (grade 5) or image not centred (grade 6) or image missing (grade 9)],

Drusen characteristics including:

Size [small (<63μm), intermediate (63 - 125μm) large (>125μm- 500μm) very large (>500μm),

Number [absent, 1-9 to >50],

Total area [<10% to >50% of macula area]

Density [**distinct** -drusen are separate one from another, **subconfluent** -drusen borders just touching and **confluent** -borders are overlapped].

Types such as the presence of **crystalline** drusen or **serogranular** druse in zone 1 were also recorded.

Hypopigmentation [presence or absence] and

Hyperpigmentation [presence and type- linear, punctate, mixed] within the three zones.

Regarding late stage ARM or AMD, the presence and overall area of the lesion was recorded with distinction between **neovascular AMD** and **geographic atrophy**. Additional features of neovascular AMD were also differentiated including **serous detachment of the RPE, neuroretinal detachment, haemorrhagic RPE detachment,** presence of a **scar** or **fibrous material** and **retinal** or **vitreous haemorrhage**. To help with subsequent sorting of data, a category of 'predominant phenotype' was also established (see table 2).

Table 2: predominant phenotype categories for grading purposes

Predominant phenotype (Grading of each eye)	Most advanced feature present
0	<5 Hard drusen
1	Hard drusen only (≥5)
2	Soft drusen
3	Geographic Atrophy
4	Retinal pigment epithelial detachment

5	Choroidal neovascularisation
7	Cannot grade- obscuring lesion
8	Cannot grade – poor photo quality

3.4.1.4 Definitions used for grading

As in other grading systems (AREDS 2001, report no 6, AJO), definitions were established for the grading terms 'absent', 'questionable', 'present', and 'cannot grade.' The term 'absent' was used when the abnormality under consideration was either 'not present' or the grader was less than 50% certain that the abnormality existed in the area under consideration. The term 'Questionable' was used to mean that the grader was at least 50% but less than 90% certain that the abnormality was present within the area under consideration. The term 'present' was used when the grader was at least 90% certain that the abnormality existed within the area being graded and lastly, 'cannot grade' was used when either an obscuring lesion or poor photographic quality did not allow assessment of any abnormality in the area under consideration.

The definitions of hard drusen, soft drusen, areas of hyperpigmentation, areas of hypopigmentation have been previously established by the International ARM Epidemiological Study Group (Bird, A. C. *et al*; 1995).

Definitions for subtypes of AMD were according to the International Classification (see grading manual). Choroidal neovascularisation and pigment epithelial detachment were both graded as neovascular AMD with the individual features classified separately. Any lesions with a subretinal neovascular complex, intraretinal or subretinal hypertrophic scar, neurosensory retinal detachment, hard exudates or retinal haemorrhages (Bird, A. C. *et al*; 1995) were classed as CNV and those with a retinal pigment epithelial detachment were categorised as PED.

Geographic atrophy was defined as an area of RPE hypopigmentation with clearly visible choroidal vessels occupying an area greater than that of a circle of 175μm in diameter. Choroidal vessels and the edges of the atrophy were considered difficult to detect in areas

smaller than this (Bird, A. C. *et al*; 1995). If choroidal neovascularisation was seen in an eye with previous geographic atrophy, it was graded as CNV. If there was evidence of previous CNV such as any fibrous tissue with adjacent areas of atrophy, this was also classified as neovascular AMD.

3.4.1.5 Validation of colour fundal image grading techniques

3.4.1.5.1 Inter- and intra-observer variability in grading ARM and AMD

The grading system used for the phenotype analysis of colour images was a revised version of that devised by the International ARM Epidemiological Study Group. It had been adapted for the study and it was therefore necessary to investigate its reliability, and inter- and intra-observer variability between graders.

Both eyes of 25 patients with ARM or AMD in at least one eye, with stereoscopic pairs of 35mm colour fundus photographs , were randomly selected from the study database. The images were graded according to the modified grading system from the International ARM Epidemiological Study Group by three graders, as described previously (see Methods).

Data were collected using grading forms and entered into a database. All stereoscopic fundus colour slides were graded separately and then re-graded by each grader with a time-delay of at least two weeks.

Finally a master-grading was established and where any disagreement between graders existed, the images were reviewed, and a consensus reached. If any uncertainty remained, final arbitration was carried out by a further retinal specialist (ACB), and these results were used for the master-grading.

Paired gradings from two respective graders (for estimating the inter-observer variability) and both gradings from each grader (for estimating the intra-observer variability) were

compared by means of tables, percentages of agreement/disagreement and kappa statistic (see methods). For abnormalities graded with a binary response (for example, absence/presence of geographic atrophy), agreement and κ-statistic were un-weighted and for abnormalities with extended scales (for example, area covered by geographic atrophy), a weighted variant was also calculated assigning a weight of 1 for perfect agreement, 0.75 for one-step disagreement, and 0 for all other disagreements. Kappa (κ) statistic was interpreted using the ranges suggested by Brennan and Silman and Landis and Koch (Brennan, P. *et al*; 1992; Landis, J. R. *et al*; 1977) (see statistics section). For features with extremely low prevalences, κ-statistic was not performed.

3.4.1.5.2 Comparison of digitised and 35mm film colour grading

In order to validate the use of digitised images for the phenotyping study, it was necessary to carry out a pilot study (Scholl, H. P. *et al*; 2004b). Gold standard 35 mm stereoscopic slide transparencies were compared to digitised non-stereoscopic images when grading for abnormalities in ARM and AMD. Kappa values were calculated to determine agreement between observers and between the digitised and 35mm film images.

Three observers utilised the modified grading system (see appendix 1) to grade 50 eyes of 25 patients randomly selected with stereoscopic colour fundus photographs taken with the Zeiss FF-series 30-degree fundus camera. Each 35 mm colour slide was then digitised using a Nikon Coolpix 990 digital camera (Nikon, Tokyo, Japan) according to previously described techniques (see methods).

These digitised images were also graded separately by three retinal specialists (HPNS, TP, and SD) using the standard digital grid template adapted from the Wisconsin age-related maculopathy grading system (see Methods section). Data were entered on grading forms and entered into a final database. To assess intra-observer variability, all colour slides and digitised images were re-graded by each grader after a time-delay of at least two weeks.

Finally a mastercopy was established for both the stereoscopic colour slides and the digitised fundus images. When the three assessments differed in any category, the graders reviewed the images to reach a consensus. If any uncertainty or disagreement remained, final arbitration was carried out by a further retinal specialist (ACB).

Inter-observer variability was estimated from gradings from pairs of graders and intra-observer variability calculated from the two gradings of each grader. For each category, the percentages of agreement/disagreement and kappa statistic were calculated. Once again abnormalities analysed as either absent or present, agreement and κ-statistic were unweighted, whereas for those with extended scales (for example, area covered by geographic atrophy), a weighted variant was also calculated assigning a weight of 1 for perfect agreement, 0.75 for one-step disagreement, and 0 for all other disagreements. For abnormalities with extremely low prevalences (1:50 or below) such as subgroups of larger drusen, serogranular drusen, hypopigmentation, and retinal haemorrhage κ-statistic was not performed.

3.4.2 Fluorescein angiograms

All images were taken digitally using Topcon digital imaging software (IMAGEnet 2000 ©) and viewed in Photoshop version 6.0. Early transit and late images were chosen for grading purposes. Fluorescein angiograms, when available were used to determine the presence of pigment epithelial detachment and to evaluate the extent of classic or occult choroidal neovascularisation using definitions provided by the Macular Photocoagulation Study (MPS, Arch Ophth 1991) (see appendix 2). When assessing the integrity of the RPE layer in CNV lesions FFA and AF image were directly compared to assess the areas of leakage. This utilised comparison software integrated within the Topcon digital imaging programme (IMAGEnet 2000 ©) (see later).

3.4.3 Autofluorescence images

These images were also viewed in Photoshop version 6.0 on a DELL 17 inch monitor with contrast and brightness standardised.

A classification system was developed (see Appendix 1) to encompass the various features of autofluorescence imaging of AMD (In particular, to evaluate the areas of **increased** and **decreased** AF in the macular region in addition to the pattern of **background** autofluorescence). Changes were divided into unifocal, multifocal, homogeneous or heterogeneous areas of abnormal AF including the presence of specific patterns such as reticular, focal or lace-like (see figure 15 [page 147-8]), as discussed by the International Fundus Autofluorescence Classification Group (In press-see section 8), were also noted.

This grading system was used to identify autofluorescence patterns in patients with unilateral visual loss. The worst eye in each case was evaluated to determine the characteristic AF patterns in GA, CNV and PED. Additionally the fellow eye in each case was assessed for patterns that may confer increased risk of visual loss.

3.5 Concordance and symmetry of phenotype between eyes

To date, symmetry has not been assessed in late stage ARM or AMD. As part of this study, the concordance between eyes in those with bilateral AMD was assessed in terms of the lesion causing visual impairment. The analysis was confined to those with bilateral AMD, rather than including those with unilateral AMD (who would inherently be classed as 'discordant'), as this latter group represents an intermediate time point where the second eye is not yet affected.

Three hundred and seventy-five patients out of the 790 that had been recruited to the study at the time of analysis were included. They were all graded with bilateral late stage disease from digital colour fundus photographs according to the modified version of the

International Classification in conjunction with the assessment of fluorescein angiograms where available (n=196).

The grading was carried out by myself and Irene Leung (certified grader), on all patients to determine the predominant AMD phenotype in each eye. In the event of disagreement, a third observer (TP) was asked to arbitrate. For the purposes of this study, late stage disease was defined as those patients with either GA, PED or CNV in both eyes or any combination of these phenotypes and 'queries'in Microsoft Access were used to identify patients.

Concordance rates for the phenotype were calculated for 'neovascular AMD', PED and CNV independently and for GA (or non-neovascular AMD). In view of the fact that FFA was not carried out on all patients, a definite diagnosis of PED or CNV could not always be determined. This was the basis for carrying out the analysis using the terms 'neovascular' and 'non-neovascular' in addition to the individual phenotypes using predominantly the grading from colour images. The justification for this was based on the international classification for ARM where no FFA images were used (Bird, A. C. *et al*; 1995).

A concordant phenotype was defined as a patient with the same phenotype in both eyes, for example those with GA in both eyes or CNV in both eyes. Patients graded with GA in one eye and PED or CNV in the other were considered to be discordant for phenotype.

In order to assist in the diagnosis of a previous PED or to determine if a patient with geographic atrophy had any evidence of previous CNV, or vice versa, historical data were obtained from the hospital notes.

The numbers of discordant phenotypes (i.e. GA in one eye and CNV or PED in the fellow eye) and concordant phenotypes (GA, CNV or PED in both eyes) from the digital grading were recorded and analysed using Microsoft Excel. Kappa (κ) statistic (see section on statistics 3.11) was then used to calculate the observed concordance between eyes above that expected due to chance assuming independence between eyes (Brennan, P. *et al*; 1992).

3.5.1 Symmetry of autofluorescence patterns in bilateral GA

With the advent of the scanning laser ophthalmoscope (SLO) and autofluorescence imaging, areas of geographic atrophy have become more readily detectable allowing quantitative analysis to be carried out. Those patients with bilateral GA and autofluorescence images were assessed for symmetry and to compare the area and pattern of atrophy between right and left eyes.

Fifty consecutive patients (100 eyes) with bilateral GA as graded from the colour photographs with autofluorescence images available of sufficient image quality to allow analysis, were assessed. Three patients were excluded from the study since only 1 eye had been imaged. Autofluorescence images of both eyes were obtained for each patient (see section 3.3.2).

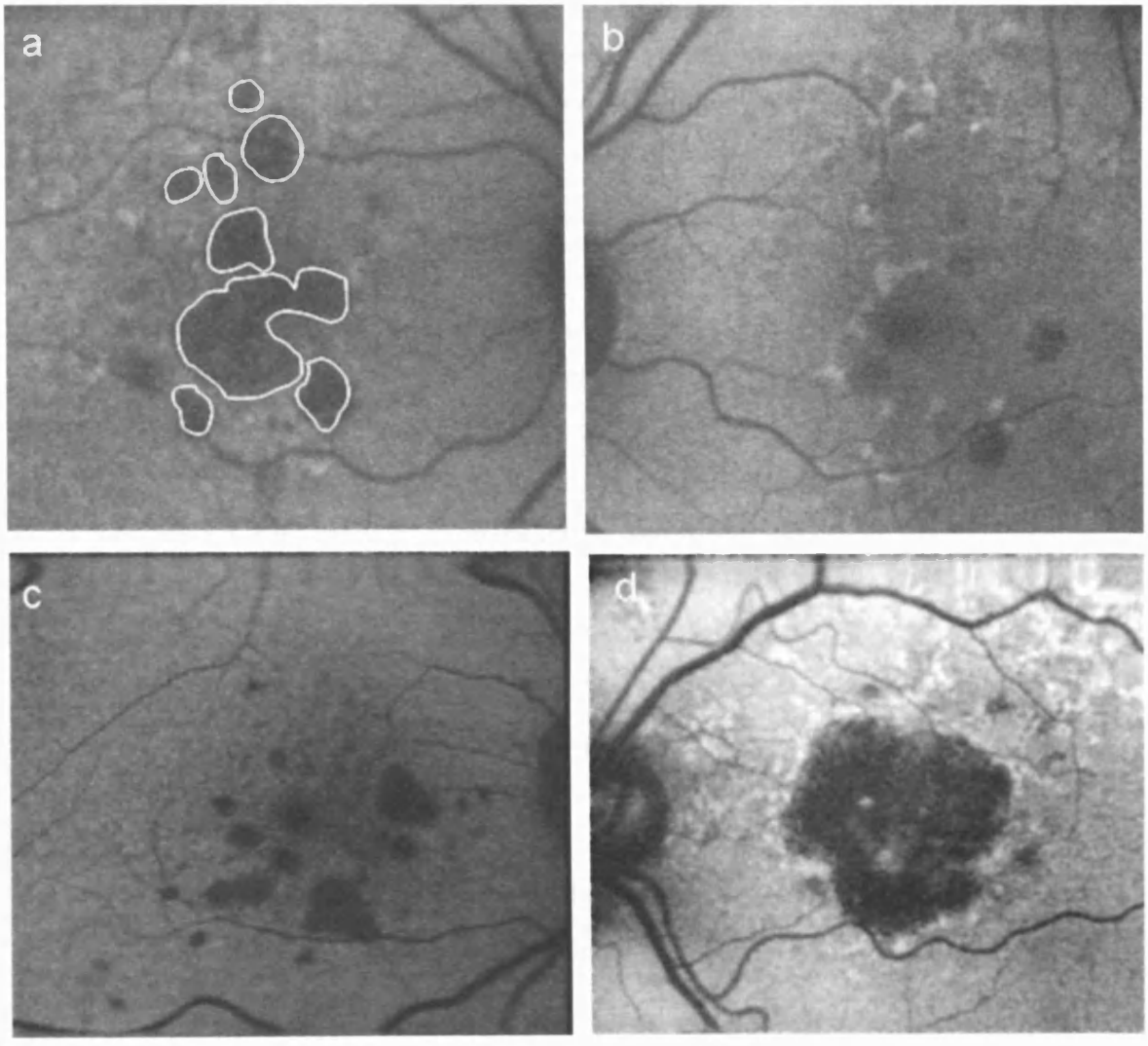
Planimetric techniques were then used by a single reader (SJ) to encircle the darkened areas of atrophy on each image (100 eyes) which are more readily seen on AF images compared to colour images especially in the presence of extensive drusen (see figure 4m-p [page 148]). A mouse driven arrow was used in conjunction with software devised 'in house' by scientists at the Institute of Ophthalmology (University of London). The area was calculated in 'square steridians' after adjusting for the size of the image. Square steridians were chosen so that images could be standardised by setting the number of pixels between the fovea and the centre of the disc to fifteen degrees. This is the measurement accepted as the standard angle between disc and fovea.

All data was analysed by myself with the total area of atrophy (see fig 7) was calculated in each eye (see table 16 {page 132}) including and excluding any areas of peripapillary atrophy to see if these areas differed between eyes. The pattern of decreased autofluorescence was also classified as 'multifocal' or unifocal' (see figure 7c&d [page 91]) depending on the number of atrophic patches in each eye.

The areas and pattern of atrophy were compared between eyes using the Pearson's correlation coefficient (r) and kappa statistic (κ) respectively (seee statistics section). The mean difference in size between right and left areas of atrophy was also tested using the paired student t-test and the results displayed with 95% confidence intervals (see table 16 {page 132}).

Figure 7

(a&b) AF images to show planimetry technique to assess area and symmetry of bilateral GA (c) AF image of GA showing 'multifocal' areas of decreased AF (d) and a predominantly 'unifocal' area of decreased AF in GA. Note the areas of increase AF surrounding the areas of atrophy which represent areas susceptible to further atrophy



3.6 Characteristics of the fellow eye in patients with unilateral visual loss

Patients with unilateral visual loss are of particular value to the study of AMD as they allow an accurate assessment of the premorbid phenotype in those with a proven susceptibility to advanced disease.

3.6.1 Colour fundus characteristics

195 patients with unilateral visual loss from AMD (affected eye worse than 6/24 and fellow eye better than 6/9- difference of 3 lines) and 30° colour fundus photographs of both eyes available for grading, were identified from the study at the time of analysis. Patients with loss of central vision due to other causes including amblyopia, retinal vascular disease or significant cataract were excluded from the initial recruitment.

The images of the worse affected eye were graded to determine the cause of visual loss with reference to the fluorescein angiograms. They were graded as either choroidal neovascularisation (CNV), avascular pigment epithelial detachment (PED), geographic atrophy (GA) or soft drusen.

The fellow eyes were graded for the number, density, area of total drusen (%) and type of drusen (using the modified international classification) and also for the presence of any other lesion relating to AMD.

These characteristics were then analysed with regard to the nature of the lesion in the worse affected eye. For data conforming to a 2x2 contingency table, statistical analysis was carried out using Fisher's exact test (see statistics section. When analysing the area of drusen, data were treated as ranked categorical data and analysed using the chi-squared test for trend. Calculations were performed using 'Medcalc®' statistical software (Belgium).

3.6.2 Autofluorescence fundus characteristics

At the time of analysis, 135 patients from the study were identified with unilateral visual loss and autofluorescence images of both eyes available for grading purposes. AF images from 40 spouse comparisons were also available for grading. Unilateral visual loss was once again defined as a visual acuity of worse than 6/24 in the affected eye and better than 6/9 in the fellow eye. This corresponds to a difference of three lines between the eyes.

Images of affected and fellow eyes from each patient (270 eyes) in addition to the right eye of each spouse comparison (n=40) were then graded by one reader (SD) to determine the AF characteristics using a pilot grading system developed for the study (see appendix 1).

The autofluorescence characteristics of the fellow eye were then compared with regard to the cause of visual loss in the affected eye (i.e. GA, CNV or PED) and to that of 40 clinically identified spouse comparisons (Right eye).

3.7 Identification of foveal location

It was found during the course of the study, that in patients with late stage AMD, the fovea was very difficult to identify just by assessing the photograph visually. Additionally data on the precise mapping of the foveal location relative to the centre of the optic disc were limited. Since the accurate position of the fovea was deemed important for many aspects of this thesis, a separate study was undertaken to establish foveal location in normal subjects. Data on the fovea's mean location in 211 young healthy eyes relative to the optic disc were collated from digital colour fundus photographs, in both disc diameters and degrees. Those patients with data from both eyes allowed assessment of symmetry between eyes.

Digital fundus photographs of 150 consecutive patients (211 eyes) from the hospital image database, with at least one healthy fovea were analysed using Topcon Imagenet 2000© measurement software. All data were collected and analysed by myself. Younger patients (subjects less than 45 years) were chosen due to the ease of locating the foveal reflex

produced by the increased reflectivity of the internal limiting membrane. One hundred patients had 35 degree field fundal photographs and 50 patients had 50 degree field images.

Using the Topcon imagenet 2000© measurement facility, the disc diameter (D), horizontal (H) and vertical (V) distance from the centre of the optic disc to the fovea and the total width of the photograph were measured in mm for each patient (see figure 8). Mean values and standard deviations for the horizontal and vertical distance from the centre of the optic disc to the fovea were calculated and expressed in terms of disc diameters and degrees. For those 61 patients with data available from both eyes, the average values were used and the oblique distances (O) between the fovea and centre of the disc in each eye were also calculated. Comparisons between eyes were made using a paired t-test.

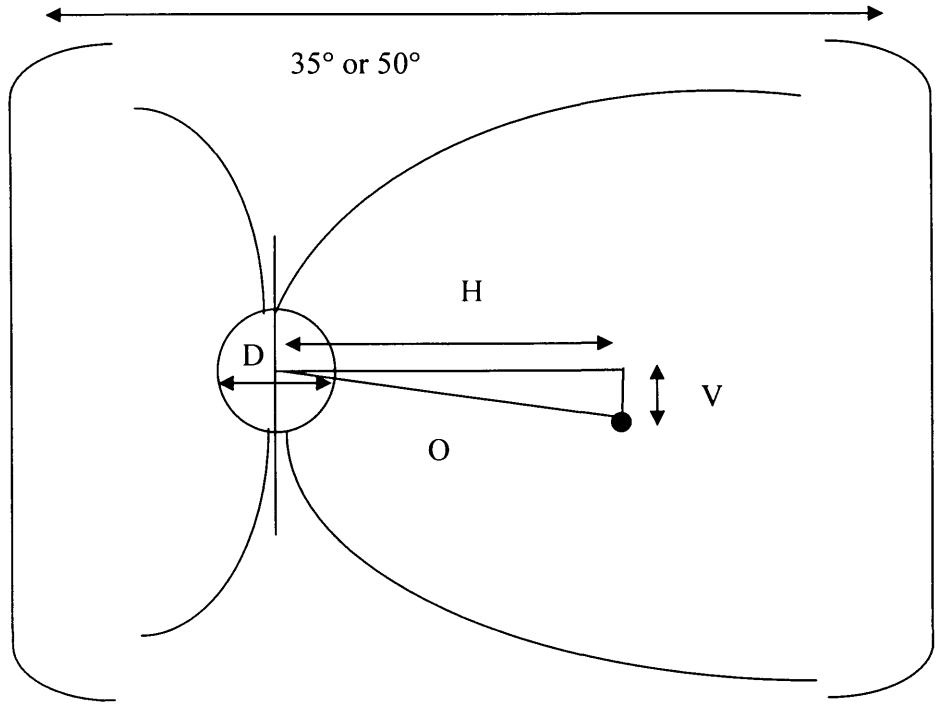


Figure 8: schematic diagram detailing measurements taken to calculate foveal location

3.8 Study of susceptibility of the macula to GA

To determine the relative susceptibility of regions of the macula to atrophy and allow the construction of a contour map, the normative data obtained for the location of the fovea in

healthy eyes (see section 4.6) was utilised. This was particularly important due to the difficulty in locating the fovea in the elderly retina affected by geographic atrophy.

Inclusion / exclusion criteria

Digital colour fundus photographs of 113 consecutive patients (200 eyes) from the study with an area of visible geographic atrophy of sufficient quality to carry out the analysis were included in the study. Forty-two patients with GA present in one or both eyes were excluded due to poor quality photographs or disciform scars with surrounding atrophy. In addition, images where the edges of the atrophy could not be clearly determined from the colour image were excluded unless an autofluorescence image was available to assist in localisation.

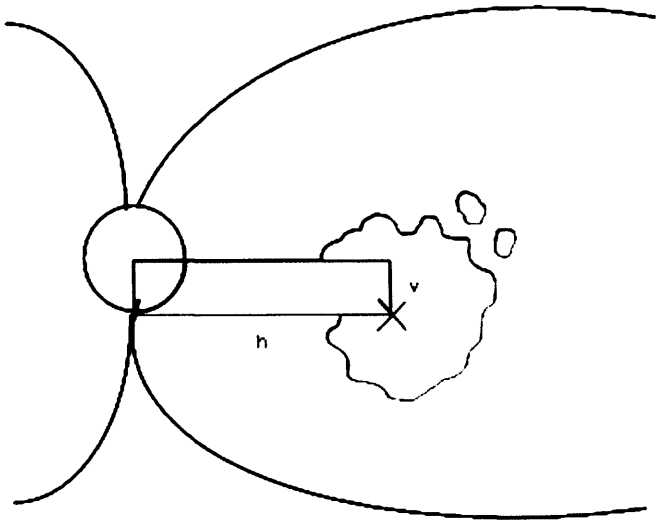
Longitudinal data from a subset of patients (n=21) was also analysed to establish progression of geographic atrophy over time.

3.8.1 Foveal marking and resizing images

Prior to analysis, each image was viewed using Adobe Photoshop version 6.0 and the fovea marked. This was performed by inserting a small square over the fovea (in those retrospective images where the fovea was identifiable) or using a partially transparent rectangle to measure a set distance between the centre of the disc and the fovea (see figure 9 [page 96]) as calculated from the normative data derived from the previous study. The images were then resized so that there were 500 pixels between the disc and fovea to allow comparison between images taken at different time points. Finally each image was compressed and saved as a JPEG (100-150KB).

Figure 9

Schematic representation of how the fovea was located using a digital rectangle overlay of known dimensions



3.8.2 Contour map construction

To produce a contour map of the susceptibility of the macula to GA, a Microsoft Excel spreadsheet was used to provide a grid for each colour image. The background was then replaced with a fundus image so that the areas of atrophy could be marked. The grid was adjusted so that 30 pixels separated the fovea from the centre of the disc. The pixels overlying the marked fovea location and centre of the disc were then filled in and marked with an arbitrary number: '2' for the fovea and a '-1' for the centre of the disc to allow comparison between images. Pixels overlying the areas of atrophy in each image were then marked with a '1'(see figure 10 [page 98]).

If the areas of atrophy could not be clearly identified on the colour image, the autofluorescence image (Zeiss or Heidelberg SLO) was used to assist in the detection of atrophy (see figures 4m-p [page 42]).

Aggregate contour maps for the distribution of atrophy were produced for i) the whole patient cohort (n=133) and ii) the patients on whom longitudinal data were available (n=21); right and left eyes. They were constructed by matrix summation of complimentary pixels for all images (see figure 16a [page 156]). The pixel matrices were aligned for the foveal centre.

Patients with longitudinal data (n=21) were included in the overall susceptibility map but the pixels overlying the initial areas of atrophy were first weighted by assigning these pixels with a '1' and subsequent areas of atrophy were assigned a fraction of '1' depending on the number of images the areas of atrophy were present in.

[Figure 10 (15)]

3.8.3 Progression of atrophy

For those patients with one or more retrospective images, of sufficient quality to analyse and taken at least 1 year prior to the latest image, progression of atrophy was also analysed (n=21, 26 eyes). Images previously taken using 35mm Ektachrome slide transparency film were digitised using a Nikon 990 digital camera as previously described.

When the foveal location could not be determined from retrospective images, the mean values from our normative data were applied to locate the foveal centre. An Excel spread sheet, was again employed to map the areas of progression of atrophy from subsequent fundus photographs. In order to assist in the alignment of images over time, the fovea location, the centre of the disc and coloured markers placed over vessel bifurcations were transposed between images.

Individual contour maps were then constructed and analysed for each patient with longitudinal data to determine whether specific patterns of progression were seen over time. These were classified as circumferential (i.e. in one direction around the fovea either clockwise or anticlockwise), centrifugal (i.e. where the atrophy progressed towards the periphery in all directions) or centripetal (where the atrophy extended towards the fovea from its initial site).

3.9 Analysis of the RPE in Neovascular AMD using AF imaging.

The ability to assess the integrity of the RPE in neovascular AMD may be important for two reasons. Firstly, it may influence the behaviour of the choroidal new vessel complex (Yamagishi, K. *et al*; 1988) (Blaauwgeers, H. G. *et al*; 1999) and secondly, visual outcome may be determined by whether or not the RPE maintains its physiological function. In geographic atrophy, AF has been shown to be decreased either because there is RPE loss or photoreceptor loss since AF reflects its metabolic activity that is driven by photoreceptor outer segment renewal, and areas of increased AF are observed at the junctional zone (Holz, F. G. *et al*; 1999a).

In this part of the study the AF findings in patients with different stages of CNV were evaluated and compared to the FFA images.

Fundus AF images of sixty-five eyes (65 patients), with choroidal neovascularisation (including PED) at various stages of disease, were analysed from the study image database.

Eligibility criteria included (1) patients with choroidal neovascularisation or pigment epithelial detachment (PED) due to AMD in at least one eye and (2) AF, colour and FFA imaging in the cases of recent onset of CNV of sufficient quality to allow evaluation.

The patients were then divided into three groups according to the timing of the AF imaging. Group 1 consisted of twenty patients who had AF imaging undertaken at the same time as CNV or PED was diagnosed using FFA. Group 2 consisted of a further 8 patients who had AF imaging performed 1-6 months following the diagnosis of CNV. The remaining 37 patients had AF imaging of late stage CNV with established disciform scars and are referred to as group 3. FFA was not performed in this latter group at the time of AF imaging.

In those with recent onset CNV (Group 1), the FFA images were directly compared to the auto-fluorescence images using Topcon IMAGEnet 2000 © software (see next section) so that areas of fluorescence could be mapped exactly to corresponding areas on the AF images (see figure 11 a&b [page 102]). Patterns of AF within and around the lesions were analysed and overall areas of abnormality on both FFA and AF were measured (see next section). Additionally, the nature of the lesion (i.e. classic, occult CNV and avascular PED) was determined from the early and late fluorescein images according to the MPS guidelines (MPS; 1991) in group 1 only.

In group 2, where the AF images were taken between 1-6 months after the FFA, areas of abnormality were not measured but areas of previous leakage on FFA were mapped to the AF images using IMAGEnet 2000 © software.

In group 3, with established disciform scars, colour and AF images were analysed for various patterns.

The colour images of all patients were graded to confirm the diagnosis of CNV by two trained graders with an arbitrator in the event of disagreement.

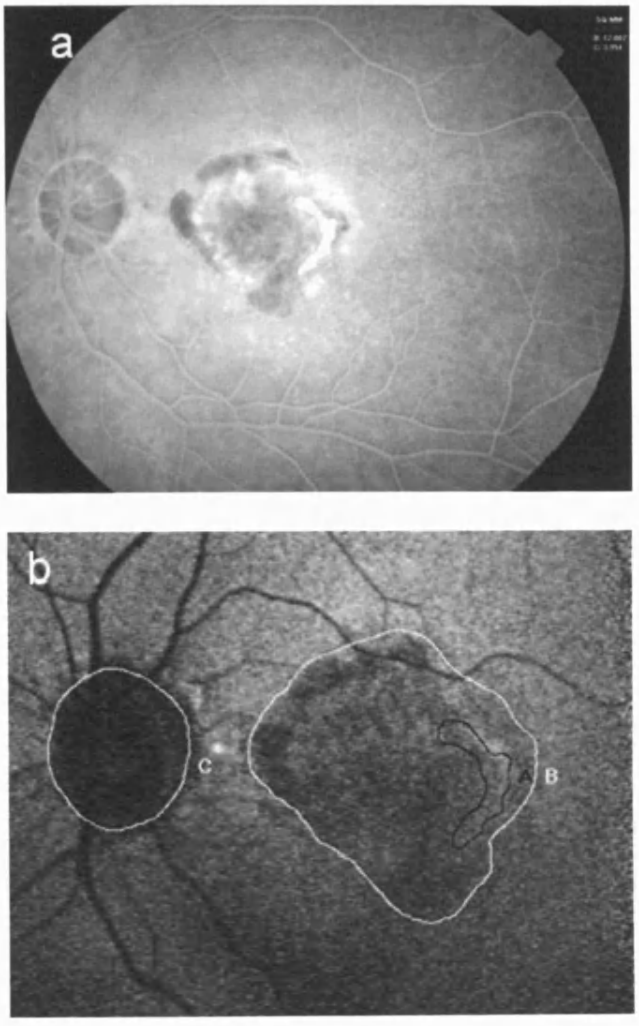
3.9.1 Topcon image analysis programme

In order to compare AF images directly with either digital fluorescein angiograms (FFA) or digital colour images, the Topcon Imagenet 2000 © image analysis software was used.

When comparing FFA images to AF images, a mid-venous phase angiogram was selected so that the site of leakage could be identified and compared directly to the AF image. It was also important that the AF image was taken just prior to FFA to avoid artefactually increased AF.

Figure 11

Planimetry technique for comparison of (a) FFA and (b) AF images of AMD using Topcon imagenet 2000© measurement facility. Note the area of hyperfluorescence on the fluorescein angiogram corresponds to a darkened area on the AF image



The two images to be compared were imported into the Topcon IMAGEnet 2000 © 'compare images' programme. At least 6 reference points at vessel bifurcations were then selected on both images to allow comparison and compensation for varying magnification. The size of various regions and areas of abnormality could then be measured by a single observer (SD) using a cursor driven measurement facility on one image with the corresponding area automatically shown and measured (in cm^2) on the second image for spatial comparison (see figure 11a&b [page 102]). Linear measurements between vessel bifurcations were also made as an internal control to ensure adequate adjustment of magnification differences between images. For each measurement, an average of 3 measures was determined.

These images were then exported to Adobe Photoshop version 6.0 and saved as JPEG or TIFF images.

3.10 Influence of smoking on type of AMD lesion

In order to analyse the influence of smoking on the type of AMD lesion (i.e. neovascular or non-neovascular) smoking data ascertained as part of the study were analysed for all those patients graded with evidence of AMD. For the purposes of the analysis, AMD was defined as either 'neovascular' where choroidal neovascularisation, disciform scar or serous detachment of the retinal pigment epithelium were present in one or both eyes or 'non neovascular' where there was no evidence of neovascular AMD in either eye but only geographic atrophy in one or both eyes.

A full smoking history was taken including data on number of cigarettes smoked per day, age when smoking began and age of stopping smoking in ex-smokers. Pack years were also calculated from the total time subjects had smoked, multiplied by the usual daily cigarette intake, and divided by 20.

A total of 711 patients were included in the study analysis (578 – graded as neovascular, 133 – graded as non neovascular).

Data were analysed using multiple logistic regression analysis (see statistics section). Age and gender were considered to be the main confounding factors for the study. Odds ratios and their 95% confidence intervals were calculated for the likelihood of neovascular AMD as the dependent variable using smoking status (categorical variable- 0= non smoker, 1= ex smoker, 2= current smoker), age (continuous variable) and gender (categorical variable - f= 0, m= 1) as independent variables, as demonstrated by the model below.

$\text{Log}_e(p/1-p) = \alpha + \beta 1$ smoking status $+ \beta 2$ age $+ \beta 3$;

where:

p = proportion of patients with neovascular AMD

α = is the y intercept

$\beta 1$, $\beta 2$, etc are the regression coefficients of the variables in the model

A further regression analysis and a chi-squared test for trend ('Medcalc®' statistical software -Belgium) were also performed using the pack year data.

3.11 Statistics

Three statistical software packages were used for analysis.

- Medcalc® (Belgium)– This was used primarily for t-tests (to test null hypothesis with continuous data), chi squared test and test for trend (for categorical data), kappa statistic (see below), Pearson's correlation coefficient calculations (where variables are continuous), Bland & Altman plots and Fisher's exact calculations (where expected values were <5 since this test does not require any distributional assumptions).
- SPSS 9.0 (SPSS Inc, statistical software, Chicago, USA) - This programme was utilised to carry out multiple regression analysis.
- STATA 7.0 (STATA corp , statistical software, Texas, USA)- This package was used to assess inter and intra-observer variability for grading and to compare 35mm and digitised images (carried out by Catey Bunce; Moorfield's Eye Hospital).

3.11.1 Kappa statistic (κ)

The κ statistic is generally used to assess the extent of observer variability in clinical measures. It assesses 'agreement' when considering data arising from nominal or ordinal scales and discriminates between actual agreement and that due to chance. It relies on the comparison between the observed and the expected amount of agreement, the latter representing that due to chance and dependent on the prevalence of the attribute being measured (Brennan, P. *et al*; 1992).

Values for κ will usually lie between zero and 1, zero indicating only chance agreement and 1 indicating perfect concordance. Negative values would suggest situations where discordance exists. Intervening values of kappa have been given different arbitrary strengths of agreement as shown in the table 3 below (Brennan, P. *et al*; 1992; Landis, J. R. *et al*; 1977).

Table 3: Kappa statistic interpretation according to Landis 1977 and Brennan 1992

κ statistic	**Strength of agreement**
<0.2	Poor
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Good/ Substantial
0.81-1.00	Very good/ Almost perfect

In this study κ was used to assess inter- and intra-observer variability, when evaluating the effect of digitising 35mm film fundal photographs and additionally to compare agreement between eyes when assessing concordance and symmetry of phenotype.

3.11.2 Multiple logistic regression analysis

This technique was used to assess the relationship between the risk of neovascular AMD (binary dependent variable) and various independent variables such as smoking, age and gender (see section 3.10).

3.11.3 Bland and Altman plots

Comparison data of measurements between AF and FFA images were displayed using a graphical method described by Bland and Altman. This plots the difference between measurements against the mean of the two measurements and is used to assess the agreement between two methods of clinical measurement (Bland, J. M. *et al*; 1986).

3.12 Functional assessment of areas of increased autofluorescence

Areas of increased AF are seen in eyes affected by both AMD and ARM (Lois, N. *et al*; 2002; Solbach, U. *et al*; 1997; von Ruckmann, A. *et al*; 1997a; von Ruckmann, A. *et al*; 1997b). In geographic atrophy, regions of increased AF have been demonstrated around areas of atrophy and are thought to precede the development of retinal pigment epithelial and photoreceptor loss (Holz, F. G. *et al*; 2001). Regarding drusen and autofluorescence, studies have been less conclusive as to the association between the distribution of AF, types of drusen and the thickening of Bruch's membrane (Solbach, U. *et al*; 1997) (Lois, N. *et al*; 2002) , (Okubo, A. *et al*; 1999). This implies that Bruch's membrane changes and AF changes may be two independent measures of ageing and ARM in the retina (Lois, N. *et al*; 2002). Since fundus autofluorescence is believed to represent the metabolic activity of the RPE, it therefore provides 'in vivo' assessment of changes in the RPE/Bruch's membrane complex and photoreceptor function (von Ruckmann, A. *et al*; 1997b).

Recently it has been shown that involvement of the rod system may precede that of the cone system in AMD (Curcio, C. A. *et al*; 2000; Curcio, C. A.; 2001; Jackson, G. R. *et al*; 2002). A study was therefore carried out predominantly by Hendrik Scholl in association with colleagues at the Institute of Ophthalmology to investigate the relative involvement of the rod and cone system and the functional implications of increases in AF using photopic and scotopic fine matrix mapping (FMM) in patients derived from the cohort of patients.

The data pertaining to this section were predominantly collected by Hendrik Scholl and colleagues at the Institute of Ophthalmology (except for patient recruitment, autofluorescence imaging and some data interpretation which were carried out by myself) and therefore represents a context report of the work of others. It is included in this thesis as although I was not the main investigator, I certainly contributed to the study and I feel that it presents important data that help to explain findings in other sections.

The inclusion criteria for patients were those with areas of increased AF at the posterior pole and a visual acuity of 20/40 or better to allow fixation in the study eye. Patients with more than mild nuclear sclerosis on slit lamp examination were also excluded so as to provide good quality images.

All study patients underwent the following protocol for the study: (1) Best corrected visual acuity with ETDRS charts, (2) Humphrey standard visual field examination using the 30-2 programme (data not shown), (3) photopic fine matrix mapping (FMM), (4) pupil dilatation with 1.0% tropicamide and 2.5% phenylephrine, (5) dark adaptation for 45 min, (6) dark-adapted 30-2 Humphrey visual field examination as described elsewhere (Chen, J. C. *et al*; 1992) (data not shown), (7) scotopic FMM, and (8) AF image recording.

3.12.1 Fine matrix mapping (FMM)

Fine matrix mapping under photopic and scotopic conditions was carried out in association with colleagues at the Institute of Ophthalmology to investigate the relative involvement of the rod and cone system and the functional implications of increases in AF in patients with age-related macular degeneration.

Depending on the location of the area of increased fundal AF, either a FMM of the centre of the macula or a FMM of the nasal superior, nasal inferior, temporal superior, or temporal inferior quadrant of the macula was obtained each of which included the fovea at one of the corners of the FMM. The technique of FMM has been described previously (Chen, J. C. *et*

al; 1990; Chen, J. C. *et al*; 1992; Chuang, E. L. *et al*; 1987; Fitzke, F. W. *et al*; 1989; Guymer, R. H. *et al*; 1997; Schachat, A. P. *et al*; 1995; Wu, D. *et al*; 1995). Briefly, the technique uses a modified Humphrey field analyser. For the photopic FMM measurements, standard Humphrey size III target white flashes were used on a standard Humphrey bowl illumination of 31.5 apostilbs. For scotopic FMM measurements, Humphrey size III target blue flashes were presented with the bowl illumination switched off. Four red light-emitting diodes in a small diamond configuration served as the fixation target. Accuracy of fixation was monitored using an infrared camera. For the FMM perimetry, retinal luminance sensitivity was tested at 100 test locations and subtended a visual angle of 9 x 9 degrees at the posterior pole. Subsequent processing of the data then produced a three dimensional contour plot showing the size and location of luminance sensitivity gradients across the grid (contour steps: 0.1 log units). These data were used to calculate the mean and the maximum threshold elevation from baseline. The higher the elevation from baseline, the greater the loss of function compared with normal values (Chen, J. C. *et al*; 1992).

These contour plots were then superimposed onto the AF images by aligning anatomical landmarks using custom image analysis software. Fundal AF images were recorded using the cSLO (Zeiss prototype SM 30-4024, Zeiss, Jena, Germany), as previously described (see above) (Lois, N. *et al*; 2002; von Ruckmann, A. *et al*; 1995; von Ruckmann, A. *et al*; 1997a).

Fine matrix mapping results were compared with age-matched data that have been published previously (Guymer, R. H. *et al*; 1997; Schachat, A. P. *et al*; 1995; Westcott, M. C. *et al*; 1997). The individual threshold or luminance sensitivity data (reciprocal of threshold) were compared over areas of normal, increased and decreased AF.

4 Results & Analysis of data

4.1 Characteristics & analysis of patients recruited to the study.

At the time of analysis, 1059 European subjects (940 patients and 119 spouses) had been recruited (90% by myself and the remaining 10% by my colleagues Sharon Jenkins, Hendrik Scholl and Tunde Peto) with colour fundus photographs, autofluorescence (AF) images and data regarding family history and smoking history.

923 of the 1059 subjects recruited to the study had colour fundus images of both eyes of reasonable quality, available for grading (839 patients, 84 spouses).

The remaining 136 subjects did not have colour fundus images available from both eyes. These 'missing images' were attributable to either a lack of photographic services on the day of recruitment (n=13), poor quality images at the time of photography regarded not suitable for grading (n=59), patients not willing to wait for images to be taken on the day of recruitment (n=38) or loss or misplacement of 35mm film images prior to digitisation (n=26).

4.1.1 Case- Comparison group definition

Once the colour fundus images had been graded, it became evident that a large proportion of subjects who had initially been recruited as the spouse 'comparison group' with fundi which appeared normal on biomicroscopy, had evidence of soft drusen (>63μm) in one or both eyes. For the purposes of this analysis, all those from the comparison group graded with soft drusen >63μm in one or both eyes were therefore reclassified as '**patients**' and only those with hard drusen <63μm in both eyes were analysed as the '**comparison group**' (see figure 4 i&j [page 42]).

Using this case –definition, 923 subjects were analysed in total; 879 as patients and 44 as spouse comparisons.

4.1.2 Demographic data

The demographic data (particularly sex and age data) for the **923** subjects with colour images of both eyes who were recruited to the study are presented here.

4.1.2.1 Sex

Of the 879 patients, 65% were female and of the 44 from the comparison group, 43% were female (see table 4). This difference in proportions was statistically significant (p=0.005: χ^2 test).

4.1.2.2 Age

The median age of the 879 patients was greater than that of the 44 from the comparison group [77 (95%CI:76-77) v 72 (95%CI: 69-74)]. There was no difference however between the median age of the females (77) and males (76) that participated in the study (Mann-Whitney U test; p=0.97). Table 4 presents the gender and age breakdown of patients and spouse comparisons.

Table 4: Age of study subjects recruited

	Age-group	Study Patients (n=879)	Comparison Group (n=44)
Female	Median Age	77 (95%CI:76-77)	70 (95%CI:62-73)
	50-59 yr	22	2
	60-69 yr	93	7
	70-79 yr	262	7
	80-89 yr	179	3
	90+ yr	14	0
	Total	**570 (65%)**	**19 (43%)**
Male	Median Age	76 (95%CI:75-78)	74 (95%CI:69-78)
	50-59 yr	12	1
	60-69 yr	49	6
	70-79 yr	137	12
	80-89 yr	105	4
	90+ yr	6	2
	Total	**309 (35%)**	**25 (57%)**

4.1.3 Smoking data

The proportion of smokers, ex-smokers and current smokers amongst the patient group and controls is given in table 5 {page 113}. The proportion of controls who are current smokers is approximately twice that of patients and there is a higher proportion of ex-smokers and non-smokers (not significant) in the patient group. These results may well be skewed by the presence of small numbers in the control group. A more detailed analysis of smoking data and the influence on the type of AMD lesion seen is shown in Chapter 2.3.1.

Table 5: Smoking status amongst patients and comparison group

	Non smoker	Ex smoker	Current smoker
Patients (n=879)	357 (41%)	415 (47%)	107 (12%)
Comparison Group (n=44)	17 (38.5%)	17 (38.5%)	10 (23%)
χ^2 test (2X2 table) =0.012, DF=1: difference of frequency of non smokers between patients and comparison group, p=0.91			

4.1.4 Family history data

The proportion of patients and spouse comparisons with a self-reported family history of AMD was 22% and 7% respectively (statistically significantly different - see table 6). In those patients graded with bilateral AMD this figure rose slightly to 25%.

Table 6: Proportion of patients and comparison group with positive family history

	Positive Family History
Patients (n=879)	199 (22%)
Patients with bilateral AMD (n=395)	100 (25%)
Comparison Group (n=44)	3 (7%)
χ^2 test = 4.769, DF = 1, p = 0.029 (difference between patients and comparison group- 2x2 table)	

4.2 Clinical phenotype of study subjects

Both eyes of 879 patients and 44 spouse comparisons were graded for the various abnormalities seen in ARM and AMD. Each patient was also assigned a predominant phenotype for each eye to represent the most advanced stage of macular abnormality present (see table 2 {page 83}).

Table 7 represents the predominant phenotype data for each eye of the 879 patients. The numbers in bold correspond to the patients with the same predominant phenotype in each eye, i.e. symmetry between eyes at the time of grading.

Table 7: Predominant phenotype graded for each eye in patient cohort (n=879)

Left Eye	Right eye	Hard drusen (1)	Soft drusen (2)	GA (3)	PED (4)	CNV (5)	Poor quality (8)	Total
	Hard drusen (1)	**0**	11	1	0	11	0	23
	Soft drusen (2)	17	**154**	17	5	111	0	304
	GA (3)	2	25	**87**	1	38	0	153
	PED (4)	1	4	0	**5**	4	0	14
	CNV (5)	11	114	48	5	**205**	1	384
	Poor quality (8)	0	0	0	0	0	**1**	1
	Total	31	308	153	16	369	2	**879**

Out of the 879 patients, 21 % were graded with drusen only in both eyes, 10% with GA in both eyes, 0.6% with PED in both eyes and 23% with CNV or a disciform scar in both eyes. All the 44 spouse comparisons were graded with bilateral hard drusen only. Kappa statistic was calculated for the overall 'agreement' or 'symmetry' between eyes in excess of that due to chance ($\kappa = 0.264$; SE = 0.025, 95% CI = 0.214-0.313).

Table 8 represents the bilaterality of the various lesions in ARM and AMD in the cohort of patients analysed. The number of unilateral cases is also given with the proportion of those with only drusen in the fellow eye given in brackets.

Table 8: Bilaterality of ARM/AMD lesions in patient cohort

Lesions	No of cases	No (%) with bilateral involvement	No (%) with unilateral involvement
Any AMD (not drusen only)	697	391 (56%)	306 (44%)
Neovascular AMD	563	219 (38%)	344 (62%)
CNV	547	205 (37%)	342 (63%) (247 (72%)of these CNV-drusen)
PED	25	5 (20%)	20 (80%) (10 (50%) of these PED-drusen)
Atrophic AMD	219	87 (40%)	132 (60%) (45 (34%) of these GA-drusen)
Any drusen (soft or hard)	484	182 (38%)	302 (62%)

4.3 Validation of fundal image grading techniques

The prevalences of the various attributes of ARM and AMD that were graded from the 50 eyes as part of the validation study were determined from the final master grading (see table 9).

Table 9: Prevalence of ARM/AMD attributes in the study eyes

	Zone 1 (central 1000μm)	Zone 2	Zone 3 (outer 3000-6000μm)
Drusen < 63 μm * (hard drusen)	39	45	48
Drusen 63-125 μm * (intermediate soft drusen)	9	32	28
Drusen 125-250 μm * (large semisolid drusen) distinct, subconfluent, confluent	4, 3, 1	13, 7, 7	13, 10, 6
Drusen 250-500 μm * (large semisolid drusen) distinct, subconfluent, confluent	0, 0, 3	5, 3, 10	1, 3, 2
Drusen > 500 μm * (large semisolid drusen) distinct, subconfluent, confluent	0, 0, 1	0, 0, 4	0, 0, 1
Crystalline drusen *	5	6	3
Serogranular drusen *	1		
Hyperpigmentation (presence)	21	19	5
Hypopigmentation (presence)	0	2	6
Geographic atrophy (presence)	11	12	6
Neovascular AMD (presence)	11	10	5
Neovascular AMD (scar/fibrous)	7	6	4
Neovascular AMD (retinal haemorrhage)	1	3	0

For large and serogranular drusen, hypopigmentation and retinal haemorrhage, the prevalence was extremely low (including values of 1:50 or less). Geographic atrophy and neovascular AMD were more prevalent in zones 1&2 compared to zone 3 (see table 9). In

zone 1, the predominant features were a subretinal scar present in 7 eyes, a serous retinal detachment in 2 eyes and a serous RPE detachment in a further 2 eyes.

4.3.1 Inter-observer variability

Photo quality

Weighted agreement between the three graders on photo quality was fair to moderate and ranged between 62% and 75% ($\kappa = 0.30 - 0.47$).

Drusen Characteristics

Section A of table 10 {page 119} presents the comparison of gradings for the various drusen characteristics. Weighted agreement, weighted kappa values and p-values are presented for the categories with an asterix (*). The p-value represents the probability that the observations made are due to chance alone. For hard drusen and intermediate soft drusen, the agreement was fair to substantial for all zones and ranged between 70% and 89% ($\kappa = 0.27 - 0.63$) for hard drusen and between 76% and 94% ($\kappa = 0.31 - 0.69$) for intermediate soft drusen. For large semisolid drusen (larger than 125 μm), agreement ranged from 87% to 100%, but the prevalence for individual types was extremely low (see table 9 {page 116}; with values of 1/50 and 0/50) and hence κ-statistic was not performed since in these circumstances it does not provide useful information. For the larger drusen, the raw data were investigated for all 81 comparisons (3 graders, 3 zones and 9 types of large drusen) and showed that the (unweighted) exact agreement was below 40/50 in only 9 cases with the smallest value being 33/50. Perfect agreement was achieved in 5 cases.

The agreement for crystalline drusen in zones 1-3 was fair to almost perfect and ranged between 95% and 100% ($\kappa = 0.25 - 0.81$). For serogranular drusen in zone 1, agreement ranged between 96% and 100%.

Pigment abnormalities

Section B of table 10 {page 119} shows the unweighted agreement or concordance and unweighted kappa values for the grading of pigment abnormalities in the 50 eyes. For the presence of hyperpigmentation, the concordance was poor to substantial ranging between 50% and 92% ($\kappa = 0.14 - 0.75$). This was also true for the type of hyperpigmentation with a range of agreement of 48% and 88% ($\kappa = 0.13 - 0.78$). For hypopigmentation, where the prevalence was low in the graded cases, agreement ranged between 78% and 100%.

Age-related macular degeneration (AMD)

Section C of table 10 {page 119} presents the agreement between the gradings of the late stages of ARM or AMD. For the presence of geographic atrophy in zones 1-3, unweighted agreement was moderate to almost perfect ranging between 88% and 98% ($\kappa = 0.60 - 0.95$) and weighted agreement for the area covered by geographic atrophy was substantial to almost perfect (93% - 99%; weighted κ 0.79- 0.97).

For the presence of CNV, the unweighted agreement was substantial to almost perfect ranging between 84% and 100% ($\kappa = 0.62 - 1.00$). However, for individual features of CNV, the comparison between different pairs of gradings resulted in substantial variability from only poor to almost perfect agreement ($\kappa = 0.09 - 1.00$; agreement between 72% and 100%).

A high concordance of grading was seen for the presence of scars and retinal haemorrhage, with unweighted agreements of between 90% and 100% ($\kappa = 0.65 - 1.00$) and between 96% and 100% respectively. Weighted agreement for the area covered by CNV was also high with agreement ranging between 86% and 100% and weighted kappa values between 0.67 and 1.00.

Table 10: Inter-Observer Agreement

		Zone 1			Zone 2			Zone 3		
		Observer 1 - 0	Observer 2 - 1	Observer 2 - 0	Observer 1 - 0	Observer 2 - 1	Observer 2 - 0	Observer 1 - 0	Observer 2 - 1	Observer 2 - 0
A	Drusen < 63 µm *	89%	75%	87%	80%	78%	70%	80%	83%	81%
	(hard drusen)	0.63	0.27	0.46	0.63	0.56	0.45	0.62	0.63	0.60
		p<0.0001	p=0.005	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
	Drusen 63-125 µm *	93%	92%	94%	89%	85%	88%	84%	77%	76%
	(intermediate soft drusen)	0.41	0.31	0.38	0.69	0.54	0.56	0.64	0.47	0.36
		p=0.003	p=0.022	p=0.006	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0003
	Drusen 125-250 µm *	98%,	96%,	94%,	97%,	92%,	92%,	89%,	92%,	87%,
	(large semisolid drusen)	99%,	95%,	95%,	95%,	94%,	94%,	97%,	92%,	92%,
	distinct, subconfluent, confluent	99%	97%	98%	98%	95%	90%	100%	93%	94%
	Drusen 250-500 µm *	100%,	98%,	98%,	99%,	93%,	94%,	100%,	95%,	95%,
	(large semisolid drusen)	99%,	97%,	100%,	99%,	95%,	95%,	99%,	94%,	95%,
	distinct, subconfluent, confluent	100%	96%	96%	97%	93%	95%	96%	92%	96%
	Drusen > 500 µm *	100%,	100%,	100%,	100%,	97%,	97%,	99%,	98%,	97%,
	(large semisolid drusen)	100%,	98%,	100%,	100%,	98%,	98%,	100%,	98%,	100%,
	distinct, subconfluent, confluent	100%	98%	98%	99%	96%	96%	99%	98%	97%
	Crystalline drusen *	99%	98%	99%	99%	95%	93%	100%	97%	97%
		0.81	0.59	0.56	0.78	0.50	0.25	0.79	0.44	0.55
		p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.005	p<0.0001	p<0.0001	p<0.0001
	Serogranular drusen *	98%	96%	98%						
B	Hyperpigmentation	86%	54%	50%	84%	72%	64%	92%	88%	80%
	(presence)	0.75	0.22	0.14	0.68	0.49	0.35	0.66	0.45	0.25
		p<0.0001	p=0.017	p=0.086	p<0.0001	p<0.0001	p=0.0004	p<0.0001	p<0.0001	p=0.007
	Hyperpigmentation	88%	52%	80%	72%	48%	56%	88%	80%	82%
	(type)	0.78	0.18	0.25	0.47	0.13	0.25	0.56	0.22	0.33
		p<0.0001	p=0.041	p=0.007	p<0.0001	p=0.079	p=0.004	p<0.0001	p=0.01	p=0.0002
	Hypopigmentation	100%	78%	78%	100%	78%	78%	100%	88%	88%
	(presence)									

C	Geographic atrophy (presence)	98% 0.94 p<0.0001	92% 0.75 p<0.0001	90% 0.70 p<0.0001	98% 0.95 p<0.0001	90% 0.71 p<0.0001	88% 0.67 p<0.0001	98% 0.90 p<0.0001	92% 0.60 p<0.0001	94% 0.72 p<0.0001
	Geographic atrophy * (area covered)	99% 0.97 p<0.0001	93% 0.79 p<0.0001	93% 0.80 p<0.0001						
	Neovascular AMD (presence)	100% 1.00 p<0.0001	84% 0.63 p<0.0001	84% 0.63 p<0.0001	100% 1.00 p<0.0001	84% 0.62 p<0.0001	84% 0.62 p<0.0001	100% 1.00 p<0.0001	96% 0.78 p<0.0001	96% 0.78 p<0.0001
	Neovascular AMD (features)	100% 1.00 p<0.0001	74% 0.10 p=0.147	74% 0.10 p=0.147	96% 0.80 p<0.0001	72% 0.09 p=0.141	74% 0.12 p=0.051	98% 0.85 p<0.0001	92% 0.31 p=0.0002	94% 0.38 p<0.0001
	Neovascular AMD (scar/fibrous)	98% 0.93 p<0.0001	90% 0.65 p<0.0001	92% 0.71 p<0.0001	100% 1.00 p<0.0001	94% 0.74 p<0.0001	94% 0.74 p<0.0001	100% 1.00 p<0.0001	96% 0.73 p<0.0001	96% 0.73 p<0.0001
	Neovascular AMD (retinal haemorrhage)	100%	96%	96%	100%	96%	96%	100%	98%	98%
	Neovascular AMD * (area covered)	100% 1.00 p<0.0001	86% 0.67 p<0.0001	86% 0.67 p<0.0001						

4.3.2 Intra-observer variability

Table 11 {page 122} presents the concordance between the master grading from initial gradings and the master grading from the re-gradings (obtained after a time-delay of at least two weeks) for the three graders. Minimal and maximal agreement, minimal and maximal kappa values, and the maximal and minimal p-value (indicating the smallest and highest statistical significance, respectively) obtained from the comparison are provided for each zone separately. As for the inter-observer variability (Table 10 {page 119}), weighted agreement and weighted kappa values are given for the drusen characteristics and the area covered by GA and CNV. For all other categories in Table 11 {page 122} unweighted (exact) agreements and unweighted kappa values are given. As for the inter-observer variability, κ-statistic was not performed for attributes with extremely low prevalences such as larger drusen, serogranular drusen, hypopigmentation, and retinal haemorrhage (Table 9 {page 116}).

Drusen characteristics & pigment abnormalities

Agreement was moderate to almost perfect for small hard drusen, and fair to almost perfect for intermediate soft drusen. Agreement for large semisolid drusen (larger than 125 μm) ranged between 84% and 100%. Agreement for both the presence and the type of hyperpigmentation was fair to substantial.

Age-related macular degeneration (GA and CNV)

Agreement was moderate to almost perfect for the presence of GA, and substantial to almost perfect for the area covered by GA. The agreement was moderate to almost perfect for the presence of CNV, and substantial for the area covered by CNV. Agreement on the individual features of neovascular AMD was lower although the agreement on the presence of scarring was moderate to almost perfect.

Table 11: Intra-Observer Agreement

	Zone 1	Zone 2	Zone 3
Drusen < 63 μm *	(min) 82%, 96% (max)	72%, 76%	78%, 81%
(hard drusen)	0.45, 0.83	0.43, 0.55	0.60, 0.64
	p=0.0001, p<0.0001	p<0.0001	p<0.0001
Drusen 63-125 μm *	88%, 98%	87%, 96%	81%, 93%
(intermediate soft drusen)	0.13, 0.75	0.51, 0.81	0.50, 0.78
	p=0.154, p<0.0001	p<0.0001	p<0.0001
Drusen > 125 μm *	84%, 100%	92%, 100%	90%, 100%
(large semisolid drusen)			
Crystalline drusen *	92%, 100%	92%, 99%	90%, 100%
	0.46, 0.94	0.67, 0.85	0.23, 0.95
	p=0.0004, p<0.0001	p<0.0001	p=0.048, p<0.0001
Serogranular drusen *	96%, 100%		
Hyperpigmentation	62%, 82%	70%, 82%	84%, 86%
(presence)	0.30, 0.68	0.39, 0.69	0.36, 0.54
	p=0.006, p<0.0001	p=0.0005, p<0.0001	p=0.0005, p<0.0001
Hyperpigmentation	54%, 68%	64%, 76%	78%, 88%
(type)	0.21, 0.46	0.30, 0.61	0.27, 0.46
	p=0.017, p<0.0001	p=0.0014, p<0.0001	p=0.002, p<0.0001
Hypopigmentation	88%, 100%	86%, 98%	82%, 90%
(presence)			
Geographic atrophy	92%, 95%	88%, 97%	94%, 96%
(presence)	0.52, 0.78	0.65, 0.77	0.69, 0.81
	p<0.0001	p<0.0001	p<0.0001
Geographic atrophy *	90%, 96%		
(area covered)	0.61, 0.88		
	p<0.0001		
Neovascular AMD	82%, 90%	88%, 96%	94%, 99%
(presence)	0.42, 0.72	0.46, 0.82	0.63, 0.90
	p=0.0008, p<0.0001	p=0.0005, p<0.0001	p<0.0001
Neovascular AMD	80%, 94%	86%, 96%	92%, 98%
(features)	0.36, 0.76	0.49, 0.84	0.18, 0.66
	p<0.0001	p<0.0001	p=0.004, p<0.0001
Neovascular AMD	86%, 96%	88%, 98%	94%, 96%
(scar/fibrous)	0.46, 0.87	0.50, 0.92	0.54, 0.78
	p=0.0003, p<0.0001	p=0.0002, p<0.0001	p=0.0001, p<0.0001
Neovascular AMD	96%, 96%	98%, 100%	96%, 100%
(retinal haemorrhage)			
Neovascular AMD *	87%, 92%		
(area covered)	0.65, 0.77		
	p<0.0001		

4.3.3 Comparison of digitised images and 35mm film grading results

The comparison of the gradings between the two mastercopies from the colour slides and digitised images from the 50 eyes used in the study are presented in Table 12 {page 125}. For binary scales exact (unweighted) agreement, kappa values and p-values are given and for abnormalities with extended scales (as e.g. for drusen characteristics), agreement and kappa values are weighted.

Section A of Table 12 presents the comparison or concordance for drusen characteristics between gradings. For small hard and intermediate soft drusen, the agreement for all zones was moderate to substantial and fair to substantial, respectively. For large semisolid drusen (larger than 125 μm), the weighted agreement was consistently above 90% for all 27 subgroups.

The agreement for crystalline drusen in zones 1-3 was fair to almost perfect. Agreement was perfect for serogranular drusen (only graded for zone 1).

In section B of Table 12, the comparison for the grading of pigment abnormalities are presented as unweighted agreement and unweighted kappa values. For the presence and type of hyperpigmentation, the concordance was only poor to fair. For the presence of hypopigmentation however, the concordance was higher (88% to 100%).

Section C of Table 12 presents the agreement between abnormalities of late stage disease (AMD). For the presence of GA, unweighted agreement was substantial to almost perfect. Weighted agreement for the area covered by GA was also substantial.

For the presence of CNV or scars, the concordance was almost perfect and for the features of CNV, agreement was fair to moderate. For the presence of retinal haemorrhage, agreement was between 96% and 98%. Weighted agreement for the area covered by CNV was also almost perfect.

To investigate possible sources of disagreement in more detail, the raw data were reviewed and analysed. For the grading of **small hard drusen**, using the grading from the stereoscopic colour slides as the gold standard, we found that the digitised grading underestimated the number of drusen in all 3 zones (9 z1, 20 z2, 15 z3).

When grading for the presence of **hyperpigmentation**, 14, 26, and 42 cases in zone 1, 2, and 3, respectively, had evidence of pigmentation on the digitised images but not on the colour slides. Conversely hyperpigmentation was seen on stereoscopic colour slides but not on the digitised images, in only 7, 2, and 0 cases in each zone respectively.

When grading for the presence of **GA**, this lesion was graded as 'absent' in three cases in zone 1 and 2 and in one case in zone 3 from the digitised images when small areas of GA were seen on the 35mm colour slides.

The presence of **CNV** from the grading of the digitised images was missed in two cases from zones 1 and 2 and in one case from zone 3; CNV was also graded as 'present' from the digitised images and 'absent' from the conventional colour slides in one case in zones 1 and 2. For the grading of CNV features, a tendency to miss neuroretinal detachments on the digitised images was also observed (two cases).

As part of a separate study, carried out by medical retinal specialists at Moorfield's Eye Hospital, 35mm transparencies of AMD were compared to digital images taken with the TOPCON imagenet 2000 © digital camera with comparable results (unpublished).

Table 12: Percentage agreements, kappa values and p-values for comparison of fundal features between the master copies of the 35mm colour slides and digitised images (* weighted kappa values) in each zone.

		Zone 1	Zone 2	Zone 3
A	**Drusen < 63 µm *** (hard drusen)	91% k=0.56 p<0.0001	86% k=0.72 p<0.0001	77% k=0.56 p<0.0001
	Drusen 63-125 µm * (intermediate soft drusen)	93% k=0.31 p=0.005	89% k=0.64 p<0.0001	83% k=0.62 p<0.0001
	Drusen 125-250 µm * (large semisolid drusen) distinct, subconfluent, confluent	98%, 98%, 100%	95%, 94%, 94%	91%, 93%, 94%
	Drusen 250-500 µm * (large semisolid drusen) distinct, subconfluent, confluent	100%, 100%, 100%	97%, 94%, 97%	97%, 97%, 97%
	Drusen > 500 µm * (large semisolid drusen) distinct, subconfluent, confluent	100%, 100%, 100%	100%, 100%, 92%	98%, 98%, 98%
	Crystalline drusen *	99% k=0.82 p<0.0001	98% k=0.72 p<0.0001	97% k=0.35 p=0.0001
	Serogranular drusen *	100%		
B	**Hyperpigmentation** (presence)	56% k=0.27 p=0.004	32% k=0.00 p=0.494	12% k=0.02 p=0.243
	Hyperpigmentation (type)	50% k=0.15 p=0.086	28% k=0.05 p=0.191	10% k=0.03 p=0.143
	Hypopigmentation (presence)	100%	98%	88%
C	**Geographic atrophy** (presence)	94% k=0.81 p<0.0001	94% k=0.82 p<0.0001	96% k=0.80 p<0.0001
	Geographic atrophy * (area covered)	93% k=0.78 p<0.0001		
	Neovascular AMD (presence)	94% k=0.82 p<0.0001	94% k=0.81 p<0.0001	98% k=0.88 p<0.0001
	Neovascular AMD (features)	90% k=0.34 p=0.0003	88% k=0.22 p=0.005	96% k=0.49 p<0.0001
	Neovascular AMD (scar/fibrous)	96% k=0.83 p<0.0001	96% k=0.81 p<0.0001	98% k=0.85 p<0.0001
	Neovascular AMD (retinal hemorhage)	98%	96%	98%
	Neovascular AMD * (area covered)	95% k=0.83, p<0.0001		

Inter- and intra-observer variability between graders

Tables 11 and 12, present the respective inter- and intra-observer agreement for the three graders for grading of the digitised colour photographs. Once again agreement and κ-statistics are weighted for abnormalities with extended scales. Section A of Tables 11 and 12 presents the comparison for the grading of drusen characteristics between and within observers, respectively. Agreement for small hard drusen was fair to substantial between observers and moderate to almost perfect within observers. For intermediate soft drusen, agreement was fair to substantial between observers and moderate to almost perfect within observers. The agreement between and within observers for larger drusen ranged from 82% to 100%, and 90% to 100%, respectively.

Agreement for pigmentary changes (Section B, Tables 11 and 12) was poor to moderate for the presence of hyperpigmentation between observers and poor to substantial within observers. The concordance for the presence of hypopigmentation ranged between 86% and 98% between observers and 84% and 100% within observers.

Agreement was moderate to almost perfect for the presence of GA between observers and substantial to almost perfect within observers (Section C, Table 12 {page 125} and 13 {page 127}). For the area covered by GA, agreement was substantial to almost perfect between observers and almost perfect within observers. For the presence of CNV, agreement was moderate to almost perfect between observers and substantial to perfect within observers. For the grading of features of CNV, agreement was poor to almost perfect between observers and fair to substantial within observers. For the area covered by CNV, weighted agreement was fair to substantial between observers and substantial within observers.

Table 13: Inter-Observer Agreement

		Zone 1	Zone 2	Zone 3
A	Drusen < 63 μm *	83%, 94%	74%, 85%	75%, 90%
	(hard drusen)	0.40, 0.73	0.52, 0.72	0.53, 0.79
		p=0.0001, p<0.0001	p<0.0001	p<0.0001
	Drusen 63-125 μm *	88%, 93%	79%, 90%	75%, 88%
	(intermediate soft drusen)	0.37, 0.52	0.52, 0.71	0.48, 0.70
		p=0.008, p<0.0001	p<0.0001	p<0.0001
	Drusen > 125 μm *	93%, 100%	82%, 100%	89%, 100%
	(large semisolid drusen)			
	Crystalline drusen *	92%, 95%	92%, 97%	90%, 95%
		0.26, 0.45	0.29, 0.38	0.02, 0.56
		p=0.0001, p<0.0001	p=0.004, p<0.0001	p=0.153, p<0.0001
	Serogranular drusen *	90%, 98%		
B	Hyperpigmentation	66%, 68%	54%, 64%	44%, 76%
	(presence)	0.47, 0.50	0.25, 0.35	0.05, 0.15
		p<0.0001	p=0.006, p=0.0001	p=0.211, p=0.041
	Hyperpigmentation	46%, 64%	44%, 74%	20%, 56%
	(type)	0.28, 0.37	0.09, 0.35	-0.08, 0.14
		p=0.0005, p<0.0001	p=0.1, p<0.0001	p=0.955, p=0.03
	Hypopigmentation	96%, 98%	92%, 96%	86%, 94%
	(presence)			
C	Geographic atrophy	88%, 94%	88%, 94%	94%, 98%
	(presence)	0.55, 0.79	0.58, 0.79	0.60, 0.89
		p<0.0001	p<0.0001	p<0.0001
	Geographic atrophy *	91%, 98%		
	(area covered)	0.70, 0.93		
		p<0.0001		
	Neovascular AMD	78%, 92%	80%, 92%	94%, 98%
	(presence)	0.41, 0.70	0.44, 0.70	0.64, 0.90
		p=0.0003, p<0.0001	p=0.0002, p<0.0001	p<0.0001
	Neovascular AMD	70%, 96%	70%, 98%	96%, 98%
	(features)	0.18, 0.73	0.13, 0.85	0.48, 0.66
		p=0.014, p<0.0001	p=0.053, p<0.0001	p<0.0001
	Neovascular AMD	84%, 92%	88%, 94%	94%, 98%
	(scar/fibrous)	0.34, 0.67	0.44, 0.74	0.55, 0.79
		p=0.005, p<0.0001	p=0.0004, p<0.0001	p<0.0001
	Neovascular AMD	92%, 96%	92%, 98%	94%, 100%
	(retinal haemorrhage)			
	Neovascular AMD *	68%, 88%		
	(area covered)	0.27, 0.62		
		p=0.002, p<0.0001		

Taken from: Scholl et al. Ophthalmology. 2004 (Scholl, H. P. *et al*; 2004b)

Table 14: Intra-Observer Agreement

		Zone 1	Zone 2	Zone 3
A	Drusen < 63 μm * (hard drusen)	91%, 95% 0.76, 0.80 p<0.0001	73%, 90% 0.45, 0.81 p<0.0001	78%, 87% 0.59, 0.76 p<0.0001
	Drusen 63-125 μm * (intermediate soft drusen)	89%, 96% 0.44, 0.75 p<0.0001	89%, 95% 0.79, 0.84 p<0.0001	85%, 95% 0.67, 0.88 p<0.0001
	Drusen > 125 μm * (large semisolid drusen)	92%, 100%	90%, 100%	75%, 100%
	Crystalline drusen *	94%, 99% 0.16, 0.88 p=0.124, p<0.0001	95%, 99% 0.30, 0.83 p=0.001, p<0.0001	95%, 99% 0.15, 0.88 p=0.156, p<0.0001
	Serogranular drusen *	94%, 100%		
B	Hyperpigmentation (presence)	54%, 84% 0.32, 0.73 p=0.0003, p<0.0001	54%, 76% 0.21, 0.44 p=0.004, p<0.0001	40%, 84% 0.08, 0.43 p=0.188, p<0.0001
	Hyperpigmentation (type)	56%, 82% 0.27, 0.71 p=0.005, p<0.0001	54%, 68% 0.13, 0.41 p=0.035, p<0.0001	42%, 72% 0.10, 0.44 p=0.056, p<0.0001
	Hypopigmentation (presence)	88%, 100%	84%, 96%	86%, 96%
C	Geographic atrophy (presence)	96%, 100% 0.85, 1.00 p<0.0001	96%, 98% 0.85, 0.94 p<0.0001	96%, 98% 0.73, 0.89 p<0.0001
	Geographic atrophy * (area covered)	95%, 97% 0.83, 0.92 p<0.0001		
	Neovascular AMD (presence)	84%, 92% 0.65, 0.70 p<0.0001	88%, 94% 0.72, 0.77 p<0.0001	94%, 100% 0.74, 1.00 p<0.0001
	Neovascular AMD (features)	80%, 90% 0.33, 0.56 p=0.002, p<0.0001	80%, 90% 0.34, 0.53 p=0.002, p<0.0001	94%, 98% 0.49, 0.74 p<0.0001
	Neovascular AMD (scar/fibrous)	92%, 96% 0.67, 0.81 p<0.0001	96%, 98% 0.84, 0.92 p<0.0001	96%, 100% 0.66, 1.00 p<0.0001
	Neovascular AMD (retinal haemorrhage)	94%, 98%	94%, 100%	98%, 100%
	Neovascular AMD * (area covered)	87%, 93% 0.67, 0.75 p<0.0001		

Taken from: Scholl et al. Ophthalmology. (Scholl, H. P. *et al*; 2004b)

4.4 Concordance and symmetry between eyes in bilateral AMD

Three-hundred and seventy-five patients (47%) out of a total of 790 patients recruited and graded at the time of analysis, had evidence of late AMD affecting both eyes. The remaining 415 patients were graded as having either drusen in both eyes (n=132;17%), GA in one eye and soft drusen in the other (n= 41; 5 %), CNV or PED in one eye and soft drusen in the other (n=222;28 %), or hard drusen only in one eye and CNV in the other (n=20; 2.5%).

4.4.1 Concordance of late AMD

Of these 375 patients (750 eyes), 67% were female. The median age at entry into the study was 78 years for both sexes (range 56 - 93). Using data from the image grading, a concordant phenotype was observed in 281/375 patients (75%); 194 (52%) patients were graded as having CNV, 82 with GA (22%) and 5 with PED (1%) in both eyes (see table 15 {page 130}). In view of the availability of fluorescein angiography in only 196 cases and the difficulty in differentiating between PED and CNV from colour photographs alone, the recalculated concordance rate with the CNV and PED phenotypes combined as neovascular AMD was 77% (290/375 patients) which included 208 patients with bilateral neovascular disease and 82 patients with bilateral GA.

The Kappa value for concordance between eyes for all three categories of disease namely CNV, GA and PED as determined from the images was 0.48, (SE 0.05, 95%CI: 0.38-0.57; $p<0.001$) and when the PED and CNV categories were combined as neovascular disease the kappa value increased to 0.49; (SE=0.049, 95%CI: 0.39-0.59; $p<0.001$).

From the grading, 143/750 (19%) eyes (33 patients both eyes, 44 Left eyes, 33 Right eyes) had features of CNV present with evidence of atrophic areas within or adjacent to the lesion. Using retrospective data (missing for 20 eyes), these areas of atrophy were shown to be the result of secondary atrophy occurring after formation of a disciform scar in 93 eyes (65%), from the resolution of PED in 12 eyes (8%) and from CNV occurring at the edge of an area of GA in 18 eyes (13%). This changing phenotype is likely to explain part of the observed discordance. For the purposes of the study these cases were graded as neovascular AMD in keeping with the International Classification.

Table 15: No of patients with a concordant and discordant phenotype between eyes according to digital grading.

	Right Eye (Grading)				
	Phenotype	GA	PED	CNV	**Total**
Left Eye (Grading)	GA	82	1	37	**120**
	PED	0	5	4	**9**
	CNV	47	5	194	**246**
	Total	**129**	**11**	**235**	**375**
	κ= 0.48; Standard Error= 0.047; 95% CI = 0.38-0.57 ($p<0.001$)				

4.4.2 Symmetry of GA from AF images

Of the 50 patients analysed, 32 (64%) were female and the median age was 76 (range 61-93). Twenty-one patients (42%) had evidence of peripapillary atrophy in one or both eyes that was measured in a similar fashion by drawing around the optic disc but not including the optic disc itself. The relevance of peri-papillary atrophy to the aetiology of AMD is at present unknown. The purpose of the study was to see if any differences existed between the eyes. In glaucoma, a study has shown that the area and extent of peripapillary atrophy is

significantly greater and more prevalent in eyes with a disc hemorrhage compared to contralateral control eyes(Ahn, J. K. *et al*; 2004).

The average areas of atrophy were calculated including and excluding the peripapillary atrophy for right and left eyes (see table 16 {page 132}). Correlation coefficients comparing right and left eyes for the atrophic area including and excluding peripapillary atrophy were 0.67 ($p<0.0001$) (95%CI 0.47-0.80) and 0.63 ($p<0.0001$) (95% CI 0.43-0.77) respectively (see figure 12a&b [page 134]). The paired t-test showed no significant difference of these average values between right and left eyes with and without the peri-papillary atrophy (see table 16 {page 132}).

When analysing the pattern of GA, 42% eyes had evidence of a unifocal atrophic lesion (3% horse shoe in shape) and 58% eyes demonstrated a multifocal pattern (see table 17 {page 132}). Kappa statistic was calculated for the agreement between the pattern of atrophy between eyes giving a value of 0.67 (95% CI; 0.46 to 0.88) which represents substantial agreement according to Landis and Koch (Landis, J. R. *et al*; 1977) (see table 3).

Table 16: Comparisons for measurements of areas of atrophy between right and left eyes (sq steridians)

	Area of atrophy including peri-papillary atrophy	Area of atrophy excluding peri-papillary atrophy
No of patients	50	50
Mean of Right Eyes	146.96 (SD 141.5)	136.1 (SD 135.7)
Mean of Left Eyes	140.54 (SD 118.3)	125.1 (SD 110.7)
Mean difference	6.42	10.96
Paired t-test (RvL)	DF=49, t=0.419, p=0.68	DF=49, t=-0.714, p=0.48
Pearson's correlation coefficient (r) (RvL)	0.665 (p<0.0001)	0.629 (p<0.0001)

Table 17: Comparison of shape of atrophic area between right and left eyes.

	L shape		
R shape	unifocal	multifocal	Grand Total
unifocal	17	5	22
multifocal	3	25	28
Grand Total	20	30	100

Kappa = 0.67 (95%CI : 0.46-0.88)

Figure 12a: Graph to show correlation of areas of atrophy (sq steridians) between the left and right eye of each patient including the peripapillary atrophy (r = 0.6650, P<0.0001)

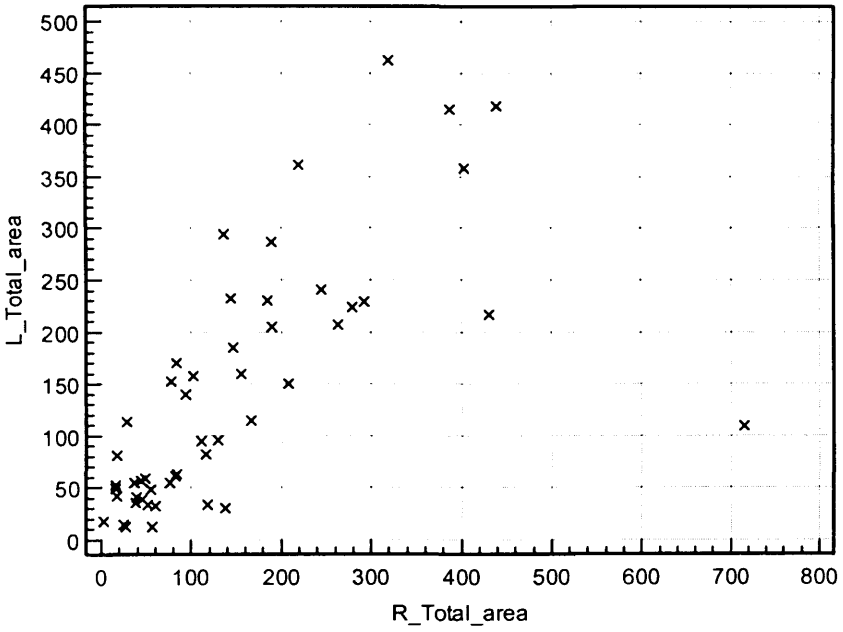
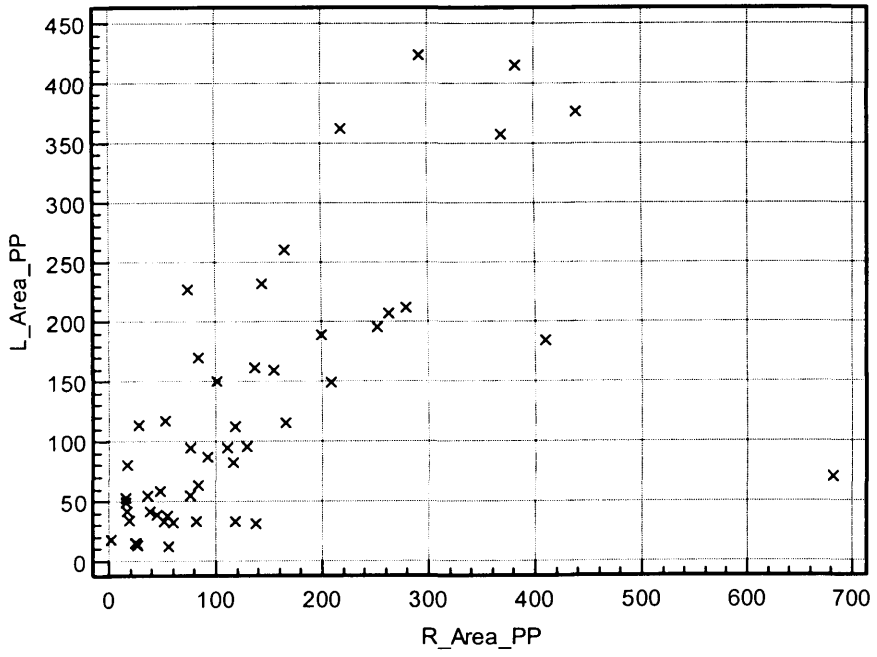


Figure 12b: Graph to show correlation of areas of atrophy (sq steridians) between the left and right eye of each patient excluding the peripapillary atrophy (r =. 0.6294 P<0.0001)



4.5 Characteristics of the fellow eye in patients with unilateral visual loss

4.5.1 Colour fundus image characteristics

Of the 195 patients analysed, 80 (41%) were male. The median age was 76 years. The cause of visual loss was graded as choroidal neovascularisation in 156 patients (CNV group), geographic atrophy in 26 (GA group), avascular pigment epithelial detachment in 3 (PED group) and soft drusen in 10 patients (Drusen group). The median ages, visual acuities and gender ratio of the individual groups are given in table 18 {page 134}. The comparative fellow eye characteristics for the two largest groups (CNV & GA) are shown in table 19 {page 137}. The fellow eyes of the PED and drusen groups were only compared to a limited degree due to small numbers.

Table 18: Demographics of patient cohort segregated by cause of visual loss

	Feature causing loss of vision			
	GA (n=26)	CNV (n=156)	PED (n=3)	Drusen (n=10)
Age (Median)	74	76	78	76
Median Visual acuity (worse eye)	6/36 – 6/60	3/60	6/36	6/36
Sex Male Female	 10 (38%) 16 (62%)	 67 (43%) 89 (57%)	 0 3 (100%)	 3 (30%) 7 (70%)

Fellow eye characteristics of GA v CNV group :

Total area of Drusen. There was a statistically significant trend for the fellow eyes of the GA group to have a greater area of the macula covered by drusen and a greater tendency for

the CNV group to have a smaller area covered by drusen. This was confirmed using the chi squared test for trend ($p<0.001$) (see table 19 {page 137}).

Confluent and Distinct Drusen. When drusen of all sizes in all zones were combined, there were significantly more GA fellow eyes with both confluent drusen (Fisher's exact test $p=0.012$) and distinct drusen (Fisher's exact test $p<0.0005$) when compared to the CNV group (see table 19 {page 137} & 24 {page 152}).

Small hard drusen (<63 μm). When considering all zones, there were 17 patients in the CNV group with only small hard drusen (<63μm), fourteen of whom had no more than 10 drusen in each zone. This was in contrast to the GA group where no patients had only small hard drusen in the fellow eye. This difference however was not statistically significant.

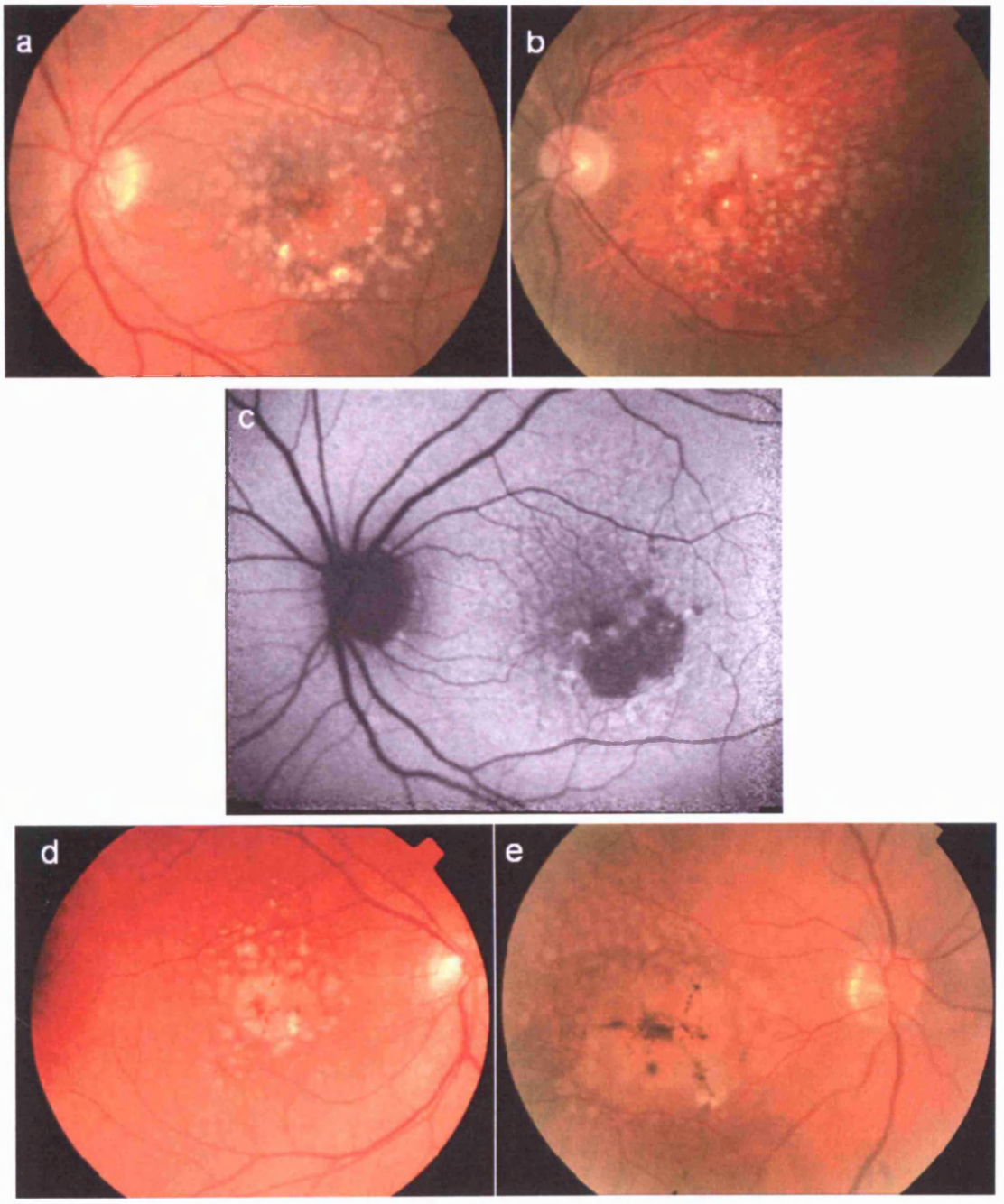
Large drusen (125 to >500μm). In zones 2&3, Drusen of size 125-250μm, whether distinct or confluent were significantly more prevalent in the fellow eye of the GA group compared to the CNV group as indeed were the larger distinct drusen, (250-500μm) in zone 3 ($p=0.0001$). Drusen over 500μm were not seen in zone 1 in either group but were statistically more prevalent in the GA group in zone 2 ($p=0.021$).

Crystalline Drusen. Crystalline or refractile drusen (see figure 13a&b [page 136]) were seen in a significantly greater frequency in GA fellow eyes compared to the CNV group in all zones but the difference was not statistically significant in zone 1(see table 20 {page 138}).

Serogranular Drusen. These large drusen present in zone 1 (see figure 13d&e [page 136]) or drusenoid PED's as they are sometimes called, were again proportionately more frequent in the GA fellow eyes compared to the CNV group (8% v 2%) but the difference was not statistically significant.

Figure 13

(a&b) Colour photographs of crystalline or refractile drusen (c) AF image of crystalline drusen showing central dark area surrounded by halo of increased AF (d&e) examples of a serogranular drusen (or drusenoid PED)



Other AMD lesions. 54% of the GA group had evidence of GA in the fellow eye as well, but not involving fixation. This was compared to 11% of CNV group with GA in the fellow eye ($p<0.00001$).

Table 19: Fellow eye characteristics of those with visual loss due to CNV & GA

Feature causing visual loss	GA (n=26)	CNV (n=156)	P value (NS- not significant)
Fellow eye			
Drusen Area (all zones)			
0%	0 (0%)	1 (1%)	Chi squared
<10%	2 (8%)	77 (49%)	test for
10-24%	10 (38%)	60 (38%)	Trend (χ^2 =
25-49%	11 (42%)	17(11%)	32.363; DF=1)
≥50%	3 (12%)	1 (1%)	P<0.0001
Any Zone			
<63u hard drusen only	0 (0%)	17 (11%)	NS
Confluent Drusen	23 (89%)	98 (63%)	P=0.01*
Distinct Drusen	17 (65%)	44 (28%)	P<0.0005*
Extrafoveal GA	14 (54%)	17 (11%)	P<0.00001*
Extrafoveal CNV	2 (8%)	10 (6%)	NS

* Fisher's exact test

Table 20: Drusen characteristics of fellow eye by zone (GA & CNV eyes)

Zone 1 presence in fellow eye of:	GA as cause of visual loss (n=26)	CNV as cause of visual loss (n=156)	Fisher's exact test (NS= not significant)
<63	25 (96 %)	143 (92 %)	NS
63-125	19 (73 %)	96 (62 %)	NS
125-250 distinct	2(8 %)	7 (4 %)	NS
125-250 confluent	7(27 %)	43(28 %)	NS
250-500 distinct	0 (0 %)	2 (1 %)	NS
250-500 confluent	4 (15 %)	9 (6 %)	NS
>500 distinct	0 (0 %)	0 (0 %)	
>500 confluent	0 (0 %)	0 (0 %)	
crystalline	2 (8 %)	2 (1 %)	NS
serogranular	2 (8 %)	3 (2 %)	NS
Zone 2			
<63	25 (96%)	150 (96%)	NS
63-125	24 (92%)	121 (78%)	NS
125-250 distinct	12 (46%)	23 (15%)	P < 0.001
125-250 confluent	19 (73%)	76 (49%)	P < 0.05
250-500 distinct	3 (12%)	5 (3%)	NS
250-500 confluent	9 (35%)	34 (22%)	NS
>500 distinct	0 (0%)	1 (0.6%)	NS
>500 confluent	3 (12%)	2 (1%)	P < 0.05
crystalline	12 (46%)	8 (5%)	P = 0.000001
Zone 3			
<63	26 (100%)	147 (94%)	NS
63-125	19 (73%)	94 (60%)	NS
125-250 distinct	11 (42%)	18 (12%)	P < 0.0005
125-250 confluent	19 (73%)	47 (30%)	P < 0.00005
250-500 distinct	6 (23%)	2 (1%)	P = 0.0001
250-500 confluent	6 (23%)	15 (10%)	NS
>500 distinct	0 (0%)	0 (0%)	
>500 confluent	1 (4%)	3 (2%)	NS
crystalline	4 (15%)	1 (0.6%)	P < 0.005

4.5.1.1.1 Fellow eye of avascular pigment epithelial detachment group

Only three cases of unilateral visual loss were due to pigment epithelial detachment. This low figure was partly due to the difficulty in diagnosing PED from colour images without fluorescein angiography in all cases. The PED fellow eyes were more likely to have larger and confluent drusen sized between 250-500μm (66% v 15% in GA group and 5% in CNV group, the latter comparison being statistically significant p=0.01) in zone 1, but no eye had

evidence of drusen over 500μm. There were also no crystalline or serogranular drusen present in any of the PED fellow eyes.

4.5.1.1.2 Fellow eye of drusen causing visual loss

Surprisingly, in 9/10 patients, there was no evidence of any other lesion other than drusen in both the eye with visual loss and the fellow eye (FFA only available in 3 cases). In the tenth patient there was evidence of GA in the fellow and interestingly only drusen in the worse affected eye. All eyes had evidence of small <63μm drusen in all zones and 6/10 eyes had intermediate sized drusen (63-125μm) in all zones.

4.5.2 Autofluorescence image characteristics

Of the 135 consecutive patients with unilateral visual loss, aged between 57 and 92 (median 76), 83 were females (61%). The median VA in the better eye was 6/9 and in the worse affected eye was 3/60. The AF images of the worse affected eye and the fellow eyes were graded in conjunction with the colour fundus photographs in order to determine the cause of visual loss and to determine any corresponding changes in the fellow eye relating to specific AF changes.

4.5.2.1 Affected Eye

4.5.2.1.1 Colour Photographs

According to the colour photographs,109 /135 patients were graded with CNV, 18 with GA, 2 with PED and 6 with drusen in the worse affected eye. The fellow eye was then analysed according to the cause of visual loss in the affected eye.

4.5.2.1.2 Autofluorescence Images

Main area of altered AF

Each of the phenotypes causing visual loss were analysed separately to identify specific autofluorescence changes (see table 21 {page 143}). CNV was most commonly associated with a unifocal, heterogeneous lesion of altered AF (60%)(see figure 14a [page 142]). GA was associated with either multifocal (56%) (see figure 7c [page 91]) or unifocal (44%) (see figure 7d), homogeneous areas of decreased AF in all 18 cases . Only 2 eyes were graded with PED and both were noted to have a unifocal, heterogeneous area of altered AF. Of the six eyes graded with drusen, 67% had evidence of homogeneous areas of decreased AF (unifocal or multifocal).

Patterns of increased AF

The most frequent patterns of increased AF associated with CNV were '**focal**' areas (see figure 15a&b [page 147])(25%) in and around the main lesion followed by a partial or complete '**band of increased AF**' in 21% (see figure 15c&d [page 147]).

In GA, a '**reticular**' pattern of increased AF was the commonest seen present in 33% of cases (see figure 15e&f [page 148]) followed by a '**mixed**' pattern in 22% of the group. A '**lace-like**' pattern (see figure 15g&h [page 148]) was seen in 17% and 3% of the GA and CNV group respectively.

In the 2 cases with PED, 1 showed a '**focal**' pattern and the other a '**band**' pattern of increased AF and in those eyes with drusen, a '**mixed**' pattern was seen in 33% cases.

Patterns of background AF

In all phenotypes the commonest background pattern of autofluorescence was a homogeneous pattern. A diffusely irregular pattern was also seen in 33% of the GA group.

Figure 14

AF images and corresponding colour images to demonstrate AF classification (a &b) unifocal heterogeneous lesion predominantly associated with CNV (c&d) multifocal homogeneous lesion predominantly associated with GA

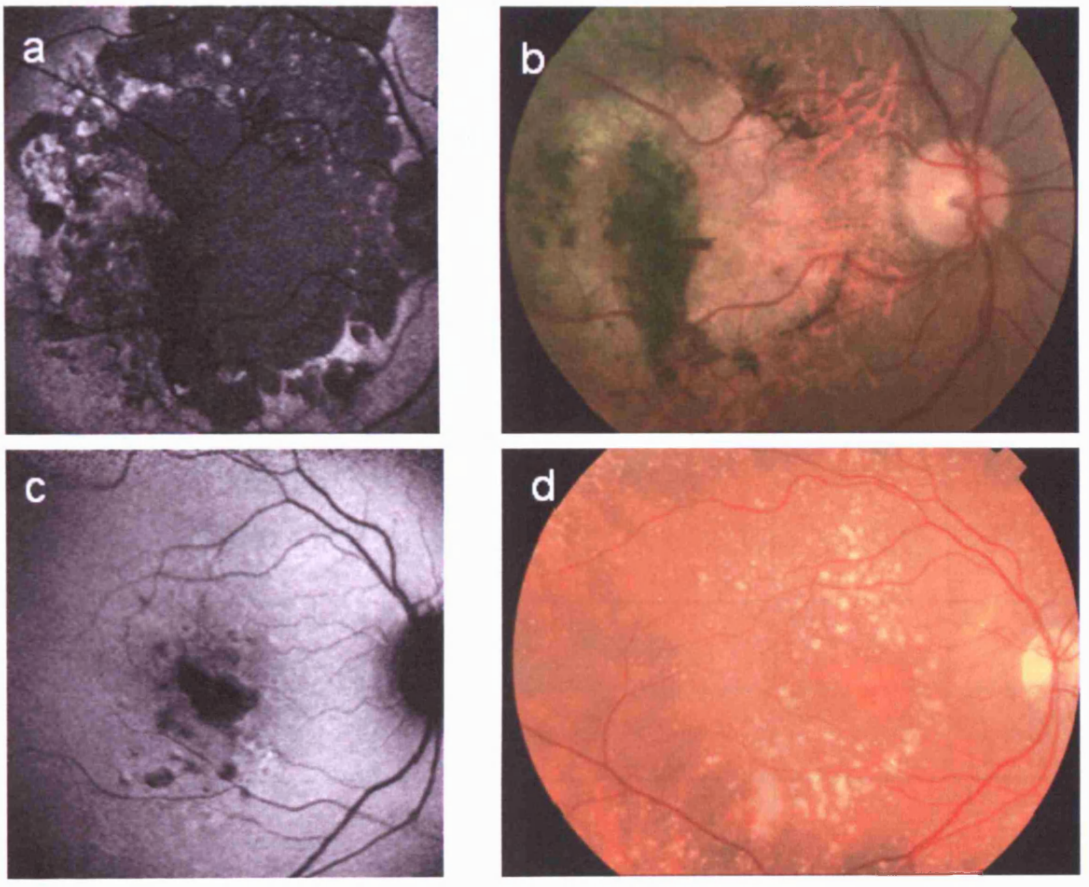


Table 21: AF characteristics for eyes with GA, CNV, PED and Drusen causing visual loss

Affected Eyes	AF characteristics	GA as cause of visual loss (n=18)	CNV as cause of visual loss (n=109)	PED as cause of visual loss (n=2)	Drusen as cause of visual loss (n=6)
Main Lesion-abnormal AF	Unifocal, homogeneous area of decreased AF	8 (44%)	15 (14%)	0	**3** (50%)
	Multifocal, homogeneous areas of decreased AF	**10** (56%)	7	0	1
	Unifocal, heterogeneous, areas of altered AF	0	**65** (60%)	**2** (100%)	0
	Multifocal, heterogeneous, areas of altered AF	0	21 (19%)	0	1
	Normal darkened area at fovea	0	0	0	0
	Too Poor Quality to Grade	0	1	0	1
Pattern of increased AF	No increase	2 (11%)	14 (13%)	0	1
	Focal Increases	2 (11%)	**27** (25%)	**1** (50%)	0
	Reticular pattern	**6** (33%)	18 (17%)	0	1
	Lace-like pattern (Inc)	3 (17%)	3 (3%)	0	0
	Partial/full band of increased	1 (6%)	23 (21%)	**1** (50%)	0
	Mixed	4 (22%)	22 (20%)	0	**2** (33%)
	Poor Quality	0	2	0	2 (33%)
Background AF	Homogeneous	**11** (61%)	**72** (66%)	**2** (100%)	**3** (50%)
	Diffusely Irregular	6 (33%)	25 (23%)	0	1
	Focal increases	1	0	0	0
	Poor Quality	0	12 (11%)	0	2 (33%)

4.5.2.2 Fellow Eye

Areas of predominantly decreased AF

A summary of autofluorescence findings in the fellow eye of the various phenotypic groups is given in table 22 {page 145}. In those fellow eyes with GA in the worse affected eye, the most frequent patterns of predominantly decreased AF were 'multifocal, heterogeneous' areas of altered AF (44%) and 'multifocal homogeneous' areas of decreased AF (28%). 1 eye demonstrated a 'normal pattern' at the fovea.

This was in contrast to the fellow eyes of the CNV group, where the majority of the group (26%) had a 'normal pattern' of decreased AF at the fovea and 8 of which (7%) had no evidence of increased AF either. The second most frequent pattern in the CNV fellow eye group (24%) were 'multifocal, heterogeneous' areas of altered AF. Of the comparison group 80% had a 'normal pattern' of decreased AF at the fovea only.

The most frequent patterns of decreased AF in the PED and drusen groups were 'unifocal, heterogeneous' (see figure 14a&b [page 142]) and 'multifocal, homogeneous'(see figure 14c&d) respectively.

Patterns of increased AF

All eyes from the GA group had evidence of abnormally increased AF. The most common pattern in this group being '**focal increases**' of AF (50%) followed by a 'reticular pattern' in 22%. In the CNV group, a '**reticular pattern**' was the most frequent (27%) with 'no increased AF' as the second most representative pattern (26%). 'Focal increases' of AF were also the most common pattern noted in the drusen group (i.e. like GA group) and 'no increase in AF' was the most frequent pattern in the comparison group (55%).

Background AF pattern

As in the affected eyes, a homogeneous background was the most frequent pattern in the fellow eye of all phenotypic groups including the comparison eyes.

Table 22: AF characteristics of fellow eyes of those with visual loss and comparison eyes

Fellow Eyes	AF characteristics	GA in affected eye (n=18)	CNV in affected eye (n=109)	PED in affected eye (n=2)	Drusen in affected eye (n=6)	Comparison Group Eyes
Areas of predominantly decreased AF	Unifocal, homogeneous area of decreased AF	0	10	0	1	0
	Multifocal, homogeneous areas of decreased AF	5 (28%)	18	0	**3** (50%)	2
	Unifocal, heterogeneous, areas of altered AF	2 (11%)	20	**2** (100%)	0	2
	Multifocal, heterogeneous, areas of altered AF	**8** (44%)	26 (24%)	0	1	2
	Normal darkened area at fovea	1	**28** (26%) (8 had no increased AF either)	0	1	**32** (80%)
	Too Poor Quality to Grade	2	7	0	0	2

Fellow Eyes cont'd	AF characteristics	GA in affected eye (n=18)	CNV in affected eye (n=109)	PED in affected eye (n=2)	Drusen in affected eye (n=6)	**Comparison Group Eyes**
Pattern of increased AF	No increase	0 (0%)	**28** (26%)	0	1	**22** (55%)
	Focal Increases	**9** (50%)	25 (23%)	0	**3** (50%)	5 (13%)
	Reticular pattern	4 (22%)	**29** (27%)	0	0	12 (30%)
	Lace-like pattern	0	3	0	0	0
	Patchy pattern (see figure 15i&j [page 148])	1	7	0	1	0
	Mixed	2 (both included lacelike)	13 (10 retic & focal)	**1** (50%) (lacelike & focal)	1 (retic & focal)	0
	Poor Quality	2	4	1	0	1
Background AF	Homogeneous	**15** (83%)	**82** (75%)	**1** (50%)	**5** (83%)	**34** (85%)
	Diffusely Irregular	1 (6%)	16 (15%)	0	0	5 (13%)
	Focal increases	0	0	0	0	0
	Poor Quality	2	11 (10%)	1 (50%)	1	1

Figure 15: (a)-(d)

AF and corresponding colour images to demonstrate various AF patterns in the affected and fellow eye (a&b) focal (c&d) band

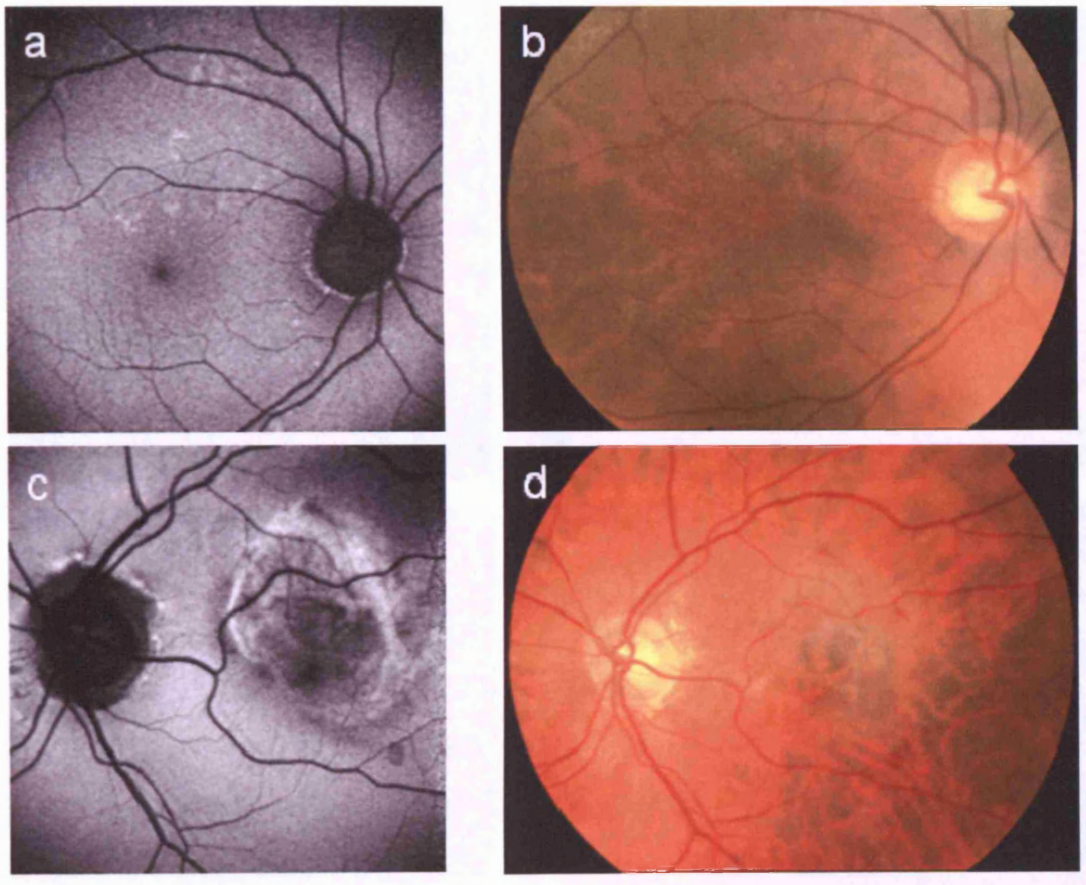
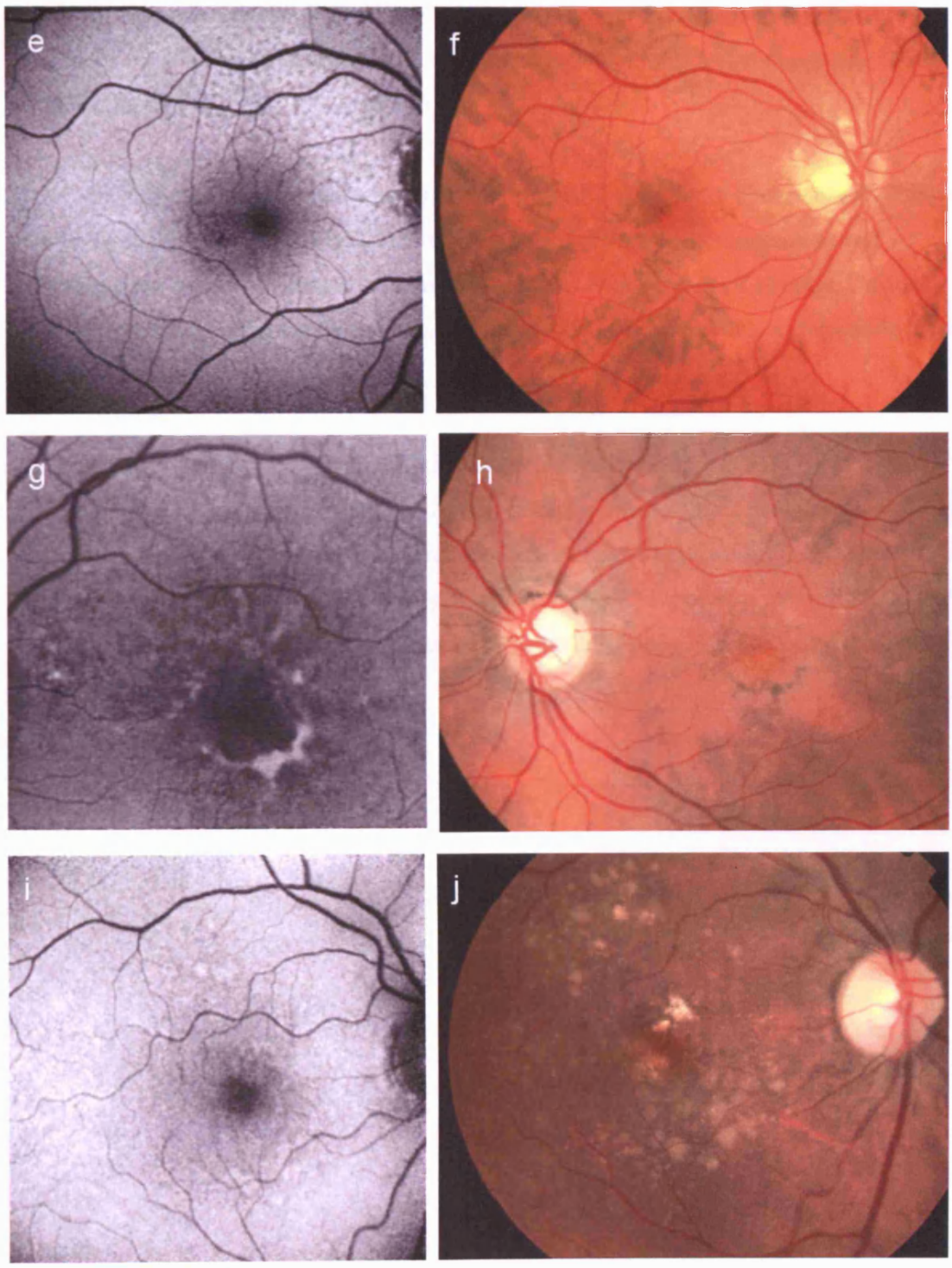


Figure 15: (e)-(j)

AF and corresponding colour images to demonstrate various AF patterns in the affected and fellow eye (e&f) reticular (g&h) lace-like (i&j) patchy



4.5.2.2.1 Corresponding lesions

Data were also analysed to determine what the various autofluorescence features corresponded to when compared to the colour images. The most frequent corresponding lesions are given in Table 23 {page 150}.

In the affected eye, homogeneous areas of decreased AF were more commonly associated with GA and heterogeneous areas with CNV. In the fellow eye, homogeneous and heterogeneous areas of decreased AF were associated with patches of atrophy. Unifocal areas of decreased AF in the fellow eye did not always correspond to any detectable lesion on the colour image.

Considering the patterns of increased AF, a 'reticular pattern' appeared to be most commonly associated with scattered drusen in both the affected and fellow eyes. A 'focal' pattern was noted to correspond most frequently with parafoveal drusen in fellow eyes and 'lacelike' changes were most commonly associated with RPE pigment change. 'Bands of increased AF' were more frequently seen at the edge of lesions in the affected eye only.

Table 23: Corresponding lesions to AF characteristics in affected and fellow eyes

Pattern of AF		Affected Eye		Fellow Eye	
		Commonest Corresponding lesion	% of cases with pattern	Commonest Corresponding lesion	% of cases with pattern
Decreased AF	Unifocal homogeneous	Area of GA (14)	54%	No corresponding lesion (9)	82%
	Multifocal homogeneous	Area of GA (9)	50%	Area of GA (12)	67%
	Unifocal heterogeneous	Area of CNV (53)	79%	No corresponding lesion (15)	63%
	Multifocal heterogeneous	Area of CNV (20)	91%	Area of GA (15)	43%
Increased AF	Reticular	Scattered drusen (5)	20%	Scattered drusen (23)	70%
	Focal	Edge of lesion (10)	33%	Large Parafoveal drusen (14)	38%
	Lacelike	RPE change (4)	67%	RPE change (3)	100%
	Band	Edge of lesion (17)	68%	N/A	0
	Patchy	N/A	0	RPE change (3)	33%
	Mixed	Scattered Drusen (12)	43%	RPE change (6) Scattered drusen (6)	35% 35%

4.6 Location of the fovea

For the 150 subjects in the study (211 eyes), the median age was 29 (age range was 1month to 43). The average horizontal distance between the fovea and the centre of the disc, as calculated from the entire group was 2.76 +/- 0.33 DD or 16.07 +/- 1.25 degrees. The average vertical distance to the fovea for the whole group was 0.31 +/- 0.21 DD or 1.83 +/- 1.19 degrees.

When comparing measurements on the subjects with 35 degree field images to those with 50 degree field images, there was no significant difference between the groups except for the horizontal measurements made in degrees where the 50 degree field measurements were significantly higher than the 35 degree field measurements (see table 24 {page 152}).

In those patients where data were available for both eyes (n=61), the results unexpectedly showed that there was a statistically significant difference between eyes in both the mean horizontal and vertical measurements especially in degrees (see table 25 {page 153}). To test whether a tortional component could be playing a role in this discrepancy, the oblique distance (O) between fovea and centre of the disc was also compared between eyes (see figure 8 [page 94]). The difference between the oblique measurements in Disc Diameters was not statistically significant between eyes. Although the Degree measurements were statistically different, they were not clinically significant with the difference only representing 0.2 degrees (see table 26 {page 153}).

Table 24: Horizontal and Vertical distances for foveal location in disc diameters and degrees (& comparison between 35 and 50 degree images)

Mean Distance		Whole sample (n=150)	35 degrees Images (n=100)	50 degrees Images (n=50)	Comparison between 35° and 50° images (t-test)
Horizontal	Disc Diameters	2.76 +/- 0.33 (12%)	2.79 +/- 0.33 (12%)	2.69 +/- 0.31 (12%)	Difference : -0.0995 95% CI : -0.2111 to 0.0121 t=1.762 DF=148 P = 0.0802 Not Sig
	Degrees	16.07 +/- 1.25 (8%)	15.53 +/- 0.86 (6%)	17.16 +/- 1.19 (7%)	Difference : 1.6306 95% CI : 1.2947 to 1.9665 t=-9.592 DF=148 P < 0.0001
Vertical (below disc)	Disc Diameters	0.31 +/- 0.21 (68%)	0.31 +/- 0.20 (65%)	0.32 +/- 0.22 (69%)	Difference : 0.0147 95% CI : -0.0562 to 0.0856 t=-0.410 DF=148 P = 0.6827 Not sig
	Degrees	1.83 +/- 1.19 (65%)	1.72 +/- 1.10 (61%)	2.06 +/- 1.34 (65%)	Difference : 0.3424 95% CI : -0.0632 to 0.7480 t=-1.668 DF=148 P = 0.0974 Not sig

Table 25: Comparison of foveal location between right and left eyes

Difference between Right and Left Eyes (paired t-tests)		Whole sample (n=61)		
		Right	Left	Difference
Horizontal	Disc Diameters	2.79 +/- 0.36	2.74 +/- 0.34	Mean difference : 0.0545 Standard deviation : 0.2372 95 % CI : -0.0062 to 0.1153 t=1.795 DF=60 P = 0.0776
	Degrees	16.31 +/- 1.40	15.94 +/- 1.53	Mean difference : 0.3744 Standard deviation : 0.5895 95 % CI : 0.2234 to 0.5253 t=4.960 DF=60 P < 0.0001
Vertical (below disc)	Disc Diameters	0.18 +/- 0.19	0.42 +/- 0.19	Mean difference : -0.2404 Standard deviation : 0.2745 95 % CI : -0.3107 to -0.1701 t=-6.840 DF=60 P < 0.0001
	Degrees	1.04+/- 1.17	2.43 +/- 1.13	Mean difference : -1.3883 Standard deviation : 1.6251 95 % CI : -1.8045 to -0.9721 t=-6.672 DF=60 P < 0.0001

Table 26: Comparison of foveal location between right and left eyes in those with bilateral data

	Sample with bilateral data (n=61)		
	Right	Left	Difference
Mean Oblique measurement (DD)	2.81	2.78	Mean difference -0.0288 +/-0.2309 95 % CI -0.0879 to 0.0304 t=-0.973 DF=60 P = 0.3343 Not statistically significant
Mean Oblique measurement (Degrees)	16.39	16.16	Mean difference -0.2255 +/- 0.5377 95 % CI -0.3632 to -0.0878 t=-3.275 DF=60 P = 0.0018 Statistically significant but not clinically significant

4.7 Regional susceptibility of the macula to GA

Entire cohort

Of the 133 patients (200 eyes) analysed, 66% were female and the median age was 76. The final analysis included 101 right eyes and 99 left eyes. Sixty-seven patients contributed both eyes to the analysis. Where only right or left eyes were included in the study, the fellow eye was excluded either due to ill-defined atrophy with no autofluorescence image (15 cases), blurred images (8 cases), CNV with surrounding atrophy (19 cases) or drusen with no visible GA (24 cases).

On analysis of the final aggregate of the entire cohort of eyes, the results show increasing susceptibility of geographic atrophy towards the fovea in both eyes with a series of concentric circles of increasing frequency towards the centre (see figure 16a [page 156]). There did not appear to be a definite predilection for one region, but the most susceptible part in both eyes was located in a parafoveal location within 2 degrees of the centre of the fovea (see figure 16a).

In order to determine more accurately where the atrophic process had started and how it progressed, those with longitudinal data were analysed separately.

Patients with longitudinal data

A separate aggregate matrix was carried out for the 26 eyes (12 R; 14 L, 21 pts) with longitudinal data with images spanning between 2 and 11 years, to establish if a more susceptible area existed in early atrophy.

The results showed a temporal and infero-temporal displacement of the centre of the contour map relative to the fovea in the right and left eyes respectively (see figure 16 b&c [page 157]), suggesting a particular susceptibility to the atrophic process in these regions of the macula.

4.7.1 Progression of atrophy

Out of the 26 eyes (12 R; 14 L) with longitudinal data, 15 eyes (9R, 6L) were classified with a circumferential pattern of progression, 10 eyes (3R, 7L) with a centrifugal pattern and 1 (L) with centripetal and centrifugal extension.

Figure 16

Figure 16a: Macular susceptibility contour map of entire cohort of patients (n=133)

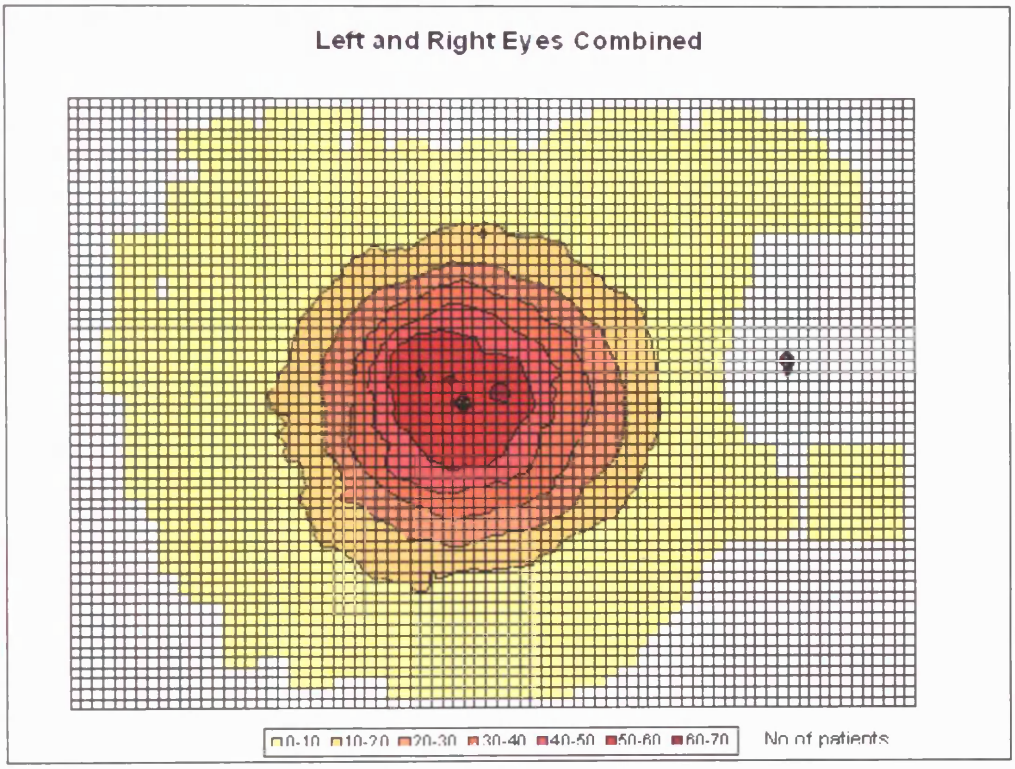
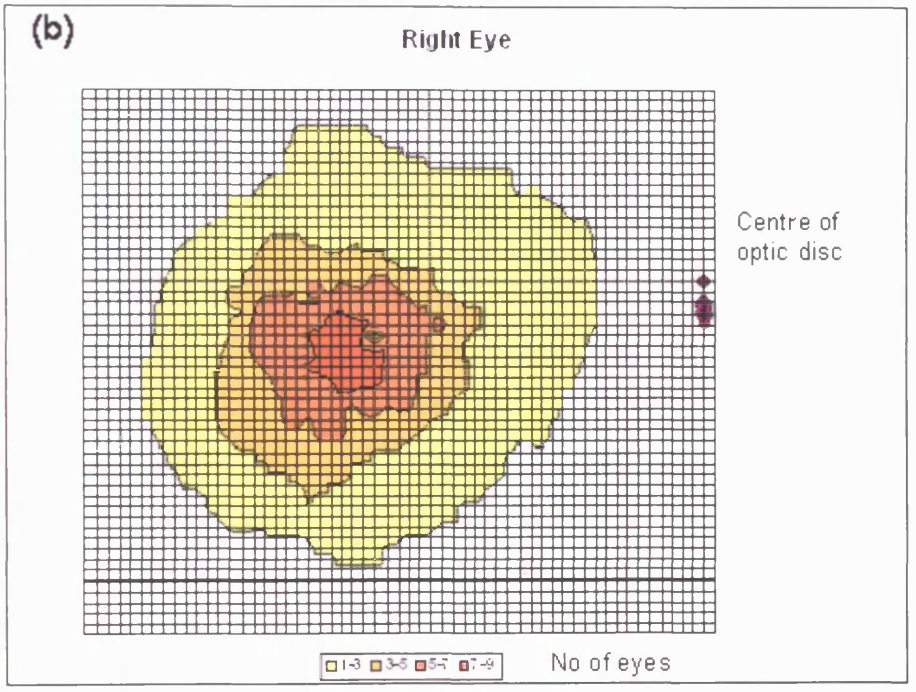
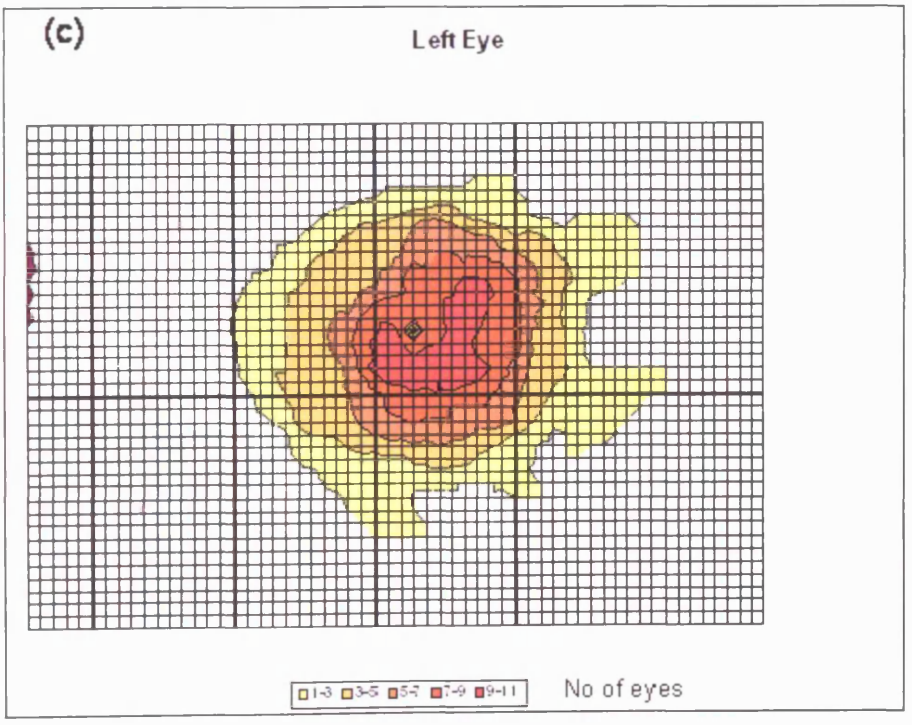


Figure 16

Figure 16 b&c: Contour maps of right and left eyes of patients with longitudinal data





4.8 Analysis of the RPE in Neovascular AMD using AF imaging.

Of the total 65 patients analysed, 27 were male and 38 female. The median age was 78 (range 58 - 90). The median ages of the three groups were 78, 75 and 78 respectively. One eye from each patient was analysed; 35 left eyes and 30 right eyes. In group 2, where the AF images were taken a few months after the diagnosis of CNV or PED, the average interval was 4 months (range 1-6 months). In group 3, all the patients had established disciform scars and had lost vision in that eye more than a year prior to presentation. The median Snellen visual acuity in the three groups was 20/60, 20/100 and 20/600 respectively.

Vessel to vessel measurements

Vessel to vessel measurements, which were taken as an internal control, compared well between autofluorescence and fluorescein images in all cases and the difference was not statistically significant (mean difference; -0.008 mm, 95%CI: -0.056, 0.040; p= 0.73). This indicated that magnification differences were adequately adjusted for using the digital Topcon IMAGEnet 2000 ©software.

Total area of lesion (Group 1 only)

The main area of abnormal autofluorescence was more widespread than the main area of abnormal fluorescence on FFA in 18 of the 20 cases, (mean difference in size = +1.63 mm^2; 95%CI= 0.58, 2.68; p=0.004). This is demonstrated with the Bland Altman plot (see figure 17 [page 159]). In three of these cases where the main abnormal area extended beyond the edges of the AF image the area was only measured up to the limits of the frame. It would be expected therefore that the difference in total area between the AF and FFA image would be even larger than quoted in these 3 cases.

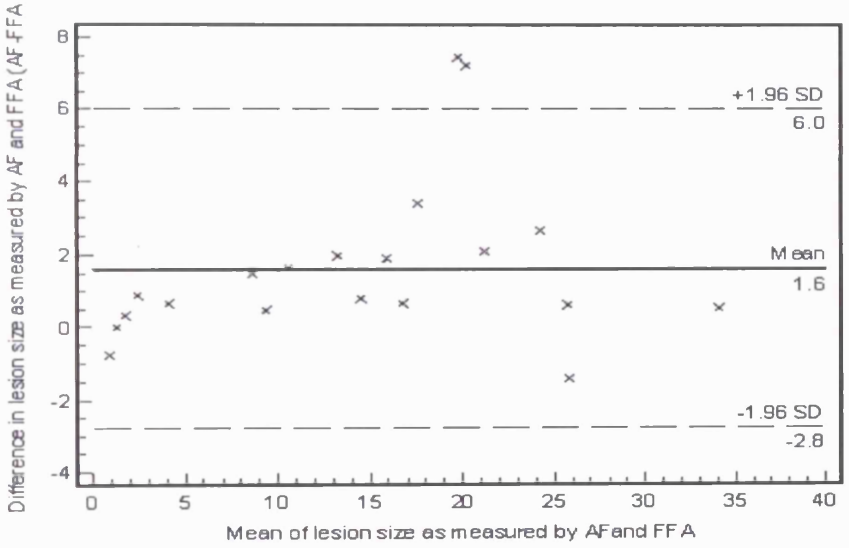


Figure 17: Bland Altman plot (see section 3.11.3) showing the difference between the extent of the AMD lesion as measured from the AF image versus the FFA image plotted against the average of these measurements. The size of the lesion was larger on the AF image in the majority of cases.

Figure 18

Comparison between FFA and AF images in group 1

(a): FFA of a neovascular lesion with areas of hyperfluorescence especially temporally and some subretinal blood superiorly and nasally. **(b)**: Corresponding AF image showing an area of increased AF at the edge of the area of leakage (area marked A) seen on the FFA image. The area marked B represents an abnormal area of AF but there are areas of more normal AF centrally.The area marked C indicates the optic disc. This latter measurement was not used in the study. **(c)**: FFA image of a neovascular lesion with a greater than 50% classic component. **(d)**: Corresponding AF image showing a ring of increased AF surrounding areas of normal AF within the complex. **(e)**: FFA image of an avascular pigment epithelial detachment. **(f)**: Corresponding AF image showing normal AF from the elevated retinal pigment epithelium and a dark rim corresponding to subretinal fluid. 'A' represents the 'normal' area of AF corresponding to the pigment epithelial detachment. 'B&C' represent corresponding abnormal areas on the FFA image and 'D' marks the optic disc. **(g)**: FFA image of a neovascular lesion with 100% occult component and multiple areas of hyperfluorescence. **(h)**: AF image showing 2 areas of decreased AF where areas of leakage are seen on the FFA (see areas labelled 'A&B'). There are also some normal patches of AF within the lesion (marked 'C') with increased AF towards the edge of the lesion. Area 'D' marks the optic disc.

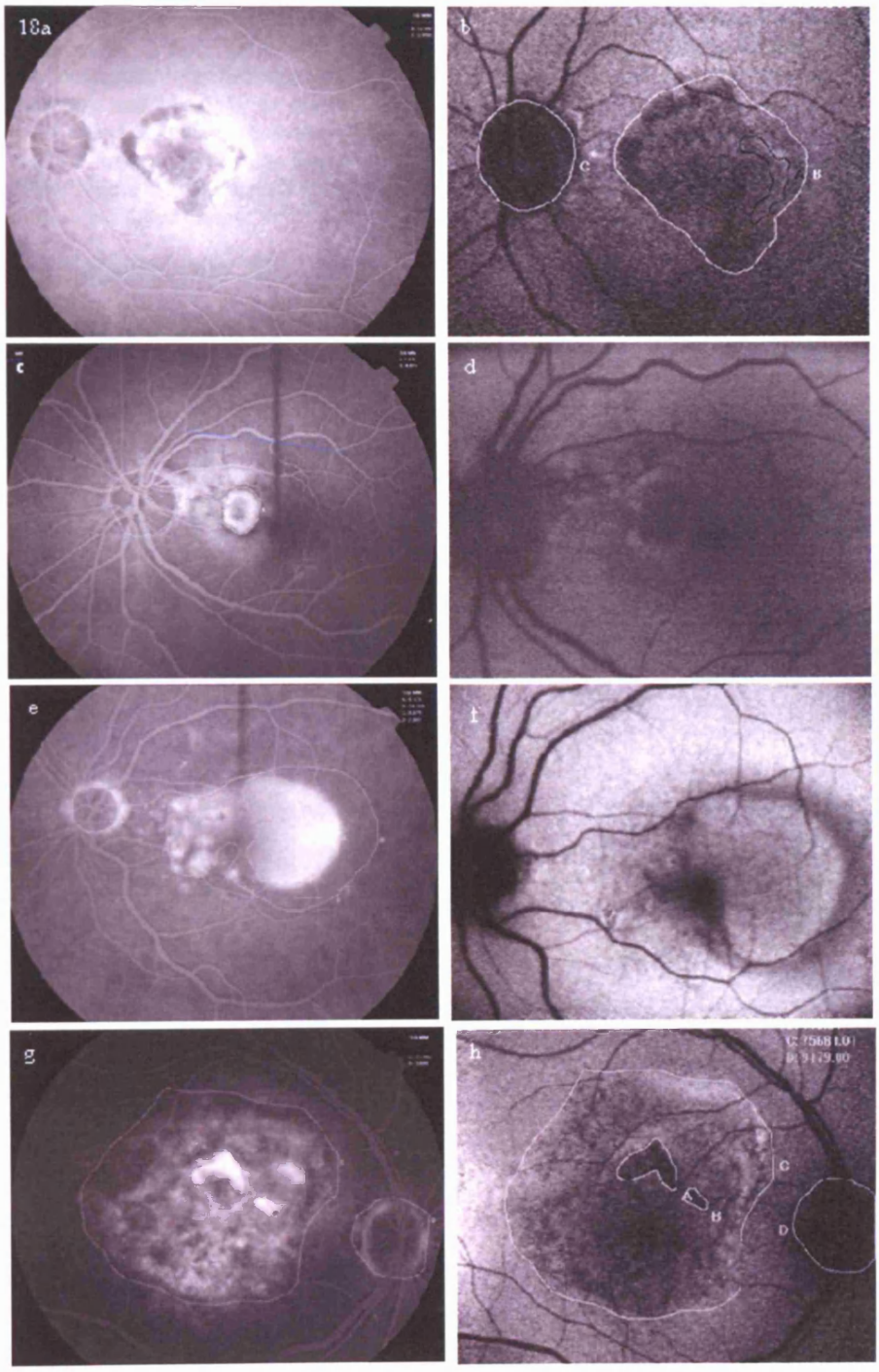


Figure 19

Comparison between FFA and AF images in group 2 (a): FFA image of a neovascular lesion showing an area of intense hyperfluorescence centrally. (b): AF image taken 6 weeks after the FFA image showing an area of decreased AF (area labelled A) corresponding to the area of intense hyperfluorescence and surrounding areas of normal AF. The linear patterns of decreased AF correspond to exudates. (c): FFA image of a neovascular lesion showing a small focus of intense hyperfluorescence adjacent to a subretinal haemorrhage. (d): AF image taken 1 month after the FFA image showing an area of decreased AF (area labelled A) corresponding to the area of intense hyperfluorescence and a surrounding band of increased AF with adjacent linear patterns of decreased AF corresponding to exudates temporally and haemorrhage nasally.

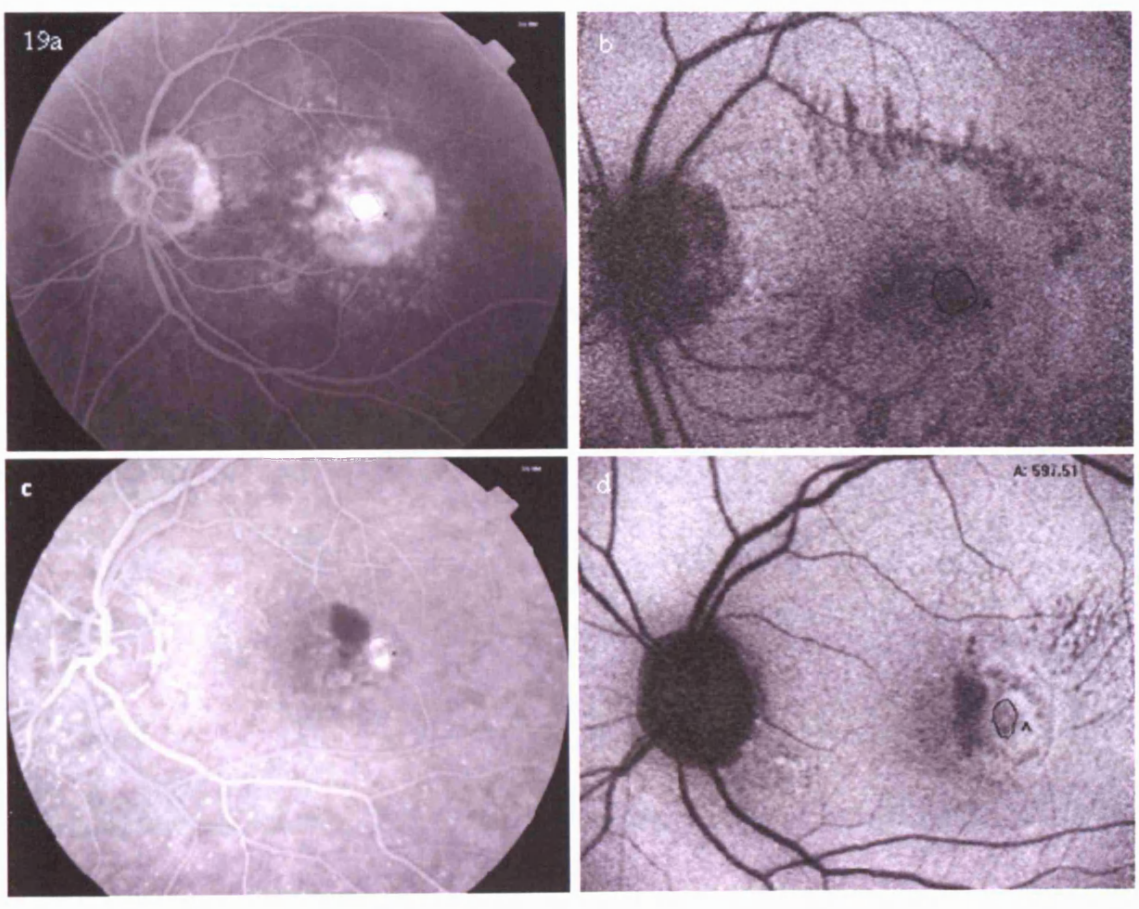
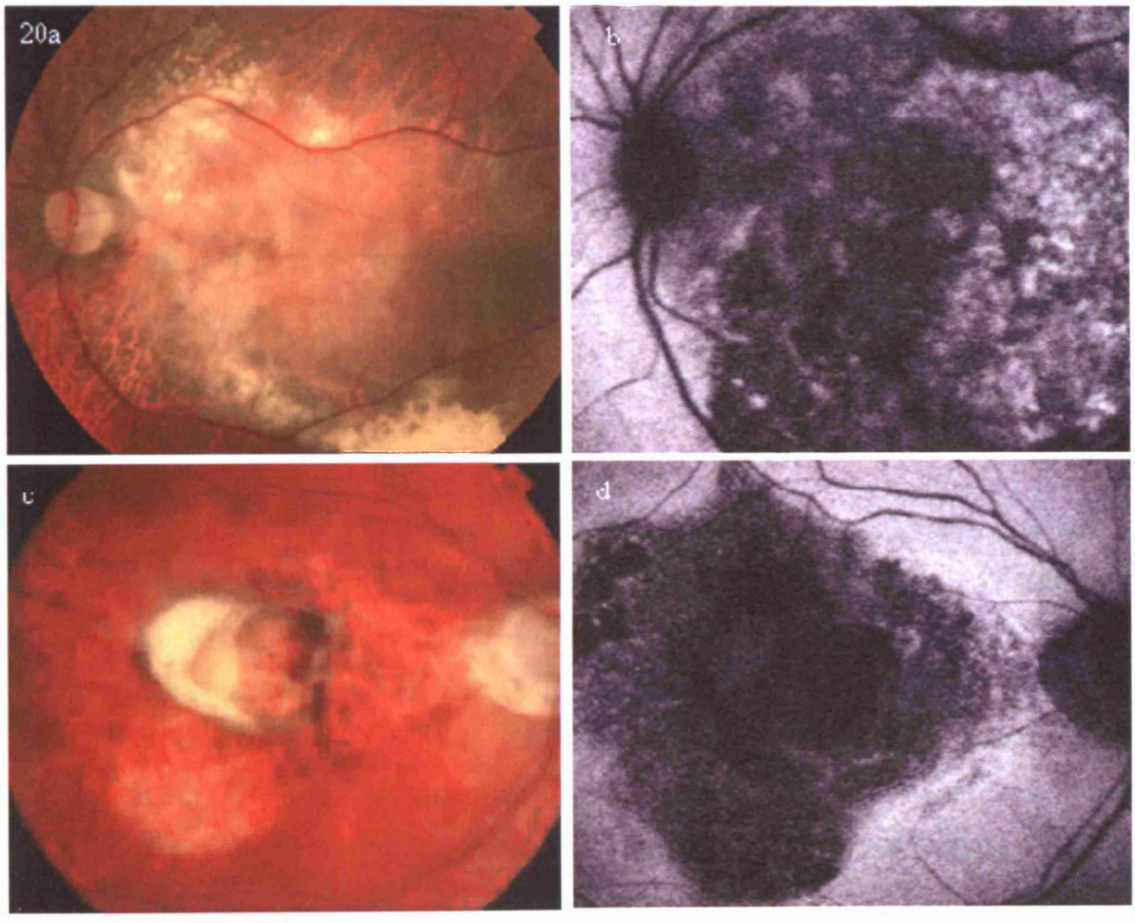


Figure 20

Comparison between fundus photographs and AF images in group 3 (a): Fundus photograph showing exudative disciform lesion. (b): Corresponding AF image showing a heterogeneous pattern of decreased and increased AF within the lesion. (c): Fundus photograph showing disciform scar with surrounding atrophy of the pigment epithelium. (d): Corresponding AF image showing a homogeneous pattern of decreased AF with no normal AF within the lesion.



Spatial correlation of leakage on FFA

In groups 1 and 2, the areas of hyperfluorescence on FFA were mapped to the AF images. In group 1, these mapped to an area of normal AF in 16 cases (see figure 18a-f [page 160]) with an adjacent area of increased AF in 13 of these cases (see figure 18a-d, g,h [page 160]). In 4 cases the hyperfluorescence on FFA corresponded to an area of decreased AF (see figure18 g&h). In group 2, the areas of previous leakage on FFA mapped to areas of decreased AF in all 8 cases (see figure 19 a-d [page 161]). In 4/8 cases an area of increased AF was present adjacent to fluorescein hyperfluorescence (see figure 19 c&d). FFA was not performed in group 3 patients.

4.8.1 Patterns of AF

In group 1, 18/20 cases, demonstrated patches of normal autofluorescence that could be seen within the lesion. In 13 of these, normal AF could be seen at the fovea and 3 had a Snellen acuity of 20/30. Five of the 20 eyes were classified as having a lesion with between 0-50% classic component (see figure 18a [page 160]), 6 patients were graded with a lesion >50% classic (see figure 18 c), 7 were graded with a 100% occult lesion (see figure 18g) and 2 patients had an avascular pigment epithelial detachment (see figure 18e). Of the seven cases with no focal increase in AF adjacent to FFA hyperfluorescence, 3 were 100% occult, 2 had an avascular PED and 2 were classified as < 50% classic.

In group 2, 7/8 cases demonstrated some areas of normal AF within the lesion-see fig 19 b (4 including the fovea). One case with normal AF at the fovea had an acuity of 20/30.

In group 3, focal increases of AF were seen in 27/37 cases (see figure 20 a&b [page 162]). Twenty nine cases consisted of a fibrous scar and 8 were graded as an exudative lesion with a serous detachment. In this group, 31/37 cases had no normal AF (see figure 20a-d [page 162]) and 6/37 cases showed patches of normal AF within the lesion but none at the fovea.

No case had a visual acuity of better than 20/50. Two patterns of AF were seen in this group; 23/37 cases had widespread areas of decreased homogeneous AF throughout the lesion (see figure 20d [page 162]). In 9 of these, surrounding secondary atrophy of the RPE was demonstrated on the colour image (see figure 20c). The second pattern was a more heterogeneous, mottled pattern with areas of increased and decreased AF (see figure 20b). This pattern was seen in 14 cases and 6 had areas of atrophy on the colour image.

In all three groups, areas of decreased autofluorescence corresponded to areas of atrophy (see figures 20 c&d) (n=21), areas of subretinal blood (n=6) (see figure 19c&d [page 161]) or areas of exudates (n=12) (see figure 19 a-d). In one case where the blood was predominantly sub RPE, the intensity of autofluorescence appeared relatively preserved.

Pigment epithelial detachments

Two cases were graded as having avascular PED from group 1. On AF imaging the sub RPE fluid appeared to show normal autofluorescence corresponding to the fundoscopic visible area of pigment epithelial detachment with the typical normal appearance of decay of AF intensity towards the foveal centre. In one case a dark rim could be seen surrounding the elevated area (see figure 18 e&f [page 160]).

4.9 Influence of smoking on type of AMD

The smoking status of the study participants by age and gender is given in Table 27 {page 166}. This table indicates that 65% of the AMD study population (n=711) were female and 46% of them had never smoked. Of the males, 28% were non-smokers and 59% ex-smokers.

Table 28 {page 166}displays the smoking status and AMD group of subjects. It shows that approximately 60% of the cohort of AMD patients were either current or ex-smokers and the remaining 40% were non-smokers.

Multiple logistic regression analysis adjusting for both age and gender, did not show an association between smoking status or increasing number of pack years and type of AMD lesion graded (see Table 29 {page 166}). The odds of 'ex-smokers' compared to 'non smokers' developing neovascular rather than non neovascular AMD when adjusted for age and gender was 0.86 (95%CI: 0.58 to 1.30) and that of current-smokers compared to 'non smokers' was 1.88 (95%CI: 0.91 to 3.89). When pack year data were analysed using the chi squared test for trend (data available on 686 pts), again no difference was seen between the neovascular and non-neovascular groups in the number of pack years smoked (see Table 30 {page 167}).

With the numbers of smokers and nonsmokers recruited, this study should have been adequately powered (80%) to detect a clinical difference of an excess prevalence of CNV in smokers compared to never smokers of 8% or more or an excess prevalence in never smokers compared to smokers of 8% or more. This calculation was based on the sample size and power calculation comparing 2 binomial proportions assuming α=0.05 in a two tailed test (Rosner, B.; 1995).

Table 27: Age, gender and smoking status of patients with late AMD

Female				
Age Group	Current smoker	Ex smoker	Non smoker	Grand Total
50-59	4	3	1	8
60-69	6	22	30	58
70-79	35	93	93	221
80-89	13	71	79	163
90-99	0	3	9	12
Grand Total	(12.5%) 58	(41.5%)192	(46%) 212	462
Male				
Age Group	Current smoker	Ex smoker	Non smoker	Grand Total
50-59	1	5	2	8
60-69	4	17	8	29
70-79	16	62	36	114
80-89	11	60	21	92
90-99	1	3	2	6
Grand Total	(13%) 33	(59%) 147	(28%) 69	249

Table 28: Smoking status according to AMD type

AMD type	current smoker	ex smoker	non smoker	Grand Total
neovascular	(89%) 81	(79%) 268	(81%)229	(81%)578
non neovascular	(11%) 10	(21%) 71	(19%) 52	(19%)133
Total with late AMD	(100%) 91	(100%) 339	(100%) 281	(100%)711

Table 29: Multiple logistic Regression table to assess the influence of smoking on the type of AMD lesion (Adjusted for Age and Gender)

Variable	b-Regression coefficient	p value	OR for neovascular AMD	95%CI (lower)	95%CI (upper)
Age	.0096	.4690	**1.0097**	.9837	1.0363
Gender (female- ref) Male	-.0321	.8753	**.9684**	.6481	1.4469
Smoking Status Ex smoker cf non smoker	-.1467	.4790	**.8636**	.5754	1.2961
Current Smoker cf non smoker	.6315	.0882	**1.8805**	.9098	3.8870
Pack years of smoking (n= 686)	1.2954	.2220	**.9996**	.9918	1.0074

Table 30: Influence of pack years of smoking on AMD lesion type

	Pack years (range)	0	<20	20-40	40-60	>60	Pack years not known	Grand Total
AMD type	neovascular	229(81%)	154(83%)	91(83%)	45(79%)	40(77%)	19(76%)	578
	non neovascular	52(19%)	32(17%)	19(17%)	12(21%)	12(23%)	6(24%)	133
	Total with late AMD	281	186	110	57	52	25	711
Chi-Square test for trend=0.465, DF=1, p=0.5 NS								

4.10 Functional assessment of areas of increased autofluorescence in AMD

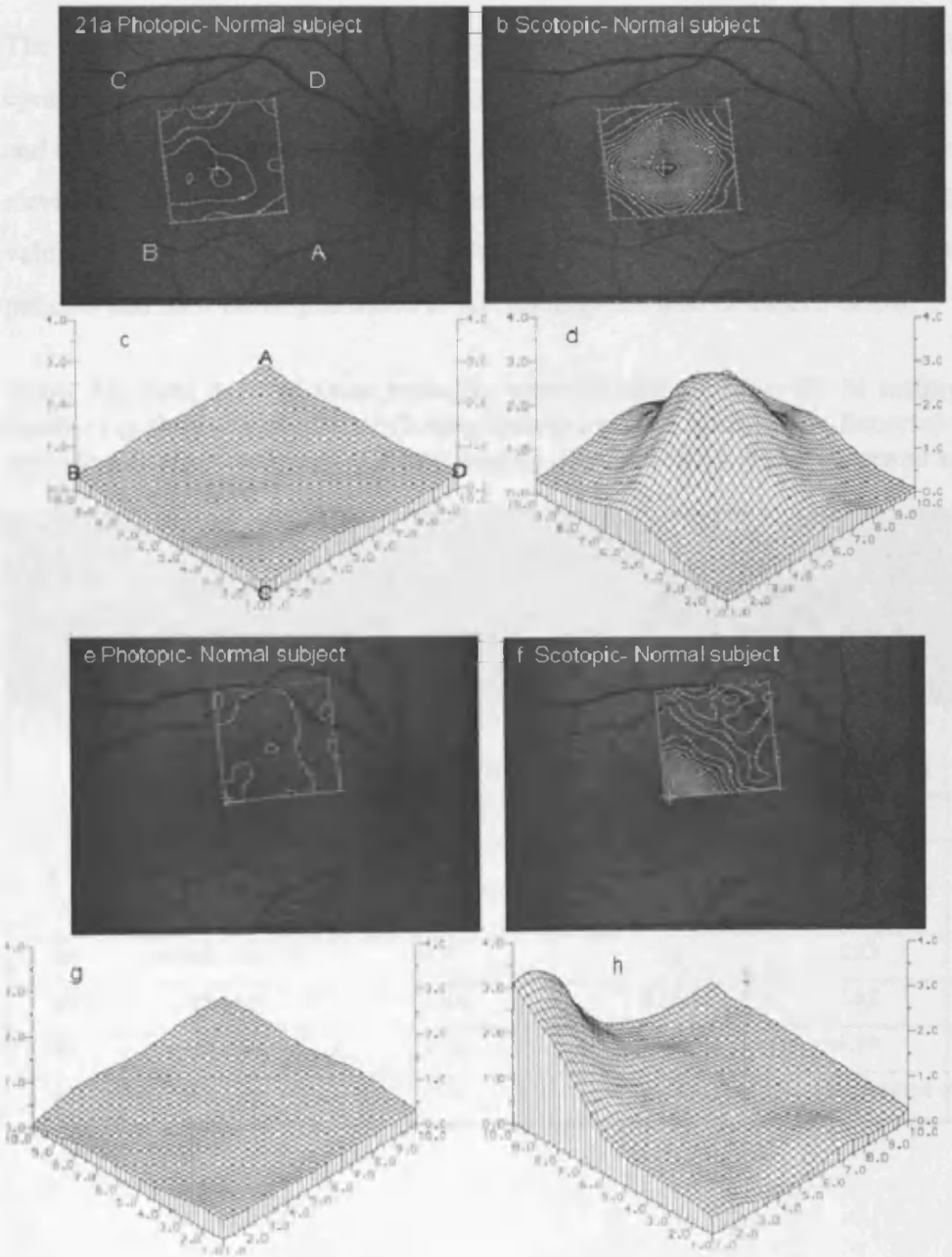
Of the 436 patients reviewed from the database, 38 ARM patients met the inclusion criteria with adequate image quality and a vision of at least 20/40. Of these, seven participated in the study (4 female and 3 male), (mean age, 75 years; range, 68-80 years) (Scholl, H. P. *et al*; 2004a). The predominant fundal features of the seven study eyes included: extrafoveal CNV (two eyes), extrafoveal GA (two eyes), and soft drusen (three eyes) (see Table 31 {page 169}). Median visual acuity amongst the seven study eyes was 20/25 (range, 20/40-20/20).

Table 31: Demographics, visual acuity and predominant fundal features of the seven study subjects

Patient	Age	Sex	Eye	FMM	Study eye	Fellow eye	VA (ETDRS)	VA fellow eye
#1	68	M	L	upper nasal macular quadrant	**Extrafoveal CNV; s/p laser treatment**	few drusen	20/20	6/5
#2	77	M	R	lower nasal macular quadrant	**Juxtafoveal CNV with disciform scar**	Few RPE pigmentary changes; no drusen	20/40	6/6
#3	69	F	R	lower temporal macular quadrant	**Secondary GA after longstanding PED; s/p prophylactic drusen laser; cellophane retinopathy**	Large disciform macular scar after RPE tear	20/25	6/60
#4	78	F	L	central macular FFM	**Extrafoveal GA; RPE hyperpigmentations; drusen**	Subfoveal CNV with disciform scar and PED	20/25	6/24
#5	80	M	L	central macular FFM	**Extensive drusen at the posterior pole including a large juxtafoveal druse**	Subfoveal CNV with disciform scar	20/25	HM
#6	79	F	R	upper temporal macular quadrant	**Large soft and small hard macular drusen**	Subfoveal CNV with large disciform scar	20/20	HM
#7	73	F	L	lower temporal macular quadrant	**Intermediate and large soft and small hard drusen**	Subfoveal CNV with PED	20/20	6/36

Figure 21

Photopic (**a, c, e, g**; left column) and scotopic (**b, d, f, h**; right column) fine matrix mapping (FMM) in a 33-year old normal subject. The first four figures (a-d) show data derived from FMM that was centered on the fovea and the second four figures (e-h) from a FMM that was placed on the nasal upper quadrant with the fovea on the lower temporal corner of the test grid. To allow orientation of the luminance sensitivity contour plots on the FMM images and the three dimensional threshold surface plots, the letters A-D have been added to figure 21c. In all AF contour maps shown, A corresponds to the right lower corner, B to the left lower corner, C to the left upper corner, and D to the right upper corner, respectively. (See Scholl et al (Scholl, H. P. *et al*; 2004a) for scotopic FMM centred on the fovea from an age-matched normal subject)



Taken from Scholl et al 2004 (Scholl, H. P. *et al*; 2004a)

Figure 21 [page 170] shows the results of photopic (a,c,e and g) and scotopic (b,d,f and h) fine matrix mapping in a normal subject (not age-matched). Two sets of fine matrix mapping were performed: The first was centred on the fovea (a-d) and the second placed on the nasal upper quadrant with the fovea on the lower temporal corner of the test grid (e-h). The sensitivity profiles are shown with contour steps of 0.1 log unit (1dB).

The data derived from both the scotopic and photopic FMM testing in all of the seven study eyes are summarised in Table 32. The global estimates of the FMM, the mean (background) and the maximum threshold elevation from baseline are given. The higher the threshold elevation from baseline, the greater the loss of function compared with established normal values. Sensitivity data (reciprocal of threshold) for a representative four of the seven patients and their correspondence to AF findings are also described below.

Table 32: Data derived from both the scotopic and photopic FMM testing in all 7 study eyes showing the mean (background) and the maximum (discrete) threshold elevation from baseline (log units) and comparison values from normal subjects.

		Photopic FFM		Scotopic FFM	
Patient	**Type of lesion in Study eye**	**Background threshold (log)**	**Maximum elevation (log)**	**Background threshold (log)**	**Maximum elevation (log)**
Normal Subject		0.27 (not aged matched)	0.51 (not age matched)	1.04 (age matched)	2.36 (age matched)
#1	Extrafoveal CNV	1.87	3.05	3.13	3.60
#2	Juxtafoveal CNV	1.92	3.11	3.46	3.63
#3	Secondary GA	1.08	2.31	2.84	3.60
#4	Extrafoveal GA	0.97	1.28	2.53	3.58
#5	Drusen	1.44	2.06	2.62	3.58
#6	Drusen	1.39	1.64	1.99	3.59
#7	Drusen	0.46	0.65	Not performed	Not performed

4.10.1 Patient with choroidal neovascularization

Patient #2 is an example of an extrafoveal occult CNV that developed 15 months prior to the study in the right eye. Visual acuity had been stable since then without any intervention. The fundus showed a disciform scar with foveal sparing surrounded by a halo of RPE atrophy. AF imaging showed centrally decreased AF surrounded by a ring of increased FAF at about 1000 μm eccentricity. Inferior to fixation there was an additional small area of increased AF. Photopic FMM (fig. 22a) revealed severely reduced sensitivity in the foveal region and moderately reduced sensitivity in the central area with mainly decreased AF. The ring of increased AF and the more eccentric portion showed only mildly reduced photopic sensitivity. Scotopic FMM (figure 22b [page 173]) revealed complete sensitivity loss in both the central area of decreased AF and the surrounding ring of increased AF.

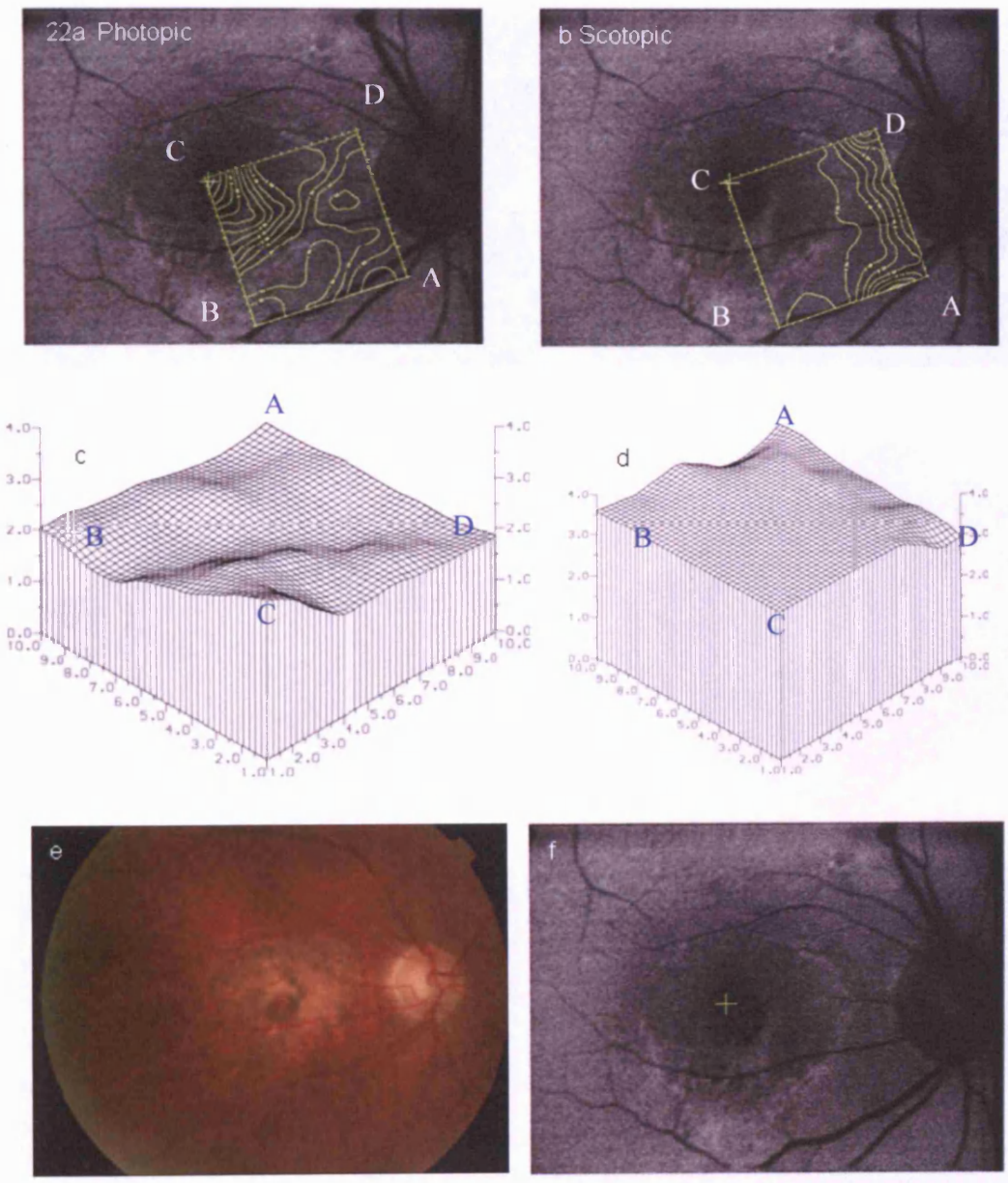
4.10.2 Patients with geographic atrophy

Patient #3 received prophylactic laser treatment for confluent soft drusen in the right eye five years prior to the study (12 spots; 200 μm, 0.2 sec; 80 mW). She then developed a longstanding RPE detachment (PED). Subsequently she developed secondary GA. AF imaging showed a central area of decreased AF with a radius of about 1000 μm surrounded by a ring of increased AF. Photopic FMM revealed normal foveal sensitivity, mildly to moderately reduced sensitivity over the adjacent area of decreased AF, but normal sensitivity over the area of increased AF and also normal photopic sensitivity eccentric to the area of increased AF (Fig. 23a). Scotopic FMM showed a complete loss of sensitivity over both the central area of decreased AF and the surrounding ring of increased AF (Figure 22b [page 173]). Eccentric to the area of increased AF the scotopic sensitivity was moderately reduced by about one log unit.

Patient #4 also exhibited a small patch of GA superior to fixation in the left eye. Although photopic FMM did not reveal reduced sensitivity (see Table 32 {page 171}), scotopic FMM showed extremely reduced sensitivity including the fovea, the area of decreased AF (GA) and the areas of increased AF.

Figure 22

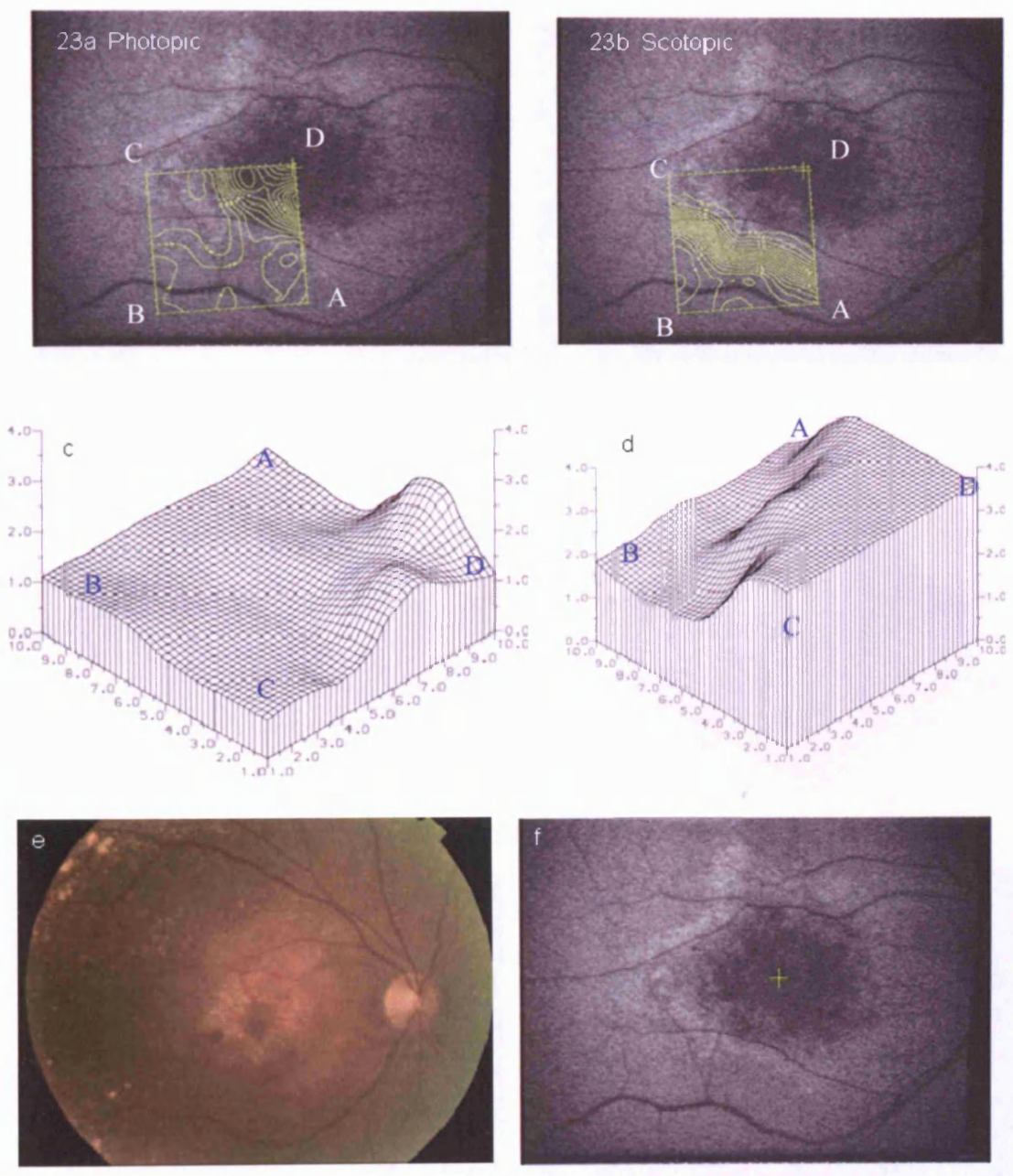
(a&c) Photopic and **(b&d)** scotopic FMM **(e)** colour fundus image **(f)** AF image from patient #2 (extrafoveal CNV)



Taken from Scholl et al 2004 (Scholl, H. P. *et al*; 2004a)

Figure 23

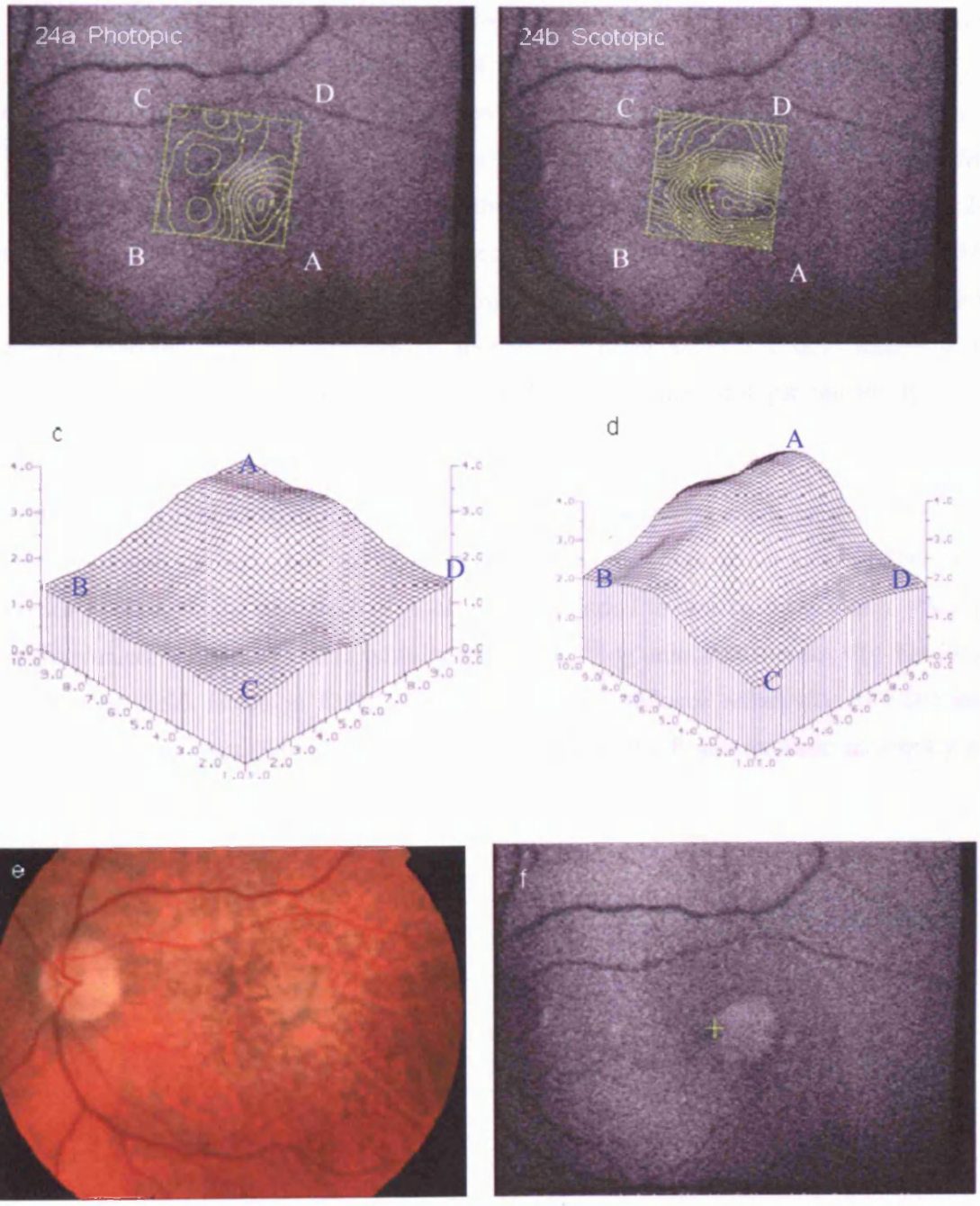
(**a&c**) Photopic and (**b&d**) scotopic FMM (**e**) colour fundus image (**f**) AF image from patient #3 (secondary GA after longstanding RPE detachment and previous prophylactic laser treatment)



Taken from Scholl et al 2004 (Scholl, H. P. *et al*; 2004a)

Figure 24

(**a&c**) Photopic and (**b&d**) scotopic FMM (**e**) colour fundus image (**f**) AF image from patient #5 (large soft foveal druse)



Taken from Scholl et al 2004 (Scholl, H. P. *et al*; 2004a)

4.10.3 Patients with drusen

Patient #5 exhibited extensive drusen at the posterior pole including a large soft foveal druse. AF imaging revealed a central area of increased FAF corresponding to the large soft foveal druse (figure 24 [page 175]). There were some additional small patches of increased AF at the posterior pole that also corresponded to the drusen distribution. Photopic FMM revealed a mildly reduced sensitivity over the test field of about 0.5 log units (figure 24). The sensitivity decrease over the area of increased AF was slightly larger. Scotopic FMM revealed considerably reduced sensitivity over the area of increased AF. This sensitivity decrease extended beyond the area of increased AF by about 1-2 deg inferiorly and temporally. The rest of the test field showed moderately reduced scotopic sensitivity.

Another patient with drusen (#6) showed many large soft and small hard drusen at the posterior pole of the right eye. AF imaging showed an area of reticular increased AF in the superior portion of the macula that poorly corresponded to drusen distribution. Photopic FMM revealed mildly reduced sensitivity of about 0.5 log units that was equally distributed over the test field. Scotopic FMM showed moderately reduced sensitivity over the areas with reticular increased AF. Outside the area of abnormal AF, the scotopic sensitivity was near normal.

5 Discussion

5.1 Characteristics of patients recruited to the study

When recruiting for the study, the gender distribution favoured females. We would expect this to be due to increased longevity but surprisingly there was no difference in the median age between the sexes. An alternative explanation for this gender difference in ascertainment may include differences in health seeking behaviour (Evans, J. R.; 2001) and a willingness to volunteer in females. Pooled data from 3 population studies, showed no significant differences in the prevalence of AMD between the genders (Smith, W. *et al*; 2001).

The spouse comparisons were predominantly male. This was again due to ascertainment bias due to the recruitment of spouses in most cases. The small number of the 'comparison group' identified from the colour grading of images and the fact that the median age of this group was lower than that of the 'patients' makes it difficult to make any definite comparisons between the two groups. The comparison group therefore have the potential to become patients over time, making interpretation of these results more difficult.

Bilaterality of the various lesions in ARM and AMD were also calculated. In those 705 cases with any AMD, 56% had bilateral involvement. Although this figure compares well with that found in the Blue Mountain Eye Study where 57% (39/69) of those with AMD were found to be bilateral (Wang, J. J. *et al*; 1998), and is slightly higher than the 42% rate found in the Beaver Dam Study (Klein, R. *et al*; 1992), we would expect our bilateral rate to be significantly higher in view of the referral bias inherent in clinic samples compared to population studies. This finding confirms the wide range of phenotypes recruited to the study and this cohort may therefore be more representative of the general population than of a typical tertiary referral centre.

When analysing the different types of AMD, 38% of the neovascular AMD cases were bilateral as were 40% of the GA cases. The corresponding values in the Blue Mountain Study were 40% and 56% respectively. Differences however exist in the way that AMD cases were classified in the two studies. In the Blue Mountain study any case with both GA and CNV in the same eye was classified with both phenotypes whereas according to the international classification used with our cohort, any patient with GA in an eye that subsequently developed CNV was reclassified as neovascular AMD for that eye.

The proportion of those with unilateral CNV and drusen graded in the fellow eye was almost twice as many as those with unilateral GA and drusen in the other eye (73 v 34%) implying that GA may be more symmetrical in terms of progression of disease. A large proportion of patients however, (n=87) graded with unilateral GA had evidence of neovascular AMD in the fellow eye. This could either be due to neovascular changes occurring at the edge of an atrophic lesion (Sunness, J. S. *et al*; 1999b)or secondly, the GA graded in the first eye could have resulted from secondary atrophy following a previous neovascular lesion.

When analysing the family history data, as expected, spouse comparisons were less likely to report a positive family history than patients. Additionally, those with bilateral AMD reported a slightly higher rate of a positive family history compared to those with unilateral disease (25% v 22%).

One of the most important findings of this part of the study was the unreliability of biomicroscopy as a way to classify patients and spouse comparisons. It is only after accurate grading of fundus images that the appropriate classification of subjects can occur.

5.2 Validation of grading techniques

The grading system used in this study represents an adapted version of that established by the International ARM Epidemiological Study Group (Bird, A. C. *et al*; 1995), and has been shown to be reliable and reproducible in the validation study. Since it represents a consensus of various groups conducting research on ARM and AMD including the Wisconsin Grading System (Klein, R. *et al*; 1991), the results of this study can be compared to other studies such as the Beaver Dam Eye Study, the Framingham Eye Study or the Age-Related Eye Disease Study. The former studies however were population-based as opposed to an ARM study population. This results in considerably different prevalences of ARM and AMD and since κ statistic measurements are dependent on the prevalence of the attribute being measured, very different values would be obtained (Thompson, W. D. *et al*; 1988), (Brennan, P. *et al*; 1992)

Important differences exist between the more recent AREDS classification (AREDS; 2001b) and the grading system used in this study. Firstly, although a similar grid was used, zones were not graded separately and secondly, drusen were only segregated by type and not semi-quantitatively as in this study. Despite these differences, similar inter-observer agreement was obtained for the presence of subretinal scarring in neovascular AMD (97%) and for the presence of GA (93%) respectively (AREDS; 2001b). In the current study agreement ranged between 90% and 98% for both types of AMD.

In this study, the inter- and intra-observer variability for the grading of drusen and for the presence of hyper- or hypopigmentation was similar. The intra-observer variability however, was slightly higher for the presence of GA, the area covered by GA, the presence of CNV and for the area covered by CNV. It was concluded that at least part of the disagreement between graders for features of AMD in this study can be explained by the disagreement within graders.

One problem with the interpretation of the 'κ-statistic' is due to the dependence on the prevalence of the attribute being measured (Thompson, W. D. *et al*; 1988). This is because when there is a very low (or very high) underlying prevalence of a particular feature, this results in a very high level of expected agreement and the κ-statistic becomes unreliable. This occurred when grading larger drusen, serogranular drusen, hypopigmentation, and retinal haemorrhage. In these cases, more emphasis was then placed on these raw data rather than the kappa statistic.

In conclusion, the revised version of the grading system established by the International ARM Epidemiological Study Group used in this study has been shown to be reproducible with inter -observer variability rates comparable to other studies. The system includes an extensive subclassification of the features of both ARM and AMD for describing the phenotype, and may therefore be used for future correlations between the phenotype and genotype of ARM and AMD patients.

5.2.1 How do conventional 35mm colour slides compare to digitised images?

The comparison between gradings showed good agreement for **drusen characteristics** with a slight tendency to underestimate drusen number on digitised images. This might be explained by the lower spatial resolution of the digitised images when compared to the original colour slides. The agreement for the grading of **hyperpigmentation** abnormalities was relatively poor and this is likely to be due to artefacts that occurred when the colour

slides were digitised. Use of this method is therefore not recommended for grading, where pigmentary abnormalities are deemed an important part of the study.

Interestingly, another recent study has compared 35mm film and 35° digital stereoscopic fundal images for grading age-related maculopathy and concluded similar findings with the lowest κ scores found for the number of small drusen and for pigmentary changes (van Leeuwen et al 2003, Ophthalmology).

The agreement or concordance for the grading of late-stage AMD (geographic atrophy and choroidal neovascularisation) was high for all features graded in the study. The only exceptions were the individual features of **CNV**. There was a tendency for neuroretinal detachments to be missed on the digitised images and this might be explained by poorer stereopsis. There was also a tendency to miss small areas of **GA** on the digitised images and a failure to detect the presence of CNV in three cases from the digitised images and in one case CNV was detected from the digitised image but not the 35mm colour slide. Resolution of the image may be one explanation but another study investigating the effects of image compression and stereopsis, using images of drusen and AMD, showed that low compression (30:1) JPEG images were almost indistinguishable from uncompressed TIFF images and that these were highly comparable to the original fundus photographs. Additionally, even high compression (80:1) JPEG images conveyed a stereoscopic quality similar to that of the original photographs especially for lesions like retinal pigment epithelial detachments (Lee et al 2000, Retina).
When all the data of inter- and intra-observer variability had been taken into account it was concluded that grading from digitised images resulted in reproducible results comparable to those from grading stereoscopic colour slides.

Comparison to other studies

Recently, studies by Lee and co-workers (Lee 2000, Retina) and van Leeuwen et al (van Leeuwen 2003) compared standard 35 mm fundus photographs and digitised and digital images respectively in patients with ARM. Even though the study population differed in the former study since it was not restricted to ARM, they found a high weighted agreement

between the grading from stereoscopic colour slides and low compression JPEG images. The second study gave weighted kappa results for the number of small hard drusen (κ; 0.41) and drusen type (κ; 0.79) similar to that found in this study and also high kappa values for atrophic and neovascular end-stage ARM (κ; 0.87 and 0.94 respectively).

Digital fundus photography has already become a sensitive tool for detecting diabetic retinopathy (Lin 1999). With the level of agreement between stereoscopic colour slides and digitised images obtained in this study, together with the results of other studies, there is increasing evidence to suggest that digital imaging will become a valuable tool for grading abnormalities in studies of ARM and AMD.

The fact that three cases of GA and two cases of CNV were missed in our study using digitised images does cause some concern but with increasing technology and image resolution, the advantages of storage, transfer of images and software manipulation, digital fundus imaging is likely to continue to improve. Additionally, using other diagnostic modalities such as digital fluorescein angiography and autofluorescence imaging would assist in determining the presence of lesions of AMD in a purely digital system.

5.3 How Concordant or symmetrical is AMD between eyes?

5.3.1 Bilateral late AMD

Assessing the degree of symmetry between eyes and the extent to which the individual phenotypes occur independently of each other may assist in determining the influence of genetic and environmental factors in AMD.

It is known that other inherited eye disorders show heterogeneity of phenotype within families although not usually between eyes of the same individual. For example, in Sorsby macular dystrophy, a monogenic disorder caused by mutations in the TIMP3 gene, a

phenotype not dissimilar to GA has been demonstrated to occur in some affected individuals in a family (Clarke, M. *et al*; 2001) whilst others show a more typical CNV-like phenotype. This shows that the effect of the primary causative mutation can be modified by other factors. Often it is impossible to determine whether such modifiers are genetic, environmental or stochastic in nature.

In order to determine the influence of genetic factors in the development of AMD, twin studies have proven useful. Hammond et al (Hammond, C. J. *et al*; 2002) demonstrated a high heritability for the presence of soft drusen and >= 20 hard drusen at the macula, by comparing concordance in MZ v DZ twins, but such patient resources are rare. This study has taken advantage of the inherent bilaterality of signs available to the ophthalmologist due to the presence of two eyes in each patient. Clearly, both eyes would share very similar genetic, developmental and environmental factors. Any phenotypic differences therefore could only be attributable to somatic mutation, skewed X-inactivation, micro-environmental differences or unknown stochastic factors.

Our results demonstrate that the symmetry or concordance between eyes of 75%-77% (kappa= 0.48) undoubtedly exceeds that expected by chance alone if the eyes were affected independently. Interestingly, this figure is less than that previously recorded for early AMD or drusen in which concordance has been described in 90% of patients (Gass, J. D.; 1973) and also for the size, number, density and fluorescence of drusen where kappa values of between 0.7 and 0.9 have been quoted (Barondes, M. *et al*; 1990). This difference in concordance may suggest that the earlier signs of ARM are a better reflection of the common genetic and environmental influences acting upon both eyes whilst the later manifestations of the disease are further downstream in the pathological process and may be affected by as yet unknown stochastic factors affecting the eyes separately, at different times during the patient's life. Early signs of drusen may therefore be more informative in studies designed to discover susceptibility genes and environmental factors.

When the entire cohort of patients (n=879) were analysed according to the predominant phenotype in each eye, a kappa value for overall concordance between eyes was calculated

as 0.264. This represents 'fair agreement' (Brennan, P. *et al*; 1992; Landis, J. R. *et al*; 1977), and is lower than the value obtained for drusen symmetry (kappa= 0.71-0.89) (Barondes, M. *et al*; 1990) and AMD symmetry (see chapter 4.4).

When considering the retrospective data of the eyes with features of CNV and adjacent areas of atrophy in those with bilateral AMD, 65% of the atrophic areas resulted from secondary atrophy following contraction of a disciform scar indicating that GA follows CNV in many cases. In a further 13%, CNV occurred at the edge of a previous GA lesion. This changing phenotype can cause difficultly in determining the initial lesion occurring in an eye and is likely to explain some of the 25% discordance observed in this study. It also emphasises the importance of examining all available historical data. It is not yet known whether environmental factors such as smoking may influence these changes.

Other locally acting factors such as ocular dominance, differences in blood flow or differing refractive error may have contributed to the observed discordance between eyes but such data were not collected in this study and no evidence at present exists to confirm these theories.

The development of CNV in an eye with GA has been well documented by Sunness et al. She reported a rate of 11% by 4 years in those with bilateral GA and in those with CNV in the fellow eye, 34% developed CNV by 4 years (Sunness, J. S. *et al*; 1999b). The development of atrophy in those eyes with CNV is less clearly defined.

In conclusion, this study demonstrates statistically significant symmetry between lesions causing visual impairment in 75%-77% patients with late stage AMD as graded from digital photos. Although this does not confirm a genetic rather than environmental influence, it is in keeping with the concept that genes may confer risk to the different manifestations of late ARM or AMD. In 23-25% cases however, patients were discordant for phenotype between eyes suggesting that other locally acting factors may modify the phenotype in a significant proportion of cases. Only with subsequent large collaborations

and accurate phenotyping in sibling, family and twin studies can the role of genetics be confirmed in the determination of phenotype in AMD but, these data do suggest that early AMD rather than the late stages may be under greater genetic influence and since the kappa values only represent moderate symmetry for late AMD, other factors acting independently on the eyes maybe modifying the phenotype.

5.3.2 Symmetry of AF in bilateral GA

The results show a high correlation for area of atrophy between right and left eyes in those with bilateral GA. This was demonstrated with and without the inclusion of peripapillary atrophy with no significant difference between the mean areas of atrophy in right and left eyes (see table 16 {page 132}). This is in accordance with a similar study measuring the extent of GA between eyes (Bellmann, C. *et al*; 2002) and serves to emphasise the non - random nature of atrophy of the pigment epithelium at the posterior pole.

Furthermore the distribution of atrophy showed marked symmetry or concordance between eyes as shown by a kappa value of 0.67 (95% CI; 0.46 to 0.88) representing substantial agreement. The pattern of atrophy was more commonly multifocal (58% eyes).

The observed peripapillary atrophy does not appear to have any relevance to the extent of GA in each eye, except to say that it occurred in 42% of patients analysed and appeared to be symmetrical. In glaucoma it has been associated with disc haemorrhage and disease progression (Jonas, J. B.; 2005), but this may be related to refractive error. We do not have sufficient data in this study to assess the effect of peripapillary atrophy on disease progression in AMD.

High rates of symmetry have been shown in AMD (see 1.6.6), all implying that individual determinants, including possible genetic or environmental factors, may play a role in the pathogenesis of the disease. Additionally, the pattern and evolution of the atrophic process may be predetermined for each individual and that random, non-specific ageing changes are

less likely to be important in disease progression. This study also suggests that later on in the course of disease when both eyes are affected, the atrophy is likely to become symmetrical.

5.4 What can the fellow eye tell us about the cause of visual loss from AMD?

5.4.1 Colour photograph features

This study show distinct differences in those with unilateral visual loss between the drusen characteristics of the fellow eye of the GA and CNV group. These include the presence of an increased number of confluent, distinct and crystalline drusen in the fellow eye of the GA group. Furthermore the overall total area covered by drusen was significantly greater in the GA fellow eyes compared to the CNV group.

These findings support the hypothesis that GA is more likely to follow the regression of pre-existing drusen. Gass (Gass, J. D.; 1973) and Sarks (Sarks, J. P. *et al*; 1988) observed that GA was often associated with the fading of drusen, but also recognised that it could occur following the resolution of a serous retinal pigment epithelial detachment.

One criticism of the current study is that it appears that GA is more frequently seen in both eyes compared to CNV. This bias may well be due to the selection criteria used for the study in that atrophy is less likely to cause unilateral visual loss due to the nature of the lesion. Similarly, eyes with CNV in the fellow eye would be more likely to have bilateral visual loss and would therefore not be included in the analysis. Assumptions on the bilaterality of GA compared to CNV cannot therefore be made from this study but need to be assessed from the entire cohort of patients.

The fewer drusen seen in the CNV fellow eyes and the existence of 14 patients (9%) with <10 (63μm) hard drusen only in each zone, is in keeping with the theory that CNV may not be necessarily related to the presence of pre-existing drusen and can occur as an event in an apparently otherwise healthy eye.

In a previous similar study comparing drusen characteristics in patients with exudative (n=89) and non-exudative AMD (n=38) (Bressler, N. M. *et al*; 1988), those younger than 75 were more likely to have confluent drusen in the fellow eyes of the exudative group (OR=6.0; CI=1.07,33.89). No difference however was found between drusen characteristics in those older than 75 years. Several differences between the two studies may explain these disparate results. Firstly, the non-exudative group included those with bilateral drusen and /or geographic atrophy in both eyes and therefore provided a different comparison group to that in our study. Additionally, there were smaller numbers of patients and the average age of the non-exudative group was 5 years younger than the exudative group. The latter factor however was adjusted for in the analysis. The small difference in median ages between the two groups (74v76) in the current study is unlikely to account for the changes seen.

The increased frequency of crystalline drusen in the GA fellow eyes, especially in zones 2 & 3, suggests that this type of drusen represents a stage of resolution associated with geographic atrophy. In histological studies, Sarks has described drusen with glistening foci of calcification within areas of atrophy (Sarks, S. H.; 1976). Although serogranular drusen or drusenoid PED's were also more frequent in the GA group compared to the CNV group, the difference was not statistically different making it difficult to implicate this type of drusen as a risk factor for GA.

Pauleikhoff (Pauleikhoff, D. *et al*; 1990a) analysed the fellow eye of 150 patients with visual loss due to PED and CNV and concluded that larger, more densely packed and less fluorescent drusen on fluorescein angiography were more likely to be present in the fellow eye when an avascular pigment epithelial detachment was the cause of visual loss in the first eye. This is consistent with the proposed neutral lipids theory of PED's (Sheraidah, G. *et al*; 1993) (Holz, F. G. *et al*; 1994a; Pauleikhoff, D. *et al*; 1990c). Our results showed

evidence of larger, more confluent drusen in the PED fellow eyes compared to the CNV group in zone 1 but not compared to the GA fellow eyes. In view of the small numbers of PED's in our study, it is difficult to make any firm comparisons between the PED and CNV group from our data.

Although visual impairment occurs with drusen alone, six of these nine individuals had visual acuities of 6/36 or worse, which is unusual. It is likely that some underlying atrophy or CNV not seen on colour photography or biomicroscopy was the cause of visual loss in these patients. Fluorescein angiography, only available in 3 of these patients, did not show evidence of underlying CNV.

These findings suggest that there are likely to be differences between the frequency and types of drusen (i.e. crystalline drusen) in the fellow eye of those with visual loss due to GA and those due to CNV. Deposition of waste material from RPE and lipids are known to cause thickening of Bruch's membrane, (Okubo, A. *et al*; 1999) (Pauleikhoff, D. *et al*; 1990a) and are likely to reflect the magnitude of metabolic activity determined by outer segment renewal. Resolution of drusen would therefore be expected to occur in geographic atrophy as the photoreceptor population falls. Further evidence to support this mechanism comes from histological(Sarks, J. P. *et al*; 1988) and autofluorescence findings (Holz, F. G. *et al*; 1999a) prior to the development of GA.

5.4.2 AF image features

Affected Eye

This part of the study demonstrated that certain autofluorescence patterns are seen with different phenotypic types of AMD. Specifically GA appears as a multifocal or unifocal pattern of decreased AF and CNV produces a more unifocal, irregular or heterogeneous pattern. These patterns are in keeping with the descriptions of autofluorescence in AMD by Von Ruckmann and colleagues (von Ruckmann, A. *et al*; 1997a) and consistent with the

theory that reduced lipofuscin generation after photoreceptor cell death in areas of atrophy produces areas of decreased autofluorescence (Delori, F. C. *et al*; 1995a; von Ruckmann, A. *et al*; 1997a). Three out of the 6 eyes, graded with drusen as the cause of visual loss, interestingly had evidence of a unifocal area of decreased AF. This may represent an area of underlying atrophy more easily detected from the AF image having been missed on the colour image and could explain the decrease in vision in these eyes (see figure 4 m-p [page 42] and figure 13 [page 136]). FFA's were not available in these cases.

It has also been shown that 'reticular' and 'lacelike' patterns of increased AF are more prevalent in GA eyes compared to CNV eyes where smaller, more 'focal' increases are more commonly seen. The reason for this is unclear but may represent more scattered drusen and RPE pigment changes in GA eyes compared to CNV eyes. Solbach et al also described areas of increased AF corresponding to drusen and RPE pigment in eyes with early AMD (Solbach, U. *et al*; 1997),but Lois et al were not able to attribute focal changes in AF to detectable soft drusen other than to large foveal soft drusen (Lois, N. *et al*; 2002). Different drusen groups are likely to have differing autofluorescence properties depending on their size and relative disruption to the RPE layer. The 'reticular' pattern of increased AF seen in this study may represent a generalised disruption of RPE metabolic activity as a result of small scattered drusen rather than being related to the drusen themselves. Furthermore, melanolipofuscin is likely to account for the high levels of autofluorescence seen in the 'lacelike' pattern at the level of the RPE (von Ruckmann, A. *et al*; 1997a).

Fellow Eye

When assessing the fellow eyes of the various phenotypes, all the GA fellow eyes had evidence of abnormally increased AF (particularly focal increases) and all but one eye had abnormal areas of decreased AF (2 eyes could not be graded due to poor quality). In contrast to this, 26% of the CNV fellow eyes did not show any abnormal increased AF and the same number had no abnormal area of decreased AF. This finding supports the theory proposed by Holz that areas of increased AF represent regions of enhanced susceptibility to

further photoreceptor loss and atrophy (Holz, F. G. *et al*; 1999a). Since GA has been shown to be symmetrical and bilateral in a number of cases (Bellmann, C. *et al*; 2002)(see ch 2.4.2), it can be argued that these 'focal' areas of increased AF in the fellow eye (also seen in 50% of PED fellow eyes) are likely to increase the risk of geographic atrophy and visual loss. The fellow eye of those with CNV showed an increased prevalence of 'reticular' change compared to the GA fellow eyes but a similar proportion of comparison eyes also demonstrated this change which implies lower risk of visual loss.

In another comparative study of autofluorescence images of patients with nonexudative AMD and fellow eyes of exudative AMD with and without retinal vascular contribution, it was shown that AF was increased in the latter groups predominantly due to increased hyperpigmentation. These differing results could be attributed to a different excitation and barrier filter wavelength to that used in our study causing excitation of different fluorophores in the RPE (Spaide, R. F.; 2003).

The background pattern of AF appeared to be homogeneous across all phenotype groups including the comparison group and is therefore less informative to differentiate between types of AMD.

In summary, AF changes appear to be characteristic for GA and CNV and areas of atrophy may be more readily detected in those eyes with only drusen according to the colour images. Additionally, it is proposed that eyes with areas of 'focal' increases in AF may be at increased risk of GA or PED compared to those eyes with more of a 'reticular' AF pattern which occurs in CNV fellow eyes as well as healthy comparison group eyes.

5.5 Location of the Fovea

From this study, the position of the human fovea in relation to the optic disc was located at an average horizontal distance of 2.76 +/- 0.33 DD or 16.07 +/- 1.25 degrees and average vertical distance of 0.31 +/- 0.21 DD or 1.83 +/- 1.19 degrees below the disc.

This compared favourably with studies by Kabanarou using psychophysical mapping techniques (Kabanarou, S. A *et al*; 2003) and with that of Barr and colleagues (Barr, D. B. *et al*; 1999) using projected colour fundus photographs. The former quoted horizontal measurements of between 15.9° +/- 1.52 (SD) and 16.6° +/- 0.8 and the latter obtained a result of 2.8 DD+/- 0.39. Vertical measurements were also similar to that obtained in the current study (range 1.54° +/- 0.8 to 1.98° +/- 0.7 inferior to fixation.) (Barr, D. B. *et al*; 1999)·(Kabanarou, S. A *et al*; 2003).

The standard deviation for the horizontal and vertical distances in degrees was smaller when compared to that of disc diameters (8% v12% and 65% v 68%) implying that the former measurement is the more accurate and should be used when the angle subtended by the image is known.

On the whole, mean differences between the 35 and 50 degree images were not statistically significant, although the mean horizontal distance calculated in degrees from the 50 degree images was significantly higher implying that the 35 degree images are more accurate.

The statistically significant difference in the measurements between right and left eyes in those patients with bilateral data (especially those in degrees and vertical measurements) was unexpected. The calculated oblique measurement (O) from the fovea to the centre of the disc however, showed that there was no significant difference between disc-diameter measurements and although the degree measurements were significantly different, they were not clinically significant. This suggests that a torsion effect may explain some of this discrepancy. Why, on average, the left eye should show greater extorsion than the right during the taking of these images remains unexplained.

Limitations of this study include lack of data on the refractive error of patients. In a study by Barr et al (Barr, D. B. *et al*; 1999) however, looking at the effect of refractive error on disc to macula measurements, there was a trend towards a greater Disc-Macula to Disc-Diameter ratio in myopia and a lower ratio in hypermetropia, but none of the inter-group correlations achieved statistical significance.

The study concluded that the fovea is located 1.83 +/- 1.19 degrees (or 0.31+/- 0.21 DD) inferior and 16.07 +/- 1.25 degrees (2.76 +/- 0.33 DD) temporal to the centre of the disc. Where possible degree rather than disc diameter measurements should be used and 35 degree colour fundus images are preferable to 50 degree colour images. Any differences between eyes may be attributed to an unexplained torsional component.

These data were then used to locate the fovea in subsequent studies particularly when this was difficult to assess due to the extent of disease.

5.6 Where is the macula most susceptible and how does GA progress?

The evolution of geographic atrophy has previously been described as starting with focal areas of RPE atrophy in a parafoveal location which subsequently spread to produce a horse-shoe-shaped and then circular lesion (Sarks, J. P. *et al*; 1988),(Maguire, P. *et al*; 1986) but no specific region within the parafoveal area has been identified as being more susceptible to the atrophic process.

Explanations for preferential involvement of the macula region in AMD include lipofuscin accumulation (Dorey, C. K. *et al*; 1989), increased retinal illumination (Katz, M. L. *et al*; 1982), and differences in metabolic rate between the fovea and more peripherally (Gass, J. D.; 1967).

This part of the study demonstrated that when the entire cohort of patients with GA were analysed, there was an increasing predilection for the atrophic process towards the fovea. Within the central region, the most susceptible areas were within 2° of the centre of the fovea.

When the 26 eyes with longitudinal data were examined separately, a different pattern emerged. Here the contour map showed displacement of increased susceptibility towards the temporal and infero-temporal region in relation to the fovea in both right and left eyes.

When analysing the progression of atrophy in these patients, it appeared that the most frequent pattern seen was in a circumferential direction. This is consistent with the development of a horse-shoe pattern in GA as described by Sarks (Sarks, J. P. *et al*; 1988).

These results suggest a greater propensity for the temporal and infero-temporal macula to the atrophic process at the earlier stages of disease followed by a circumferential spread to involve the parafoveal macula regions. Macular susceptibility then appears to reduce equally in all directions away from the fovea towards the vascular arcades.

The central foveal zone with horizontal diameter of between 1.25° and 2.75° (Curcio, C. A. *et al*; 1990),(Curcio, C. A.; 2001) is described as being relatively rod free with a rod dominated parafovea. This distribution of photoreceptors may therefore play a role in susceptibility of the macula. The preferential vulnerability of the rod system in ageing and ARM has been shown by various studies including work described in the latter part of this thesis involving fine matrix mapping carried out predominantly by Hendrik Scholl and colleagues (Scholl, H. P. *et al*; 2004a).

In histological studies of the maculae of older adults with no visible drusen or pigmentary change, the number of cones in the macula has been shown to remain stable until the ninth decade (Curcio, C. A. *et al*; 1993) with the number of rods reducing by 30% especially at the parafovea. Another study of early and late ARM eyes, demonstrated preferential loss of rods in 3 out of 4 eyes (Curcio, C. A. *et al*; 2000).

This selective vulnerability of rods may be explained simply by the relatively large number of rods in the peripheral macula. In the young adult, rods outnumber cones in the macula by 9:1(Curcio, C. A. *et al*; 2000). Another explanation may result from the ability of the cone

system to utilise an alternate visual cycle to regenerate opsin photopigments involving Müller cells (Mata, N. L. *et al*; 2002) rather than relying on recycled products of phagosomal degradation from the RPE. The increased cone density in the nasal retina (40-45% higher) compared to temporal retina may also play a role in the relative protection of this region (Curcio, C. A. *et al*; 1990).

The subset of patients with longitudinal data showed an increased susceptibility of the temporal and infero-temporal areas of the macula. An explanation for this increased susceptibility could be related to the increased photoreceptor to RPE cell ratio in this region compared to more nasal regions, the difference being more significant in those over the age of 50 (Dorey, C. K. *et al*; 1989). This would result in a higher phagocytic and metabolic load to the RPE cell, causing preferential accumulation of lipofuscin, a toxic by-product of outer segment renewal, which may ultimately lead to photoreceptor death (Dorey, C. K. *et al*; 1989), (Holz, F. G. *et al*; 1999b), (Schutt, F. *et al*; 2000), (Okubo, A. *et al*; 2000). Evidence to support this comes from spatial distribution studies of lipofuscin using spectrophotometry and autofluorescence imaging. Delori has shown higher rates of accumulation of lipofuscin temporally compared to at the fovea with maximum fluorescence occurring at 7° eccentricity (Delori, F. C. *et al*; 2001).

Light exposure has previously been put forward as a possible aetiological factor. It would be expected that the retina exposed to the most light would be in the inferonasal region. However studies by Kooijman and Pflibsen and colleagues, revealed no evidence of increased illumination at the posterior pole, but rather a homogeneous retinal light distribution regardless of pupil size (Kooijman, A. C.; 1983; Pflibsen, K. P. *et al*; 1988).

A potential source of bias in this study comes from patient ascertainment. It is more likely that patients with foveal atrophy rather than extrafoveal atrophy would be referred to hospital clinics. In spite of this, the study population contained patients with a spectrum of atrophic changes with a significant proportion with no significant visual impairment and areas of non-foveal atrophy.

Refractive error could not be corrected in our patients due to lack of data, but those with significant refractive error (> +/-6D) would have been excluded from the original study. Additionally, rotational misalignment between images of those patients with longitudinal data in this study may have affected the results, but every effort was made to match up subsequent images as accurately as possible using markers on vessel bifurcations.

In summary, the results show that the area most susceptible to early GA lies at a temporal and infero-temporal location in relation to the fovea, progressing most commonly in a circumferential direction. The risk of atrophy also reduces outwards towards the vascular arcades. The process appears to occur in a predominantly rod rich area and the increased susceptibility of the temporal region may be related to the increased density and turnover of rod outer segments suggested by an increase in photoreceptor to RPE ratio and lipofuscin accumulation rather than light exposure.

5.7 What does AF imaging tell us about the RPE in CNV development?

This part of the study demonstrated and characterised the inherent retinal autofluorescence of eyes with CNV or PED and showed that the RPE appeared to remain viable at least initially. It has been assumed that a continuous area of autofluorescence represents surviving RPE and there is good evidence to support this assumption (Holz, F. G. *et al*; 1999a).

Of interest is that those eyes in groups 1 & 2 often had preservation of RPE autofluorescence, in comparison to those eyes in group 3 (those with earlier visual loss) where patches of decreased AF were seen. These results may indicate preservation of RPE viability in the initial lesion and in some cases for a long period, which would have implications for treatment interventions and long term visual prognosis.

The fact that the median visual acuity in group 1 was better than group 3 suggests that visual outcome may be determined by maintenance of RPE function and these conclusions are supported by the good visual acuity in those with intact autofluorescence at the fovea. Unfortunately the time frame to RPE dysfunction and cell death is not known, but is likely to occur in the first few weeks or months following onset of leakage from new vessel complexes as shown by the results of this study.

Evidence that the RPE may determine the behaviour of the CNV comes from work by Uyama (Yamagishi, K. *et al*; 1988) where sodium iodate (NaIO3) was used to damage RPE cells in primates while inducing CNV with photocoagulation. Viable RPE was required to induce new vessel growth and later in the course of disease, it suppressed the neovascular process. It was thought that the RPE produced diffusible factors that then influenced the integrity and permeability of the choroid (Blaauwgeers, H. G. *et al*; 1999). Experiments in rodents have demonstrated that over expression of Vascular Endothelial Growth Factor (VEGF) in the RPE can induce intra-choroidal neovascularisation (Schwesinger, C. *et al*; 2001) and high concentrations of VEGF and VEGF receptors have been detected in choroidal neovascular membranes and in the RPE (Kliffen, M. *et al*; 1997; Kvanta, A. *et al*; 1996; Wada, M. *et al*; 1999). The fact that RPE can be seen to be present in the early stages of CNV development and overlies the area of maximum leakage, supports this hypothesis. Subsequently there may be upregulation of pigment epithelial derived growth factor (PEDF) expression (Ohno-Matsui, K. *et al*; 2003), which is believed to be antiangiogenic, and may cause the lesion to form an inactive scar.

The extent of the main abnormal areas as measured on AF imaging in group 1 was larger than that on FFA in 18/20 cases. Although the difference was small it was statistically significant and has been interpreted as implying that the RPE abnormality is more extensive than the main area of FFA hyperfluorescence. AF imaging may therefore more accurately delineate areas of choroidal neovascularisation. In support of this, a histological study (Bynoe, L. A. *et al*; 1994)of subfoveal neovascular membranes from six patients (4 with AMD), showed that the excised CNV complexes were significantly larger than expected from fluorescein angiogram. Moreover, they demonstrated that the distribution of blood

vessels was irregular and large areas of the complex periphery were avascular and hence would not show up on fluorescein angiography. If this interpretation is correct it has implications for recurrence of CNV following laser treatment based on the fluorescein findings, and supports the need to include a surrounding area of apparently normal retina in any treatment.

Classic & occult lesions

In 13/20 eyes from group 1 and 4/8 from group 2, areas of increased AF were seen adjacent to areas of FFA hyperfluorescence. These areas did appear to be more prevalent in those eyes with a classic component on fluorescein angiography. This observation may be explained by the fact that increased autofluorescence may be due to phagocytosis of laterally diffused debris derived from the new vessel complex that would be greater in those with classic new vessels, and would not necessarily only define the area of leakage.

The differences in the anatomical site of neovascular complexes in eyes with classic and occult CNV was investigated in a clinicopathological study of 28 eyes by Lafaut and colleagues (Lafaut, B. A. *et al*; 2000). They showed that 18/19 classic complexes had a predominantly sub-retinal fibrovascular component as opposed to occult lesions (n=10) that all contained a sub RPE fibrovascular component. It is hypothesised that classic membranes start in the sub RPE space and then grow through the plane of the RPE to proliferate in the subretinal space (Green, W. R. *et al*; 1993). If this were the case we would expect to see more abnormalities on the AF images of classic membranes than on those of occult membranes.

Areas of decreased AF

In all three groups, areas of decreased AF imaging corresponded to areas of atrophy, subretinal blood or intra-retinal exudate. In 3 eyes an increase in AF was seen adjacent to

areas of RPE atrophy. This phenomenon is known to occur in primary GA in eyes with AMD (Holz, F. G. *et al*; 1999a) and represents areas of RPE with abnormal function and vulnerability to subsequent atrophic change.

Pigment epithelial detachments

Also of interest is the normal autofluorescence intensity from RPE overlying avascular pigment-epithelial detachments. This would be in accord with the view that these lesions occur due to changes in the structure of the underlying Bruch's membrane and that the anatomy and function of the overlying RPE would remain relatively undisturbed (Bird, A. C.; 1991; Pauleikhoff, D. *et al*; 1990c). In one of these cases, a rim of decreased autofluorescence on AF imaging surrounded the pigment epithelial lesion and corresponded to an area of subretinal fluid noticed on biomicroscopic examination (see figure 18 e&f [page 160]).

Autofluorescent imaging, unlike other forms of retinal examination, exclusively examines the retinal pigment epithelial layer, and in this study provides further information on integrity of this layer in excess of that provided by fluorescein angiography alone. It also gives support to the theory that the RPE is important in modifying the behaviour of CNV. Further longitudinal studies, using AF imaging, of eyes presenting with CNV may allow us to document the extent of RPE viability during the evolution of these lesions and also the changes consequent upon treatments such as photodynamic therapy. Additionally, the presence of some residual AF in long standing lesions implies preserved integrity of the RPE and photoreceptor cells and may suggest that vision could be rescued in such cases, and questions the disappointing results of treatment to-date.

5.8 How does smoking influence the lesion occurring in late AMD?

This study demonstrates no bias in the distribution of CNV and geographic atrophy amongst smokers and non-smokers even when pack years (which represents a measure of

lifetime exposure to smoking) were considered, despite high numbers of patients with AMD. Additionally age and gender did not appear to show any association with the type of lesion occurring in patients with AMD.

The lack of association between smoking status and type of AMD lesion confirms the absence of a consensus of opinion in the literature. (Klein, R. *et al*; 1993; Vinding, T. *et al*; 1992; Vingerling, J. R. *et al*; 1995a) (Seddon, J. M. *et al*; 1996). Additionally, the power of the study should have been sufficient to detect a clinically significant difference if one existed.

The demographic data of our cohort of AMD patients showed that 72% males and 54% of females had smoked at some stage in their lives. Our figure is similar to the findings of other studies for males but much higher for females (i.e. the Blue Mountain study where 68% of the males and 39% of the females had smoked (Smith, W. *et al*; 1996) and the POLA study where 74% males and 15% of females had smoked at some point (Delcourt, C. *et al*; 1998). This difference may represent smoking trends in the UK compared to Europe and Australia.

Smoking has been proposed to promote the development and progression of subretinal new vessels (Klein, R. *et al*; 1993) and promote atherosclerotic and hypoxic damage to the choroidal vasculature (Vingerling, J. R. *et al*; 1995a),(Paetkau, M. E. *et al*; 1978). This would suggest a greater association with CNV compared to GA but it could be argued that choroidal damage could affect the viability of the RPE and Bruch's membrane, causing atrophy to occur. This study however, showed no evidence to suggest that smoking predisposes to subretinal neovsacularisation over geographic atrophy or vice versa.

Other confounding factors such as history of coronary artery disease, hypertension, lipid or cholesterol levels cannot be ignored as they could have affected the chances of finding an effect, but these factors have not been found to be important in late AMD in other studies (Klein, R. *et al*; 1993) .

In conclusion, smoking is known to be a significant risk factor for AMD but at present there is no evidence to suggest that there is a difference in the type of AMD occurring in smokers compared to non smokers.

5.9 What do areas of increased AF tell us about photoreceptor function?

Areas of increased AF in the ARM & AMD patients studied showed moderate to severe scotopic sensitivity loss with consistently less reduced photopic sensitivity; either being normal or showing a minor decrease. Thus, in all cases in whom scotopic and photopic FMM was available (n=6) rod sensitivity loss exceeded cone sensitivity loss. This suggests that areas of increased AF can be correlated with photoreceptor function and there is preferential dysfunction of the rod system. This substantiates the findings from the work on macular susceptibility discussed earlier.

Although evidence exists as to the origin of fundal AF (Delori, F. C. *et al*; 1995a), the functional significance of focal lipofuscin accumulation within the RPE is still unresolved. Holz et al. have shown that areas of increased AF precedes the development and enlargement of GA (Holz, F. G. *et al*; 2001). Further observations by Dorey et al have demonstrated that the number of photoreceptor cells is reduced in the presence of increased lipofuscin content in the RPE thus preceding cell loss (Dorey, C. K. *et al*; 1989).

The results of this study are particularly interesting as it appears that scotopic sensitivity loss exceeds photopic sensitivity loss in all types of ARM and AMD including CNV, GA, and drusen. It is however, not clear if the primary event occurs in the RPE and then subsequently affects photoreceptor function or if the initial event is in the photoreceptors with the RPE being affected later. Longitudinal studies of both AF and rod and cone photoreceptor function are required to answer this question.

In the seven patients studied, it was observed that the distribution of drusen and AF was poorly correlated and that photoreceptor dysfunction deficits followed the distribution of both increased and decreased AF rather than the distribution of drusen. In previous psychophysical studies including both scotopic and photopic sensitivity testing, no correlation between drusen and photoreceptor dysfunction was found (Owsley, C. *et al*; 2000; Sunness, J. S. *et al*; 1988).(Sunness, J. S. *et al*; 1985; Sunness, J. S. *et al*; 1988) The sensitivity loss therefore seems to reflect a more diffuse disease of the retina and retinal pigment epithelium and is not a direct effect of drusen. It can be concluded that drusen and AF have different functional implications. This supports the hypothesis that they represent two independent measures of ageing in the retina (Lois, N. *et al*; 2002).

The one exception to this was the large soft foveal druse in patient #5 which appeared to correspond to areas of increased AF as observed previously (Lois, N. *et al*; 2002). Moreover, the area covered by the large soft druse showed mildly reduced photopic and considerably reduced scotopic sensitivity. One explanation for this is that large foveal drusen often represent small RPE detachments and therefore it is not surprising that scotopic sensitivity is affected. Furthermore, these drusen may be somewhat different to soft drusen elsewhere (Lois, N. *et al*; 2002).

These data are in accord with recent functional studies that showed that the rod system is preferentially affected in ageing and ARM. In a recent psychophysical study it was found that in early ARM there can be prominent dark-adapted dysfunction (Owsley, C. *et al*; 2000). The mean scotopic sensitivity at the posterior pole was significantly lower in early ARM patients compared to age-matched controls. (Owsley, C. *et al*; 2000).

The preferential vulnerability of the rod system in ageing has been discussed in the previous section (see section 4.7) and appears to be substantiated by these results.

A hypothesis to explain the sensitivity loss in rods proposes that diffuse deposits of abnormal material derived from the RPE, deposited into the inner portion of Bruch's membrane might account for both choroidal perfusion abnormality and functional loss by acting as a diffusion barrier against exchange between the choriocapillaris and the RPE (Chen, J. C. *et al*; 1992). In those patients with ARM and manifest choroidal perfusion abnormality, scotopic FMM has shown discrete areas of scotopic sensitivity loss compared with age-matched controls (Chen, J. C. *et al*; 1992).

Vitamin A deprivation in the visual cycle is known to lead to outer segment degeneration and photoreceptor death in vivo (Katz, M. L. *et al*; 1991; Katz, M. L. *et al*; 1993).(Katz, M. L. *et al*; 1993) In ARM, a lack of vitamin A may occur due to changes in the diffusion characteristics of Bruch's membrane or an inability to recycle products of phagosomal degradation in the RPE (Okubo, A. *et al*; 2000). It is also known that vitamin A deficiency affects primarily rods but eventually impacts on cones as well (Carter-Dawson, L. *et al*; 1979; Kemp, C. M. *et al*; 1988; Kemp, C. M. *et al*; 1989). Relative retinoid deficiency at the level of the RPE and Bruch's membrane would therefore explain our observation that there is topographic correspondence of ARM-related photoreceptor dysfunction and RPE dysfunction reflected by increased AF. Moreover, there is recent evidence from Mata and co-workers of an alternate visual cycle that allows pigment regeneration in cones. This alternate cycle is independent from the RPE and may involve Müller cells (Mata, N. L. *et al*; 2002). This implies that visual-pigment regeneration in cones is not exclusively dependent on RPE function in contrast to the rod system. Therefore it is proposed that retinoid deficiency due to RPE dysfunction, as reflected by abnormal AF, would explain the higher susceptibility of the rod system relative to the cone system observed in this study.

These findings may have implications for future intervention to maximize cone survival in ARM. Rod photoreceptors serve as an early indicator of impending cone dysfunction and are known to contribute a diffusible substance essential to preserve cone survival (Fintz, A.

C. *et al*; 2003; Hicks, D. *et al*; 1999). In this study, since cone photoreceptor function was either normal or only mildly reduced, fine matrix mapping may serve to indicate an appropriate disease stage for early intervention to preserve the cone system.

6 Conclusions

This thesis provides a descriptive study of 879 patients with ARM and AMD and 44 spouses with normal maculae, acting as a comparison group. Fundal features have been characterised from colour photographs, autofluorescence imaging and fluorescein angiography (where indicated) to allow a more detailed phenotyping of study subjects than has previously been described.

Furthermore, a revised grading system for use with digital colour fundus photography has been introduced and been shown to be reliable for use in future studies. A pilot grading system for autofluorescence images has also been introduced.

During the collection of these phenotypic data, other important contributions have been made in terms of understanding underlying disease mechanisms:

(A) The demonstration of greater discordance between eyes in those with bilateral AMD than has been previously documented for eyes with drusen (ARM) suggests that late AMD may be less informative than early signs of drusen for studies designed to discover susceptibility genes and environmental risk factors. Other locally acting factors may therefore be influencing the final phenotype in each eye.
(B) Drusen characteristics and AF changes in the fellow eye may predict the cause of visual loss in the first eye, especially in GA. These may also indicate the nature of the pathogenetic mechanisms involved.
(C) Particular areas of the macula may be more susceptible to damage in GA and may be related to the distribution of rod photoreceptors. The additional demonstration of a loss of scotopic rather than photopic sensitivity in areas of increased autofluorescence in ARM and AMD confirms a preferential vulnerability of the rod system in ageing.

(D) The preservation of the integrity of the RPE shown during the early stages of CNV development, from autofluorescence images, may have important implications for treatment of AMD.

(E) Lastly, although smoking is a known environmental risk factor for AMD, as yet we have no evidence to suggest that it is more likely to cause a CNV lesion compared to GA in the late stages of disease.

7 Future Work

In view of the lack of a suitable animal model for AMD, different approaches are required to increase our understanding of the disease process. To date, it is felt that genetics may play an important role in susceptibility to AMD, especially with the finding of the CFH (complement factor H) polymorphism. So far, approaches to search for genes that confer risk for ARM and AMD have yielded limited results, most likely due to the complex nature of the disease and the fact that it comprises a group of very different phenotypes.

As part of this study, in addition to the clinical data, DNA samples have been collected from all 879 patients resulting in a large panel of DNA from subjects where the phenotype has been well characterised. This will allow the segregation of patients according to phenotype to produce purer samples of disease such that genetic investigation is likely to be more successful. This cohort will therefore serve as an invaluable resource for future association studies and phenotype- genotype correlations, pushing us towards identifying the important environmental and genetic aetiological factors for AMD. Defining the genetic influences would point to the relevant pathogenic mechanisms involved and help identify those at high risk of visual loss. Ultimately to try to reduce the burden of blindness produced by this progressive disease.

8 Publications based on this work

In press

Dandekar.SS, Jenkins S.A, Peto T, Scholl HPN, Sehmi KS, Fitzke FW, Bird A.C, Webster AR. Autofluorescence imaging of Choroidal neovascularisation due to Age-related macular degeneration. Archives of Ophthalmology (MS# ECS30174). In Press.

Bindewald, A. Bird A.C, Dandekar S, Dolar-Szczasny.J, Fitzke,F.W, Einbock. W, Holtz. F.G (IFACG) Classification of Fundus Autofluorescence Patterns in early Age-related Macular Disease. Invest Ophthalmol Vis Sci (MS# 04-0430.R2) In Press

Published

Scholl HP, Bellmann C, Dandekar SS, Bird AC, Fitzke FW. Photopic and scotopic fine matrix mapping of retinal areas of increased fundus autofluorescence in patients with age-related maculopathy. Invest Ophthalmol Vis Sci. 2004 Feb;45(2):574-83.

Scholl HP, Dandekar SS, Peto T, Bunce C, Xing W, Jenkins S, Bird AC. What is lost by digitizing stereoscopic fundus color slides for macular grading in age-related maculopathy and degeneration? Ophthalmology. 2004 Jan;111(1):125-32.

Scholl HP, Peto T, Dandekar S, Bunce C, Xing W, Jenkins S, Bird AC. Inter- and intra-observer variability in grading lesions of age-related maculopathy and macular degeneration. Graefes Arch Clin Exp Ophthalmol. 2003 Jan;241(1):39-47.

See Appendix 11.4 for copies

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11 Appendices

11.1 Appendix 1

Coding Manuals

- Colour fundus photograph grading
- Fluorescein angiography image grading
- Autofluorescence image grading (Eye with visual loss)
- Autofluorescence image grading (Fellow eye)

APPENDIX 1: CODING MANUAL FOR AMD STUDY: COLOUR PHOTOGRAPHS

1. Photo quality	2. Drusen size	ANSWER FOR DRUSEN SIZE	ZONE 1-3	ZONE 1 ONLY
		Grade separately for each size Drusen		
	Size	**Number**	**Crystalline Drusen**	**Serogranular Drusen**
	0. Absent	0. Absent	0. Absent	0. Absent
1. Perfect image (Perfect stereo)	1. Questionable	1. Questionable	1. Questionable	1. Questionable
2. Reasonable image Reasonable stereo	2. < **63μm** (Hard drusen)	2. 1-9	2. 1-9	2. Present
3. Acceptable image (Acceptable stereo)	3. **63μm - 125μm** intermediate, soft drusen	3. 10-19	3. >10	
4. Poor, but main features still gradable (Poor stereo)	**125- 250 μm** 41 large semisolid distinct 42 large semisolid subconfluent 43 large semisolid confluent	4. 20-50		
5. Very poor image, no grading possible **(No stereo)**	**250-500 μm** 51 large semisolid distinct 52 large semisolid subconfluent 53 large semisolid confluent	5. >50		
6. Not centred, but grading of at least one zone possible	**>500 μm** 61 large semisolid distinct 62 large semisolid subconfluent 63 large semisolid confluent			
	7. Cannot grade- obscuring lesions	7. Cannot grade - obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade - obscuring lesions
	8. Cannot grade- photo quality	8. Cannot grade - photo quality	8. Cannot grade - photo quality	8. Cannot grade - photo quality
9. Missing	9. Missing	9. Missing	9. Missing	9. Missing

3. Hyper-pigmentation		**4.Hypopigmentation**	**Zone 1-3 TOTAL area covered by Drusen**	**5. Geographic Atrophy**		**6. Neovascular AMD**
Presence	Type	Presence		5.1 presence: Zone 1, 2 and 3	5.3 TOTAL area covered	6.1 presence
0.absent	0.Absent	0. Absent	0. Absent	0.Absent	0.Absent	0.Absent
1. Questionable	1.Questionable	1.questionable	1. Questionable	1. Questionable	1. < 250 μm	1. Questionable
Present 2. <63μm	2.linear	2. <63μm	2. <10%	2. Present	2.250μm- 500μm	2. Present
3. >63μm	3.punctate	3. > 63μm	3. <25%	3. GA due to CNV	3. 500μm - 1000 μm	
	4. Mixed		4. <50%		4. 1000-3000μm	
	5. Peripapillary		5. ≥50%		5. 3000-6000μm	
	6. Artefact				6. > 6000μm	
7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade – Obscuring lesions	7. Cannot grade – obscuring Lesions	7.cannot grade – Obscuring lesions
8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo Quality	8. Cannot grade – photo quality
9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing

6.2 Features	6.3 Scar/fibrous	6.4 Retinal haemorrhage	6.5 TOTAL area of Neovascular lesion	7.0 Predominant Phenotype
0.absent	0.absent	0. Absent	0.absent	0. Normal
1. Questionable	1. Questionable	1. Questionable	1. < 250 μm	1. Hard drusen only
2. Hard exudates	2. Subretinal	2. Subretinal	2. 250-500	2. Soft drusen
3. Serous neuroretinal detachment	3. Preretinal	3. In plane of retina	3. 500 - 1000μm	3. GA
4. Serous RPE detachment	4. Neovascular membrane	4. Sub-hyaloid	4. 1000μm - 3000μm	4. Pigment Epithelial Detachment
5. Haemorrhagic RPE detachment		5. Intravitreal	5. 3000μm - 6000μm	5. CNV
			6. > 6000μm	6. Other
7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions
8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo quality
9. Missing	9. Missing	9. Missing	9. Missing	9. Missing

Coding manual is based on Survey of Ophthalmology, 39(5): 367-374

CODING MANUAL FOR AMD STUDY: FLUORESCEIN ANGIOGRAPHY
Grade EARLY and LATE phases

1. Photo quality	**6. Neovascular AMD (Grade proof sheet/late photo)**	**Grade proof sheet/late photo**	**Grade proof sheet**	**Grade late photo**
	Presence	**Features**	**Classification of CNV**	**Total area of neovascular lesion**
1. Perfect image	0.absent	0.absent	0.absent	0.absent
2. Reasonable image	1. Questionable	1. Questionable	1. Questionable	1. <250 μm
3. Acceptable image	2. Present	2. Hard exudates	2. 100% classic	2. 250-500
4. Poor, but main features still gradable		3. Serous neuroretinal detachment	3. 50-99% classic	3. 500 - 1000μm
5. Very poor image, no grading possible		4. Serous RPE detachment	4. 1-50% classic	4. 1000μm - 3000μm
6. not centred, but grading of at least one zone possible		5. Haemorrhagic RPE detachment	5. 100% occult	5. 3000μm - 6000μm
		6. RPE tear		6. > 6000μm
	7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions	7. Cannot grade – obscuring lesions
9. Missing	8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo quality	8. Cannot grade – photo quality
	9. Missing	9. Missing	9. Missing	9. Missing

CODING MANUAL FOR AMD STUDY: AUTOFLUORESCENCE (SLO) IMAGES (grade whole photo)

1. Photo quality	**2. Predominant Phenotype**	3. Main area of altered Auto-fluorescence (Decreased)	4. Main area of decreased Auto-Fluorescence (Total area)	5.Corresponding decreased Autofluorescence **(colour)**	6. Fovea involved in lesion	7. Increased Auto-fluorescence	8. Corresponding increase Auto-fluorescence **(colour)**	9. Background fluorescence
	0. Normal	0. Absent	0. Absent	0. Absent	0. Absent	0. Absent	0. Absent: no corr	0. Homogeneous
1. Perfect image	1. Hard drusen only	1. Questionable	1. < 250 μm	1. Geographic Atrophy	1. Questionable	1. Questionable	Corresponds with 11. Scattered Drusen 12. Parafoveal drusen	1. Questionable
2. Reasonable image	2. Soft drusen	2. Unifocal Homogeneous Decreased AF	2. 250-500 μm	2. Neovascular lesion	2. Yes	21: reticular pattern	2. Corresponds with pigmentary RPE changes	2. Diffusely irregular
3. Acceptable image	3. GA	3. Multifocal Homogeneous Decreased AF	3. 500 – 1000 μm	3. Soft drusen		22::focal	3. Edge of lesion	3. Focal increases
4. Poor, but main features still gradable	4. Pigment Epithelial Detachment	4. Normal decreased AF at fovea 41. homogeneous 42 heterogeneous	4. 1000- 3000μm	4. blood		23: lacelike pattern	4. Scar / fibrous	
5. Very poor image, no grading possible	5. CNV	5. Unifocal Heterogeneous AF	5. 3000- 6000μm	5. exudates		32: partial band (<50%) 33: band (>50%)	5. Detached retina (SRF)	
6. Not centred, but grading of at least one zone possible	6. Other	6. Multifocal Heterogeneous AF	6. >6000 μm	6.edge		34: mixed	6. Exudates	
	7. Cannot grade obscuring lesions	7. Obscuring lesion	7. Obscuring lesion	7. Obscuring lesion	7. Obscuring lesion	7. Obscuring lesion	7. Obscuring lesion	7. Obscuring lesion
	8. Cannot grade – photo quality	8. Cannot grade- photo quality	8. Cannot grade- photo quality	8. Cannot grade- photo quality	8. Cannot	8. Cannot grade-	8. Cannot grade- photo quality	8. Cannot grade- photo quality
9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing

CODING MANUAL FOR AMD STUDY: AUTOFLUORESCENCE FELLOW EYE (SLO) IMAGES (grade whole photo)

1. Photo quality	**2. Predominant Phenotype**	3. Decreased Auto-fluorescence	4. Decreased Auto-Fluorescence (Total area)	5.Corresponding decreased Autofluorescence **(colour)**	6. Fovea involved in lesion	7. Increased Auto-fluorescence	8. Corresponding increase Auto-fluorescence **(colour)**	9. Background fluorescence
	0. Normal	0. Absent	0. Absent	0. Absent	0. No	0. Absent	0. Absent: no corr	0. Homogeneous
1. Perfect image	1. Hard drusen only	1. Questionable	1. < 250 μm	1. Geographic Atrophy	1. Questionable	1. Questionable	1. Geographic Atrophy	1. Questionable
2. Reasonable image	2. Soft drusen	2. Unifocal Homogeneous	2. 250-500 μm	2. Neovascular lesion	2. Yes	2. focal increases only	2. Neovascular lesion	2. Diffusely irregular
3. Acceptable image	3. GA	3. Multifocal Homogeneous	3. 500 – 1000 μm	3. Soft drusen		3. reticular pattern	3. Soft Drusen 31. scattered drusen 32. parafoveal drusen 33. foveal drusen	3. Focal increases
4. Poor, but main features still gradable	4. Pigment Epithelial Detachment	4. Normal decreased AF at fovea 41. homogeneous 42 heterogeneous	4. 1000- 3000μm	4. hard drusen		4. lacelike pattern	4. Hard Drusen 41. scattered drusen 42. parafoveal drusen 43. foveal drusen	
5. Very poor image, no grading possible	5. CNV	5. Unifocal Heterogeneous	5. 3000- 6000μm	5. RPE pigment changes		51. reticular and focal 52. reticular and lacelike 53. lacelike and focal 54. speckled (mixed)	5. RPE pigment changes	
6. Not centred, but grading of at least one zone possible	6. Other	6. Multifocal Heterogeneous	6. >6000 μm	6.blood		6. patchy increase (>200μ-larger than focal increases	6. blood	
	7. Cannot grade obscuring lesions	7. Obscuring lesion	7. Obscuring lesion	7. other	7. Obscuring lesion	7. Obscuring lesion	7. other	7. Obscuring lesion
	8. Cannot grade – photo quality	8. Cannot grade- photo quality	8. Cannot grade- photo quality	8. Cannot grade- photo quality	8. Cannot	8. Cannot grade-	8. Cannot grade- photo quality	8. Cannot grade- photo quality
9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing	9. Missing

11.2 Appendix 2

Instructions for digital grading

1. Open selected colour image for grading in Adobe Photoshop (CD)
2. Adjust size of image to 80% on right side of screen and press return (Navigator). This is to maximise the image on the screen.
3. Minimise or Close windows on right of screen (colour, history and layers **NOT Navigator**)
4. **Open** file 'transparent template' for grading grid
5. Go to **Select 'all'**
6. Then go to **Edit 'Copy'** (to copy the grid)
7. Go back to the colour image by clicking on it, maximise it and make sure it is sized correctly to fit the image size using the navigator
8. Centre image using scroll bars
9. Then **Edit, 'Paste'** grid onto photo
10. Go to **Edit 'free transform'**
11. This allows you to move the grid over the underlying picture to centre it with the mouse
12. To increase the size of the grid, go to the '**link**' button on the top tool bar (looks like two interlocking chains) and select it
13. Then go to the **width** or **height** window next to it and either type in **160** or **170%** or use the '**up**' arrow to increase the size without distortion. It should be sized so that the distance between the centre of the disc and the fovea is 3000μm (two disc diameters).
14. You can move the grid around to centre it again using the mouse
15. If you want to **remove the grid** while grading, select the grid by double clicking it and go to **Layer** on the tool bar and '**delete layer**'. It will ask you if you want to delete layer 1, say 'yes' to the prompt. Go to '**undo**' to get the grid back or **ctrl+Z**.
16. **Note**: You cannot then resize the grid or move it without going to **edit, 'free transform'** again.

17. You are now ready to start grading in each of the three zones, the same way as conventional grading.
18. To open the next image go to **file 'close', don't apply transformation, don't save changes to image** and **don't close the grid** and then **open** the next image
19. After re-sizing and maximising the colour image you should be able to just press '**paste**' to apply the grid and continue from no 12.

11.3 Appendix 3

Letters of Ethics Committee Approval

Moorfields Eye Hospital NHS
NHS Trust
Patron: Her Majesty The Queen

Chairman
Sir Thomas Boyd-Carpenter

Chief Executive
I.A.J Balmer

City Road
London
EC1V 2PD

Tel 020 7253 3411
Minicom 020 7566 2279

11 July 2002

Professor Alan Bird MD FRCS FRCOphth
Professor of Clinical Ophthalmology
Moorfields Eye Hospital

Direct dial 020 7566 2455
E mail :frances.stobbs@moorfields.nhs.uk

Dear Professor Bird

BIRA1008
Genetic investigation of age-related macular disease
Professor Alan Bird, Mr Andrew Webster et al

The revised patient information sheet and consent form under cover of the letter from Dr Dandekar and Ms Jenkins dated 10 June 2002 relating to establishing DNA cell lines was approved at the Moorfields Local Research Ethics Committee meeting on 26 June 2002. You had declared your interest in the protocol and took no part in the approval process.

Yours sincerely

Frances Stobbs
Administrator, Moorfields Eye Hospital LREC

MOORFIELDS EYE HOSPITAL

N.H.S. TRUST

Patron: Her Majesty The Queen

CITY ROAD
LONDON
EC1V 2PD

Chairman
Leila Lessof

House Governor and Chief Executive
I. A. J. Balmer

www.moorfields.org.uk

Telephone:
020 7253 3411

Minicom:
020 7566 2279

Direct Dial
Email frances.stobbs@moorfields.nthames.nhs.uk

Professor Alan C Bird MD FRCS FRCOphth
Professor of Clinical Ophthalmology
Moorfields Eye Hospital

30 August 2000

Dear Professor Bird

Characterisation of the phenotype in age-related macular disease (AMD) in patients with unilateral visual loss
Project No

I am writing to confirm that this project has approval by the Ethics Committee at Moorfields.

Yours sincerely

Miss Linda Ficker
Deputy Chairman
Ethics Committee

Request for approval for change of protocol

Characterisation of phenotype in age-related AMD in patients with unilateral visual loss
Characterisation of phenotype in age related macular disease in patients with unilateral visual loss
Genetic investigation of age-related macular disease

Project 498/BIRA 1003 was approved in May 1998. It covered 500 individuals and their relatives. It was to run from April 1998 for 3 years.
Project BIRA1008 was approved in October 2000. It is an extension of Project 498. The time period has been extended for the study to run from October 2000 for 4 years. The number of subjects has extended to 3000 which necessitated an application to the MRC for funding. That funding has been awarded.

There is now a request for ethical approval for a protocol amendment to BIRA1008 to take blood not just from patients and their blood relatives but also from the patient's spouse. The numbers involved will be 2500 patients and 500 spouses. Professor Bird has drafted an explanation to the spouse and consent form.

This request for a protocol amendment is to be raised as Any Other Business at the Moorfields LREC meeting on Wednesday 25 April. The Committee will also consider the explanation to the spouse and consent form.

Frances Stobbs
LREC Administrator
24 April 2001

H:\LREC\AGENDA\

11.4 Appendix 4

Publications from this work

Dandekar SS, Jenkins S, Peto T, Bunce C, Halfyard A, Scholl HPN, Fitzke FW, Webster AR, Bird A. Autofluorescence imaging of choroidal neovascularisation due to age-related macular degeneration. ***Archives of Ophthalmology***. Nov 2005: *In Press*

Scholl H, Dandekar SS, Peto T, Bunce C, Xing W, Jenkins S, Bird A. What is lost by digitising stereoscopic fundus colour slides for macular grading in Age-related maculopathy (ARM) and degeneration (AMD)? ***Ophthalmology***. 2004 Jan;111(1):125-32

Scholl HPN, Bellman C, Luong V, Dandekar SS, Bird A, Fitzke FW. Photopic and scotopic fine matrix mapping of retinal areas of increased fundus autofluorescence in patients with Age-related maculopathy. ***Invest Ophthalmol Vis Sci***. 2004 Feb;45(2):574-83

Scholl H, Peto T, Dandekar S, Bunce C, Wen Xing, Webster A, Bird A. Inter and Intra-Observer variability in grading lesions of age-related maculopathy (ARM) and macular degeneration (AMD). ***Graefe's Archives of clinical and experimental ophthalmology*** 2003; 241:39-47

Dandekar SS, Jenkins SA, PetoT, Bird AC, Webster AR. Does smoking influence the type of age-related macular degeneration? ***BJO*** **(Submitted).**