Investigations of a Possible Link between Age-Related Macular Degeneration and Atherosclerosis

SOBHA SIVAPRASAD

INSTITUTE OF BIOSCIENCE AND TECHNOLOGY

CRANFIELD UNIVERSITY

AND

KING'S COLLEGE HOSPITAL

2005

SUPERVISORS

Dr. Tracey A Bailey
Mr. Victor NH Chong
Prof. Anthony Woodman

ABSTRACT

Increasing epidemiological evidence suggests a link between atherosclerosis and age-related macular degeneration (AMD). There are two main hypotheses that explain the link. One hypothesis is that AMD and atherosclerosis are tissue responses to injury. The 'Response to Injury' hypothesis defined as 'abnormal reparative response to chronic, recurrent injurious stimuli' speculates that AMD and atherosclerosis may be cellular effects of chronic or repetitive risk factors. The second hypothesis suggests that AMD is secondary to vascular insufficiency caused by atherosclerosis.

The research in this thesis is an attempt to test these hypotheses to better understand the correlation of AMD and atherosclerosis. Both basic and clinical science approaches are employed.

Part one focuses on the role of extracellular matrix (ECM) proteins in the pathogenesis of AMD. Both serum elastin derived peptides (S-EDP) and matrix metalloproteinases (MMP-2 and MMP-9) are raised in subjects with atherosclerosis. The circulating levels of these matrix components were tested in patients with varying degree of severity of AMD and compared to age-matched controls. Both S-EDP and MMP-9 were found to be significantly raised in patients with AMD, while MMP-2 did not correlate with it.

S-EDP is elevated in abdominal aortic aneurysm (AAA), a manifestation of atherosclerosis. Since S-EDP correlates with size of both AAA and severity of AMD, this research looked at a possible association between the two diseases but found no significant correlation.

The second part of this thesis investigates whether chronic inflammation may explain the co-existence of the two diseases. With the recent finding that Complement Factor H (CFH)

is related to AMD, this study focused on the role of complement activation in AMD, speculating that the final common pathway of both diseases may be chronic inflammation. Increased systemic complement activation was found in neovascular AMD, as assessed by the measurement of C3a des Arg.

Part three of this thesis tested the second hypothesis that AMD is secondary to the vascular insufficiency caused by atherosclerosis. Both the choroidal blood flow and retinal vessel calibre in patients with asymmetric AMD were studied and no significant changes in ocular haemodynamics were noted.

In conclusion, this research favours the concept that atherosclerosis and AMD are parallel responses to chronic, recurrent injurious stimuli, with extracellular matrix remodelling and probably inflammatory response being the common cellular responses. This research did not find any significant ocular haemodynamic changes in subjects with asymmetric AMD.

ACKNOWLEDGEMENT

At the very outset, I would like to thank my clinical supervisor, Mr. Victor Chong for his faith and confidence in me to pursue this project. He shared novel ideas and thoughts with me and spent invaluable time and effort to make this project a success.

I greatly appreciate Dr. Tracey Bailey and Dr. Anthony Woodman for all their advice, ideas, thoughts, suggestions, invaluable time and patience with my project.

My sincere thanks also go to Dr. Sarah Morgan who was instrumental in enhancing my laboratory skills and solving my many hurdles in the lab.

Undoubtedly, my thanks to all my colleagues in the IBST lab as well as my research colleagues at the Retinal Research Unit in King's College Hospital for their untainted support, encouragement and assistance. I like to particularly thank Jane Watkins who helped me juggle my clinical commitments with my lab work.

I acknowledge and appreciate the Mercer fund and King's College Ophthalmic fund for providing me with the financial support to carry out my DM. I also acknowledge the Medical Research Council grant that was utilised for pheno-typing of patients with age related macular degeneration and collection of their serum samples.

I would like to express my gratitude and appreciation to all the patients who participated in my project.

Last but not least, I thank my family who has always been the beacon of my hope and inspiration. Their best wishes, prayers, patience and understanding during my project have been thoughtful, invaluable and immeasurable.

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

ABBREVIATIONS AND SYMBOLS

µl	-	microlitre
µm	-	micrometre
^{0}C	-	Degrees Celsius
AAA	-	abdominal aortic aneurysm
ABTS	-	3-ethyl-benzthiazoline-6-sulphonic acid
AL	-	axial length
AMD	-	age-related macular disease / degeneration
ANOVA	-	analysis of variance
ApoE	-	apolipoprotein E
AREDS	-	Age Related Eye Disease Study
ARIC	-	Atherosclerosis Risk in Communities study
ARM	-	age-related maculopathy
AVR	-	Arteriole to venule ratio
BDES	-	Beaver Dam Eye Study
BlamD	-	Basal laminar deposits
BM	-	Bruch's membrane
BMI	-	Body mass index
Bo	-	zero standard
BSA	-	Bovine serum Albumin
C3	-	Complement 3
C5	-	Complement 5
C5b-9	-	Complement terminal complexes

C3a des Arg	-	Complement 3a des Arginine
CCR2	-	chemokine receptor 2
CD 46 and CD 40	-	membrane cofactor protein
CFH	-	Complement Factor H
CHARM	-	Cardiovascular Health and Age-Related Maculopathy
CHD	-	coronary heart disease
CMV	-	cytomegalovirus
CNV	-	choroidal neovascularisation
COAD	-	Chronic obstructive airway disease
COX-2	-	cyclo-oxygenase-2
CR1	-	Complement receptor type 1
CRAE	-	Central retinal arteriolar equivalent
CRP	-	C-reactive protein
CRVE	-	Central retinal venular equivalent
CV	-	Coefficient of variation
ε2	-	Epsilon 2
ε3	-	Epsilon 3
ε4	-	Epsilon 4
ECM	-	extracellular matrix
EDCCS	-	Eye Disease Case Control Study
EDP	-	elastin derived peptides
ELISA	-	Enzyme linked immunosorbent assay
FFA	-	Fundus Fluorescein Angiography

FHL	-	An alternatively spliced variant of the factor H gene
FPA	-	Fundus pulsations amplitude
GAG	-	glycosaminoglycans
HCL	-	hydrochloric acid
HDL	-	high density lipoproteins
HF1/ CFH	-	Complement Factor H gene
HLA	-	Human Leucocyte Antigen
HMG-CoA	-	3-hydroxy-3-methylglutaryl coenzyme A
HRF	-	Heidelberg retinal flowmeter
HRP	-	Horseradish peroxidase
ICAM-1	-	Intercellular adhesion molecule-1
ICG	-	Indocyanine angiography
ICZ	-	inner collagenous zone
IgG	-	immunoglobulin G
IL	-	interleukin
IMT	-	Intima-media thickness
INF	-	interferons
IOP	-	intra ocular pressure
KCH	-	King's College Hospital
LDL	-	low density lipoproteins
MAC	-	Membrane attack complex
MCP-1	-	monocyte chemoattractant protein-1
MHC	-	major histocompatibility complex

MMPs	-	matrix metalloproteinases
MMP-2	-	matrix metalloproteinase-2
MMP-7	-	matrix metalloproteinase-7
MMP-9	-	matrix metalloproteinase-9
MMP-12	-	matrix metalloproteinase-12
MPGN II	-	membrano-proliferative glomerulonephritis type II
mRNA	-	messenger ribo-nucleic acid
Na_2CO_3	-	sodium carbonate
NaOH	-	sodium hydroxide
NSAID	-	non-steroidal anti-inflammatory agents
NSB	-	non-specific binding
OBF	-	ocular blood flow
OCZ	-	outer collagenous zone
OD	-	optical density
oxLDL	-	oxidised low density lipoprotein
p-Npp	-	p-nitrophenylphosphate in buffer
PA	-	pulse amplitude
PAS	-	Periodic Acid Schiff
PBS	-	phosphate buffered saline
PBST	-	phosphate buffered saline with 0.05% v/v Tween
PMWF	-	presumed macular watershed filling
POAG	-	Primary open angle glaucoma
POBF	-	pulsatile ocular blood flow

PON	-	Paraoxanase gene
PR	-	pulse rate
PV	-	pulse volume
ROS	-	reactive oxygen species
RPE	-	retinal pigment epithelium
RPE-BM	-	retinal pigment epithelium-Bruch's membrane complex
rpm	-	revolutions per minute
R/T	-	room temperature
SD	-	standard deviation
SEM	-	Standard error of mean
SLO	-	scanning laser ophthalmoscope
SSPS	-	Statistical Package of Social Science
SST BD tubes	-	Serum separator tubes (BD Vacutainer system)
TBS	-	Tris buffered saline
TBST	-	Tris buffered saline with 0.05% v/v Tween
TIMPs	-	Tissue Inhibitor of metalloproteinases
TIMP-3	-	Tissue Inhibitor of metalloproteinase- 3
TNF	-	tumour necrosis factor
UCLA	-	University of California, Los Angeles
UK	-	United Kingdom
USA	-	United States of America
VCAM-I	-	vascular cell adhesion molecule-I
VEGF	-	vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

1.1 Age-related macular degeneration

1.1.1 Definition of age-related macular degeneration

Age related macular degeneration (AMD) is a progressive disease affecting the retina of the elderly. The macula lutea, meaning 'yellow spot' is the central part of the retina (fig.1.1) and the dysfunction of this area results in the inability to read, recognize faces and drive, thus compromising the lifestyle. The visual disability it imposes on the elderly depends on the extent and nature of the disease.

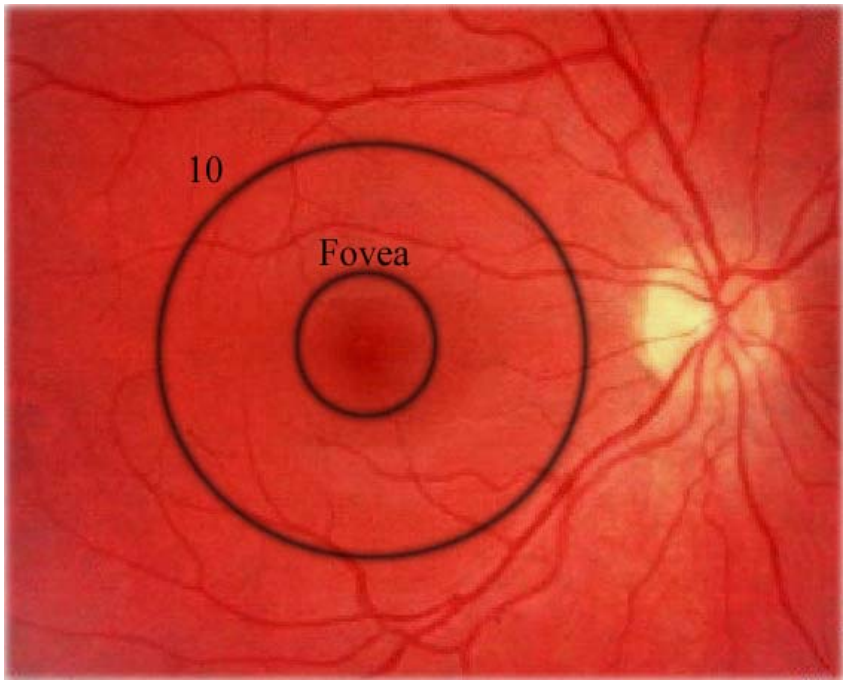

Fig 1.1: Colour photograph of fundus indicating Macula (the outer circle).

1.1.2 Diagnosis of age-related macular degeneration

AMD is a heterogeneous disorder and not all patients suffer degeneration of the macula. Bird proposed a more appropriate name for the condition as 'age related macular disease' (Bird, 2003).

According to the current nomenclature of the International AMD Epidemiological Study Group, all early and late signs of AMD are called age-related maculopathy (ARM) (Bird, 1995). Drusen are yellow spots that appear on the fundus. There is almost no risk of progression to AMD if these drusen are small (<63μm diameter) and few. However, with time some patients develop larger drusen (>125μm diameter) or develop retinal pigmentary abnormalities (with hyper- or hypo-pigmentation). These changes are termed early age-related maculopathy (fig 1.2). **In this thesis, ARM is used to denote early disease.**

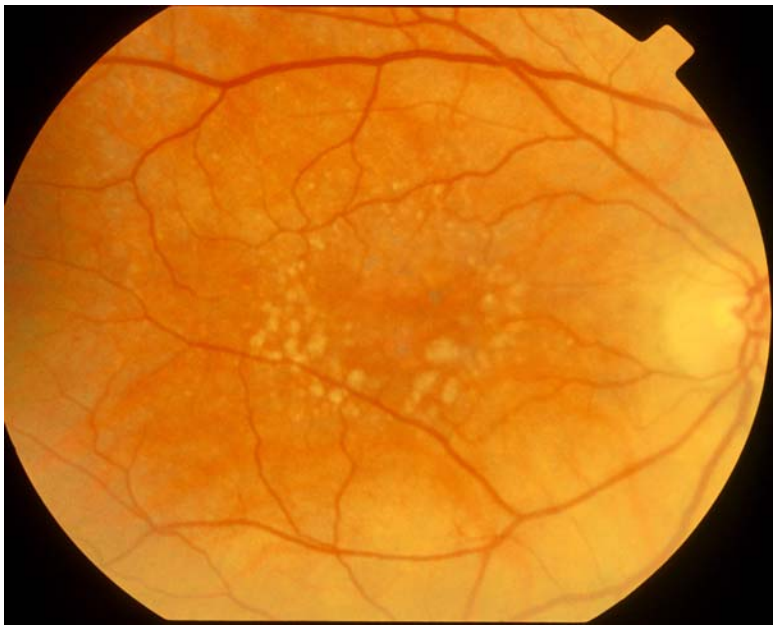

Fig 1.2 : Early age-related maculopathy

The two late stages of the disease are the dry type, also called geographic atrophy (fig. 1.3), and the wet type, called neovascular AMD. Fig. 1.3 shows a central area of geographic atrophy.

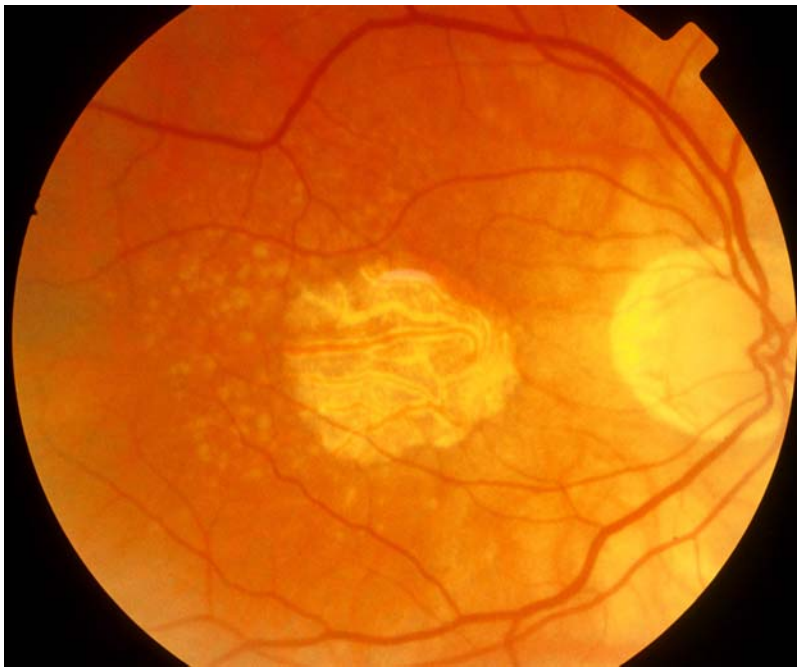

Fig 1.3: Geographic atrophy

Choroidal neovascularisation (CNV) is the hallmark of wet or neovascular AMD. Fig 1.4 shows how the CNV breaks through the Bruch's membrane (BM) to lie external to the retinal pigment epithelium (RPE) or under the sensory retina. These subtypes of CNV are clinically diagnosed by fundus fluorescein angiography as shown in fig.1.5. The fluorescein angiographic leakage patterns of CNV are classified according to the proportion of classic component defined as area of early hyper-fluorescence that increases in intensity and area in late frames. Eligibility criteria for treatment of these CNVs are at present determined by the pattern of angiographic leakage, site and size of lesion.

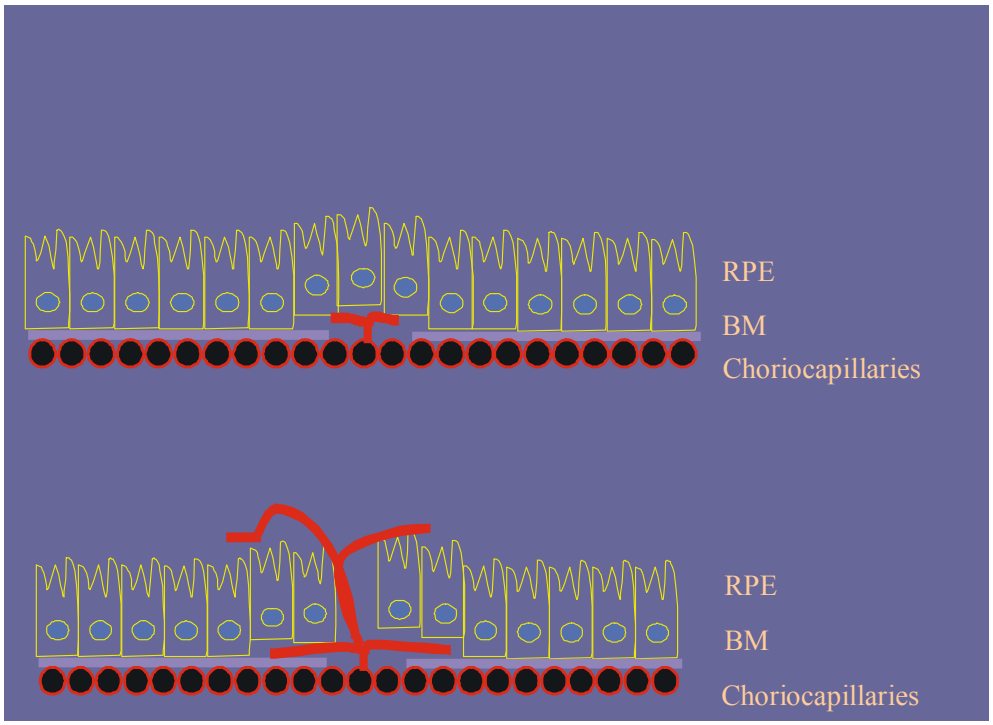

Fig 1.4: Schematic representation of CNV

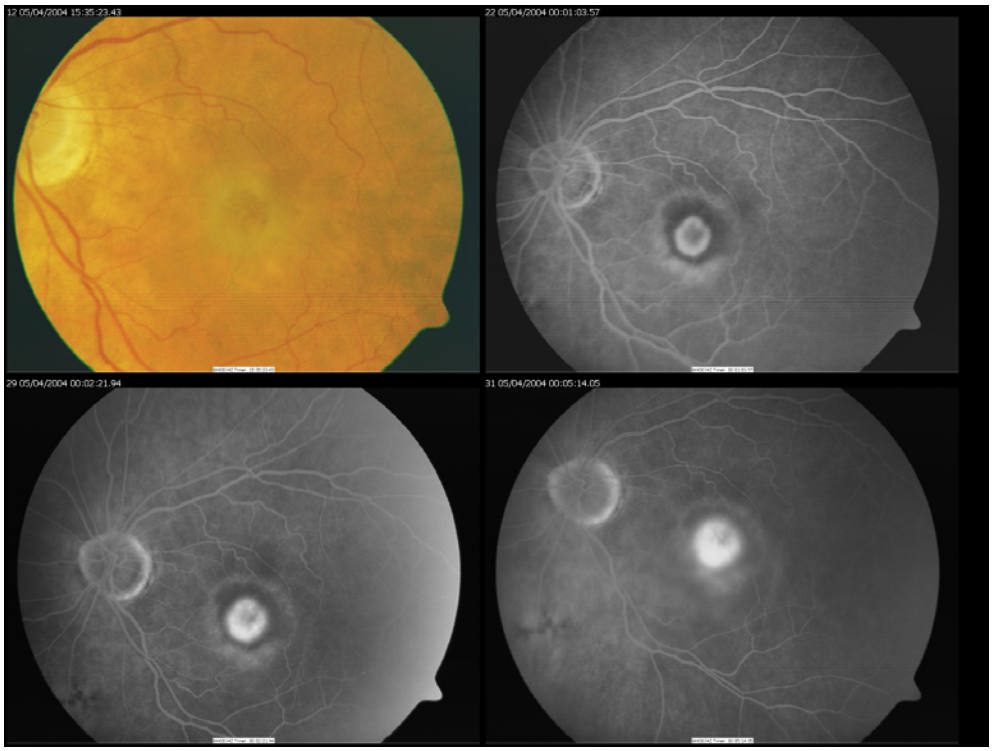

Fig.1. 5: Colour photograph and fluorescein angiographic evidence of CNV

1.1.3 The patho-physiology of AMD

Numerous epidemiological and basic science studies have provided suggestions for potential causal mechanisms for AMD. These include genetic influence, cumulative exposure to oxidative stress, vascular insufficiency secondary to atherosclerosis and immune and inflammatory mediated process (Ambati et al. 2003a).

Several genes have been studied in AMD and the latest evidence suggests increased risk of AMD in subjects with CFH gene mutation (Klein et al. 2005; Edwards et al. 2005; Haines et al. 2005). The exact role of this gene in AMD remains to be demonstrated though many elegant studies have demonstrated immune mediated biogenesis of drusen (Anderson et al. 2002; Crabb et al. 2002; Johnson et al. 2001).

AMD, like many other age-related diseases, have also been attributed to oxidative stress caused by reactive oxygen intermediates. The retina is particularly susceptible to oxidative damage due to the high oxygen tension, life time exposure to irradiation, high proportion of polyunsaturated fat, the presence of numerous chromophores in the retina and the phagocytic function of the RPE. Oxidative damage results from an mbalanace between reactive oxidative intermediates and thelevels of anti-oxidants in the retina. Superoxide dismutase, catalase and glutathione peroxidase and vitamins C, E, carotenoids and zinc form part of the complex system that protects the retina from oxidative damage while the pro-oxidants include lipofuscin, glycation and oxidation of lipoproteins (Beatty et al. 2000).

Hydrodynamic changes of the Bruch's membrane with age can also impede the two-way conduit through this strategically situated membrane and influence the development of AMD (Moore et al. 1995).

Ocular blood flow alterations may also contribute to the pathogenesis of AMD though the exact nature of impairment has not been elucidated (Friedman, 2000).

The intricate interplay and complexity of the multiple molecular processes involved make the understanding of AMD still quite elusive.

1.2 Atherosclerosis

1.2.1 Definition

Atherosclerosis is a degenerative disease of blood vessels and is a major cause of morbidity and mortality in the elderly.

1.2.2 Pathogenesis

The primary site of injury is thought to be the endothelial cells. The transmigration of lipids and monocytes through the dysfunctional endothelium initiate the initial lesion of atherosclerosis, namely fatty streak. The fatty streak evolves to the complex atherosclerotic plaque, which consists of a lipid core covered by a fibrous cap. Plaques are prone to rupture, leading to atherosclerotic advanced lesions. The traditional cardiovascular risk factors activate a complex cascade of events including inflammation, ECM remodelling and release of growth factors and cytokines and adhesion molecules.

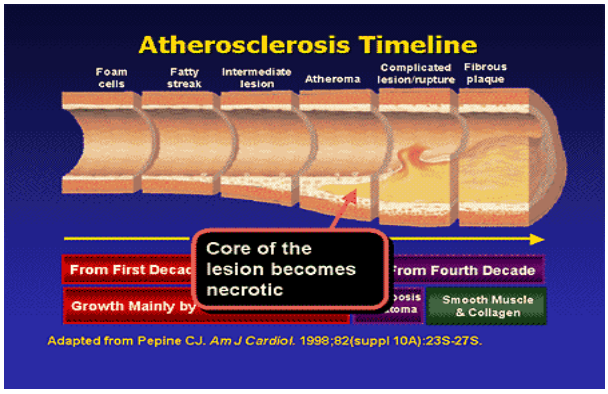

Fig.1. 6: Schematic representation of an atherosclerotic vascular wall

1.3 The link between atherosclerosis and AMD

1.3.1 Hypotheses

There are two main hypotheses that link atherosclerosis and AMD. The first hypothesis is that AMD is a tissue response to injurious stimuli parallel to the 'Response to Injury' hypothesis that explains atherosclerosis (Lim, 2002). The response to injury is defined as 'abnormal reparative response induced by exposure to chronic, recurrent injurious stimuli' (Ross, 1999). Both genetic and environmental factors can contribute to injury or repair. In AMD, the photoreceptors, RPE, BM and choriocapillaris may all be relevant potential targets. Similarly, the endothelial cells of the blood vessel wall were once thought to be the prime site of injury. However, recent research has shown that all layers of the vessel wall undergo significant changes due to the continuous or recurrent insults (Libby, 2003).

Cellular repair responses of the blood vessel wall and the BM are manifested by a wide spectrum of changes such as alteration in the synthesis of collagen and elastin, matrix metalloproteinases and other matrix molecules; by increasing production of chemotactic signals and angiogenic factors; and inflammatory and immune responses.

Regardless of the stimuli, the extracellular deposits that occur in both AMD and atherosclerosis share several common constituents (Mullins, 2000). These deposits may act as substrate for activation of a non-specific chronic inflammatory process (Hageman, 2001). The recent evidence that genetic polymorphism of Complement Factor H (CFH) contributes to the pathogenesis of AMD suggests that complement activation and inflammation may be crucial in the genesis of AMD (Klein et al, 2005; Haines et al, 2005; Edwards et al, 2005). This complement regulator has also been observed in the vascular intima in atherosclerosis and is thought to bind to proteoglycans (Oksjoki et al. 2003).

The second hypothesis is explained by Friedman's vascular model (Friedman, 2000). This haemodynamic model that focuses on the co-morbidity of atherosclerosis and AMD, proposes that AMD is secondary to vascular insufficiency induced by atherosclerosis.

Arterial intimal thickening, which is a hallmark for atherosclerosis may affect the choroidal vessels. The thickening and stiffening of the walls of the choroidal vessels result in decreased lumen diameter, increased blood flow resistance, elevated hydrostatic pressure and resultant RPE dysfunction (Friedman, 1997). This process is accelerated by age-related decreased density of choriocapillaris (Ramrattan al, 1999). Moreover, carotid arteriosclerosis can cause diminution of blood flow through the retinal and choroidal circulation and thereby interfere with the high metabolic rate of RPE resulting in sub-RPE deposits and RPE dysfunction (van Leeuwen, 2003).

1.3.2 Epidemiological evidence of co-morbidity of Atherosclerosis and AMD

Although both diseases are thought to be multi-factorial, the aetiology of AMD remains largely unclear and treatment options are limited. In contrast, several risk factors have been identified for atherosclerosis resulting in effective primary prevention and improved treatment. Increasing epidemiological evidence suggest a link between AMD and atherosclerosis.

About 300 risk factors have been identified for atherosclerosis. The established risk factors include advancing age, smoking, dyslipidemia, hypertension, obesity, impaired glucose metabolism and sedentary life. These risk factors have also been studied in AMD and only increasing age and smoking are found to be the most consistent risk factors.

a) Age

Of all the factors considered to affect atherogenesis, age has the strongest and most consistent association. This holds good for AMD as well. It is not possible to state that aging itself is implicated in the disease process or whether the effects of age represent a longer period of exposure to other risk factors such as smoking.

Several case studies have examined the incidence and prevalence of AMD with age. Different case definitions and diagnostic procedures have made it difficult to assess the exact risk involved. However, it is universally shown that risk of AMD increases exponentially with advancing age in men and post-menopausal women (Holz, 2003).

b) Post-menopausal women

Age and oestrogen deficiency are together the most important cause of risk for atherosclerosis in post-menopausal women. The risk is due to the abrupt interruption of oestrogen that has indirect protective effects on lipid metabolism and direct effect on blood vessel function. Studies that looked at the risk of AMD in post-menopausal women showed consistent results for various definitions of AMD and menopause. Most of the studies suggested positive co-relation of AMD with early menopause (EDCCS, 1992; Smith, 1997; Vingerling, 1995). The role of hormone replacement therapy on AMD remains unclear (EDCCS, 1992; Klein 1994; Klein 2000).

c) Smoking

Smoking is a strong modifiable dose-dependent risk factor for both atherosclerosis and AMD. Smoking more than doubles the risk for coronary heart disease (Snow and Sneddon, 1999).

The accumulated evidence from epidemiological studies provides strong causal relationship between cigarette smoking and AMD (Deblack, 2003). This established risk factor affects the eye mainly through its role in oxidative stress by reducing the levels of anti-oxidants. It can also decrease the choroidal blood flow, increase platelet adhesiveness, increase fibrinogen levels, decrease high-density lipoproteins levels and increase carboxyhaemoglobin in blood. In addition, it may affect the eye by decreasing CFH levels. The dose-dependent relationship indicates that these effects depend on cumulative lifetime exposure to smoking. The finding that former smokers have a higher risk compared to non-smokers shows that these effects diminish slowly unlike in atherosclerosis where cessation of smoking reduces the risk immediately and the risk declines to level of non-smokers within 2-5 years after cessation.

d) Hypertension

Hypertension is a strong modifiable risk factor for atherosclerosis. It increases cardiovascular morbidity and mortality two to four fold. However, many case control and cohort studies addressed the association between AMD and systolic or diastolic hypertension, hypertension or use of anti-hypertensives with inconclusive results (Sperduto, 1986; Evans, 2001). The risk of hypertension seems to be more concordant for neovascular AMD than for ARM (AREDS, 2000; Hyman, 2000; MPS, 1997; Klein et al, 1997a). In conclusion, there seems to be only a weak association of hypertension and AMD. It seems that hypertension and atherosclerosis may be independent risk factors for AMD.

e) Hyperlipidemia

Hyperlipidemia is another modifiable dose dependent risk factor for atherosclerosis, especially total serum lipids and low-density lipoprotein and an inverse relation with the high-density lipoprotein fraction (Zemel and Sowers, 1990).

The association between serum cholesterol and AMD is inconsistent. A positive association between AMD and serum cholesterol have been implied in two studies (EDCCS, 1992; Belda Sanchis et al. 2001). However, most studies on total serum cholesterol and AMD did not find any association (Smith et al. 2001; Klein et al. 1997a; Smith et al. 1998; Sanders et al. 1993; Cruickshanks et al. 1997; Klein et al. 1993; Delcourt et al. 2001). A few studies have also reported increased risk of early AMD with high serum HDL level (Abalain et al. 2000; Delcourt et al. 2001, Hyman et al. 2000; Klein et al. 2003c, van Leeuwen et al. 2004a, Watcher et al. 2004). These studies imply that serum HDL has an adverse effect on AMD in contrast to its protective effect on atherosclerosis.

f) Dietary intake

An inverse association was noted with omega 3 fatty acids intake and fish with early disease (Seddon et al. 2001, Smith et al. 2000). Similar protective roles of these dietary components have been suggested in atherosclerosis.

A number of studies have linked increased dietary fat intake to both early and late AMD (Heuberger et al. 2001, Hyman et al. 2000; Mares-Perlman et al. 1995; Seddon et al. 2001). These studies indicate a possible association between atherosclerosis and AMD. However, the inconsistent association with hyperlipidemia also hints that dietary fat may induce a local effect in the RPE-Bruch's membrane complex and so the effects may not totally correspond with serum levels. This fact is substantiated by the inconclusive role of lipid

lowering agents such as statins in AMD (Guymer et al. 2005). The anti-inflammatory effects of statins may also be responsible for the effect of statins on AMD (McGwin et al, 2005).

g) Genetic link

These speculations are further substantiated by genetic studies on AMD. A candidate gene for AMD is the cholesterol transporter gene apolipoprotein E (ApoE) that plays an important role in lipoprotein metabolism. This gene is polymorphic with 3 alleles ε2, ε3 and ε4. ε3 allele is neutral, while ε2 allele is associated with lower and ε4 allele with higher serum LDL levels. Thus, the presence of ε2 allele is considered a protective factor against atherosclerosis while ε4 is associated with coronary heart disease (Davignon et al. 1999). However, for AMD, studies have consistently reported the opposite findings with a significant protective effect of ε4 (Baird et al. 2002, Klaver et al, 1998, Souied et al. 1998). Some studies have also reported a weak increased risk of AMD associated with ε2 (Baird et al. 2002, Klaver et al. 1998). Souied proposed two different explanations to explain the protective effect of ε4 in AMD. ε4, unlike ε2 and ε3, does not contain disulfide bridges and the resultant smaller size may allow more efficient lipid transport across the BM. Secondly, ε4 presents positive charges which reduces the hydrophobicity of BM thereby favouring clearance of debris (Souied et al. 1998). Therefore, the varied effect of apoE polymorphism in the two diseases may be best explained again by its local effect in different tissues.

Another lipid related gene, the paraoxonase gene (PON 1) has been implicated in AMD. Paraoxonase is a polymorphic protein that prevents LDL oxidation. BB and LL genotype of this gene has been found more frequently in Japanese subjects with neovascular AMD

(Ikeda et al. 2001). In conclusion, it seems that though the two diseases are dependent on lipoprotein metabolism, the cellular effects may be different.

h) Diabetes mellitus

Diabetes accelerates atherosclerosis. The risk of myocardial infarction is two to three times more for diabetic men and three to seven times higher in diabetic women. Through its effects on the choroidal circulation and local effects on the RPE, diabetes has been hypothesized to increase the risk of AMD (Kohner et al. 1995). However, only a weak positive association between diabetes and AMD can be established from a number of studies that investigated the relationship (Kahn et al. 1977, EDCCS, 1992; Hyman et al. 1983; Delaney and Oates, 1982; Blumenkranz et al. 1986, Vidaurri et al. 1984, Klein et al. 1997a, Klein et al. 1992b, Watcher et al. 2004).

i) Obesity and physical activity

Obesity is an established cause of atherosclerosis. The risk of coronary artery disease is 2-3 times higher in obese subjects compared to lean subjects. Though the link is not as well established as in atherosclerosis, a positive link between AMD and body mass index (BMI) have been noted in several studies (Delcourt et al. 2001, Hirvela et al. 1996, Klein et al. 2001, Schaumberg et al. 2001, Smith et al. 1998). Waist to hip ratio, another marker of adiposity has been found to be increased in early AMD in women (Klein et al. 2001) However, lean individuals also appeared to be at increased risk of early AMD suggesting that the relationship between adiposity and AMD is J-shaped (Smith et al. 1998). Sedentary lifestyle is a modifiable risk factor for atherosclerosis. The relative risk of death from coronary artery disease (CHD) is two times higher than in active subjects. However there are only two case control studies that looked at physical activity in AMD and the results are

inconsistent (Seddon et al. 2003; Klein et al. 2003b). The mechanism by which obesity increases the risk of AMD may be related to the physiologic changes that occur with this condition. These include increased oxidative stress, changes in the lipoprotein profile, and increased inflammation. These changes would also result in an increased destruction and a decreased circulatory delivery of lutein and zeaxanthin to the macula of the eye. Therefore, the mechanism by which obesity is related to AMD risk may be through indirect effects on changes in lutein and zeaxanthin status and metabolism.

j) Alcohol intake

While non-alcoholics and heavy alcoholics have increased risk of cardiovascular disease, moderate alcohol intake is cardio-protective indicating a J-shaped relationship. However, the evidence for an association between alcohol intake and AMD is weak (Smith and Mitchell, 1996; Ajani et al. 1999, Cho et al. 2000, Ritter et al. 1995, Klein et al. 2002a).

k) History of cardiovascular disease

Self reported history of cardiovascular disease is potentially biased by misclassification. Most of the studies on the association of AMD and history of cardiovascular disease did not find an association (McCarty et al. 2001, EDCCS, 1992; Hyman et al. 2000; AREDS 2000). Positive associations were found by two groups (Hyman et al. 1983; Goldberg et al. 1998). No association was found with low dose aspirin, a cardioprotective drug, and the incidence of AMD (Christen et al. 2001). No increased risk of mortality has been associated with AMD after adjusting for risk factors that affect mortality (Borger et al. 2003).

l) Objective evidence of atherosclerosis

Several studies looked at objective evidence of atherosclerosis in patients with AMD. Vingerling at al reported that subjects below the age of 85 years with plaques at the carotid bifurcation were 4.7 times as likely to have late AMD as those without plaques. An ankle-arm index below 0.9 was also significantly associated with the presence of AMD. They did not find an association between intima-medial wall thickness (IMT) of the common carotid artery and AMD (Vingerling et al. 1995). The Rotterdam study also noted that calcifications in the abdominal aorta and peripheral arterial disease were not associated with AMD. These findings made them postulate that the atherosclerosis affecting the cerebral circulation may be more associated with AMD rather than atherosclerosis of aorta or the peripheral arteries (Klein et al. 1997a).

Their findings were not supported by other studies. The ARIC study found only retinal pigment epithelial depigmentation to be associated with carotid artery plaque and focal retinal arteriolar narrowing.

However, many other investigators found different indicators of atherosclerosis to be associated with AMD. Left ventricular hypertrophy was associated with AMD in the Framingham Eye Study (Kahn et al. 1977). A 2.5 fold increased risk of AMD was noted in patients with lower extremity disease in one study (Klein et al. 1999). The Beaver Dam study found that higher pulse pressure to be associated with 30% increased 5-year incidence and 25% increased progression of neovascular AMD in subjects aged 65 years or older (Klein et al. 1997a). The Cardiovascular Health and Age Related Maculopathy (CHARM) Study estimated pulse wave velocity, IMT and augmentation index in subjects

with AMD and found an association between poor cardiovascular parameters and progression of AMD (Guymer et al. 2005).

However, retinal arteriosclerosis defined as generalized narrowing of retinal arteriolar diameter has been studied by different groups with varying results. Hirvela et al., using a subjective analysis of retinal arteriolar calibre, found higher prevalence of AMD in subjects with retinal arterial narrowing (Hirvela et al. 1996). This is supported by two studies that used newer objective analysis of retinal vascular calibre (Klein et al. 2004, Wang et al. 2004) but not by the Rotterdam study group (Ikram et al. 2005).

Summary of evidence to date

Epidemiologic studies have evaluated several cardiovascular risk factors in patients with AMD and suggest an overlap of some of these vascular factors and AMD. The differential susceptibility of AMD to cardiovascular risk factors suggests that the nature of the injurious stimuli of these risk factors is more parallel than convergent. They seem to produce different cellular effects. The findings also suggest a possible relationship between carotid atherosclerosis and AMD. Further research in these areas may possibly lead to new insights into the aetiologic factors for AMD and identify methods of preventing or slowing the progression of AMD in susceptible individuals.

1.4 Aims and Objectives of this project

The aim of the first part of the project was to assess the correlation of systemic ECM proteins with the severity of AMD. A review that compares the changes of the vascular wall in atherosclerosis with the Bruch's membrane in AMD was done to better understand the similarities and differences between these two structures with particular emphasis on ECM proteins. The subsequent experimental objectives were:

a) Analysis of serum elastin-derived peptides (S-EDP) by ELISA to investigate the elastic tissue degradation of the ECM in AMD patients and age-matched controls.

b) Measurement of serum MMP-2 and MMP-9 by ELISA, to investigate the role of matrix metalloproteinases in AMD patients and age-matched controls.

c) Assessment of the correlation between S-EDP and MMP-2 and MMP-9 to note whether MMPs are responsible for the degradation of elastin in AMD

d) Assessment of the possible link between atherosclerosis and AMD by retinal examination of patients with abdominal aortic aneurysm (AAA).

The aim of the second part of the project was to assess the role of complement activation in AMD. As atherosclerosis is redefined as an inflammatory disease, the role of inflammation in AMD was reviewed. Experimentally, serum C3a des Arg, which determines the role of complement pathway in the pathogenesis of AMD, was analysed in subjects with varying severity of AMD and compared to age matched controls.

The aim of the third part of the project was to assess ocular blood flow in AMD. A review of ocular haemodynamics in AMD was performed. Choroidal blood flow and retinal

vascular calibre was measured and compared in both eyes in patients with asymmetric AMD. The choroidal blood flow was assessed by measuring the pulsatile ocular blood flow. The retinal vascular calibre was measured using computer software adapted from the Atherosclerosis Risk in Communities study (ARIC) study.

Chapter II

EXTRACELLULAR MATRIX

Is the concurrence of atherosclerosis and age related macular degeneration due to similar extracellular tissue remodelling?

CHAPTER 2: EXTRACELLULAR MATRIX

This chapter reviews the evidence published to date that suggests a link between the BM and the vascular intima, then describes experimental investigations carried out in attempt to provide further evidence for this link.

2.1 Bruch's Membrane and the Vascular Intima: Is There a Common Basis for Age-related Changes and Disease?

This review examined the morphological and biochemical alterations in the senescent BM and its analogy to the vascular wall to evaluate the concurrence of atherosclerosis and AMD.

2.1.1 Structural Comparison of the Bruch's Membrane and the Vascular Wall

The BM is a thin (2-4μm) connective tissue interposed between the metabolically active RPE and its source of nutrition, the choriocapillaris. This penta-laminar structure is classically described as consisting of the basement membrane of the RPE, an inner collagenous zone (ICZ), a fenestrated elastic layer, an outer collagenous zone (OCZ) and the basement membrane of the endothelium of the choriocapillaris (Hogan and Alvarado, 1967). In contrast, a healthy arterial wall of a major blood vessel such as aorta is composed of only three histologically specific layers: the innermost layer (intima) consists of a single layer of endothelial cells; the media is composed of smooth muscle cells, collagen and elastic fibrils and the third distinct layer is a fibrous adventitia (Lakatta and Levy, 2003).

In order to investigate the co-morbidity of atherosclerosis and AMD, a useful analogy of the BM is the arterial wall. The fenestrated choriocapillaris and the endothelial monolayer

of the arterial wall possess a similar sub-cellular stratified ECM: the BM for the choriocapillaris and the media and adventitia for the vascular intima. However, the key difference is the virtual absence of smooth muscle cells in the BM. Moreover, unlike the vascular ECM, the BM is strategically located between an endothelium and an epithelium (RPE) and is therefore, subject to changes from both sides. In contrast, the vascular wall matrix is affected by changes caused by endothelial dysfunction only.

While the causes for endothelial dysfunction of the choriocapillaris may be akin to that of atherosclerosis, RPE dysfunction may arise secondary to causes that do not primarily affect its nutrition from the choricapillaris, and this includes oxidative insults, local inflammatory processes, senescence and genetic defects. This may further explain the differential susceptibility of AMD to cardiovascular risk factors.

2.1.2 Macroscopic Changes of the BM and the Vascular Wall

The fundamental age-related change in both structures is typified by increased thickness. The age-associated thickening of the arterial wall consists mainly of intimal thickening. The carotid IMT increases 2-3 fold between 20 and 90 years of age (Virmani et al. 2000). Similarly, the BM undergoes diffuse thickening, with thickness being reported to increase by 135% in ten decades (Ramrattan et al. 1994). The maximum thickness occurs in the substrata of the OCZ (Killingsworth, 1987). However, there is marked heterogeneity in the intimal and BM thickness among individuals at a given age (Lakatta and Levy, 2003; Bird, 1992).

Disease risk is associated with age-related thickness of these structures. Carotid intima-media thickness is used as a predictive non-invasive test for atherosclerotic burden (Meschia and Gerber, 2003) Likewise, a prolonged choroidal filling phase during

fluorescein angiography signals the presence of diffuse BM thickening (Pauleikhoff et al. 1992). In addition, serial measurement of the thickness of the BM may be a useful prognostic parameter in longitudinal studies of AMD.

2.1.3 Cell biology of the BM and the vascular wall

The three anatomical changes that occur in the BM with age are the progressive accumulation of debris, lipid deposition and alteration of the ECM. A similar build up is seen in the arterial wall with accumulation of lipids, cellular waste products, and fibrin and calcium deposits resulting in plaque formation (Virmani et al. 2000).

The deposition of PAS positive granular, vesicular and filamentary deposits in the ICZ of BM has been identified as early as the first decade of life (Hogan and Alvarado, 1967).

As age advances, the debris accumulates and contaminates all the collagenous layer of the BM, and is seen on both sides of the elastic lamina thus forming the bulk of the age-related debris in the collagenous layers, especially in the OCZ. Depending on their proximity to the RPE, deposits may represent incompletely digested waste material emanating from a dysfunctional RPE (Burns and Feeney-Burns, 1980; Ishibashi et al. 1986; Young, 1987; El Baba et al. 1986) or it may be the sequelae of dysfunctional endothelium of the choriocapillaries (Friedman et al.1963). Experimental evidence also suggests that these deposits may be due to altered remodelling of the membrane (Newsome et al. 1987) or an inappropriately directed immune response (Hageman et al, 2001; Penfold et al. 2001).

The cell biology of the arterial intima suggests abundant granulo-vesicular debris in the intima, probably originates from disintegration of vascular smooth muscle cells and dead macrophage foam cells (Klurfield, 1985). Though the source of the deposits in both

diseases may not be directly linked, a common component may be that the cellular debris in both structures acts as a focus for inflammation and local immune response.

The link between atherosclerosis and AMD is further substantiated by the similarity of molecular composition of drusen and atherosclerotic deposits (Mullins et al. 2000). The proteins identified in the drusen/ BM include both locally derived (neural retina, RPE and choroid) and extracellular non-ocular components. The oxidative modifications of some of these components may also be the primary catalyst in drusen formation. Most of these non-ocular components are also present in atherosclerotic lesions.

The complement system also plays a role in both the diseases. C5b-9 complexes have been isolated in intact cells, disintegrated cells and cell debris enmeshed in the extracellular matrix (ECM) of thickened vascular intima and atherosclerotic plaque (Rus et al. 1988). Some of these cells represent activated or dead macrophages. They trigger inflammatory events and progression of atherosclerotic lesions. Likewise, immunohistochemical analyses of all phenotypes of drusen and the BM have also revealed C3, the terminal complement complex C5 and the membrane attack complex C5b-9 terminal complexes. While C3 and C5 mRNA are present in the neural retina, RPE and choroid (Anderson et al. 2002), the source of most of the complement factors may be from plasma. In addition, several complement inhibitors have been identified in drusen such as clusterin, CD46 and CR1 (Johnson et al. 2001). The localisation of these complements proteins and their regulators provide evidence of local inflammatory response. The granulo-vesicular nuclear fragments, oxidized lipoproteins and AGE may be potential activators of the complement cascade in both the vascular intima and BM (Libby, 2002; Hageman et al. 2001). The fact that immunological injury may be an initiating factor in the development of AMD and

atherosclerosis is further substantiated by the isolation of HLA antigen markers in drusen and atherosclerotic lesions (Dahlen et al. 1994; Goverdhan et al. 2004). However, the multifactorial nature of both disease processes makes a simple relationship with a single MHC determinant unlikely. The recent evidence that CFH polymorphism is associated with increased risk of AMD (Klein et al. 2005; Haines et al. 2005, Edwards et al.2005) suggest that that complement activation is a crucial step in AMD. The role of CFH in atherosclerosis showed that proteoglycans, because of their ability to bind the CFH, may inhibit complement activation in the superficial layer of the arterial intima. In contrast, deeper in the intima, where CFH and proteoglycans are absent, complement may be activated and proceed to C5b-9. Thus, the superficial and the deep layers of the human coronary artery atherosclerotic lesions appear to differ in their ability to regulate complement activation (Oksjoki et al. 2003).

2.1.4 Lipid accumulation

An important age-related change that forms the base for atherosclerosis is lipid accumulation in the thickened intima. In the arterial intima, plasma lipoproteins are transported across the endothelium and trapped amongst fibrils of ECM (Berliner et al. 1995). These initial lesions are characterised by microscopically visible lipid droplets which coalesce to form fatty streaks, and finally stratified pools of lipid are found in the intimal thickening.

A similar age-related exponential accumulation of lipids occurs in the BM. The BM becomes sudanophilic and exhibits increased staining with Oil red O (Holz et al. 1994; Spaide et al. 1999; Farkas et al. 1971; Sheraidah et al. 1993; Pauleikhoff et al. 1992). In eyes of patients less than 60 years old, these lipid-containing small round solid particles are

scarcely scattered in both collagen layers. In older eyes, the lipid droplets occupy up to one third of the ICL and also form a thin lipid layer external to the RPE basement membrane (Ruberti et. 2003).

Although both the BM and the arterial intima become lipid laden with age, the lipid composition is dissimilar. Unlike the plasma-derived low density lipoproteins (LDL) infiltration in the vascular intima, the predominant lipids in the BM consist of phospholipids and fatty acids (Holz et al. 1994; Spaide et al. 1999; Farkas et al. 1971; Sheraidah et al. 1993; Pauleikhoff et al. 1992). About 50% of the phospholipids are phosphatidylcholine suggesting that the lipids in the BM are more likely of a cellular origin (a potential source being the photoreceptor outer segment membranes) than from plasma. However, recent studies using filipin histochemistry and hot stage polarizing microscopy revealed that lipid content of BM also consist of both esterified and unesterified cholesterol but a vascular origin for the lipids is unlikely (Curcio et al. 2001; Ruberti et al. 2003; Haimovici et al. 2001).

2.1.5 Matrix dysregulation

The matrix changes associated with age-related arterial changes include: increased collagen content, increased collagen cross-linking, increased fibronectin, decreased elastin associated with calcification and fragmentation of the elastic lamina, and increased glycosaminoglycans (Goncalves et al. 2003). Similar changes are observed in the aging BM.

Collagen synthesis increases with age in both structures (Barnes and Farndale, 1999; Karwatowski et al. 1995). Type I and III collagens form the bulk of the total plaque protein in atherosclerosis. Likewise, Newsome et al found an age-related increase in collagen I in

the BM with age (Newsome et al. 1987). Other collagens noted in the BM include types III, IV and V (Karwatowski et al. 1995). In addition, atypical banding periodicity has been observed in the collagen produced. The ICZ consists more of 640A type collagen while the OCZ contains more 1000A type collagen with age (Hogan and Alvarado, 1967). The meshes formed by the tightly interwoven collagen fibres in the ICZ also become irregular and coarse with age (Yamamoto et al. 1989). Increased cross-linking results in a linear decline of solubility of the collagens with age (Karwatowski et al.1995). The long spacing collagen is a material with periodicity ranging between 100-140nm found mainly in the OCZ and extends as intercapillary pegs to areas where choriocapillaris have undergone age-related atrophy (Krey, 1975). This material has also been isolated in the aortic media (Morris et al. 1978). In both structures, the increased insoluble collagen may contribute to debris accumulation and serve as a depot for lipoproteins, growth factors and cytokines.

The amount of non-collagen protein in BM also increases significantly with age as suggested by an increase in deposition of non-collagen amino acids in the macular area (Karwatowski et al. 1995). The elastic layer becomes basophilic; the elastic fibres increase in number and become more electron dense. Needle like crystals are deposited within the fibres (Hogan and Alvarado, 1967) The BM also undergoes calcification and fragmentation, rendering the membrane to loose its elasticity and become more brittle. The degree of calcification and fragmentation of BM correlates with the severity of wet AMD, although it is seen in both types of AMD (Spraul et al.1999).

Similar changes occur in the elastic layer of the arterial wall. The exact mechanism of calcification of the elastic fibres is not known, but it may be the result of production of abnormal elastic fibres, degeneration of elastic fibres or other alterations in the ECM

(Walker et al. 1989). Degradation of the arterial wall elastin is a characteristic feature in atherogenesis. Serum concentrations of elastin-derived peptides (S-EDP) are elevated in atherosclerotic patients and reflects elastin turnover (Baydanoff et al. 1987). Increased S-EDP is a potential indicator of advanced atherosclerosis such as plaque instability and is also a predictor of rupture in atherosclerotic aortic aneurysms (Petersen et al. 2002).

Changes in glycosaminoglycans (GAG) also occur in the ECM of both the vascular intima and Bruch's membrane. Sulfated GAG, which are components of the vascular matrix, increase during the early stages of atherosclerosis (Stevens et al. 1976). Binding of dermatan sulfates to low density lipoproteins correlates positively with lipid accumulation in the intima and progression of atherosclerotic lesions respectively. Chondroitin sulphate normally retards the passage of plasma particles and maintains the viscoelastic property of the vessel wall which is structurally altered in advanced lesions (Stary et al, 1994). On the contrary, heparan sulphate, a major component of basement membrane, reduces with the severity of atherosclerotic lesions (Williams and Tabas, 1995). These changes do not correspond with changes in the Bruch's membrane. An increase in the content and structure of GAG in BM has been identified (Kliffen et al. 1996). The increased volume of GAG may partially be responsible for the altered metabolism of the collagens and the resultant increased negative field may also contribute to the decreased filtration across the BM (Hewitt et al. 1989). An increased proportion of heparan sulfate in the basement membranes of the BM and simultaneous decrease in proteoglycan filaments containing chondroitin sulfate and dermatan sulfate associated with the collagen fibrils have been noted (Hewitt et al. 1989, Takada et al.1994). It may be that the main source of dermatan sulphate in the intima is the smooth muscle cells and the absence of these cells in the BM

correlates with the scarcity of this GAG. In addition, there may be differences in macrophage recruitment and accumulation in the two structures and proteolytic degradation by the macrophages may account for the significant differences in changes in proteoglycan subtypes. The difference in the proteoglycan subtypes may partially be also responsible for the altered lipid content in these structures.

2.1.6 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are required both by BM and arterial intima for ECM remodelling, protein processing and angiogenesis (Visse and Nagase, 2003) The age-related increased collagen content, the release of reactive oxygen radicals by the lipid-laden BM and intima, and the presence of inflammatory cytokines, cellular transformation and growth hormones in both the structures may upregulate these enzymes, especially MMP-2 and MMP-9 (Sethi et al. 2000; George, 1998). Both MMP-2 and MMP-9 are gelatinases and readily digest denatured collagen. In addition MMP-2 digests type I, II and III collagens (Visse and Nagase, 2003). Localisation of MMP-2 and MMP-9 expression to areas of new vessel formation in the BM and to areas of adventitial vasovasorum suggests their role in the growth of neovascular complexes in both AMD and atherosclerosis (George, 1998; Steen et al. 1998).

One of the endogenous tissue inhibitors of MMPs (TIMPs) regulates the activation of MMPs and also has other independent actions. Of the four TIMPs characterised to date, TIMP-3 is the only member found exclusively in ECM explaining the presence of TIMP-3 in both the intima and BM (George, 1998). In addition, it binds tightly to sulphated glycosaminoglcans. Western blotting and quantitative reverse zymography have demonstrated an age-related increase in TIMP-3 in BM and its concentration is shown to

correlate with the amount of ECM and the quantity of drusen (Kamei and Hollyfield, 1999). In BM, TIMP-3 controls ECM turnover, limits neovascularisation (Moses and Langer, 1991) and may play a role in apoptosis (Baker et al.1998). Mutations of TIMP-3 associated with Sorsby's fundus dystrophy suggest that TIMP-3 may result in aberrant protein interaction and increased cell adhesiveness, which may cause defective turnover of the BM (Yeow et al. 2002). A similar up-regulation of TIMP-3 in atherosclerotic aorta has been noted (Carrell et al. 2002).

2.1.7 Conclusion from Literature Review

It is clear that the vascular intima and the Bruch's membrane share several age-related changes with the involvement of several common molecules. This may be because they are exposed to similar genetic variations, oxidative stress or they may be exposed to the same immune or inflammatory disease complexes. On the basis of this review, one may argue that atherosclerosis and AMD may share some aspects in a response to the resultant tissue damage.

2.2 Serum Elastin Derived Peptides in Age-related Macular Degeneration

2.2.1 Introduction

The pathogenesis of AMD is undoubtedly complex and multifactorial (Ambati et al. 2003a). Several investigators have shown that changes in matrix biology play an important role in the pathogenesis of AMD (Johnson and Anderson, 2004, Newsome et al. 1987). Elastin is an extremely insoluble fibrous protein constituent of the ECM (Partridge, 1962). It is the main protein of the elastic fibre and contributes to the elastic property of several tissues such as the vascular wall, lungs and skin. In the eyes, elastin is present in the BM and choroidal vessels (Chong et al. 2000). Partial proteolysis of elastin by activated proteinases results in the release of soluble elastin derived peptides (S-EDP) into the circulation. Therefore, S-EDP measurement is indicative of systemic elastin turnover (Bouissou et al. 1985).

Large amounts of S-EDP are produced during several pathological processes (Fulop et al. 1990, Bizbiz et al. 1997). The measurement of S-EDP has been proposed as a method for monitoring disease processes such as emphysema and as a predictor of expansion of small abdominal aortic aneurysms (Pardo et al. 1999, Lindolt et al. 1997). Accelerated elastolysis has also been reported in patients with various manifestations of atherosclerosis (Petersen et al, 2002). Several clinical and epidemiological studies have concurrently illuminated established cardiovascular risk factors and markers of atherosclerosis with AMD (Snow and Sneddon, 1999). Therefore, we postulated that both the vascular wall matrix and the Bruch's membrane may share several common changes including degradation of elastin

and release of S-EDP into the circulation. In this study, we analysed S-EDP levels in patients with AMD compared to S-EDP in age matched controls.

2.2.2 Materials and Methods

2.2.2.a) Subjects

Fifty-six subjects with a clinical diagnosis of AMD were included in the study. The control group consisted of 15 healthy subjects without AMD (defined as the absence of drusen, pigmentary abnormalities and neovascular AMD). All enrolled subjects underwent a complete ophthalmic examination by the recruiting retinal specialist: visual acuity, slit lamp examination and retinal examination after pupil dilation were documented. Each subject also had 30° colour stereo fundus photographs of both eyes taken (Topcon TRC-50X, Topcon Ltd. Tokyo, Japan). Fluorescein angiography was also performed if there was a clinical suspicion of CNV. Subjects with co-existent fundus pathologies and subjects with un-gradable photographs were excluded from the study.

A detailed medical history of each subject was taken. Particular care was taken to note history of hypertension (defined as being prescribed anti-hypertensives), angina pectoris, myocardiac infarction, intermittent claudication, chronic obstructive airway disease (COAD), abdominal aortic aneurysm (AAA) and hypercholesterolemia (defined as being prescribed statins). History of smoking was classified into: never smoked, ex-smoker, or current smoker. All current smokers were excluded.

This research adhered to the tenets of the Declaration of Helsinki. Institutional ethics committee approval was obtained and all subjects gave their full informed consent. The patients in this study were recruited as part of a Medical Research Council (UK) funded project on AMD.

2.2.2.b) Grading of AMD

Colour fundus photographs of the subjects were graded by two graders using the nomenclature and classification recommended by The International ARM Epidemiological Study Group (Bird et al. 1995). Fundus fluorescein angiography was available to exclude CNV in patients classified as early ARM by fundus appearance. The graders were masked for the age and clinical history of the participants. Double grading for intra-observer and inter-observer variability was performed. Discrepancies were resolved by discussion. The subjects were classified as either early age related maculopathy (ARM) or neovascular AMD. Early age-related maculopathy was defined as the presence of intermediate soft distinct drusen (>63μm) with retinal pigment epithelial depigmentation or hyperpigmentation, soft indistinct drusen (>125μm), or reticular drusen; Neovascular AMD consisted of subjects with CNV. If the grades in the two eyes were different, the subject was categorized according to the severity of changes in the worse eye.

2.2.2.c) Blood samples

Venous blood was collected from all subjects in serum separator tube (SST-BD Vacutainer, BD Diagnostics**,** Oxford UK), allowed to clot for 30 minutes and then centrifuged for 10 minutes at 1500 g at room temperature. Serum was aliquoted and stored at –70°C within an hour of collection and then thawed when required. The samples were randomized so that the scientist who analyzed the samples was masked of the clinical history of the subjects.

2.2.2.d) Estimation of S-EDP

a) Optimization of ELISA

i) Defining the starting condition

Construction of a direct binding of α-elastin at 1.25μg/l with varying concentration of primary antibody (0-3μg/ml) was performed. The initial capture antibody tested was mouse monoclonal antibody to bovine α-elastin (Abcam Ltd, Cambridge, UK). This antibody recognizes insoluble elastin and soluble α-elastin and cross-reacts with human elastin. Binding was carried out at various incubation periods of 30mins, 1 hr, 2 hr and 24 hours and at varying temperature of 4^0C, 20^0C and 37^0C. The wells were rinsed with PBST and binding was detected by HRP-labelled goat anti-mouse IgG antibody (Santa Cruz Technology, Autogen Bioclear, Wiltshire, UK) at 1: 1000 dilutions at 1 hr in room temperature. The plates were rinsed and reacted with the substrate (100μl/well of ABTS). Absorbance was read at 495nm.

ii) Optimization of the sensitisation stage

A varying range of α-elastin concentration (1-3 μg/ml) without modification of the sodium carbonate buffer was used to coat the microtiter plate and the test repeated with all other variables unchanged.

iii) Optimization of secondary antibody

The same protocol was followed with α-elastin concentration fixed at 1.25μg/ml and capture antibody at 2μg/ml. The concentration of secondary antibody was tested at 1:500, 1:1000, 1:2000 and 1:4000.

iv) Confirmation of antibody specificity by dot-blot

This procedure was carried out to check the suitability of capture antibodies for use in enzyme-linked immuno-sorbent assay (ELISA). On a membrane (Hybond C, Amersham

Bioscience, Buckinghamshire, UK), 10µl aliquots of 1.25µg/ml of α-elastin from human aorta (Sigma-Aldrich, Poole, UK) in 0.1M sodium carbonate (pH 9) were placed in dots in grid formation and allowed to dry. The membrane was blocked with blocking solution consisting of 5% w/v low fat milk powder ('Marvel', Premier Foods, St. Albans, UK) in TBS (Tris-Buffered Saline) for one hour on an orbital shaker. The capture antibodies tested included a mouse monoclonal antibody to α-elastin (Abcam Ltd, Cambridge, UK) and a rabbit polyclonal antibody to α-elastin (Elastin products Co, Missouri, USA). Each capture antibody was added to blocking solution (described above) at a range of dilutions, and applied to a strip of membrane with an α-elastin 'dot' for one hour at room temperature. The membrane was then washed with TBST (Tris-Buffered Saline containing 0.05% v/v Tween 20) five times, for five minutes each. The strips were then incubated with appropriate detection antibody (both HRP-conjugated antibodies purchased from Santa Cruz Technology, Autogen Bioclear, Wiltshire, UK). Donkey anti-mouse detection antibody was used for the monoclonal capture antibody (1:1000 in 5% w/v milk in TBS), and goat anti-rabbit detection antibody for the polyclonal capture antibody (1:1000 in 5% w/v low-fat milk powder in TBS), both incubated for one hour at room temperature. Membranes were then washed again in TBST for five minutes five times. Chemiluminesence detection was then performed using an ECL detection kit (Amersham Bioscience, Buckinghamshire, UK) and the membrane strips were placed in a cassette with a high performance chemiluminesence hyperfilm ECL (Amersham Bioscience, Buckinghamshire, UK) prior to developing the film for analysis.

b) Competitive Enzyme linked Immunosorbent assay (ELISA)

The concentration of S-EDP was determined using a competitive enzyme-linked immunoassay (ELISA) adapted from a protocol described by Peterson et al (Petersen et al. 2002). Soluble α-elastin was obtained from human aorta (Sigma-Aldrich, Poole, UK). The rabbit anti-human elastin IgG (Elastin Products Company, MO, USA) was raised against α-elastin prepared from human aorta.

The wells of a microtitre plate (Nunc, Fischer Scientific, UK) were coated with 150 µl of α-elastin (1.25 µg/ml) in 0.1M sodium carbonate, pH 9 by incubating at 4^0C for 12 hours. The plate was then washed four times with 150 µl phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20 (PBST). A standard curve was generated using dilutions of human aortic α-elastin (0-100 ng/ml). Anti α-elastin antibodies (1:1000) in PBST and 7% (w/v) bovine serum albumin (PBST-7% BSA) were pre-incubated with variable dilutions of α-elastin in PBST-7% BSA overnight at 4^0C. These solutions were then transferred to the coated plate and incubated at room temperature for one hour. The pre-incubation period prior to addition to the α-elastin coated microtitre plate therefore creates the 'competitive' aspect of the ELISA. Before addition of the detection antibody, the microtitre plate wells were washed with PBST four times. Then, 150 µl of HRP-conjugated goat anti-rabbit IgG antibody (Santa Cruz technology / Autogen Bioclear, Wiltshire, UK) was added to each well (1:2000 dilution in PBST-7% BSA). The plate was incubated for 1 hr at room temperature and again washed with PBST four times. Thereafter, 100 µl of ABTS (3-ethyl-benzthiazoline-6-sulphonic acid; Roche, Lewes, UK) buffer was added and the plate incubated for a further hour at room temperature. Absorbance was read at 495 nm

using an automated plate reader (Dynex Technologies, West Sussex, UK). A standard curve was generated from the absorbance readings obtained from the α-elastin dilutions.

During the assay optimization process, pooled human sera from healthy volunteers were used as a diluent for the standard curve instead of the assay buffer (PBST-7% BSA). This was done to examine the potential effect of serum components on the ELISA. The standard curves generated with standards in pooled human sera and assay buffer were correlated and the level of significance tested by sign test. Since the standard curves were well correlated and there was no significant difference between them, subsequent standard curves were based on α-elastin in assay buffer.

Standards and serum samples were analyzed in duplicate. The S-EDP concentrations were calculated from the standard curve and expressed as ng/ml.

The following controls of the reaction were employed: a) substrate control: only assay buffer, wash solutions and ABTS substrate were added to the polystyrene wells coated with α-elastin; b) no capture antibody control: the detection antibody was added directly to the wells coated with α-elastin and then the wells were incubated with the ABTS substrate solution; c) negative control to assess the specificity of the reaction: The tested samples were replaced with human albumin solution and serum samples from healthy volunteers and assayed using the standard protocol; d) positive control: the tested sample was replaced with human aortic α-elastin at 1g/ml in assay buffer.

Intra-plate and inter-plate reproducibility tests were also performed. For the former, a pair of positive and negative controls was each tested 30 times in the same ELISA plate (intra-plate). In the latter test, the positive and the negative serum controls were tested 30 times using different plates from the same lot on different dates (inter-plate). The mean, standard

deviation (SD) and coefficient of variation (CV) were calculated for both the inter-plate and intra-plate optical density (OD) values. The ELISA was considered reproducible if the variations of each of the 30 positive and 30 negative control OD value was within ±2 SD of the mean of the individual runs. Sample results were discounted if the duplicates disagreed by more than 5%.

2.2.2.e) Statistical methods

Analysis was done using the Statistical Package of the Social Sciences (SSPS version 11.0). Results were reported as mean ± standard error of mean (SEM). The normal distribution of S-EDP values was assessed using the Shapiro-Wilk test. The standard curves generated with standards in pooled human sera and assay buffer were correlated and the level of significance tested by sign test. Multiple regression analysis was performed using S-EDP as the dependent variable and the disease status of the subjects as independent variable. The relationship of aging to S-EDP levels was analyzed independently and in the three groups using correlation coefficient. Student's t-test and ANOVA one-way was used for analysis of results. Probability values less than 0.05 were considered statistically significant.

2.2.3 Results

2.2.3.a) Characteristics of subjects

Serum was analysed from 71 subjects consisting of 30 with ARM, 26 with neovascular AMD, and 15 controls. Table 2.1 presents the demographic details and baseline clinical data of the population. Age and gender distribution were similar in both groups of AMD

and controls. No statistically significant differences in clinical data were observed between subjects in the three groups.

Table 2.1: Characteristics of the subjects

	ARM (n=30)	**Neovascular AMD (n=26)**	**Controls (n=15)**	**p-value**
Age(years) Mean [range]	77 [65-88]	79 [62-92]	75.2 [61-91]	0.7
Gender M:F	12:18	10:16	6:9	0.5
Ex-smoker (%)	3.3	3.8	0	0.9
Hypertension (%)	16.6	15.4	13.3	0.8
Myocardiac infarction (%)	3.3	3.8	0	0.9
Angina Pectoris (%)	0	0	6.6	0.2
Intermittent Claudication (%)	0	3.8	0	0.4
Stroke (%)	3.3	0	0	0.5
COAD (%)	3.3	0	0	0.5
AAA (%)	0	0	0	1.0
Hypercholesterolemia (%)	3.3	3.8	0	0.9

2.2.3.b) Optimization of ELISA

i) Defining the starting condition

Construction of a direct binding of α-elastin at 1.25µg/l with varying concentration of primary antibody (0-3µg/ml) was performed. There was no significant binding in any well and no standard curve could be plotted. It was inferred that the initial concentration of α-elastin 1.25ug/ml may not be sufficient or the secondary antibody concentration was fixed

using the manufacturer's recommended dilution and that stronger concentration of secondary antibody may be required to detect the binding.

ii) Optimization of the sensitisation stage

A varying range of α-elastin concentration (1-3 μg/ml) was used to coat the microtiter plate and the test repeated with all other variables unchanged. However, no binding was noted. In addition, the incubation of the α-elastin coated plates was varied from 1 hr, 2hr and 24 hr. No binding was again noted. So it was inferred that the optimal concentration for α-elastin is 1.25μg/ml but the concentration of secondary antibody had to be optimized.

iii) Optimization of secondary antibody

The same protocol was followed with α-elastin concentration fixed at 1.25μg/ml and primary antibody at 2μg/ml. The concentration of secondary antibody was tested at 1:500, 1:1000, 1:2000 and 1:4000. A level of absorbance was noted in all wells at 1:500 suggesting too high a concentration of secondary antibody. The best colour and absorbance was at 1:2000.

iv) Antibody Specificity

The results of dot blot analysis demonstrated that only the polyclonal capture antibody tested would consistently detect human α-elastin. The mouse monoclonal antibody tested failed to indicate binding to the antigen using either a dot-blot or ELISA protocol.

Figure 2.1 shows the consistent binding of the rabbit polyclonal antibody of various concentrations with α-elastin**.**

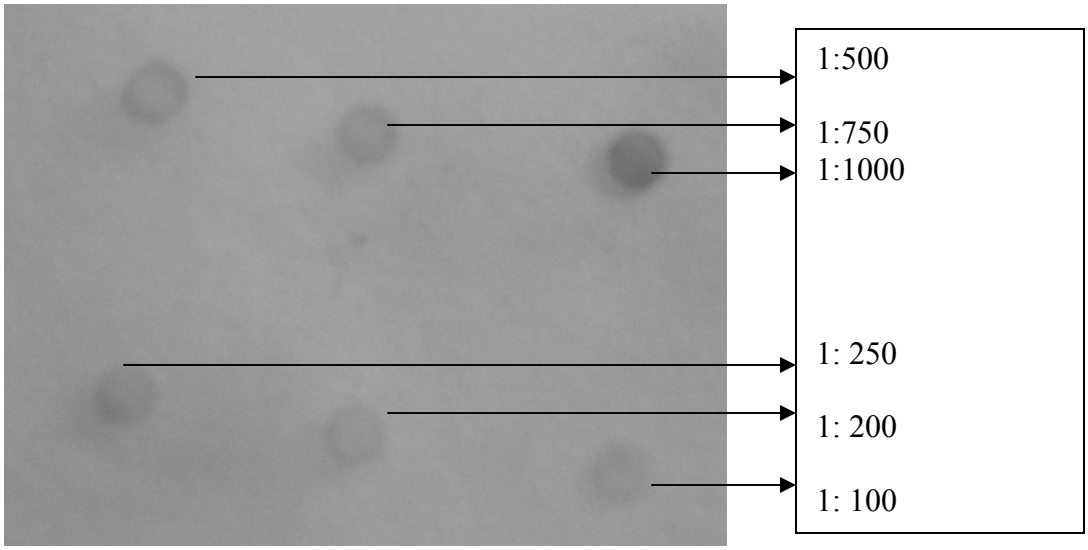

Fig 2.1: Dot blot test showing the binding of various concentrations of polyclonal capture antibody with soluble α-elastin of human aorta. The binding with α-elastin was best with the antibody concentration of 1:1000. So polyclonal rabbit anti human antibody 1:1000 was used for the ELISA.

v) Optimisation with different α-elastin

The ELISA was repeated in triplicate with different makes of α-elastin (Sigma soluble α-elastin of human aorta, Sigma soluble α-elastin to rat lung and elastin derived peptides.) The binding was best and the gradient steepest with soluble α-elastin of human aorta (Sigma) (Fig. 2.2)

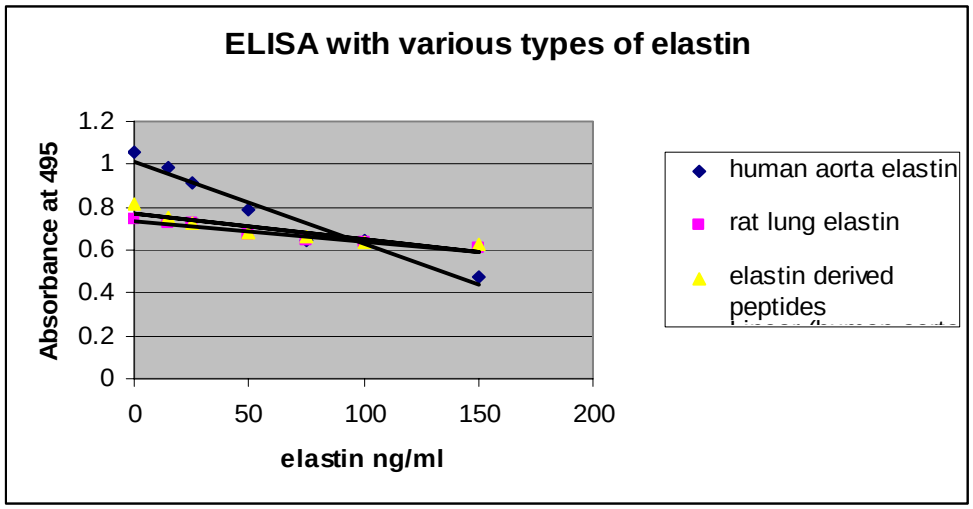

Fig. 2.2: Graph showing the standard curve with various types of elastin.

vi) Introduction of matrix

There was an excellent correlation between the standard curve created with α-elastin in PBS containing BSA and a standard curve created with α-elastin in pooled serum from healthy volunteers. Levels of α-elastin detected were slightly higher in pooled sera than in PBS containing BSA, but the difference was not statistically significant (p=0.13, sign test). Figure 2.3 compares the standard curve with assay buffers with that in pooled human sera.

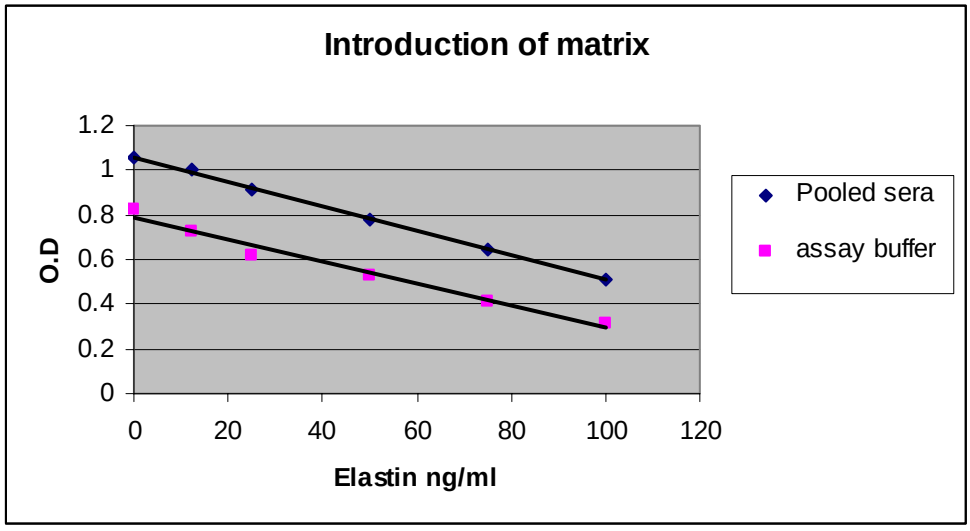

Fig. 2.3: Graph comparing the standard curve with assay buffers with that in pooled human sera.

vii) Standard curve

The typical standard curve shown below (fig. 2.4) is obtained with soluble α-elastin from human aorta, capture antibody used was rabbit anti human elastin IgG 1:1000 and secondary antibody used was goat anti-rabbit antibody at concentration of 1: 2000. The standard curve is plotted with optical density in y-axis and the concentrations of α-elastin in x-axis. A linear standard curve was obtained.

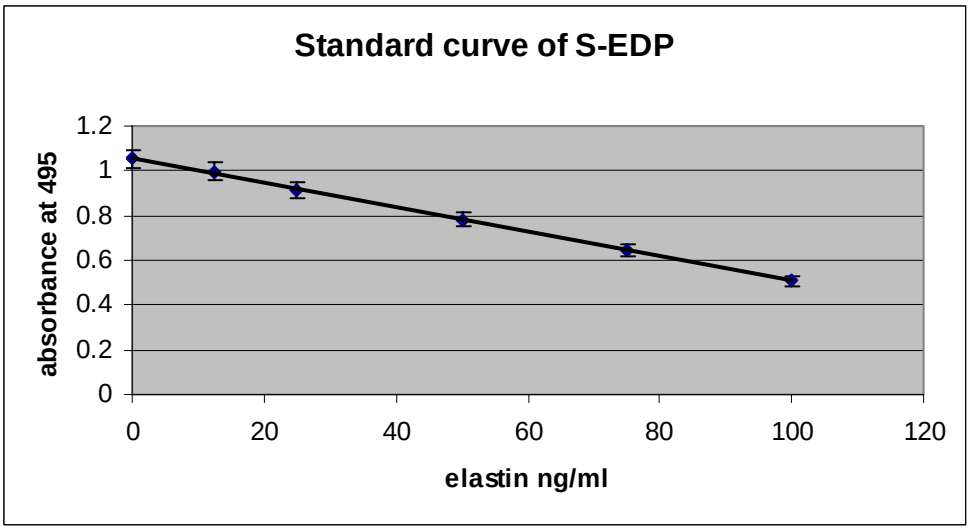

Fig. 2.4: Typical standard curve of competitive ELISA for S-EDP.
(Error bars: ±2 standard deviations)

2.2.3.c) Assay Reproducibility

The assay was found to be sensitive from 0.5-100ng/ml of α-elastin. For the intra-plate and inter-plate reproducibility studies, OD values of the individual positive and negative control were all within ±2 SD of the mean OD values as shown in Table 2.2.

Table 2.2: Reproducibility of S-EDP ELISA

S-EDP	**Intra-plate precision**			**Inter-plate precision**		
0 ng/ml	n	OD mean ± S.D	CV (%)	n	OD mean ± S.D	CV (%)
	30	1.125 ± 1.67	31.0 %	30	1.089 ± 0.53	12.5 %
75 ng/ml	30	0.645 ± 0.42	11.3 %	30	0.625 ± 0.18	6.7%

S.D: standard deviation, CV: Coefficient of variation

2.2.3.d) Estimation of S-EDP in AMD and controls

The mean ± SEM of S-EDP in AMD (n=56) was 29.6± 3.95ng/ml compared to control group (n=15) with 15.4± 2.71ng/ml ($p<0.05$, student's t-test). The mean ± SEM of S-EDP in ARM (n=30) and neovascular AMD (n=26) was 23.9 ± 1.75 ng /ml and 36.2 ng ± 4.52ng /ml respectively (p, 0.05, student's t-test).

Figure 2.5 depicts the progressive increase in S-EDP with increase in severity of AMD ($p<0.05$, ANOVA).

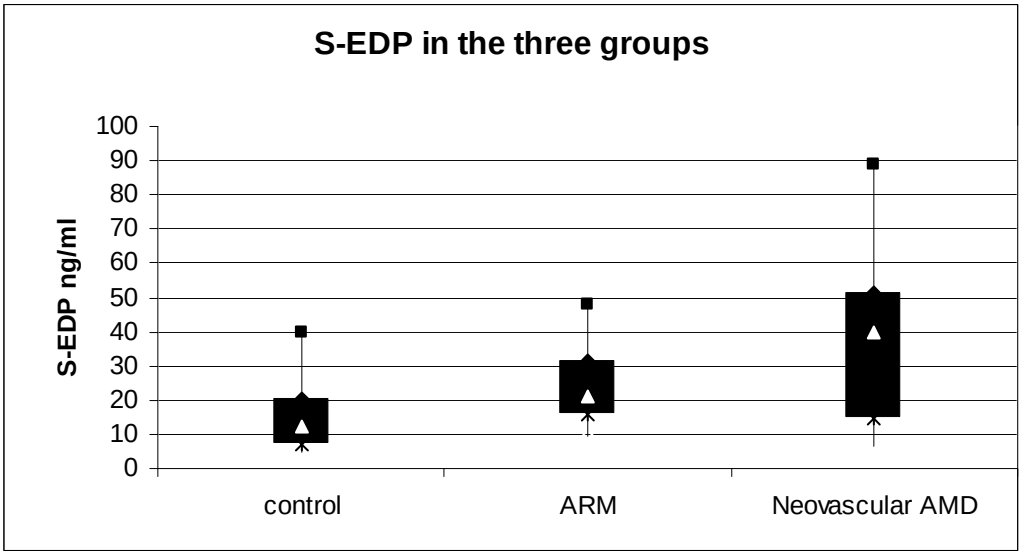

Fig. 2.5: **Box-chart showing progressive increase of S-EDP with increasing severity of AMD**

The multiple regression analysis showed that the various clinical conditions did not significantly influence the levels of S-EDP in this cohort (Table 2.3).

Table 2.3: Robustness check of S-EDP

Independent variable	Multiple regression analysis with S-EDP as dependent variable
Age(years) Mean [range]	0.53
Gender (females)	0.61
Ex-smoker	0.68
Hypertension	0.31
Myocardial infarction	0.56
Angina Pectoris	0.35
Intermittent Claudication	0.62
Stroke	0.63
COAD	0.54
AAA	1.00
Hypercholesterolemia	0.54

2.2.3.e) Correlation of S-EDP with aging

The levels of S-EDP of control and pathological sera did not correlate with age of the subjects. There was a strong dispersion of results (r=-0.01). The separate evaluation of AMD patient groups did not yield a significant age-dependent trend for the values of S-EDP (r=0.02).

2.2.4 Discussion

Elastin is the main protein of elastic tissue contributing to the elastic properties of many tissues such as vascular wall, skin and lung (Partridge, 1962). In the eye, elastin is found in many structures including the trabecular meshwork, uvea and optic nerve. The major sites of elastin deposition in the uvea include the choroidal blood vessels and the BM. The elastic lamina of the BM is thinner and less abundant in the macula than the periphery and it is also thinner and less abundant in the macular region for AMD eyes (Chong et al. 2005). Age-related changes in the BM include lipid accumulation, calcification and fragmentation (Guymer et al. 1999). In a study by van der Schaft and colleagues, age-related calcification of the BM was noted to commence as early as the third decade of life (van der Schaft et al. 1992). Calcification and fragmentation of BM facilitates ingrowth of choroidal neovascular membranes with consecutive development of neovascular AMD (Spraul et al.1999). Moreover, elastin complexed with lipids and calcium favours its degradation compared to native elastin (Saulnier et al. 1991). In a case-controlled study, Blumenkranz et al noted elastotic degeneration in non-sun exposed skin in AMD patients suggesting that photic insults are not primarily responsible for this change but that there are inherent, systemic differences in elastin synthesis/ degradation. This change in elastic fibres is an important risk factor for CNV (Blumenkranz et al. 1986).

Elastolytic enzymes mediate the degradation of elastic fibres. These enzymes include: elastase from neutrophils and platelets, Cathepsin G and metalloproteinases (MMPs) such as gelatinase A (MMP-2), gelatinase B (MMP-9), matrilysin (MMP-7) and the macrophage metalloelastase (MMP-12) (Robert et al. 1998). Degradation of the elastic fibres by these

enzymes results in the release of S-EDP into the circulation. Therefore, the quantification of increased S-EDP may reflect the increased production or activity of these enzymes.

Several immunological methods have been used to estimate S-EDP (Baydanoff et al. 1987). As elastin is insoluble, it cannot be used directly in immunoassays. Therefore, elastin has to be solubilized before use. Insoluble elastin can be solubilized by treatment with 0.25M oxalic acid to obtain soluble α elastin, treatment with potassium hydroxide to obtain κ-elastin, or by enzymatic digestion of elastin using pancreatic or leucocyte elastase, or other enzymes such as pepsin, papin and ficin (Mecham and Lange, 1982). However, a major problem in elastin histochemistry is that the purification and preparation of elastin and the reactivity of the antibodies influence the reported normal value of mean S-EDP (Wei et al. 1993). This phenomenon may explain, in part, the difference between the reported mean of control samples in our study versus that obtained by Petersen et al (15 ng/ml compared to 26 ng/ml respectively), since different capture antibodies were used (Petersen et al. 2002).. An improvement on our methodology would have been to include antibodies to peptides other than α-elastin, but the current study was limited to use of those antibodies that were readily commercially available. Although the absolute S-EDP concentrations have been found to be variable, other authors suggest that it is the variation of S-EDP between individuals which is more significant than the absolute values (Baydanoff et al. 1987; Kucich et al. 1983).

It is also important to consider factors that may influence the levels of S-EDP that can be detected by an immunoassay: Circulating elastin auto-antibodies may play a role in the interpretation of our results. Natural auto-antibodies constitute a proportion of normal circulating immunoglobulins (Dighiero et al. 1986), and S-EDP has been shown to induce

production of anti-elastin antibodies (Colburn et al. 2003). The levels of these antibodies in the serum or the BM may therefore indirectly influence the levels of S-EDP. Moreover, our results could also be influenced by the presence of elastin-binding proteins and elastin-receptor interactions.

Despite the potential limitations of the immunoassay as described, this study suggested that S-EDP was significantly higher in subjects with AMD compared to controls indicating increased elastin turnover in subjects with AMD. S-EDP was also found to increase progressively from early disease to neovascular AMD.

The precise source of circulating S-EDP is unclear, but there would seem to be several possibilities: Although tissues such as lung parenchyma and skin could contribute to elastin degradation, most of the elastic fibres are present in the vascular wall, with thoracic aorta containing 30-40% elastin and abdominal aorta about 20%, indicating the most likely source of increased circulating S-EDP (Bizbiz et al. 1997). Increased elastin degradation may help explain the association of AMD with atherosclerosis (van Leeuwen et al. 2003a) and emphysema (Klein et al. 2003a). The contribution of the BM and choroidal vessels to the circulating S-EDP is presumably negligible, so it is not thought likely that increased S-EDP occurs as a result of AMD progression in the first instance. However, higher levels of MMP-2 and MMP-9 have been found in BM of patients with AMD compared to age matched controls, and these enzymes may have a role in the degradation of elastic fibres of the BM (Lambert et al. 2002, Lambert et al. 2003). This suggests that higher levels of EDP at a local level in patients with neovascular AMD may be relevant. Fragmentation of the BM is a pre-requisite for the invasion of CNV into the sub-retinal space. Higher levels of

S-EDP in neovascular AMD suggest that S-EDP may be directly involved in the pathogenesis of CNV.

S-EDP interacts with cell membrane receptors such as the elastin-laminin receptor and the integrins (Hynes, 1987, Hornebeck et al. 1986). These interactions activate intracellular signalling pathways that lead to diverse cellular events (Labat-Robert, 2004). Thus, S-EDP are defined as matrikines as they originate from fragmentation of a matrix protein, and have distinct cellular effects such as increased elastase production, free radical release, induction of LDL (low density lipoproteins) oxidation, stimulation of endogenous cholesterol production and chemotactic activity (Hunninghake et al. 1981; Senior et al. 1980; Fulop et al. 1998}. These peptides also act with various growth factors; cytokines and vasoactive molecules released as a response to injury and stimulate endothelial cells to proliferate (Anderson et al. 2004; Nackman et al. 1996). Thus, the disruption of elastin is not just an end-product of elastin turnover. It may be an important contributor to the pathogenesis of neovascular AMD.

Several reports indicate the role of inflammation in AMD. Vine and Powell showed a correlation between elastolytic activity and inflammatory cell infiltrates in degenerative vascular disease (Vine and Powell, 1991). Likewise, the Bruch's membrane in AMD is also populated by macrophages and lymphocytes in AMD (Penfold et al. 2001; Hageman et al. 2001). Macrophages elaborate an array of proteinases including metalloelastase (MMP-12). In addition, macrophages and lymphocytes are potential sources of cytokines which can activate other cells to produce degradative enzymes. Therefore, the release of leucocyte elastase can also lead to a collapse of the elastin network and fragmentation of the BM.

Another mechanism that should be considered in light of our results is the possibility of altered elastin gene transcription in AMD. Indeed, many cytokines already studied in AMD have the ability to modulate elastin expression (Carreras et al. 2002; Kuang et al. 2003). Further insight into transcriptional mechanisms accompanying tissue responses to the elastolytic events can lead to the design and testing of intervention strategies pertinent to AMD.

Although further studies are needed to clarify the influence of S-EDP on AMD, this work demonstrates a potential role for elastin peptides in the pathogenesis of AMD. Larger studies are also needed to determine whether the serum level of S-EDP can be used as a predictor for the conversion of early ARM to neovascular AMD.

2.3 Plasma levels of Matrix Metalloproteinase- 2 and 9 (MMP-2 and MMP-9) in Age-related Macular Degeneration

2.3.1 Introduction

The data linking aberrations in ECM biology and AMD are mounting. Numerous histopathological studies have shown that each component of the ECM in Bruch's membrane undergoes substantial changes in AMD.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that possess catalytic activity against ECM components such as elastin and collagen. These enzymes are expressed constitutively but under pathological states their expression can be increased. An accumulation of MMP-2 and MMP-9 and TIMP-3 have been identified in the Bruch's membrane of AMD eyes, suggesting the presence of a pathologic local enzymatic process (Guo et al. 1999; Kamei and Hollyfield, 1999). In addition, elegant studies have document the synergistic effect of MMP-2 and MMP-9 in the development of CNV (Lambert et al. 2002; Lambert et al. 2003).

Compositional similarity between drusen and atherosclerotic plaques may indicate that AMD and atherosclerosis may be parallel tissue responses to abnormal ECM homeostasis (Mullins et al. 2000).

MMPs have been implicated in several stages of atherosclerosis. Among them, circulating MMP-9 (gelatinase-B) has been identified as a predictor of cardiovascular mortality in patients with coronary artery disease (Blankenberg et al. 2003). Likewise, MMP-2 (gelatinase-A) plays a pivotal role in oxLDL-induced activation of various signalling pathways potentially involved in atherosclerosis (Auge et al. 2004).

We hypothesize that if atherosclerosis and AMD are tissue responses to altered ECM turnover, circulating MMP-2 and MMP-9 may also be elevated in patients with AMD compared to controls.

2.3.2 Materials and Methods

2.3.2.a) Subjects

Thirty three subjects with varying grades of AMD were recruited for the study from the Macula Clinic of King's College Hospital. Participants of the control group (n=17) were selected among subjects from the Cataract Clinic. They were included if they were 50 years or older and fundus examination revealed the absence of drusen, pigmentary abnormalities and late AMD. Exclusion criteria were: subjects with co-existent fundus pathology and subjects with un-gradable photographs. The study did not include any subject with a history of neoplastic, hepatic, infectious or autoimmune disease; or any surgical procedure in the preceding 6 months.

All enrolled subjects underwent a complete ophthalmic examination by the recruiting retinal specialist: visual acuity, slit lamp examination and retinal examination after pupil dilation were documented. Each subject had 35° colour stereo fundus photographs of both eyes taken (Topcon TRC 50IX, Topcon Ltd. Tokyo, Japan). Fluorescein angiography was performed if there was a clinical suspicion of CNV. A detailed medical history of each patient was taken with particular care taken to note history of cardiovascular disease.

This research adhered to the tenets of the Declaration of Helsinki. Institutional ethics committee approval was obtained and all subjects gave their full informed consent. The subjects in this study were recruited as part of a Medical Research Council (UK) funded project on AMD.

2.3.2.b) Grading of AMD

Fundus photographs of these subjects were randomized and graded by two retinal specialists using the nomenclature and classification recommended by the International ARM Epidemiological Study Group (Bird et al. 1995). The graders were masked of the age and clinical history of the participants. Double grading for intra-observer and inter-observer variability was performed. Discrepancies were resolved by discussion.

Briefly, we classified the subjects into three groups: a) control group consisted of subjects with no evidence of drusen, retinal pigmentary abnormalities or late AMD b) ARM group included subjects with the presence of either large (≥63 μm) soft distinct drusen with pigmentary abnormalities, or indistinct drusen (≥125 μm) or reticular drusen) c) Neovascular AMD group included subjects with fluorescein angiographic evidence of CNV. If the grades in the two eyes were different, the subject was categorized according to the severity of changes in the worse eye.

2.3.2.c) Plasma Collection

Venous samples were collected into Heparin tubes (Vacutainer, BD Diagnostics**,** Oxford UK), centrifuged at 1000 x g for 15 minutes at room temperature and plasma was transferred to a fresh tube within an hour of collection and kept frozen at -80'C. Plasma samples were thawed on ice and used for MMP measurements. The samples were randomized so that the scientist who analyzed the samples was blinded of the clinical history of the subjects.

2.3.2.d) Measurement of total plasma protein concentration

The Coomassie Blue Protein Assay reagent (Pierce, Rockford IL) was used to determine the total protein level of each plasma sample. 2ul of BSA standards (0-2mg/ml) and samples were added to 100ul of substrate solution and incubated for 10 minutes at room temperature. The absorbance was read at 575nm. A standard curve was prepared by plotting the average blank-corrected 595nm reading for each BSA standard against its concentration. The standard curve was used to determine the protein concentration of each sample. Any sample with a reading that was significantly different from the average value was excluded.

2.3.2.e) Measurement of total plasma MMP-2 concentration

Plasma MMP-2 levels were quantified with a sandwich ELISA using the Quantikine total human MMP-2 kit (R&D Systems Inc, Minneapolis MN). The kit assayed the proactive and active forms of the MMP-2. The assays were processed according to the manufacturer's instruction (see protocol in appendix 2).

In short, the standards of 7 serial dilutions of recombinant standard (rMMP-2) and the samples of 3 serial dilutions 1:45, 1:90 and 1:180 were prepared. A polyclonal antibody specific for MMP-2 was pre-coated in the microtiter plates. 100 µl of assay diluent and 50 µl of standard and sample were added to each well and incubated for 2 hours on the shaker at 250 rpm at room temperature. The wells are then aspirated and washed twice.

Then to each well 200 µl of the MMP-2 Conjugate was added (enzyme linked polyclonal antibody specific for MMP-2). The plates were incubated for 2 hours on the shaker at 250 rpm at room temperature and then washed three times. The substrate solution (200 µl) was

added and incubated for a further 30 minutes and the reaction was terminated with 50 µl of stop solution. The absorbance was read at 450 nm. The standard curve was constructed in each assay for sample calculation. All samples were measured in duplicate and in 3 serial dilutions and the results were averaged as mean ± standard error of mean (SEM) ng/ml.

2.3.2.f) Measurement of total plasma MMP-9 concentration

Plasma MMP-9 levels were also quantified with a sandwich ELISA using the Quantikine total human MMP-9 kit (R&D Systems Inc, Minneapolis MN). The kit assayed the proactive and active forms of the MMP-9. The assays were processed according to the manufacturer's instruction (see protocol in appendix 2). The standards of 6 serial dilutions of recombinant standard (rMMP-9) and the samples of 3 serial dilutions 1:25, 1:75 and 1:225 respectively were prepared.

This assay utilized specific mouse monoclonal antibodies against MMP-9 coated in 96-well microtitre plates. 100 µl of assay diluent and 100 µl of standard and sample were added to each well and incubated for 2 hours on the shaker at 250 rpm at room temperature. The wells are then aspirated and washed twice.

Then to each well 200 µl of the MMP-9 Conjugate was added (enzyme linked polyclonal antibody specific for MMP-9). The plates were incubated for 1 hour on the shaker at 250 rpm at room temperature and then washed three times. The substrate solution (200 µl) was added and incubated for 30 minutes. The reaction was terminated with 50 µl of stop solution and the absorbance read at 450 nm. The standard curve was constructed in each assay for sample calculation. All samples were measured in duplicate and in 3 serial dilutions and the results were averaged as mean ± standard error of mean (SEM) ng/ml.

2.3.2.g) Statistical methods

MMP-2 and MMP-9 levels in the three groups were analysed using Student's t-test. The ANOVA one way was also used to identify relationship between patient parameters. Statistical significance was set as $p<0.05$.

2.3.3 Results

2.3.3.a) Characteristics of the subjects

Plasma was analysed from 50 subjects consisting of 15 with ARM, 18 with neovascular AMD, and 17 controls. There were no significant differences in average age, gender distribution or prevalence of major risk factors among the three groups (Table 2.4).

Table 2.4: Characteristics of subjects in the three groups

	ARM (n=15)	Neovascular AMD (n=18)	Controls (n=17)	p-value
Age(years with range)	74 [61-81]	75 [62-91]	76 [61-81]	0.5
Gender M:F	5:10	7:11	6:11	0.5
Smoker (%)	6.6	5.5	0	0.7
Hypertension (%)	33.3	27.7	35.3	0.7
Myocardial infarction (%)	6.6	11.11	5.9	0.6
Angina Pectoris (%)	0	0	5.9	0.3
Stroke (%)	6.6	0	0	0.3
Hypercholesterolemia (%)	13.3	11.11	17.6	0.6

2.3.3.b) Quality of plasma samples

Total protein measurements were performed as a preliminary test for the quality of plasma samples collected. Statistical analysis showed that there was no significant difference

(p>0.05) in the protein level of the samples between subjects in the 3 identified groups. The average measurement is 3.3 ± 0.3 mg/ml.

2.3.3.c) Total Plasma MMP-2 levels

The typical standard curve for plasma MMP-2 is shown in fig 2.6. The assay was reproducible in both the intra-plate and inter-plate studies. OD of the individual positive and negative control were within ± 2 SD of the mean OD values.

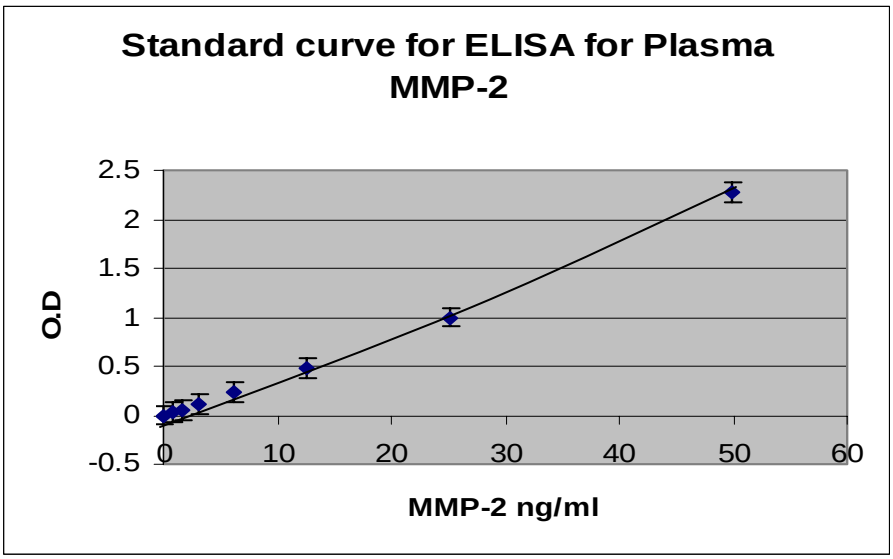

Fig. 2.6: Typical standard curve of ELISA for plasma MMP-2 (error bars denote 2 SD).

The mean plasma levels of MMP-2 in control (495 ± 124 ng/ml), ARM (507 ± 145 ng/ml) and CNV patients (523 ± 100 ng/ml) were not significantly different (p=0.8) as shown in fig. 2.7.

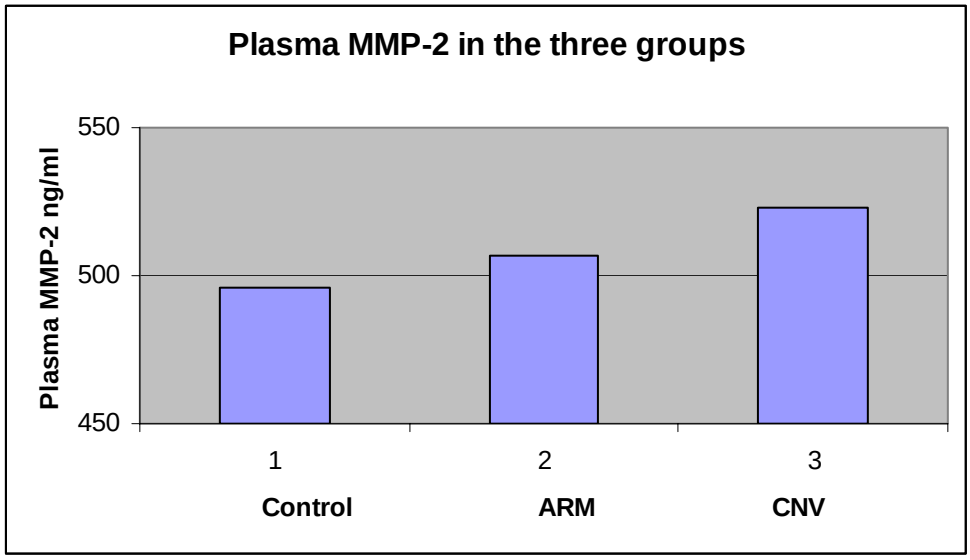

Fig 2.7: Mean plasma MMP-2 levels in the three groups

2.3.3 d) Total MMP-9 levels

Fig. 2.8 shows the standard curve for the MMP-9 assay. The assay was reproducible in both the intra-plate and inter-plate studies. OD of the individual positive and negative control was within ± 2 SD of the mean OD values.

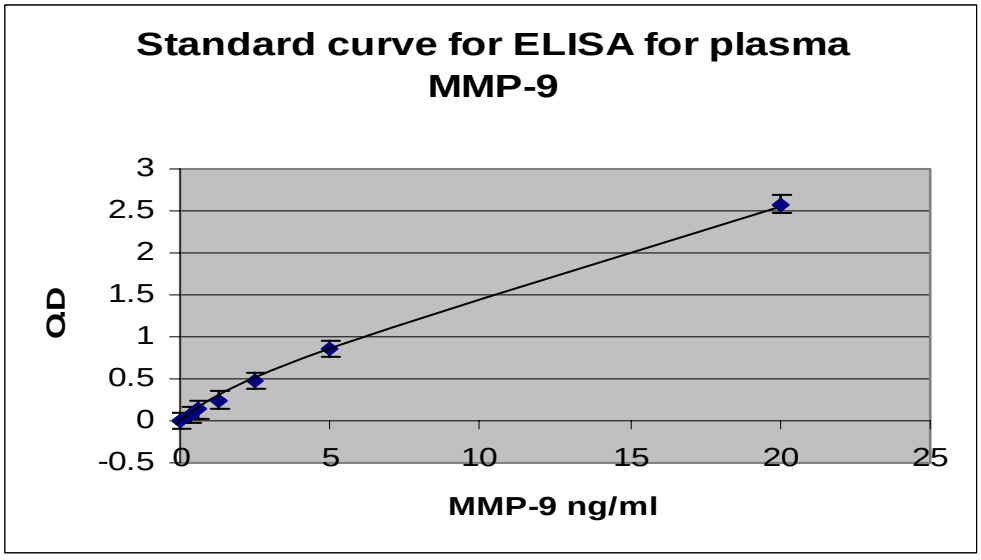

Fig 2.8: Typical standard curve of ELISA for plasma MMP-9

The mean plasma MMP-9 levels were significantly higher in ARM (659 ± 315 ng/ml) and CNV (740 ± 494 ng/ml) compared to that of control patients (265 ± 134 ng/ml) (p=0.008), as demonstrated by fig. 2.9.

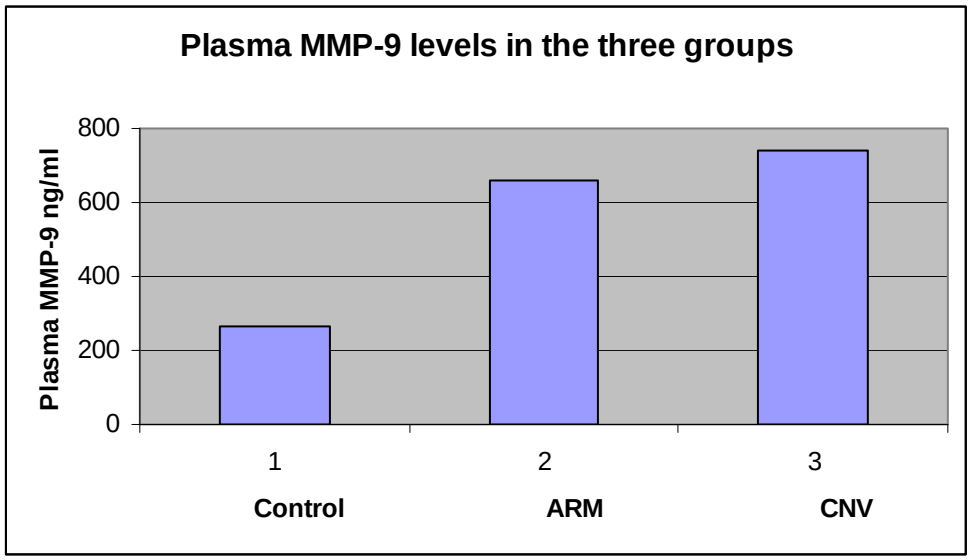

Fig. 2.9: Mean serum plasma MMP-9 levels in the three groups

2.3.4 Discussion

In this study, plasma MMP-9 levels in AMD subjects were found to be approximately 3 fold higher than controls. However, there was no significant difference in MMP-9 levels between the ARM and CNV groups. Plasma MMP-2 levels were noted to be similar in the three groups

There is growing evidence that MMPs play important roles in many disease processes, but most of these enzymes are likely to be active at a local tissue level.

Nonetheless, raised plasma MMP-9 levels have been documented in cancer, hepatic and lung diseases and rheumatoid arthritis (Farias et al. 2000; Chung et al. 2004; Ohbayashi, 2002; Tchetverikov et al. 2004). Subjects with history of these conditions were excluded from this study. The level of circulating MMP-9 is raised in several stages of

atherosclerosis (Kai et al. 1998). No statistically significant differences in clinical data related to atherosclerosis were observed between subjects in the three groups in this study. The levels of plasma MMP-9 also vary in different ethnic groups but all the patients in this cohort were Caucasians (Tayebjee et al. 2005). Therefore, these findings of raised plasma MMP-9 in this study is most probably associated with AMD. There may be other unknown confounding factors that may influence the results. However, they should be highly associated with MMP-9 to explain these results.

Total MMP-2 and MMP-9 (pro and active forms) were measured in this study. All MMPs require activation from precursors to attain enzymatic activity. The antibodies used did not distinguish the active form of these enzymes from their proenzyme forms. Gelatin zymography is a more sensitive assay for detection of both latent and active forms of MMP-2 and MMP-9. In addition, TIMPs dissociate from MMPs during electrophoresis thus preventing TIMPs from inhibiting enzymatic activity (Kleiner and Stetler-Stevenson, 1994). Therefore, increased MMP-9 immunoreactivity in this study does not necessarily correspond to its augmented enzymatic activity.

Nevertheless, the source of increased MMP-9 in the circulation in AMD patients remains unclear. The contribution of the RPE-BM complex to the circulating MMP-9 is presumably negligible, so it is not thought likely that increased MMP-9 occurs as a result of AMD in the first instance. Other important sources of MMPs include astrocytes, neurons, microglia, leucocytes and macrophages (Lorenzl et al. 2003). It is therefore possible that circulating leucocytes may contribute to the increased levels of MMP-9. The recent evidence of raised C-reactive protein (CRP) in intermediate and advanced AMD suggests a state of low grade inflammation (Seddon et al. 2004). Proinflammatory cytokines may also account for

macrophage activation and subsequent release of MMP-9 (Beuche et al. 2000). Furthermore, oxidative stress can result in enhanced expression and activation of MMPs (Okamoto et al. 2001). Further investigations are needed to address these issues.

The lack of an increase in MMP-2 levels may reflect its more constitutive expression, whereas MMP-9 levels are more responsive to reactive oxygen species and inflammatory cytokines (Girolama et al. 2004).

In summary, this is the first study that reveals an association of circulating MMP-9 with AMD. It is difficult to make wide ranging conclusions/ assumptions based on these observations in view of the small sample size. However, this is an important starting point. Larger scale future studies will be required to clarify these findings including the link with systemic inflammatory markers.

2.4 The correlation of S-EDP and MMPs in Age-related macular degeneration

2.4.1 Introduction

Dysregulation of the ECM plays an important role in the pathogenesis of AMD. In the earlier part of this chapter (section 2.2), we found that elastin degradation products in serum (S-EDP) increases with increasing severity of AMD. Elastolytic enzymes mediate the degradation of elastin. These enzymes include: elastase from neutrophils and platelets, Cathepsin G and metalloproteinases (MMPs) such as gelatinase A (MMP-2), gelatinase B (MMP-9), matrilysin (MMP-7) and the macrophage metalloelastase (MMP-12) (Robert et al. 1998). We also noted that plasma MMP-9 is increased in patients with AMD (both early and late disease) (section 2.3). From these findings, we postulated that increased S-EDP in patients with AMD may be due to an increase in MMP-9 in circulation. Therefore, in this pilot study, we estimated and compared the S-EDP, MMP-2 and MMP-9 levels in the sera of patients with AMD and age matched controls.

2.4.2 Materials and Methods

2.4.2.a) Subjects

Thirty subjects with a clinical diagnosis of AMD were included in the study. The control group consisted of 10 healthy subjects without AMD (defined as the absence of drusen, pigmentary abnormalities and neovascular AMD). All enrolled subjects underwent a complete ophthalmic examination and subjects with AMD were graded as described in section 2.2.2.

2.4.2.b) Blood samples

Venous blood was collected from all subjects into two tubes: serum separator tube and heparin tubes (Vacutainer, BD Diagnostics**,** Oxford UK), then centrifuged for 10 minutes at 1000 g at room temperature. Serum was aliquoted and stored at –70°C within an hour of collection and then thawed for S-EDP estimation while plasma was transferred from the heparin tubes and used for MMP measurements. The samples were randomized so that the scientist who analyzed the samples was blinded of the clinical history of the subjects.

2.4.2.c) Estimation of S-EDP

The concentration of S-EDP was determined using a competitive enzyme-linked immunoassay (ELISA) as described in chapter 2.2 of this thesis.

2.4.2.d) Measurement of plasma MMP-2 AND MMP-9 levels

MMP-2 and MMP-9 were quantified with the enzyme-linked immunosorbent assay (ELISA) using the Quantikine total human MMP-2 and MMP-9 ELISA kits (R&D Systems Inc, Minneapolis MN) as described in chapter 2.3 of this thesis.

2.4.2.e) Statistical methods

Pearson's correlation coefficient (r^2) was used to compare the S-EDP and MMP-2 and MMP-9 in the three groups. Statistical significance was set as $p<0.05$.

2.4.3 Results

2.4.3.a) Characteristics of subjects

Serum was analysed from 30 subjects consisting of 10 with ARM, 20 with neovascular AMD, and 10 controls. Age and gender distribution were similar in both groups of AMD

and controls. No statistically significant differences in clinical data were observed between subjects in the three groups.

2.4.3.b) Mean S-EDP levels

The mean serum levels of S-EDP in CNV group (39.08 ± 4.62ng/ml) were significantly higher than ARM (21.23 ± 3.21ng/ml) and control group (20.99 ± 2.4ng/ml) ($p < 0.05$).

2.4.3.c) Mean MMP-2 levels

The mean plasma levels of MMP-2 in control (523 ± 31ng/ml), ARM (423 ± 22ng/ml) and CNV patients (498 ± 26ng/ml) were not significantly different ($p > 0.05$).

2.4.3.d) Mean MMP-9 levels

The mean plasma MMP-9 levels were significantly higher in ARM (888 ± 126ng/ml) and CNV (729 ± 130ng/ml) compared to that of control patients (413 ± 39ng/ml) ($p < 0.05$).

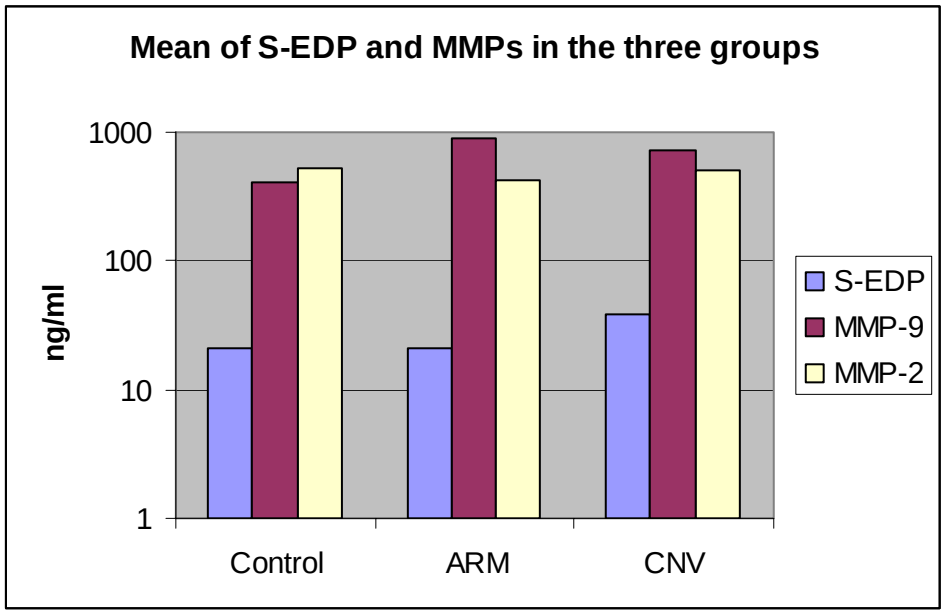

Fig. 2.10 Means of S-EDP and MMP2 and MMP-9 in the three groups

Fig 2.10 demonstrates the relation between the means of S-EDP and the MMPs. Though S-EDP and MMP-9 are raised in the AMD subjects, their levels do not correspond showing no significant relation between their levels.

2.4.3.e) Correlation of S-EDP with MMP-2 and MMP-9

Using Pearson's correlation coefficient, the control group showed a positive trend between MMP-9 and S-EDP that disappeared completely in the ARM and neovascular groups as shown in figs. 2.11, 2.12 and 2.13. However, MMP-2 did not show any correlation with S-EDP in any groups.

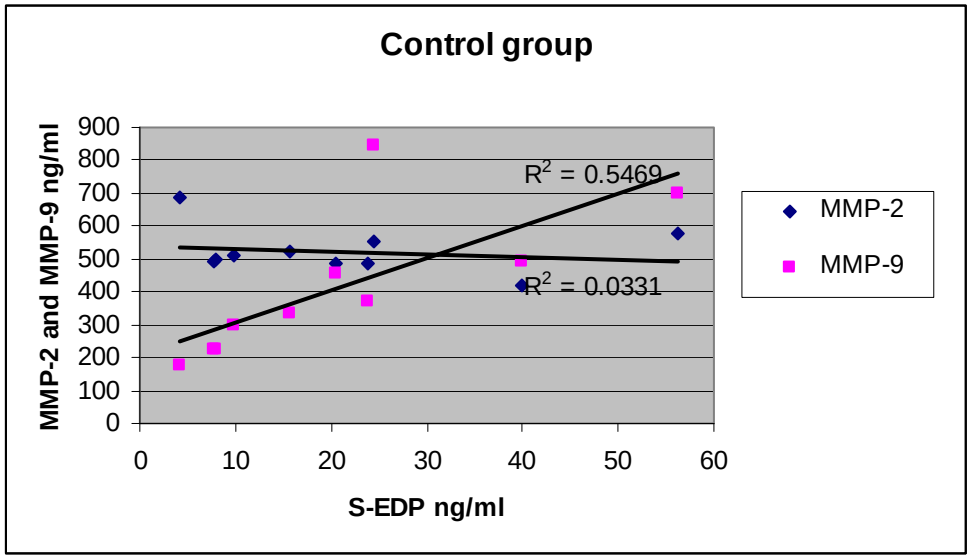

Figure 2.11: Correlation between MMP-9, MMP-2 and S-EDP in controls

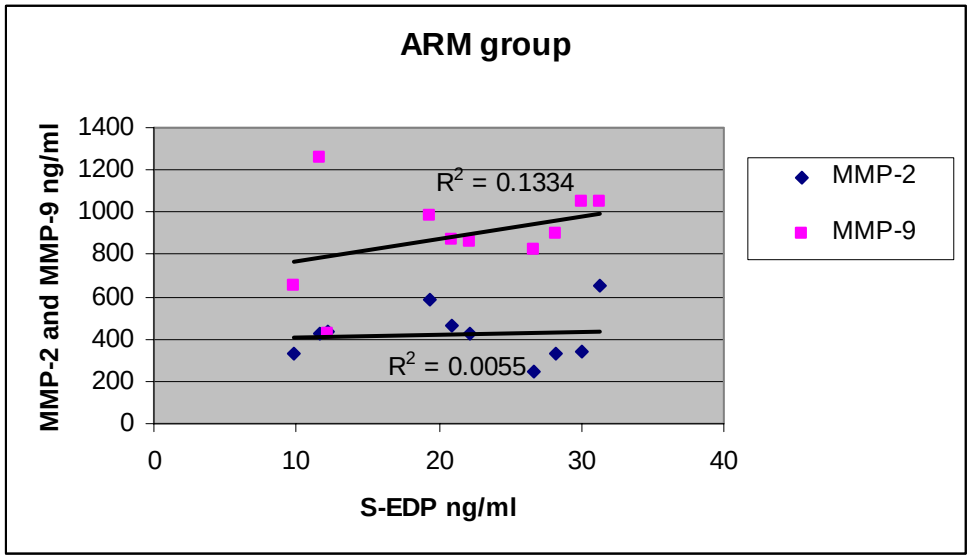

Fig. 2.12: Correlation between MMP-9, MMP-2 and S-EDP in early disease

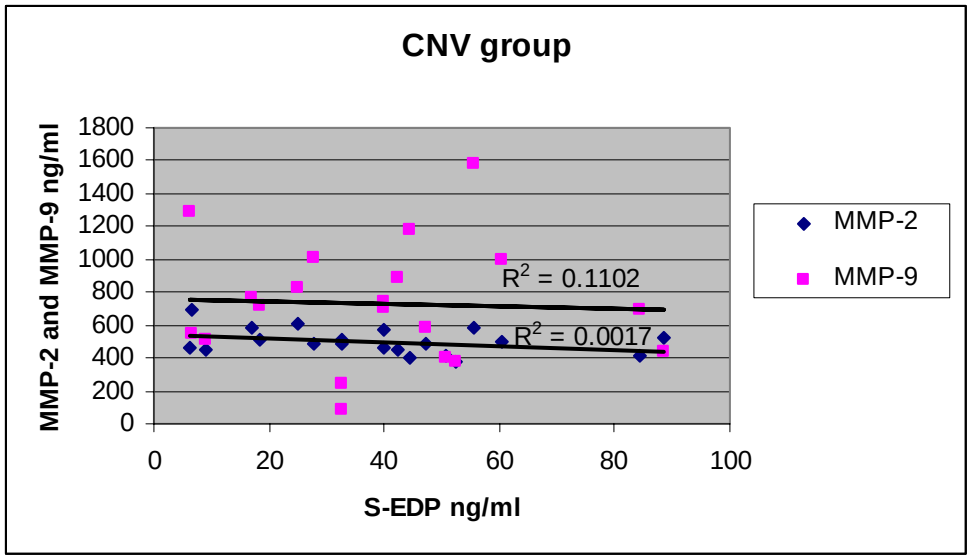

Fig. 2.12: Correlation between MMP-9, MMP-2 and S-EDP in neovascular AMD

2.4.4 Discussion

The three dimensional organization of elastin, collagen and proteoglycans is optimal for the integrity and function of the BM. In AMD, the BM is subjected to injury: lipid deposition, hypoxia, macrophage infiltration and accumulation of reactive oxygen species and

phagocytic material (Ambati et al. 2003a). The ECM molecules are hydrolysed by proteases with resultant degradation of the framework and invasion of the CNV.

The MMPs are zinc dependent proteolytic enzymes. Both MMP-2 and MMP-9 have been isolated in CNV suggesting that they are involved in the degradation of the Bruch's membrane (Guo et al. 1999; Lambert et al. 2003). The proteolytic activities of MMPs are precisely controlled at the level of transcription, activation of the precursor zymogens interaction with specific ECM molecules and inhibition by endogenous inhibitors.

In this study, we found that the levels of systemic MMP-9 levels showed a trend with the elastin degradation in the control group indicating the precise controlled activity of MMP-9 in physiological conditions. The balance tilts to increased elastin degradation that does not correlate with MMP-9 levels in the neovascular AMD though both S-EDP and serum MMP-9 levels are increased. The results suggest that other elastolytic enzymes may also play an important role in neovascular AMD. Both MMP-7 and MMP-9 have been shown to participate in the degradation of dermal elastic fibres (Ghomrasseni et al. 2003). Blumenkranz et al noted that senile dermal elastosis is associated with AMD (Blumenkranz et al. 1986). It may be that additional upregulation of other metalloproteinases such as MMP-7 may account for increased S-EDP in neovascular AMD.

Many elastolytic enzymes are confined to areas of inflammatory cell infiltrations and these cells have been shown to infiltrate the BM and CNV (Penfold et al. 2001). The elastolytic enzymes are formed of four groups of proteinases (serine-, metallo-, aspartic and cysteine proteinases). They are released from different cells including granulocytes, monocytes, lymphocytes, skin fibroblasts, cancer cells and others. Among cysteine proteases, cathepsins S and K have been considered as the most potent elastolytic activities with

cathepsin K exhibiting a slightly higher activity than cathepsin S (Sukhova et al. 1998). Glycosaminoglycans (GAGs) such as chondroitin sulfate specifically inhibit the elastolytic activities of cathepsins V and K via the formation of specific cathepsin-GAG complexes (Yasuda et al. 2004). Because the GAG content is reduced in AMD, an increase of cathepsins may also accelerate the destruction of the elastin matrix in the BM.

An increased elastin turnover can occur in several disorders such as atherosclerosis and emphysema. These diseases are associated with AMD indicating that ECM dysregulation may be the common factor. Most studies concentrate on the balance of MMP and their endogenous inhibitors in assessing ECM degradation in AMD. Although the analyzed population is small, this study demonstrates that MMPs alone cannot explain the increased ECM turnover and it may also help explain why MMP inhibitors (Prinomastat) were found to inefficient in the management of AMD (Behrendt, 2004). Statins and doxycycline also inhibit MMPs (Kadoglou and Liapis, 2004; Guymer et al. 2005).

Their roles in AMD are also inconclusive Further studies on the up-regulation of other elastases and the systemic MMP-TIMP balance may help to better understand ECM turnover in AMD.

2.5 Abdominal aortic aneurysms and age related macular degeneration

2.5.1 Introduction

Abdominal aortic aneurysms (AAAs) represent a degenerative process of the abdominal aorta that is often attributed to atherosclerosis (Reed et al. 1992); however, the exact cause is not known (Wanhainen et al. 2005). Other causes include infection, cystic medial necrosis, arteritis, trauma and inherited connective-tissue disorders. The disease generally affects elderly white men. Smoking and hypertension are the risk factors that are strongly associated with AAA (Fassiadis et al. 2005).

The most important mechanisms involved in the pathogenesis of AAA are proteolytic degradation of aortic wall connective tissue, inflammation and immune responses, biomechanical wall stress, and molecular genetics. The aortic wall contains smooth muscle, elastin, and collagen arranged in concentric layers in order to withstand arterial pressure. Elastin is the principal load-bearing element in the aorta. Elastin fragmentation and degeneration are observed in aneurysm walls. The decrease in content coupled with the histological changes of this matrix protein contributes to the development of AAA (Carmo et al. 2002). S-EDP is an important predictor of rupture of AAA (Petersen et al. 2002).

The aortic media appear to degrade in AAA by means of a proteolytic process. This implies an increase in the concentration of proteolytic enzymes relative to their inhibitors in the abdominal aorta as the individual ages. Reports have documented increased expression and activity of MMPs in subjects with AAAs. The amount of circulating MMP-9 has not only been reported to be significantly higher in patients with AAA (McMillan and Pearce,

1999), but has also been significantly associated with the size and expansion rate of these aneurysms (Lindholt et al. 2000).

The BM consists of a similar composition of extracellular matrix proteins as the vascular matrix, with a central core of elastin sandwiched between two collagenous layers. This penta-laminar membrane is strategically located between the RPE and its nutrition (the choriocapillaris). Age related changes in the BM are akin to the changes in the vascular wall (section 2.1). Earlier studies in this thesis also show that S-EDP correlates with severity of AMD (section 2.2) and it was also observed that circulating MMP-9 is raised in subjects with AMD compared to age matched controls (section 2.3).

The hypothesis in this study is that if AAA and AMD undergo similar remodelling of ECM, subjects with AAA may have higher incidence of AMD compared to age matched controls. It is uncertain whether this link exists. If it does, it might help to identify high risk individuals of developing AMD from the vascular clinic and likewise, identify high risk individuals of developing AAA from the eye clinic. This study looked at the risk of AMD in subjects with AAA compared to age matched controls.

2.5.2 Materials and Methods

Subjects with AAA were recruited from the vascular laboratory of King's College Hospital during the year April 2003 to April 2004. Inclusion criteria included: age ≥ 55 years; ultrasound evidence of AAA (≥ 4 cm); hypertension defined as being on antihypertensives. The controls included age-matched hypertensive subjects recruited from oculoplastic clinics of King's College Hospital. Hypertension was defined as being on anti-

hypertensives. All enrolled subjects underwent a complete ophthalmic examination including dilated fundus examination by the recruiting retinal specialist.

AMD was graded using the nomenclature and classification recommended by The International ARM Epidemiological Study Group. The subjects were classified as either early age related maculopathy (ARM) or neovascular AMD. Early age-related maculopathy was defined as the presence of intermediate soft distinct drusen (> 63 μm) with retinal pigment epithelial depigmentation or hyperpigmentation, soft indistinct drusen (> 125 μm), or reticular drusen; Neovascular AMD consisted of subjects with CNV. If the grades in the two eyes were different, the subject was categorized according to the severity of changes in the worse eye.

In addition, all co-existent posterior segment pathology were documented in each subject.
The Statistical Package of the Social Sciences (SSPS version 11.0) was used for the analysis. The Mann Whitney-U test was used to compare groups and statistical significance was set at 95% ($p<0.05$).

This research adhered to the tenets of the Declaration of Helsinki. Institutional ethics committee approval was obtained and all patients gave their full informed consent.

2.5.3 Results

2.5.3.a) Characteristics of subjects

During the year April 2003 to April 2004, the vascular laboratory had a potential group of seventy subjects with AAA of diameter ≥ 4 cm. However, 28 of them died prior to the recruitment for this study. Eight subjects were too ill for the eye examination and 2 subjects were non-hypertensive. So, 32 subjects in the AAA group were included in the study. A total of 32 hypertensive controls were also included in the study.

Table 2.5: Age stratification in the two groups

Age groups (years)	AAA	control
55-65	10	2
66-75	16	18
>76	6	11

2.5.3.b) Grading of AMD

One subject from each group had ARM. The subjects with ARM had intermediate soft distinct drusen (> 63 μm) with retinal pigment epithelial changes. None of them had soft indistinct drusen (> 125 μm), or reticular drusen. There were no subjects with geographic atrophy or neovascular AMD.

2.5.3.c) Other ocular morbidity

Many ocular diseases were common in the two groups so the associations were compared. Table 2.6 shows the associated ocular co-morbidity in the two groups.

Table 2.6: Associated ocular co-morbidity in the two groups

	AAA	Control	p-value
Cataract/ pseudophakia	25	30	NS
POAG/ LTG	4	2	NS
CRVO	2	0	NS
BRVO	2	0	NS
Macular hole	1	0	NS
NPDR	0	1	NS
AION	0	1	NS
Iritis	0	1	NS
Amblyopia	1	1	NS

2.5.4 Discussion

This study showed no significant association of AMD with AAA. Since systemic elastic turnover is increased in both AAA and AMD, it was hypothesized that a pilot study like this would have established a relation between AAA and AMD. In addition, the two conditions share risk factors such as smoking, age and atherosclerosis. Failure to find any significant association between AAA and AMD may be due statistical power constraints. A reasonable overall estimate of the prevalence of AMD in subjects aged 65-74 years is 1%, increasing to 5% in subjects aged 74-84 years and 13% in subjects 85 years and older. Therefore, a larger cohort of subjects with AAA is required to obtain a significant association.

However, such comparison is hampered by a survivor cohort effect. That is, those who survive to an age when AMD is likely to develop have survived the AAA; those who did not survive the AAA may have been more likely to develop AMD if they survived. The AAA gradually enlarges (0.2-0.8 mm/y) and eventually ruptures (Brown and Powell, 1999). The risk for rupture of large AAA increases with age. Additionally, AAA in women has been shown to rupture at smaller diameters in comparison with men; therefore, a lower threshold for elective repair has been advocated in this patient population. Other contributing factors for rupture include hypertension, continued smoking, or chronic obstructive pulmonary disease (COPD) (Sakalihasan et al. 2005). The mortality rate is 1.8-5% if elective surgery is opted and 85% if ruptured (Thompson, 2003). In general, the survival rate of people with successful aortic aneurysm repair is comparable to that of people in the age-matched population at large who have never had an aneurysm. So future studies of larger cohorts aimed at persons who have survived AAA repair may give a definitive answer as to whether there is an association between the two conditions. However, the feasibility of recruitment of such patients from a single institution is doubtful.

Moreover, most persons with AAAs are asymptomatic. This cohort consisted of those who have been diagnosed either incidentally or because they are symptomatic. So this cohort may not be a true representation of the population with AAA.

A final possibility is that there is no true association between the two conditions.

An increased trend was found for open angle glaucoma and retinal veno-occlusive diseases in the AAA group compared to controls despite the relatively small number of patients. As both groups are matched for hypertension, it appears that AAA may be an independent risk

factor for the development of open angle glaucoma (POAG) and veno-occlusive disease. It is difficult to make assumptions from the observations made from this study with a small sample size. It may be that the lamina cribrosa in patients with AAA undergoes similar elastolysis and is therefore more susceptible to intraocular pressure changes and venous obstruction. In aneurysmal tissue, a tendency exists for increased MMP activity favouring the degradation of elastin and collagen. The mechanism that tips the balance in favour of degradation of elastin and collagen in the aortic wall of AAAs by MMPs and other proteases is presently unknown. Further research in the role of extracellular matrix remodelling in the vascular wall, lamina cribrosa and Bruch's membrane may help us to better understand the pathogenesis of AAA, POAG and AMD.

Chapter III

INFLAMMATION

Does inflammation link atherosclerosis and age related macular degeneration?

CHAPTER 3: INFLAMMATION

This chapter reviews the evidence of the role of inflammation in AMD, and then presents data obtained through investigation of one of the factors which may be involved in progression of the disease.

3.1 Inflammation and Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of severe visual loss in patients above the age of 65 years in the western world (Vingerling et al. 1995c; Mitchell et al. 1995; Klein et al. 1997b). Despite an exponential increase in research on AMD in the last two decades, the aetiology of this heterogeneous condition remains obscure. Several clinical and epidemiological studies have suggested that inflammation may play a role in AMD (Penfold, 2001, Hageman, 2001). With the recent evidence of increased risk of AMD in subjects with genetic polymorphism of CFH (Klein et al. 2005; Edwards et al. 2005; Haines et al. 2005, Hageman et al, 2005), the inflammatory paradigm in AMD is revisited to better understand its role in the pathophysiology of AMD

3.1.1 The concept of inflammation

Inflammation is a local response to a site of injury. The cardinal signs of inflammation, dolor, tumor, calor and rubor actually represent secondary reaction of blood vessels to an initiating stimulus and merely amplify the inflammatory response (Metchnikoff, 1892). Chronic inflammation occurs when there is a failure to eliminate the initiating targets and tissue destruction and attempts to repair proceed simultaneously (Cotran et al. 1999). The fundamental reaction in AMD represents a chronic fibroproliferative response, the hallmark of chronic inflammation.

3.1.2 Inciting Stimuli for inflammation

The inciting stimuli in AMD remain speculative. Basal laminar deposits (BlamD), RPE cell debris and focal lipid accumulation in the Bruch's membrane may serve as powerful chemotactic stimuli for inflammation (Anderson et al. 2002). BlamD consist of diffuse heterogeneous material that lies between the plasma membrane of RPE and its basement membrane. Ultrastructural and histochemical analyses suggest wide-spacing collagen to be a dominant constituent of these deposits. Long spacing collagen is selectively phagocytosed by macrophages in AMD indicating its chemotactic property (Loeffler and Lee, 1986).

Another potential nidus for inflammation is RPE debris. Though the exact aetiology for RPE dysfunction in AMD remains obscure, progressive accumulation of RPE cell debris occurs in all layers of the Bruch's membrane. Drusen, the visible hallmark of AMD, is thought to be derived from this debris. A series of elegant studies has documented that the accumulation of drusen elicits an inflammatory response (Penfold et al. 1985; Anderson et al. 2002; Crabb et al. 2002; Johnson et al. 2001). Similarly, an age-related exponential accumulation of lipid occurs in the Bruch's membrane (Sheraidah et al. 1993; Curcio et al. 2001; Haimovivi et al 2000). As in atherosclerosis, this lipid pool and its modification may also contribute to the inflammatory process.

Other than these extracellular deposits, vascular injury induced by mechanical or biochemical stimuli can also trigger an immune response. Recent evidence of systemic chronic infections in AMD suggests that these agents might initiate the vascular injury in AMD (Kalayoglu et al. 2003).

3.1.3 Evidence of Inflammation

a) Cellular response

Macrophages are the principal inflammatory cells in all stages of AMD. Several histological studies have suggested the spatiotemporal correlation of macrophages with drusen, geographic atrophy, CNV and breaks in the Bruch's membrane (Caicedo et al. 2005; Cousins et al. 2004; Sarks et al. 1997, Penfold et al. 1985; Killingsworth et al. 1990). The precise role of macrophages in AMD is still not fully understood. Though they play an important role as scavengers to remove cell debris in the RPE-choroid complex, they are also antigen presenting cells for T-lymphocytes and are inflammatory effector cells. The macrophages are mainly recruited from the circulating monocytes but resident macrophages in retinal microglia also contribute significantly to the inflammatory and angiogenic response (Forrester et al. 2005). The activation of monocytes (defined as TNF-alpha expression) is a pre-requisite for entering the leucocyte inflammatory cascade. Activation of macrophage results in accumulation of inflammatory mediators, free radicals, pro-apoptotic factors and matrix metalloproteinase's. Patients with CNV have more activated monocytes than controls suggesting a pro-inflammatory state with resultant damage to host tissues (Cousins et al. 2004). These activated macrophages usually transform to epitheloid cells and multinucleated giant cells and these cells co-exist in both atrophic AMD and neovascular AMD.

AMD lesions are also infiltrated by T-lymphocytes, suggesting a local immune response (Penfold et al. 1985). T-cells play a key role in initiating and perpetuating inflammation, not only via the production of soluble mediators but also via inter-cellular interactions through membrane receptors and their ligands. Other than T-lymphocytes, a distinct array

of active participants of the humoral and cellular immune processes has also been identified in drusen such as dendritic cells, immune antigen-presenting cells, HLA-DR and immunoglobulin light chains indicating the role of immune mediated biogenesis of drusen (Forrester et al, 2005, Anderson et al, 2002). Mast cells induce angiogenesis and mediate cell injury and have been shown to accumulate in the choroidal vasculature and the Bruch's membrane in CNV due to AMD (Penfold et al. 1984).

b) Cytokines

Cytokines are a heterogeneous group of small molecules that act in combination in inflammatory reactions. They include interleukins (ILs), interferons (INFs), tumour necrosis factors (TNFs), growth factors, colony-stimulating factors and chemokines. Cytokines activates T-cells which in turn activate monocytes/ macrophages, endothelial cells and fibroblasts to produce chemokines and matrix metalloproteinase's, responsible for tissue destruction. In addition, CD40 ligand at inflammatory sites stimulates fibroblasts and tissue monocyte/macrophage production of VEGF, leading to angiogenesis, which promotes and maintains the chronic inflammatory process (Tsutsumi et al. 2003). The RPE can produce certain cytokines such as the chemoattractant molecule, monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) (Bian et al. 2004). MCP-1 binds to its receptor CCR2 and this interaction is responsible for the transendothelial migration and differentiation of monocytes into lesional macrophages. High levels of MCP-1 have been shown in RPE cells in AMD probably acting as the chemoattractant for the macrophages. It has also been demonstrated that mice deficient either in MCP-1 or its receptor CCR2 develop cardinal features of AMD, including accumulation of lipofuscin in and drusen beneath RPE, photoreceptor atrophy and CNV

(Ambati et al. 2003b). Moreover, the number of infiltrating macrophage and the area of CNV are reduced in CCR2 knock-out mice indicating the active role of macrophages in the development of CNV.

c) Adhesion molecules

A family of adhesion molecules is expressed on the surface of vascular endothelial cells. They are essential for the adherence and transmigration of leucocytes through the vascular endothelium. These adhesion molecules are membrane bound and their soluble counterparts in plasma indicate their presence in circulation. At least four super families of adhesion molecules participate in these events: the selectins, the integrins, certain members of the immunoglobulin super family and cadherins. In CNV secondary to AMD, the activated vascular endothelial cells express various cellular adhesion molecules such as ICAM-1 and VCAM-1 and selectin-E (Yeh et al. 2004). A recent study on serum ICAM-1 in AMD did not show a significant association with the development of CNV (Seddon et al. 2005; Klein et al. 2005).

d) Complement activation

Recent evidence suggests that the complement system may play a significant role in the pathogenesis of AMD. Inflammatory and immune mediated events involving complement proteins have been implicated in the biogenesis of drusen.

The complement system consists of a group of soluble plasma proteins, mainly serine proteases that interact in three enzymatic activation cascades: classical, alternative and lectin pathways. When activated, the complement cascade performs diverse biological functions including pro-inflammatory and immunoregulatory functions.

CFH is an immunoregulatory protein. It controls the complement system in the fluid phase and on cellular surfaces. It inhibits the activation of C3 to C3a and C3b and also directly controls C3b. It is a major inhibitor of the alternate complement pathway. It also binds to C-reactive protein (CRP) which may help to arrest CRP-dependent complement activation induced by damaged tissues (Jarva et al. 1999).

Linkage studies and candidate gene screening have suggested that a locus associated with AMD may exist at the region 1q25-32. Though many other loci are also associated with AMD, the gene of CFH (HF1/CFH) is also located in 1q31. The mutational screening of CFH gene in independent series of patients resulted in the identification of a polymorphism in the CFH gene that confers susceptibility to AMD. (Klein et al. 2005; Edwards et al. 2005; Haines et al. 2005, Hageman et al, 2005). The risk allele has been identified as a tyrosine-histidine substitution at amino acid 402. Although the genetic studies have clearly demonstrated the involvement of CFH in AMD, the precise role the CFH plays in this heterogeneous condition is unknown. The cause of AMD is probably still multifactorial. Inherited complement defects may represent a predisposing condition that increases the risk of the condition in combination with other intercurrent environmental or acquired factors. Established risk factors for AMD such as smoking have been shown to decrease the plasma CFH levels (Klein et al. 2005). The exposure of the RPE-choroid complex to these risk factors may initiate local tissue response that activates complement. In normal conditions, CFH may effectively limit the complement activation and extension of tissue damage. When the bioavailability or activity of CFH is congenitally defective, it may lead to uncontrolled complement activation and further tissue damage.

CFH deficiency can also cause type II membranoproliferative glomerulonephritis (MPGN II), a rare renal inflammatory disease with coexistent drusen that share similar molecular composition to those in AMD (Mullins et al. 2000). A high proportion of these patients are also shown to harbour the AMD at-risk haplotype of CFH suggesting that both diseases may be the result of uncontrolled trigger of the alternate complement pathway.

In addition, up-regulation of the mRNAs for the complement proteins and immunohistochemical evidence of their presence have been found in the RPE-choroid complex in AMD (Anderson et al. 2002; Crabb et al. 2002; Johnson et al. 2001). A murine model of laser-induced CNV in C57BL/6 mice revealed the deposition of C3 and membrane attack complex (MAC) in the neovascular complex. The CNV was inhibited by complement depletion using cobra venom factor. Thus, activation of complement, specifically the formation of MAC, is essential for the development of laser- induced choroidal angiogenesis in mice (Bora et al. 2005). Though most of the complementary proteins are derived from the circulation, local sources include macrophages and T-lymphocytes, RPE cells in AMD. These proteins may be activated by modified lipoproteins, cholesterol, fibrillar proteins, and damaged cell debris that exists in RPE-choroid complex. The activation of complement induces cell lysis which in turn may account for at least a part of the cell debris characteristic of drusen. Moreover, sub lethal injury by complement proteins permits release of growth factors and serve as powerful chemotactic stimulus, reinforcing the inflammatory process and angiogenesis (Johnson et al. 2000).

e) Inflammatory markers

The inflammatory process in AMD is further strengthened by the presence of inflammatory markers in the circulation in AMD. Pentraxins are important activators of the complement system. C-reactive protein (CRP) is a non-specific marker of chronic low-grade inflammation. A strong link has been shown between baseline elevations of CRP and risk of AMD (Seddon et al. 2004; Seddon et al. 2005).

Other acute phase proteins that are raised in AMD include fibrinogen (Smith et al. 1998; Klein et al. 2003a) and ceruloplasmin (Newsome et al. 1986). High leucocyte count has been noted in AMD (Klein et al. 1993). Other indirect evidence of systemic inflammation in AMD is the association with raised serum oxLDL and leptin levels (Evereklioglu et al. 2003, Ikeda et al. 2001).

f) Association with infectious agents

Epidemiological studies have implicated periodontal disease, Chlamydia pneumonia, and CMV virus as risk factors for the development of AMD (Miller et al. 2004; Kalayoglu et al. 2004). Neovascular AMD has been associated with serological anti-Chlamydia pneumonia antibodies, and high cytomegalovirus IgG titres. It remains obscure whether these infectious agents have a causal effect on AMD or are merely an epiphenomenon of the inflammatory process. It may be that localized persistent infection may influence systemic levels of inflammatory mediators which in turn potentially impact inflammation-associated processes including AMD or it may be that these agents induce vascular endothelial injury and predispose the eyes to CNV.

g) Co-morbidity with other diseases

Age-related multifactorial conditions such as atherosclerosis and Alzheimer's disease seem to share some aetiological factors with AMD in which inflammatory mechanisms provide a possible common basis. Though chronic inflammation may not be causative, it may greatly influence the pathogenesis of these disorders. The Beaver Dam study found that subjects with gout and emphysema were associated with increased risk of advanced AMD (Klein et al. 2003). Aging by itself is accompanied by an increase in systemic inflammatory mediators indicating that low-grade inflammation provide a common link between aging on the one hand and age-related diseases on the other (Krabbe et al. 2004). It is still controversial on how systemic low grade inflammation is related to local disease in peripheral tissues such as the RPE-choroid complex. McGeer et al introduced the term 'auto toxicity' to define a macrophage –directed attack on self proteins. It may be that a similar mechanism of action exists in AMD (McGeer et al. 2001).

h) Anti-inflammatory agents in AMD

The inflammatory response in AMD may represent an epiphenomenon or a true inflammatory process. A true inflammatory process would suggest the possible use of anti-inflammatory drugs as therapeutic agents.

Several investigators have studied the role of steroids as a treatment option for neovascular AMD (Penfold et al. 1995; Challa et al. 1998; Danis et al. 2000). Steroids have both anti-inflammatory and anti-angiogenic properties. Initial reports on the use of intravitreal triamcinolne in neovascular AMD were promising. However, a prospective randomised controlled study failed to demonstrate a significant treatment effect in the long term (Gilles et al. 2003). Despite these equivocal results, intravitreal triamcinolone is being investigated

as an adjuvant to other therapies in various trials on the management of neovascular AMD (Spaide et al. 2005).

Studies that examined a possible relation between the use of Aspirin and AMD found no relation (Christen et al, 2001).

The recent evidence of the serological association of anti-chlamydia pneumonia antibodies and AMD makes oral tetracyclines a potential anti-inflammatory agent in AMD. Tetracyclines suppress leucocyte chemotaxis, inhibit lymphocyte proliferation, limit cytokines, inhibit angiogenesis and inactivate MMPs, collagenases and lipases. Wirostko et al proposed a trial with oral minocycline for early AMD as this drug is rapidly absorbed and attains high intraocular concentration (Wirostko et al 2004).

The association of non-steroidal anti-inflammatory agents (NSAIDs) and the incidence of early ARM was investigated in a pooled cohort from three population based, prospective studies and no association was noted (van Leeuven et al. 2004b). However, a recent study indicated that patients with rheumatoid arthritis had reduced risk of AMD compared to controls suggesting that NSAIDs may be protective in AMD (McGeer and Sibley, 2005). COX-2 inhibitors such as celecoxib are known to be effective in arthritis. Clearly, more positive evidence of the role of NSAIDs in AMD is required before embarking on properly designed clinical trials on these agents in AMD.

Statins are used universally in the prevention of cardiovascular end-points secondary to atherosclerosis. In addition to its action as a lipid lowering agent by inhibiting HMG-CoA reductase, statins display a pleiotropic effect on several mechanisms involved in the atherosclerotic plaque formation. Their anti-inflammatory activity and particularly their ability to down regulate endothelial cell activation induced by different stimuli strongly

suggest their possible use in conditions in which the systemic inflammation and the endothelial activation/damage are thought to represent key pathogenic mechanisms. Statins exert their anti-inflammatory action by inhibiting mononuclear cell proliferation, decreasing secretion of cytokines, modulating lymphocyte function and reducing serum CRP levels. In addition, it is anti-angiogenic (Bocan, 2002). Despite having the ideal properties required to prevent and/ or treat AMD, the role of statins in AMD remains inconclusive. Though retrospective studies on the use of statins in AMD show a protective effect, prospective population based studies failed to show any benefit (Guymer et al. 2005).

3.1.4 Conclusion from Literature Review

Much interest has recently been shown on the possible association between polymorphisms in genes encoding inflammatory mediators (CFH gene) and AMD. It is possible that the CFH gene is a permissive gene that plays a vital role in the elderly with low-grade systemic inflammation. We believe it would be appropriate to evaluate the biological effects of CFH gene and other inflammatory mediators in the elderly versus the young population (without low grade inflammation). Such studies will contribute to the development of new strategies in preventing and controlling chronic diseases in the growing elderly population.

3.2 Plasma Complement C3a des Arg in Age-related Macular Degeneration

3.2.1. Introduction

The complement system comprises of a complex of at least thirty enzymes and regulators and provides an innate immune defence mechanism. Three principal pathways are involved in complement activation: the classical, alternate and the mannose-binding lectin (MBL) pathways. Activation occurs when the components are cleaved into smaller pro-inflammatory and larger proteolytic fragments, which subsequently activates the next step. Initiation of the classical complement pathway relies on the adaptive immune system and occurs by the interaction of antigen-antibody complexes with the C1q portion of the complement activation (fig. 3.1). The alternate pathway complement pathway is activated in an antibody-independent manner on microbial surfaces, biomaterials and by tissue type plasminogen activator leading to the formation of the converatase C3bBb. This convertase is also formed when factor D cleaves factor B bound to the C3b generated by the classical and the MBL pathway. In this way, the C3 convertase accelerates the classical and the MBL pathway. The MBL pathway is activated when MBL binds to carbohydrate structures on microbial surfaces.

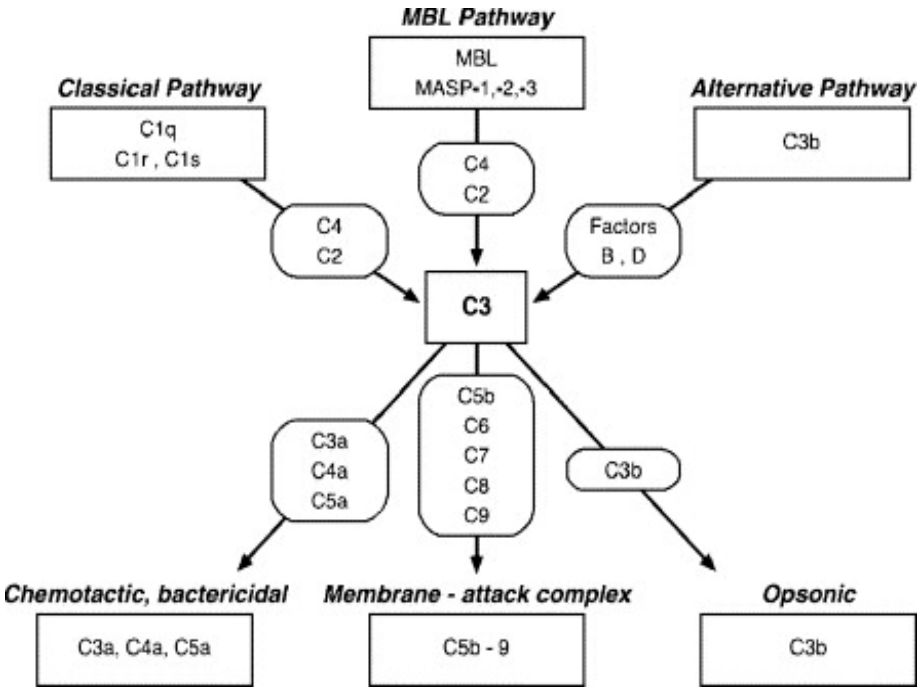

Fig 3.1: The complement pathways.

All the pathways converge on the activation of the third component C3. Activation of C3 results in a common trail to the development of membrane attack complexes (MAC) and other smaller pro-inflammatory molecules. C3 undergoes proteolytic cleavage into C3a and C3b. The C3a is a chemokine and is also called anaphylatoxins. C3a des Arg is produced through a two-step process involving three proteins of the alternate pathway: C3, adipsin (Factor D) and Factor B all of which are produced and stored in the adipocytes. The terminal Arg (arginine) of the parent molecule C3a is cleaved at the carboxy terminus by carboxypeptidase N to generate C3a des Arg. Though C3a des Arg by itself is not an active immunomodulator, only the des Arg form of C3a is present in normal human plasma and therefore, its plasma concentration is an indirectly reflection of complement activation, independent of individual complement component levels. It should be pointed out that C3a des Arg can potentially be generated from all three complement pathways.

This protein also has a distinct metabolic property from the parent C3a molecule in adipocyte lipogenesis. It is also called acylation stimulating protein (ASP) and stimulates triacylglycerol synthesis. By estimation of plasma complement C3a des Arg in subjects with age related macular degeneration (AMD) compared to age-matched controls, it is therefore possible to determine indirectly the role of systemic complement activation with particular emphasis on the pathways that link the host defence to the adipocyte biology in the pathogenesis of AMD.

3.2.2 Materials and Methods

3.2.2 a) Subjects

Eighty one subjects with a clinical diagnosis of AMD were included in the study. The control group consisted of 38 healthy subjects without AMD (defined as the absence of drusen, pigmentary abnormalities and neovascular AMD). All enrolled subjects underwent a complete ophthalmic examination as described in section 2.2.2.

3.2.2 b) Blood samples

Venous blood was collected from all subjects in heparin tubes (SST-BD Vacutainer, BD Diagnostics**,** Oxford UK), centrifuged for 10 minutes at 1500 g at room temperature and the plasma was aliquoted and stored at –70°C within an hour of collection and then thawed when required. The samples were randomized so that the scientist who analyzed the samples was masked of the clinical history of the subjects.

3.2.2 c) Competitive Enzyme linked Immunosorbent assay (ELISA)

A commercially available competitive ELISA kit was used for the assay of C3a des Arg (Assay designs, Metachem, Northampton, UK). A brief outline of the assay design is given below (see protocol in appendix 2 for details).

i) Plasma precipitation: Frozen sera were thawed and plasma proteins were precipitated from the sample as whole proteins compete with the complement in the assay. The supernatant was then diluted 1:200 fold in fresh tubes.

ii) ELISA: Microtiter plates coated with goat antibody specific to rabbit IgG were used in this competitive ELISA. First, 100µl of the serially diluted standards and the diluted samples were placed in each well in duplicate. Controls included zero standard (B_0) and assay buffer only (for non-specific binding or NSB). Then 50µl alkaline phosphatase conjugated with C3a des Arg was added to each well except the blank wells. The capture antibody used was 50µl of rabbit polyclonal antibody to C3a des Arg. The plate was then incubated at room temperature for 2 hours at 500 rpm. Following 3 washes with 200µl wash buffer (0.5ml v/v Tween 0.05% in l litre of tris buffered saline), 200µl of p-Npp substrate (p-nitrophenylphosphate in buffer) solution was added to each well and incubated at 37^0C for one hour. The reaction was stopped by the addition of 50µl of stop solution (Trisodium phosphate in water) to each well. Absorbance was read at 405nm using an automated plate reader (Dynex Technologies, UK). The ELISA was repeated thrice on the same day and on 3 separate days to note precision and reproducibility of the ELISA.

iii) Calculation of results: The net OD bound for each standard and sample was calculated by subtracting the average NSB OD from the average OD bound. The percent bound was calculated as Net OD/ Net B_o OD x 100.

The standard curve was plotted with the percent bound in the y-axis and the log of the concentrations of the standards in the x-axis. The concentration of C3a des Arg in the samples were determined by interpolation.

3.2.2.d) Statistical Analysis

The Statistical Package of the Social Sciences (SSPS version 11.0) was used for the analysis. The age and sex match was determined by ANOVA one-way. Results were reported as mean ± standard error of means (SEM). Plasma C3a des Arg concentrations between groups were tested by student's t-test. Statistical significance was set at 95% ($p<0.05$).

3.2.3. Results

3.2.3.a) Characteristics of subjects

Plasma complement C3a des Arg was analysed from 119 subjects consisting of 39 with ARM, 42 with neovascular AMD, and 38 controls. Age and gender distribution were similar in both groups of AMD and controls as shown in table 3.1.

Table 3.1: Patient demographics

	Control	**ARM**	**CNV**
Age in years (SD)	78 (8)	76 (10)	81 (6)
Sex (M:F)	16:22	15:24	21:21

3.2.3.b) Estimation of Complement C3a des Arg

The standard curve was consistent with the manufacturer's values (Fig. 3.2.). The ELISA was reproducible and accurate.

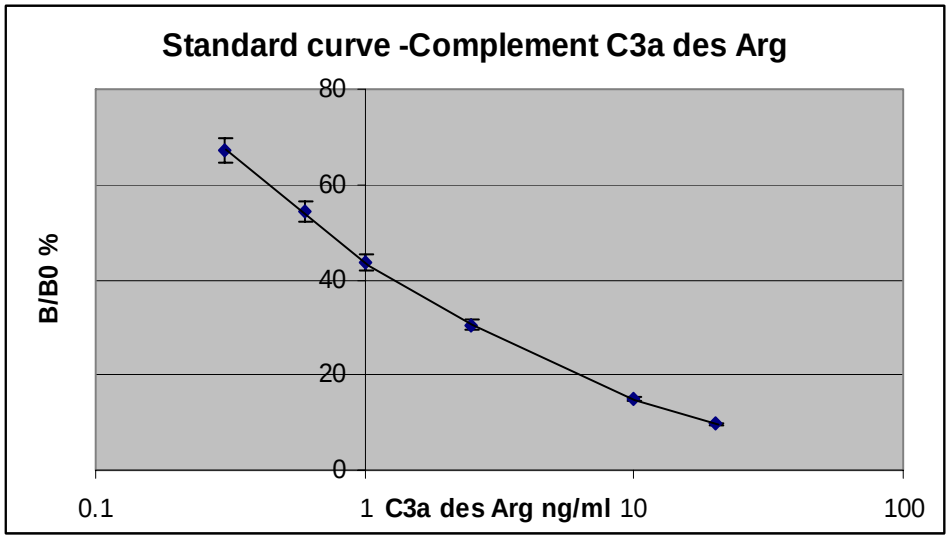

Fig 3.2 Typical standard curve for C3a des Arg

The mean ± SEM of plasma complement C3a des Arg in ARM (n=39) was 70.98 ± 8.97 ng/ml compared to control group (n=38) with 65.98 ± 8.59 ng/ml (p=0.67, student's t-test). The mean ± SEM of plasma complement C3a des Arg in neovascular AMD (n=42) was 97.07 ± 14.68 ng/ml and was significantly raised compared to the control group (p=0.05, student's t-test) and showed a positive trend compared to the ARM group (p=0.1, student's t-test). The descriptive statistics of the plasma complement C3a des Arg in the three groups is shown in table 3.2 and depicted by the box-plot in fig. 3.3

Table 3.2: Plasma C3a des Arg in the three groups

	CONTROL	ARM	CNV
Ist Quartile	23.6	23.6	38.2
Minimum	6.51	2.78	3.13
Median	54.6	54.7	71.9
Maximum	198	198	250
3rd Quartile	78.1	81.7	168

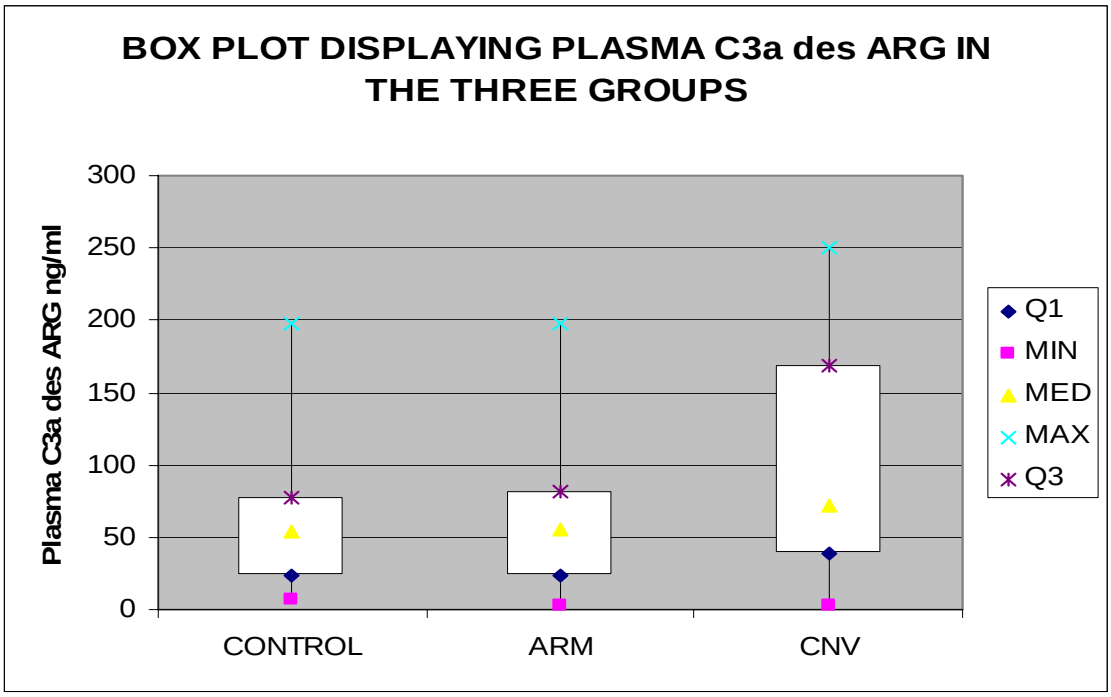

Fig 3.3 Plasma C3a des ARG in the three groups

3.2.4 Discussion

The estimation of plasma C3a des Arg indirectly reflects systemic complement activation, independent of individual complement component levels. The liver is the main source of complement synthesis and the complement molecules constitute approximately 5% of the total serum proteins. Many extra-hepatic cells such as monocytes, endothelial cells, epithelial cells, adipocytes, glial cells and neurons also produce complements (Laufer et al. 2001).

This study showed an increase of plasma complement C3a des Arg in neovascular AMD compared to controls and patients with early disease. It suggests that systemic activation of the complement system may play a role in the development of CNV. Angiogenesis is a complex multi-step process that leads to neovascularization generated from pre-existing blood vessels. Sub-lethal injury by complement proteins permits release of growth factors.

In addition, products of the complement cascade also serve as powerful chemotactic stimulus, reinforcing the inflammatory process required for angiogenesis. A murine model of laser-induced CNV in mice revealed the deposition of C3 and membrane attack complex (MAC) in the neovascular complex and the CNV was inhibited by complement depletion using cobra venom factor. It is also interesting to note that CNV did not develop in C3 (-/-) mice (Bora et al. 2005). It may be that the systemic complement activation compounds the local complement concentration to initiate the process of angiogenesis. This is substantiated by studies that showed that systemic acute phase proteins such as C-reactive protein (CRP) are raised in AMD especially in advanced disease (Seddon et al. 2004). CRP activates the classic pathway and inhibits binding of C5b-9 through the direct binding of CFH (Mold et al. 1999).

Moreover, Chlamydia pneumonia and cytomegalovirus which are also activators of complement system were recently implicated in the pathogenesis of AMD especially in the neovascular stage (Kalayoglu et al. 2003; Miller et al. 2004). These data are suggestive of the existence of multiple activators of complement system in neovascular AMD.

The study also demonstrated that the plasma C3a des Arg did not alter significantly in the patients with early disease indicating that systemic activation of complements is not involved in ARM. Drusen are the hallmark for early disease. Several components of the complement cascade including C3 complement fragments, C5 and the membrane attack complex C5b-9 have been identified in drusen, the sub-RPE space and within the capillary pillars of the choroid. In addition, both fluid phase and membrane bound regulatory proteins such as vitronectin, clusterin, MCP and CR1 have also been immunolocalised in drusen (Johnson et al. 2000; Johnson et al. 2001; Crabb et al. 2002). Recent evidence also

indicates that transcripts for CFH and FHL1 (truncated isoform gene) in RPE-choroid complex approaches levels observed in liver (Hageman et al. 2005). The results of this study suggest that the complements identified in the drusen and surrounding area in the early stage of the disease may be from a local source and not from the circulation. Several components of the chronically sequestrated debris in AMD may be potential activators of the proteolytic cascade including apoptotic cells, nuclear fragments and membrane bound vesicles. Metabolic end products such as lipofuscin, phospholipids, advanced glycation end products, cholesterol and microfibrillar proteins may also serve as powerful chemotactic stimuli for leucocytes via the complement cascade (Johnson et al. 2001).

The multiple biological activities of this complement cascade include control of inflammatory reactions and chemotaxis, clearance of immune complexes, cellular activation and antimicrobial defence. This depends on the balance between complement activators and regulatory proteins. Recent studies have shown that polymorphism in the regulatory protein, CFH is a risk factor for the pathogenesis of AMD (Klein et al. 2005; Edwards et al. 2005; Haines et al. 2005, Hageman et al, 2005). It may be that the biogenesis of drusen is a result of the imbalance between potential activators and inhibitors of the complement cascade. The activation of complement induces cell lysis which in turn may account for at least a part of the cell debris characterised of drusen thus initiating a vicious cycle.

It is also important to note the the complement component C3a des Arg has an active role in adipocyte lipogenesis. It is also called acylation stimulating protein (ASP). It is produced through cleavage of its precursor complement C3 by interaction of adipsin and factor B. All

three factors are made by adipocytes in a differentiation-dependent manner which results in an increased production of C3a des Arg.

This protein in turn, increases triglyceride synthesis as well as glucose transport, therefore increasing overall fat storage. These changes can occur without changes in lipid parameters. Both C3a des Arg and C3 have been shown to be associated with cardiovascular disease in adults (Cianflone et al. 2003). It is interesting to note that that C3 may be a strong predictor of cardiovascular disease (Cianflone et al. 2003). C3a des Arg has also been used as a systemic marker of lipid peroxidation.

The conversion of C3 to C3a des Arg requires multiple factors. Adipsin is needed to cleave factor B to generate the membrane-attached active convertase (C3bBb), a properidin dependent pathway. Factor H, the main regulator of this activation, prevents formation and promotes dissociation of this C3 convertase enzyme, and, together with factor I, mediates the proteolytic inactivation of C3b. Therefore, Factor H deficiency, allows unhindered activation of C3. The recent evidence of CFH mutation in AMD may explain the increased C3 activation in AMD. Increased C3 activation is also reported in membranoproliferative glomerulonephritis. Although, the role of C3 dysregulation in the pathogenesis of MPGN is not fully understood, it has been reported that uncontrolled C3 activation is essential for the development of MPGN associated with deficiency of factor H (Pickering et al, 2002). In conclusion, this work demonstrates a potential role for systemic complement activation in the pathogenesis of neovascular AMD. With the recent evidence of the risk of AMD in subjects with CFH mutation, it may be that C3 dyregulation mainly through the alternate pathway may be primary source of insult in AMD. Further studies are needed to clarify the influence of C3 activation in AMD.

Chapter IV

RETINAL VESSEL CALIBRE AND PULSATILE OCULAR BLOOD FLOW

Do changes in ocular haemodynamics contribute to the pathogenesis of age related macular degeneration?

Chapter 4: OCULAR BLOOD FLOW

This chapter reviews the evidence published to date which suggests that AMD is associated with ocular perfusion defects, then describes experimental investigations carried out in attempt to provide further evidence of these defects in various stages of AMD.

4.1 Ocular haemodynamics in age related macular degeneration

4.1.1 Introduction

Several studies have observed ocular perfusion defects in AMD. It is not possible to determine if these perfusion abnormalities play a causal role or is a consequence of AMD. Despite this, many therapeutic options for AMD are based on improving the macular perfusion in order to prevent or delay disease progression. This section reviews the ocular haemodynamics and the various techniques used to measure ocular blood flow in AMD.

4.1.2 Anatomy of the ocular circulation

Approximately 98% of the total ocular blood flow supplies the uvea, with 85% to the choroid making it the most vascular tissue in the human body. The choroid is responsible for the nutrition of the RPE and outer layers of the retina. In addition to providing the metabolic requirements of the outer retina, it also aids in temperature regulation.

The choroidal circulation is characterized by high blood flow rate (approximately 1400 ml/100 g per minute), low oxygen extraction and low vascular resistance. The choroidal blood flow is mainly controlled by sympathetic innervations and is not auto regulated.

Because of these features the choroidal vessels may be more susceptible to systemic vascular changes than the retinal circulation. The retinal circulation is characterized by low blood flow and high oxygen extraction. Retinal circulation lacks autonomic innervation,

shows an efficient auto regulation and is mainly influenced by local factors. The retinal circulation provides oxygen and nutrients to the inner aspect of the neural retina. The retinal capillary microvasculature has two distinct beds: the superficial capillary layer in the nerve fibre/ganglion cell layer, and the deeper capillary layer extending into the inner nuclear and outer plexiform layers. Unlike the choroidal circulation, the retinal blood vessels are end arteries.

4.1.3 Aging changes in the ocular circulation

Aging changes in the ocular circulation are similar to those found in general circulation. Arteriosclerosis affects both the retinal and choroidal circulation. In the adult retinal circulation, the endothelial cell: pericyte ratio is 1:1. Aging is associated with a decrease in the endothelial cells followed by a decrease in pericytes leading to sectors of acellular channels. Thickening of the basement membrane with the cellular loss leads to narrowing of the vascular lumen resulting in decreased retinal microcirculatory flow and reduced tissue perfusion (Lee et al. 1987). Decreased retinal macular circulation have been observed clinically using the Blue Field Entoptic Phenomenon in which a reduction of leucocyte density and velocity have been noted in the retinal macular capillaries with increasing age (Grunwald et al. 1993).

Age related changes of the choroid may compound to RPE dysfunction. The thickness of the choroid and the diameter of the lumen of choriocapillaris vessels decrease with age (Ramrattan et al.1994). Moreover, there is an increase and elongation of the intercapillary space and a decrease in number of choroidal capillaries with resultant decreased choroidal blood flow with age (Sarks, 1976; Grunwald et al. 1998).

4.1.4 Physiology of ocular blood flow

Blood flow through an organ is determined by perfusion pressure and local resistance to blood flow. The perfusion pressure of the ocular circulation is related to systemic blood pressure and intraocular pressure by a relationship: ocular blood flow = (mean arterial blood pressure –intraocular pressure)/vascular resistance (Riva et al. 1985).

The potential causes of reduced ocular blood flow can therefore be caused by reduced perfusion pressure or increased local resistance. Increased resistance may be due to structural changes in the vessel wall or extrinsic anatomical changes that induce increase resistance to blood flow through the vessels.

4.1.5 Ocular perfusion defects in AMD

Ocular perfusion defects in AMD may either be a cause or effect of AMD. In support of primary ocular perfusion anomalies, Friedman proposed a vascular model (Friedman, 1997). He suggested that increased lipid accumulation in the Bruch's membrane and sclera leads to increased vascular resistance and impaired choroidal perfusion, which in turn affects the function of the RPE. This theory is supported by studies demonstrating an association between increased scleral rigidity and AMD (Friedman, 1989).

The heterogeneous nature of AMD can also be explained based on increased choroidal resistance. An increase of choroidal resistance more than the cerebral vascular resistance leads to the accumulation of drusen due to an increased osmotic gradient against which the RPE must pump. On the contrary, if the increased choroidal vascular resistance is less than the cerebral vascular resistance, the high choroidal perfusion pressure facilitates the development of CNV in the presence of Bruch's membrane cracks and an angiogenic growth factor milieu.

Earlier proponents of this theory thought 'angiosclerosis of the choriocapillaris' is a predisposing factor for disciform macular degeneration (Verhoeff and Grossman, 1937). The relationship of drusen to choriocapillaris associated with the collecting venules of the vortex vein points further in the direction of primary increased vascular resistance (Friedman, 1963). A recent study also demonstrated that equatorial autofluorescent drusen had specific spatial relationship to choroidal vessel walls (Lengyel et al, 2004).

On the other hand, ocular perfusion defects may be secondary to the RPE senescence. The RPE plays a permissive role in the maintenance of the choriocapillaris. The growth factors such as VEGF that are physiologically secreted by the RPE cells maintain the integrity and conformation of the choroidal circulation. So RPE senescence coupled with the thickened and lipid laden Bruch's membrane in AMD may result in the decreased density and diameter of the choriocapillaris and secondary ocular perfusion defects (Henkind et al. 1983).

4.1.6 Measurement of ocular blood flow

It is difficult to quantify ocular perfusion defects. Different techniques have been tried to assess various aspects of the ocular perfusion to better understand the changes induced in AMD. They include fundus fluorescein angiography, indocyanine angiography, laser Doppler flowmetry, Colour Doppler Imaging, pulastile ocular blood flow, retinal laser Doppler and Heidelberg retinal flowmeter (HRF).

a) Fluorescein angiography

Fluorescein angiographic evidence of choroidal filling delay in AMD has been noted in several studies (Pauleikhoff et al 1990; Piguet et al. 1992; Chen et al. 1992; Holz et al.

1994). Morphologically, delayed choroidal perfusion correlates with diffuse thickening of Bruch's membrane and functionally it correlates with discrete areas of increased threshold on static perimetry (Pauleikhoff et al 1990; Piguet et al. 1992). These eyes with choroidal perfusion defects are at risk of unfavourable visual prognosis (Chen et al. 1992). Holz et al noted that subjects with coexistent soft drusen and delayed choroidal filling were prone to progress to geographic atrophy than neovascular AMD (Holz et al. 1994). This finding substantiates the theory of primary ocular perfusion defects causing secondary RPE defects.

b) Indocyanine angiography (ICG)

The ICG better delineates the choroidal circulation than fluorescein because ICG is bound to plasma proteins which prevent its diffusion through the fenestrated choroidal capillaries. Secondly, the near infrared light absorbed by ICG penetrates the RPE better than the shorter wavelength absorbed by fluorescein and is therefore a better modality to assess choroidal circulation. Dilated arterial loops in the macula of patients with AMD substantiate the fact that macular choriocapillaries are subjected to relatively high intramural pressure. Increased frequency of presumed macular watershed filling (PMWF) in early phase indocyanine videoangiographic has been associated with neovascular AMD. These watershed areas represent 'characteristic vertical, angled or stellate-shaped zones of assumed hypoperfusion'. Choroidal neovascularisation was found to arise from these zones (Ross et al. 1998).

This subjective evaluation of choroidal perfusion defects has been supported by more objective area dilution analysis using ICG and scanning laser ophthalmoscope. The researchers evaluated the relative concentrations of ICG in four areas in the macula and

two areas in the temporal peripapillary retina and showed macular predilection of delayed choroidal filling in subjects with non-exudative AMD, with correlation to the severity of the disease and visual acuity (Ciulla et al. 2002). This technique has also been used to evaluate blood flow in feeder vessels of CNV (Yamamoto et al. 2003).

c) Laser Doppler flowmetry

Pathologic changes in AMD have a predilection for the macula. The short posterior ciliary arteries that supply the choroid enter the globe in the macular area, each artery having its own territory in the choroid. The macula is also a watershed zone to the drainage of blood by the four vortex veins. Therefore, an age related decrease of macular blood flow by about 20% may have a significant effect on the macular perfusion. The laser Doppler flowmetry measures the foveal blood flow and is a more specific test of the compromise of macular choroidal circulation in AMD. This technique cannot be readily applied outside the foveal centre as the overlying retinal circulation would Doppler shift the reflected light from the laser and prevent analysis of the choroids (Grunwald et al, 1998).

Grunwald et al. confirmed alterations in the foveal blood flow in non-exudative AMD. In a recent study, they also observed that the mean foveolar choroidal blood velocity, foveolar choroidal blood volume and foveolar choroidal blood flow decreases with increased risk for CNV, suggesting a role for ischemia in the development of CNV (Grunwald et al. 2005).

d) Colour Doppler Imaging

Evaluation of the retrobulbar vasculature gives an indirect estimation of ocular blood flow velocities. Studies using this technique have noted a reduction of blood flow velocities in both the central retinal artery and short posterior ciliary arteries indicating a global

reduction of blood flow favouring primary ocular perfusion defect in AMD (Friedman et al. 1995; Ciulla et al. 1999). Other research groups observed significant increase in resistance in the posterior ciliary arteries in patients with unilateral exudative AMD compared to normal controls (Uretman et al. 2003; Hosal et al. 1998).

e) Laser interferometric method

Local fundus pulsation amplitudes (FPAs) are reduced at classic neovascular membranes in patients with AMD (Schmetterer et al. 1998). The mechanism behind this finding remains unclear. Future studies have to ascertain whether this observation is associated with changes in choroidal perfusion abnormalities.

f) Pulsatile ocular blood flow (POBF)

POBF reflects the total pulsatile component of ocular blood flow. The pulsatile component of the total blood flow ranges from 50% to 80% and is thought to represent the choroidal circulation. It is measured by a pneumotonometer based on a pressure volume relationship. This provides a non-invasive, reliable, reproducible and inexpensive method of calculating the average POBF from the IOP (Silver et al. 1989). POBF may be influenced by various factors such as age, sex, blood pressure, scleral rigidity, refractive error and axial length (Mori et al. 2001). A group-wise comparison between exudative AMD, non-exudative AMD and age matched controls in a Japanese cohort using pneumo-tonometry showed a significant decrease in POBF in exudative AMD (Mori et al. 2001b). In addition, significant difference in POBF was found between eyes in asymmetric AMD in a Taiwanese group (Chen et al. 2001). This supports the notion that there is a global decrease in choroidal blood flow in AMD.

g) Retinal laser Doppler

Using the retinal laser Doppler, increased retinal blood flow pulsatility was observed in the retinal arteries of eyes with AMD compared to controls with no differences noted in mean blood velocity, arterial diameter or blood flow rate among the groups. The researchers suggested that an increasing vascular rigidity in the systemic arterial circulation is directly associated with an increasing severity of AMD (Sato et al. 2005).

h) Heidelberg retinal flowmeter (HRF)

HRF measurements in patients with AMD indicated an increased macular retinal capillary blood flow in patients with the exudative AMD and a decreased macular perfusion in those with the fibrotic form (Remsch et al. 2000).

4.1.7 Retinal microvascular anomalies in AMD

Arteriosclerosis results in generalised retinal arteriolar narrowing and is caused by intimal thickening, medial hyperplasia and hyalinisation and sclerosis. As the retinal arterioles can be visualised, the measurement of retinal arteriolar narrowing appears a feasible method of assessing arteriosclerosis. However, retinal arterioles are small (50μm-200μm in width) and a wide variation in vessel calibre exists in the normal population. Moreover, retinal vascular calibre alters with age, sex, hypertension, smoking, diabetes and variations of intraocular pressure. Therefore significant confounding factors exist in the quantitative estimation of retinal vessel calibre. Several techniques have been used to assess blood vessel calibre in AMD.

Hirvela et al. measured retinal vasculature using enlarged projected images (micrometric

methods) and observed significant retinal arteriolar narrowing in AMD (Hirvela et al. 1996). However, the technique is rather subjective. With the advent of digital photography, newer techniques have been developed for retinal image acquisition. The retinal vessel analyzer (Seifertl and Vilser, 2002; Vilser et al. 2002) allows real-time assessment of retinal vascular diameters at a maximum frequency of 50 Hz and has demonstrated reproducible results (Polak et al. 2000; Pache et al. 2002). A major limitation of the retinal vessel analyzer is that it does not adjust for refractive errors.

The scanning laser ophthalmoscope (SLO) (Nagel et al. 1992) provides a high-quality image of the fundus using less than 1: 1000 of the light necessary to illuminate the fundus with conventional light ophthalmoscopy. Because the SLO only illuminates a small area of the fundus at any one time, only a small amount of the patient's pupil is used for illumination. This means that pupil dilation is not usually necessary when acquiring fundal images with the SLO. However, the optical resolution of the SLO is currently only 10–20 μm per pixel, and therefore is currently insufficient to be able to produce accurate measurements of retinal vessels.

The laser doppler flow meter also measures individual vessels and it takes numerous measurements over a 2-s time period, and therefore can be averaged to account for the different stages of the cardiac cycle. However, a significant drawback of the technique is that the resolution is limited to vessels greater than approximately 60 μm in diameter (Guan et al. 2003; Yoshida et al. 2003; Jonescu- Cuypers et al. 2004).

Recently, computer driven micro densitometry based on the vessel's gray scale profile have been used by Hubbard et al to evaluate arteriolar and venular diameters (Hubbard et al.

1991). They reported good intra-observer and inter-observer reliability. The ARIC study, Blue Mountain Eye Study and the Rotterdam study have used this technique to note retinal microvascular changes in AMD. Data from these epidemiological studies are consistent with respect to the retinal microvascular abnormalities associated with incident early ARM. The Beaver Dam study reported that retinal arteriolar diameters were not related with 10-year incidence of early ARM (Klein et al. 2004). The Blue mountain eye study noted borderline significant association between retinal arteriolar focal narrowing and arteriovenous nicking with incident ARM but found no relation with generalized retinal arteriolar diameter (Wang et al. 2004b). The Rotterdam study found that smaller arterioles are associated with higher blood pressure while larger venules were related to atherosclerosis and inflammation (Ikram et al. 2005). However, both the retinal arteriolar and venular diameters were not related to the risk of incident ARM.

4.1.8 Therapies based on ocular blood flow

Although most of the studies above indicate the presence of ocular perfusion defects in AMD, it is not possible to determine if these perfusion abnormalities play a causal role or is a consequence of AMD. Despite this, many therapeutic options for AMD are based on directly or indirectly improving the macular perfusion in order to prevent or delay disease progression.

Friedman suggested that lamellar scleral resection could decrease scleral rigidity and thereby enhance choroidal perfusion and in turn arrest the progression of the disease (Friedman et al. 1989). Similarly, rheopheresis is a safe and effective modality of therapeutic apheresis to treat microcirculatory disorders and represents a novel treatment option for patients with dry AMD. Elimination of a defined spectrum of high molecular

weight proteins from human plasma including pathophysiologically relevant risk factors for AMD such as fibrinogen, cholesterol, von Willebrand factor, and alpha 2-macroglobulin results in the reduction of blood and plasma viscosity as well as erythrocyte and thrombocyte aggregation. It also decreases the circulating macromolecules involved in drusen formation, Bruch's membrane degradation, and retinal pigment endothelial cell dysfunction. Lowering blood and plasma viscosity also increases ocular blood flow, subsequently inducing sustained improvement of microcirculation and recovery of retinal function (Pulido et al. 2005a). Two controlled randomized clinical trials demonstrated the safety and efficacy of rheopheresis for the treatment of AMD patients, especially for those with the dry form (Klingel et al. 2003). Recently the interim analysis of the sham-controlled, double blind, randomized multicenter Multicenter Investigation of Rheopheresis for AMD (MIRA-I) trial confirmed these results (Pulido 2005b).

Though rheopheresis is postulated to decrease drusen biogenesis by increasing blood flow, laser induced resolution of drusen is not associated with an increase in choroidal blood flow. The Complications of AMD Prevention Trial (CNVPT research group, 1998) used low intensity laser treatment to enable resolution of the drusen load. A study aimed at determining whether choroidal blood flow alterations were responsible for the resolution of drusen found no significant alterations in choroidal blood flow at three months after low intensity laser therapy (Figueroa et al. 2004).

Several topical and systemic pharmaceutical agents that enhance ocular blood flow have been evaluated as treatment option in AMD. A Cochrane review on the role of Ginkgo Biloba, a drug that is postulated to increase blood flow and prevent oxidative stress, found

no evidence to inform clinical practice on the role of this drug in AMD (Evans, 2000). Therapeutic doses of niacin were also found to have no change in the net choroidal blood flow in AMD patients (Metelitsina et al. 2004). Similarly, Dorzolamide, a topical carbonic anhydrase inhibitor, was observed to increase peripapillary choroidal perfusion in non-exudative AMD patients (Harris et al. 2003) while animal studies showed that drugs that increase nitric oxide production could specifically increase the choroidal blood flow (Xuan et al. 2003). Crocin analogs isolated from *Crocus sativus L*. were found to significantly increase the blood flow in the retina and choroid and to facilitate retinal function recovery (Xuan et al. 1999). Increased blood flow due to vasodilation presumably improves oxygenation and nutrient supply of retinal structures. A 3-month course of oral pentoxifylline treatment has been shown to increase choroidal but not retinal blood flow in patients with AMD (Kruger et al. 1998).

A theoretical model that showed that occlusion of either Sattler arteriole or venules in the vicinity of the capillary-like vessels connecting a CNV to the underlying choriocapillaris resulted in significant blood flow reduction in the CNV (Flower et al. 2001). These results suggest that reduction of blood flow in the choriocapillaris may be the common haemodynamic event associated with the successful application of several currently practiced methods of treatment of CNV, including feeder vessel photocoagulation, photodynamic therapy and transpupillary thermotherapy. Ciulla et al. noted that transpupillary thermal therapy is associated with transiently decreased volumetric blood flow in the retinal circulation 24 hours after treatment while alterations in choridal blood flow existed at one month post-treatment (Ciulla et al. 2001, Chen et al. 2004).

4.1.9 Conclusion from Literature Review

Several techniques have been used to indirectly assess the changes in retinal and choroidal blood flow in AMD. Most studies provide qualitative evidence of choroidal perfusion defects at the macula. The fact that some studies show decreased choroidal perfusion in the stage of early disease suggests that choroidal insufficiency may be a primary cause rather than result of AMD. Nevertheless, none of the techniques seem to give an accurate assessment of the vascular disturbances. The results from epidemiological studies on retinal vascular disturbances in early ARM suggest that retinal blood flow is not affected in early disease though decreased blood flow through central retinal artery were noted using colour Doppler imaging. Further studies are required to accurately assess the choroidal and retinal circulatory changes in various subtypes of ARM to better understand its role in the pathogenesis of ARM.

4.2 Pulsatile blood flow and retinal microvascular calibre changes in patients with asymmetric age related macular degeneration

4.2.1 Introduction

Evidence from epidemiological studies has suggested associations between atherosclerosis, cardiovascular risk factors and AMD. One of the hypotheses that explain this association is Friedman's vascular model (Friedman, 2000). He reported increased scleral rigidity, decreased blood velocity and increased blood pulsatility in the central retinal artery and short posterior ciliary arteries in subjects with AMD (Friedman, 1995). Based on these findings, he proposed that AMD is associated with an increase in resistance of the choroidal vasculature caused by a decrease in compliance of the sclera and the choroidal vessels. The resultant decreased choroidal blood flow is thought to alter the metabolism in the macular region impeding supply and removal of waste products.

Several techniques have been used to measure ocular blood flow in AMD. Most techniques assessed the focal macular perfusion abnormalities. This study aimed at measuring the pulsatile ocular blood flow (POBF) in patients with asymmetric AMD. In addition, the retinal microvascular calibre changes especially generalized retinal arteriolar narrowing has been shown to predict the development of stroke, dementia and sub clinical cerebral vascular diseases (Ikram et al, 2005). Therefore, the retinal vascular calibre was assessed in this cohort to note if eyes with neovascular AMD (active CNV and/or disciform disease) have significantly more generalized arteriolar narrowing than fellow eyes with early disease (ARM).

4.2.2 Materials and Methods

4.2.2.a) Sample size

The sample size was calculated using UCLA power calculator. We designed this study to have a power of 80% to detect a difference in mean POBF of 20% between fellow eyes and a difference of mean equivalent arteriole: venule ratio (AVR) of 0.02 between eyes in asymmetric AMD subjects, at the 5% significance level. The sample size calculated was 15 per group.

4.2.2.b) Subjects

The inclusion criteria were: age ≥50 years; Caucasian; evidence of asymmetric AMD. Exclusion criteria were: high myopia, history of any associated ocular conditions such as coexistent glaucoma, diabetic retinopathy and vascular disorders including veno-occlusive disease. Eyes with intra-ocular surgeries done in the previous year or laser treatment for CNV were also excluded.

All subjects underwent slit-lamp biomicroscopy, fundus photography and fluorescein angiography to classify them into two groups. In addition, each subject had applanation tonometry, pulse rate, blood pressure and axial length measurement using the IOL Master (Carl-Zeiss, Germany).

4.2.2.c) Definition of asymmetric AMD

Group 1: Subjects with active CNV in one eye and early ARM in the fellow eye.

Group 2: Subjects with disciform scar in one eye and early ARM in the fellow eye

Early ARM was defined as the absence of late stage ARM and presence of either: (1) large (>125 μm diameter) indistinct soft or reticular drusen or (2) both large distinct soft drusen and retinal pigment abnormalities (hyper or hypopigmenation) within the macular area (Stage 2a to 3 as per Rotterdam study criteria). Disciform macular scar was defined as

presence of sub retinal fibrosis at the macula with blocked fluorescence and/ or staining on angiography with no associated haemorrhage or hard exudates. Active CNV was defined as the presence of serous or haemorrhagic detachment of the RPE or sensory retina, presence of subretinal or sub-RPE haemorrhages or exudates and angiographic evidence of classic and/ or occult CNV with a fibrotic component of less than 25% of the total lesion.

4.2.2.d) Assessment of Choroidal blood flow

The pulsatile ocular blood flow (POBF) was measured using the OBF Analyser (Ocular Blood Flow Analyser, Dicon Diagnostics, Paradigm, U.S.A, and Fig 4.1). Subjects were assessed in the sitting position by the same examiner who was masked to the diagnosis in each eye. The measurements were taken with a mounted probe after instillation of topical anaesthetic (0.5 % proxymetacaine). The computer software of the OBA identifies five pressure pulses out of a longer train of pulses that are closest to each other in beat to beat variation over 10-14 second interval. These five pulses are then averaged and used to calculate the pulse amplitude (PA). The software also calculated the pulse volume (PV) and POBF were also calculated from five pulses using previously described theory and methods (Silver et al. 1989).

Fig 4.1 OBF analyser

4.2.2.e) Retinal microvascular changes

Retinal microvascular abnormalities were assessed using the methodology adapted from the ARIC Study (Klein et al. 2004). A 35° colour retinal photograph of each eye of each participant was taken after pharmacological mydriasis using a fundus camera (TOPCON fundus camera, Topcon Optical Co., Tokyo, Japan.). Centred between the optic disc and the macula, the photograph documented the optic disc, the macula, substantial portions of the temporal vascular arcades, and approximately 2 disc diameters of retina nasal to the optic disc. Using Adobe Photoshop 6.0 computer software, the photographs were cropped to include the disc and surrounding blood vessels and the ARIC grid (Fig. 4.1) was overlaid on the photograph to establish the measurement zone, the annulus between 1/2 and 1 disc diameter from the disc margin (zone B) as shown in fig. 4.2.

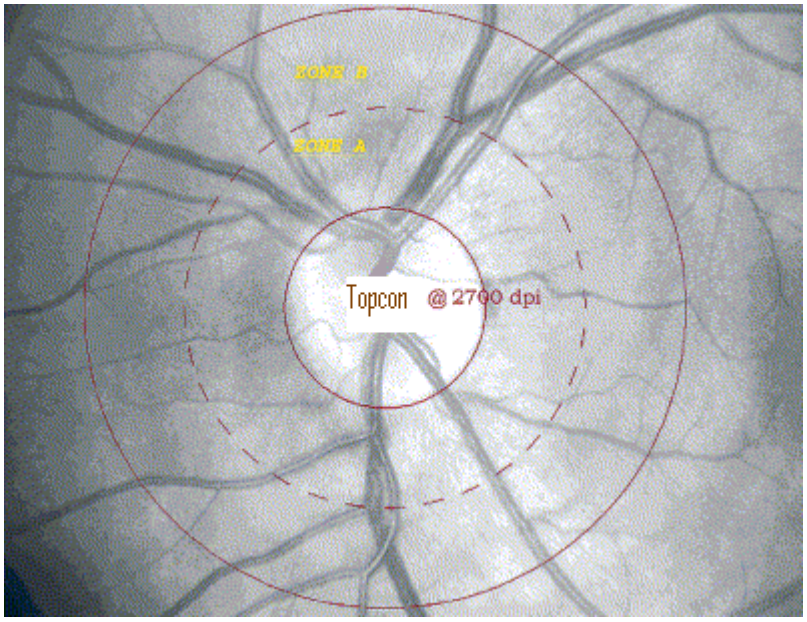

Fig 4.2: ARIC grid overlaid on fundus photograph

Before measuring the vessels coursing through the zone, the arterioles and venules were identified in the original colour slide and checked to avoid focally narrowed or distended parts. Vessels < 25 μm were ignored, since their edges often are not sufficiently resolved to measure accurately, and they have little impact on the central arteriolar or venular equivalent. It was then magnified four times in a separate window. The vessel edges were located by identifying the points at which the vessel became uniformly darker than the adjacent retinal pigment epithelium. Then, the segment of each vessel within zone B most suitable for measurement (based on image quality and straightness of the vessel) was identified. A mouse was used to mark the two edges of the vessel, drawing a circle with its centre on one edge of the vessel and its circumference on the opposite edge so that the radius gave the perpendicular width). The measurement of generalized arteriolar narrowing took approximately 10 to 15 minutes per eye. To monitor reproducibility of the grading technique, grading of selected samples were replicated.

Computation of summary variables

To quantify generalized narrowing as an arteriole to venule ratio (AVR), measurements of individual arterioles and venules were combined according to the formulas developed by Parr et al (1974) and Hubbard et al (1999) respectively:

$$Arterioles\ W_c = \sqrt{0.87\,W_a^2 + 1.01\,W_b^2 - 0.22\,W_aW_b - 10.76}$$

$$Venules\ W_c = \sqrt{0.72\,W_a^2 + 0.91\,W_b^2 + 450.05}$$

in which W_c is the calibre of the trunk vessel, W_a the calibre of the smaller branch, and W_b the calibre of the larger branch. The largest vessel was combined with the smallest, the next largest with the next smallest, and so on until all were accounted for. If the number of vessels to be combined was odd, the single remaining vessel was carried over to the next iteration. The pairing process was repeated until the central equivalents of the arteriolar and venular diameters (CRAE and CRVE) were obtained, and their ratio (AVR) was calculated. (See appendix for details).

4.2.2.f) Statistical analysis

Multivariate logistic regression analysis was used to adjust the POBF for axial length (AL), intra-ocular pressure (IOP) and pulse rate (PR). POBF and its parameters were taken as dependant variables and IOP, AL and PR taken as the independent variables. Multiple regression analysis was also done to adjust CRAE, CRVE and AVR for age, sex and blood pressure. The paired Student t- test was used to compare the mean POBF between fellow eyes of each subject. P values of <0.05 were considered statistically significant.

4.2.3 Results

4.2.3.a) Characteristics of subjects

Thirty nine subjects with asymmetric AMD in the fellow eye were included in the study. This included 12 (31%) males and 27 (69%) females. 21 subjects were included in group 1 (active CNV in one eye, drusen in the fellow eye) and 18 were included in group 2 (disciform scar in one eye with drusen in the fellow eye). The demographic details of subjects are given in table 4.1. No statistically significant difference in age or sex was noted between the groups.

Table 4.1: Patient demographics

Characteristics	**Group 1 CNV/ARM**	**Group 2 Disciform/ARM**	**p-value**
No. of pts	21	18	
Age in years (SD)	80 (7.87)	80.05 (6.2)	0.9
Sex (M/F)	8/13	6/12	0.8

4.2.3.b) POBF

Table 4.2 shows the mean value of IOP, PA and POBF in the two groups. The mean IOP and PA showed no significant differences between the fellow eyes in both groups. The adjusted mean POBF was not statistically different between fellow eyes in either group.

Table 4.2 Mean value of IOP, PA and POBF in the two groups

	Mean IOP mmHg (SD) in each eye in the two groups	**Mean PA (SD) in each eye in the two groups**	**Mean POBF µl/min (SD) in each eye in the two groups**
Group 1 (n=21) CNV ARM	 12.3 (4.6) 11.7 (3.4) *p=0.3*	 3.2 (0.9) 3.2 (1.0) *p=0.8*	 1153.2 (402.8) 1148.6 (369.0) *p= 0.9* **p= 0.3*
Group 1 (n=18) Disciform scar ARM	 13.3 (3.4) 13.8 (3.4) *p= 0.4*	 3.0 (0.9) 3.0 (0.7) *p= 0.9*	 1052.0 (439.7) 1000.8 (338.6) *p=0.4* **p=0.4*

*p= adjusted p value for IOP, PR and axial length; SD=standard deviation

The mean % increase in POBF in the CNV eyes compared to the fellow eye with early disease was 7% while the mean decrease in POBF in the eyes with disciform scar was 0.01%. As the statistical significance was set at 20%, the change of POBF did not reach clinical significance in either group.

Table 4.3: The mean differences in the PA and POBF in the two eyes in each group

	Mean % change in PA between eyes	**Mean % change in POBF between eyes**
Group 1 (n=21) CNV/ARM	-1%	+7%
Group 2 (n=18) Disciform scar/ ARM	0.09%	-0.01%

4.2.3.c) Changes in retinal vessels calibre

Retinal microvascular changes are shown in table 4.3. The photographic quality was too poor to measure the vascular diameters in one subject in the ARM-CNV group and three

subjects in the ARM- disciform degeneration group. Therefore, a total of 20 patients were included in the ARM-CNV group and 15 patients in the ARM- disciform scar group.

Table 4.4 illustrates that there is no significant change in calibre of the equivalent venular or arteriolar diameter in both groups.

Table 4.4: Mean retinal vascular calibre in the two eyes of the patients in each group

	Mean CRVE (SD)	**Mean CRAE (SD)**	**Mean AVR (SD)**
Group 1			
CNV	212.27 (34.86)	134.32 (29.91)	0.65 (0.18)
ARM	215.17 (34.31)	139.94 (28.43)	0.66 (0.14)
Group 2			
Disciform scar	222.91 (17.20)	147.86 (29.52)	0.66 (0.11)
ARM	221.21 (17.60)	148.77 (29.84)	0.67 (0.11)

4.2.4 Discussion

The results of this study show that there is no significant change in POBF in the various stages of AMD in patients with asymmetric AMD. POBF measures the global choroidal blood flow. So the total choroidal blood flow remains practically unaltered in the various stages of AMD. However, various studies have observed focal perfusion defects in the macula in AMD. This may be because the macula is the watershed zone for the short posterior ciliary arteries and is therefore more susceptible to subtle changes in choroidal circulation.

Studies assessing POBF in AMD show inconsistent results. Mori et al compared POBF between single eyes of Japanese subjects with neovascular AMD (11 eyes), non-neovascular (10 eyes) and age matched healthy controls (69 eyes). The results were expressed as median with POBF being significantly lower in neovascular group (30%

decrease) compared to non-neovascular group AMD (p=0.02) and healthy controls (p=0.01). No significant difference was noted between non-neovascular AMD and controls (Mori et al. 2001b). When the data of the present study are analysed similar to the study by Mori et al., the median POBF in the neovascular group (CNV group 1087 µl/min and disciform group 950 µl/min) averaged at 1019 µl/min. The median POBF of all the eyes with early disease in this study was 1016 µl/min and therefore this study did not show any statistical difference in POBF on group wise comparison. However, group-wise inter-individual comparison of POBF shows high inter-individual variation and therefore the validity of the methodology is threatened. In addition, Mori et al. did not define the neovascular group as eyes with disciform scar in this study were found to have lower POBF than the eyes with active CNV.

A second study by Chen et al investigated differences in POBF in a Taiwanese cohort with asymmetric AMD between fellow eyes of the same subject thus eliminating the high inter-individual variation in POBF (Chen et al. 2004). As the methodology and sample size was similar to this study, it enabled us to compare the blood flow characteristics in the two groups. In the Taiwanese cohort, the mean value of POBF, after adjusting for IOP and PR, was significantly lower in the eyes with drusen compared to fellow eyes with CNV but higher than fellow eyes with disciform scar. Though my study also adjusted for these factors, I found only a trend of increased POBF in the CNV group (mean increase of 7%) compared to fellow eyes with ARM. No change in POBF was found between eyes with disciform scar and ARM. It may be that other confounding factors exist. Both studies did not adjust for scleral rigidity and refractive errors that may influence the readings obtained by POBF.

Moreover, despite stringent measures in accurate assessment of POBF, the OBA may not be the ideal tool for assessment of total choroidal blood flow. The pulsatile component of the total ocular blood flow ranges from 50 to 80%. Further studies using other techniques to assess total choroidal blood flow may explain these inconsistencies and help us to understand better choroidal blood flow in AMD.

In brief, even if the total choroidal blood flow may be reduced in AMD due to atherosclerosis, this study shows that the severity of AMD does not correlate with the amount of total choroidal blood flow in asymmetric AMD measured by OBA.

This study also assessed the retinal vascular calibre in the same cohort. A semi-automated technique of computer analysis of the arteriolar and venular diameter was adapted from the ARIC study (Klein et al. 2004). There are inherent advantages in using AVR to quantify retinal vascular calibre. It adjusts for the wide range of vessel diameter in the normal population and offers protection against variable magnification from different refractive errors, poor photographic focus or ocular media clarity and inter-observer variations.

An AVR of 1.0 indicates the equivalent arteriolar diameter is equivalent to the venular diameter. Smaller AVR, in general, indicates retinal arteriolar narrowing though

Ikram et al. noted that arteriolar narrowing may be an indicator of hypertension while venular dilation may be due to inflammation or atherosclerosis (Ikram et al, 2004). The mean AVR is smaller in men than women probably due to hormonal influence. Changes in AVR is less significant in the elderly population (>80 years old). This may be because the rigidity of the arteriolar wall caused by age related involutional sclerosis of the retinal arterioles may prevent the same degree of narrowing as seen in the younger counter parts. Logistic regression analysis was done to adjust for age and sex of the cohort and the retinal

vascular calibre did not alter significantly between eyes with asymmetric AMD in this study.

Epidemiological studies such as the ARIC study, Blue Mountain Eye Study and the Rotterdam study have used this technique to note retinal microvascular changes in AMD and consistently found no association of generalized arteriolar narrowing with incident early ARM. This study, though of small sample size further points out that there are no significant changes in retinal microvascular abnormalities between eyes with varying severity of AMD. However, it should be stressed that this study is cross-sectional and it does not clarify the natural history of retinal changes and their temporal relationship with the progression of AMD. Another limitation of this study is that adjustment for history of smoking was not done. Measurement of AVR using the methodology in this study is practical only in a research setting and is too tedious to be used in clinical setting. As a fully-computerized retinal analysis software package is now available, it may be useful to consider a longitudinal study to assess the changes in retinal vascular calibre in late incident ARM.

4.2.5 Conclusion

This study shows that there is no significant change in pulsatile component of the ocular blood flow or retinal microvascular abnormalities between eyes of patients with asymmetric AMD. Even if generalized retinal and choroidal vascular insufficiency due to atherosclerosis may play a role in AMD, this study shows that the severity of AMD does not correlate with generalized ocular blood flow or retinal vascular calibre changes.

CHAPTER 5: GENERAL DISCUSSION

Since the introduction of the vascular hypothesis by Verhoff and Gossmann, the exact contribution of atherosclerosis to the development of AMD still remains unclear (Verhoff and Gossmann, 1937). Most epidemiological studies have addressed this hypothesis by studying various cardiovascular risk factors in AMD and the resultant data is inconsistent.

5.1 Objectives

The objectives in this thesis were to address ways in which AMD is linked to atherosclerosis. In the review on the disease related changes in the Bruch's membrane and the vascular wall, it is noted that the common primary pathogenic event is the accumulation of debris in both structures and this is postulated to initiate a pathologic cascade resulting in the ultimate clinical syndrome associated with atherosclerosis and the various subtypes of AMD.

From an early life, the vascular wall is subject to injury such as lipid deposition, hypoxia, reactive oxygen species, enzymatic degradation and products of inflammation. ECM remodelling is an important response to injury. Similar insults affect the RPE-Bruch's membrane complex. So the first hypothesis in this thesis is that the pathogenesis of AMD, akin to atherosclerosis, is a response to injury that partly manifest as ECM remodelling.

Secondly, atherosclerosis is re-defined as an inflammatory disease. The recent finding of a strong link of CFH gene with AMD supports our hypothesis that the injury in AMD similar to atherosclerosis may be due to inflammation induced by the various insults mentioned above. So both diseases are parallel response to injury as shown if fig 5.1.

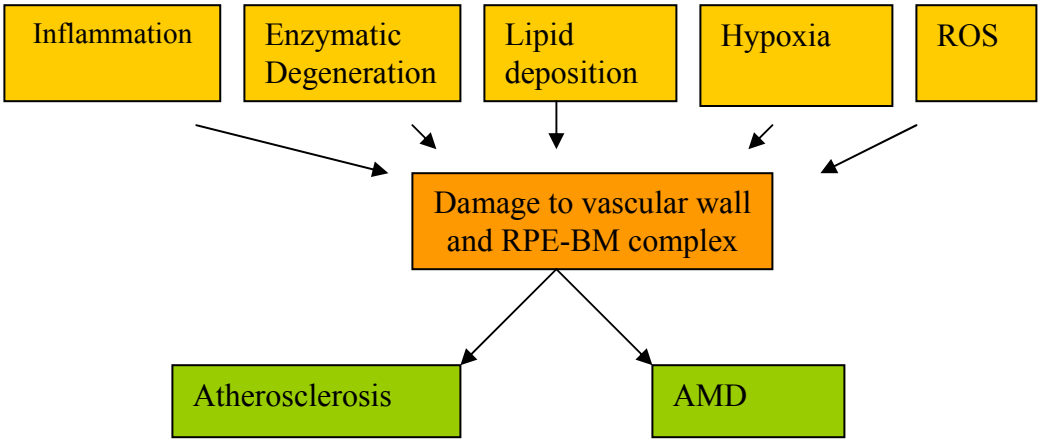

Fig 5.1: Schematic diagram of the first hypothesis that link atherosclerosis and AMD

The second hypothesis is that AMD may be secondary to vascular insufficiency induced by atherosclerosis as depicted in fig 5.2. Thickening and stiffening of the ophthalmic artery and retinal and choroidal circulation by atherosclerosis which may lead to decreased vascular lumen diameter, an increased blood flow resistance, diminished blood flow and reduced tissue perfusion. This process may then directly impair the metabolism of the RPE or lead to leakage and deposition of proteins and lipids due to elevated hydrostatic pressure as proposed by Friedman (Friedman, 1997).

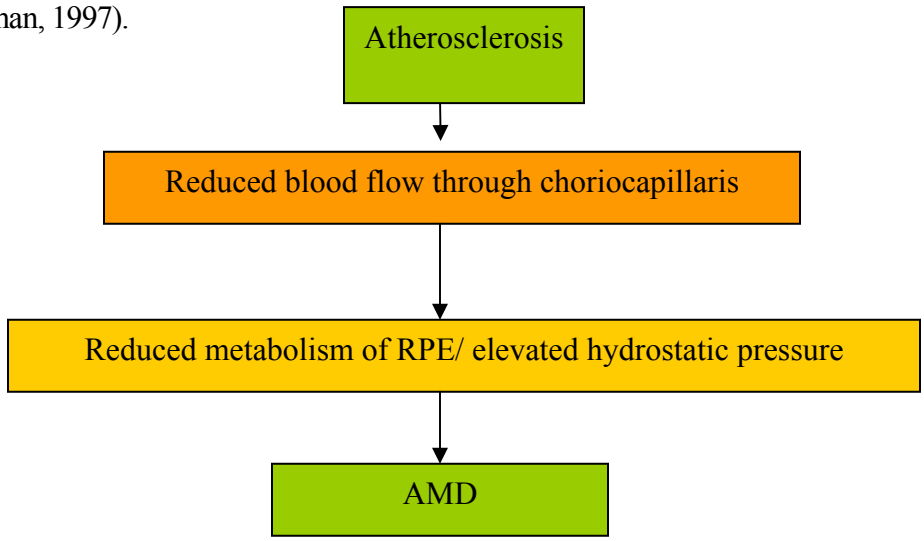

Fig 5.2: Schematic diagram of the second hypothesis

5.2 Methodology

5.2.1 Study design: All studies in this thesis are prospective case control studies. The prospective study made it possible to determine a temporal relationship. However, the studies were clinical based and had a potential for selection bias. To the best of our knowledge, all basic science studies done to test the first hypothesis in this thesis are novel and have not been done before. Therefore, these were pilot projects done with small sample sizes though they were sufficiently powered to obtain a statistical significance.

5.2.2 Definition of ARM: Definition of ARM was based on the nomenclature suggested by the International Epidemiological Study Group for ARM. The Rotterdam study divided ARM into 5 mutually exclusive groups to quantify the progression of ARM. In the basic science projects in this thesis, ARM was classified into two groups: ARM group signified early disease (group 2 and 3 of Rotterdam study) and CNV signified the neovascular AMD group. Larger sample sizes will be required to classify the subjects into the 5 groups of Rotterdam study in order to obtain statistical power. Secondly, subjects with geographic atrophy were excluded from the study because of the relatively low incidence of these subjects in clinics.

5.2.3 Confounding factors: Even though many potential confounders were adjusted, the possibility of insufficient measurement of confounders or the presence of unknown confounders should be considered in these cross-sectional studies. None of the studies in this thesis adjusted for factors related to oxidative stress. In Chapter 4.2, the work would have been more robust if smoking and refractive error were adjusted for the calculation of retinal vessel calibre in this cohort though it could be argued that it should not affect the results as the comparison was between eyes of the same subject..

5.3 Results

In the second chapter, S-EDP and circulating MMP-9 were found to be increased in both early and neovascular AMD compared to age- matched controls. These findings support the hypothesis that AMD share similar extra cellular matrix changes to atherosclerosis. The degradation of ECM protein is tightly regulated and is essential for cellular migration. Macrophages are the main effector cell types in chronic inflammation and several studies have observed increased activity of macrophages in AMD. Macrophages are important sources of MMPs especially MMP-9. The leucocyte derived MMPs are predominant early mediators of matrix degradation. The finding in this thesis that circulating MMP-9 is raised in AMD may indicate that a state of chronic low-grade inflammation exists in these subjects elaborating sufficient systemic MMP-9 that may partly be responsible for the matrix turnover as evidenced by increased S-EDP in AMD. AMD may contribute to or is the result of systemic elastin turnover.

The precise source of circulating S-EDP is unclear though the vascular wall is the most likely source. This may help explain the association of AMD with atherosclerosis. Higher levels of S-EDP in neovascular AMD suggest that S-EDP may be involved in the pathogenesis of CNV. The precise mechanisms of ECM remodelling in AMD remains speculative and require further investigations. By correlating MMP-9 and S-EDP, it is noted that MMP-9 is not the sole proteolytic enzyme that is responsible for elastin degradation. Systemic MMP-2 is predominantly secreted by the endothelial cells and this may explain why it is not raised in AMD. Previous studies on other systemic endothelial cell markers have also not observed any significant relation to AMD. It is important to investigate the potential role of other elastolytic enzymes and the balance of systemic MMP-TIMP in AMD.

Although S-EDP and plasma MMP-9 were found to be elevated in AMD in this thesis and similar elevated levels have been noted in AAA, there was no significant association between AMD and

AAA in the cohort studied. However, these findings may be attributable to survivor cohort effect and statistical constraints. The incidence of neovascular AMD increases with age while patients with AAA unless screened early have a high mortality rate at an age at which the rate of neovascular AMD is low. The association can only be determined in a cohort of patients who have survived AAA repair.

In order to elucidate whether atherosclerosis and AMD are the common result of low grade chronic inflammation, the role of systemic complement activation in varying grades of AMD was observed. Raised C3a des Arg was noted in neovascular AMD. The recent evidence that CFH mutations are associated with increased risk of AMD in combination with the results of this study indicate the need for estimation of other complement proteins in the alternate pathway in larger cohorts to better understand the role of complements in AMD. Previous elegant studies have shown local complement activation in drusen biogenesis but more work is needed to note the role of systemic complements especially in neovascular AMD.

The second hypothesis is that vascular insufficiency induced by atherosclerosis is associated with AMD. I looked at whether the severity of AMD correlates with reduction of global ocular blood flow by measuring the POBF in patients with asymmetric AMD. There were no significant changes in POBF between eyes in a group of patients with early ARM in one eye and CNV in the other or in the second group with early changes in one eye and disciform degeneration in the other. As this technique of measurement of ocular blood flow shows high inter-individual variations, POBF was compared between eyes of the same subject negating that effect. However, no adjustments were done for refractive errors although these factors should not affect the results significantly given that, adjustments were made for axial length and pulse variations. Therefore, from these findings on this small cohort of patients, it is logical to assume that global choroidal blood flow does not correlate with severity of AMD.

Retinal vascular calibre changes were also measured in the same cohort using semi-automated computer software adapted from the ARIC study and this study found no significant difference in retinal vascular calibre between the two eyes of these patients with asymmetric AMD.

Therefore, from the studies in this thesis, it may be inferred that atherosclerosis and AMD are parallel tissue responses to similar noxious stimuli supporting the first hypothesis. The findings from this study did not support the second hypothesis. Measures used to test the second hypothesis are indirect approaches to measure the effect of vascular insufficiency on the severity of AMD but no correlation was obtained.

CHAPTER 6: CONCLUSION

Considering the results of the clinical and basic investigations in this thesis, it may be postulated that AMD should not be regarded as a single entity. The disease may be part of an age-related multi-system disorder probably linked by low-grade inflammation and/ or oxidative stress that induce similar ECM changes in various tissues in the body such as the vascular wall and the Bruch's membrane. The effect of multiple inflammatory markers on measures of oxidative stress and ECM degradation may be influenced by many genetic and environmental factors. The combination of these determinants may decide the age of onset, rate of progression and final stage of AMD.

These issues have to be evaluated together in larger prospective cohorts to test this hypothesis.

The recent emergence of the CFH gene in the pathogenesis of AMD supports the role of inflammation in AMD. Complement activation also plays an important role in atherosclerosis. It may be that this gene plays a permissive role in the above postulation.

Although vascular insufficiency due to atherosclerosis may be related to AMD, the results of this study on ocular blood flow and retinal microvascular changes suggest that the severity of AMD does not correlate with the degree of vascular insufficiency.

CHAPTER 7: FUTURE WORK

The studies in this thesis have shown that S-EDP and MMP-9 are raised in AMD suggesting that ECM changes in AMD may be of a systemic process. However, correlation between S-EDP and MMP-9 was not demonstrated indicating that other elastolytic enzymes or the balance of systemic MMP-TIMP may play a role.

These findings deserve further investigations in a large prospective cohort. An ideal prospective study should simultaneously evaluate a number of elastolytic enzymes and the role of systemic MMP-TIMP in AMD in order to evaluate if one of these is superior to the others or if these enzymes complement each other in elastin degradation.

In addition, it will be useful to estimate the role of circulating anti-elastin antibodies in patients with AMD of varying severity. Another important work related to elastin would be to look at the presence of altered elastin gene transcription in patients with AMD compared to an age matched control group.

The study on the association of AAA and AMD did not reveal a link despite the fact that S-EDP increases with severity in both conditions. Failure to find any significant association between AAA and AMD may be due statistical power constraints and survival bias. So future studies of larger cohorts aimed at persons who have survived AAA repair may give a definitive answer as to whether there is an association between the two conditions. However, recruitment of subjects from a single institution may prove difficult.

Systemic complement activation as measured by plasma C3a des Arg was found to be increased in patients with neovascular AMD. Complement C3 is the central protein in the complement cascade. It will be worthwhile to measure multiple complement proteins

especially in the alternate pathway, given the recent evidence of CFH gene in the pathogenesis of AMD.

Moreover, oxidative stress activates complement through the lectin pathway and as oxidative stress plays an important role in the pathogenesis of AMD, it will be useful to estimate the role of the serine proteases in the lectin pathway such as serum MBL (mannose binding lectin) in patients with AMD.

Genetically-engineered deficiencies of specific complement components such as C3 knockout mice may also offer opportunities for specific evaluation of the role of different components of the complement system in the pathogenesis of both early disease and neovascular disease.

APPENDIX-1

Ethics Committee Approval I

King's College Hospital Research Ethics Committee
Research Ethics Office
2nd Floor Hambledon Wing
King's College Hospital
Denmark Hill
London SE5 9RS
Email: Sophie.Bonser@kingsch.nhs.uk
Website:www.corec.org.uk
Tel: 020 7346 3923 Fax 020 7346 5085

07 October 2003

Mr Victor Chong
King's College Hospital
Consultant Ophthalmic Surgeon
King's College Hospital
Denmark Hill
London
SE5 9RS

Dear Mr Chong

Re: LREC Protocol No. 02-156
Human DNA and serum sample collection and phenotypic characterisation of age-related macular degeneration.

Thank you for your letter regarding clarification on the use of blood samples from this collection outside King's College Hospital.

I am happy to confirm that if your protocol allows laboratory analysis of samples in this study at laboratories outside King's, this is satisfactory ethically from our point of view.

King's College Hospital is compliant with ICH GCP guidelines.

Yours sincerely

Dr. D Jewitt
Chairman, Research Ethics Committee
King's College Hospital

King's College Hospital NHS
NHS Trust

King's College Hospital NHS Trust
King's College Hospital
Denmark Hill London SE5 9RS

Direct tel: 020 7346 4548
Direct fax: 020 7346 3738

Patient information Sheet

Title: Human DNA and serum sample collection and phenotypic characterisation of age-related macular degeneration

Lead Researcher: Victor Chong

King's protocol number: 02-156

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information and discuss it with others as necessary. Do ask us if there is anything that is not clear or if you would like further information. Take time to decide whether or not you wish to take part.

Background and purpose of the study

Age-related macular degeneration (AMD) is a disease affecting the central portion (macula) of the light sensitive layer of the eye (retina) which mediates accurate vision. During ageing, the macula is susceptible to a loss of retinal tissue (atrophy) and growth of new blood vessels which destroys the retinal tissue. The condition causes loss of the ability to see accurately so that tasks such as driving, reading, watching television, and recognising faces are difficult or impossible to perform. Peripheral vision is usually preserved and so navigation is possible in familiar surroundings, although more difficult. AMD is the commonest cause of visual impairment in the Western world. It accounts for over 50% of blind registration and is estimated to affect over approximately 3 million people in the UK. Unfortunately, there is no effective treatment for the disorder and presently no way of preventing the disease from occurring in those who may be susceptible.

Although AMD is not usually inherited in a straight-forward way in families, previous work does suggest an important genetic component to the disease, such that a person's chances of developing the disease in old age depends strongly on the genes that they are born with and inherit from their parents. Presently the specific genes that may be

important in AMD are unknown. The discovery of these genes and their further study provides the clearest hope of developing treatments and preventative measures for people who suffer from the disease.

In order to help investigators throughout the country and abroad discover these important genes, we at King's College Hospital in collaboration with Moorfields Eye Hospital, are asking the help of patients with AMD and those without the disease (controls) and if they are willing, entering them into a national study. The study involves taking photographs of the eyes after having dilated the pupils with eye-drops and taking a blood sample for the research. The study intends to recruit 3000 patients over 3 years. The blood samples will then be used by other researchers to detect any differences in the genes that may cause AMD, in patients with the disease compared to those without.

Why have I been chosen?

You have been chosen either because you have age-related macular degeneration, or because you have some changes that are associated with ageing but do not presently threaten your vision, or because you have entirely normal retinas and so would be suitable as a control in the study. The doctors and nurses in the clinic will explain to you which of these categories you are in.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you decide not to take part this will not affect the management of your eye problem in the clinic in anyway.

What will happen to me if I take part?

If you do decide to take part, your examination is exactly the same as would happen anyway when you are seen in the eye-clinic. The routine examination involves a doctor examining your eyes by using a microscope and having some photographs taken after your pupils have been dilated with eye-drops. These drops sting for a few seconds but otherwise are not uncomfortable. After your examination, we would like to ask you a few questions and take a small blood sample from you for our research study. This sample will then be available to other scientists who are investigating the genes that may cause AMD in the future.

What are the side-effects and possible disadvantages of taking part?

The only side-effects are those of the eye-drops. If you take part in the study you will spend longer in the clinic than if you do not as the doctors will be spending time explaining the study to you and taking the blood sample. There are no other disadvantages to taking part in the study.

Will my taking part in this study be confidential?

If you consent to take part in the research, your identity will be known only to the small number of doctors at the hospital who have been involved with you during the examination. Otherwise the study is entirely confidential. Each patient is assigned a research number and the link between this number and your name will be accessible only to the doctors who have been involved in your examination. It is intended to make your DNA available to other investigators who are trying to discover the genes that might cause AMD. However, they will not be able to trace your name or other personal details as they will only have the research number for each of the blood samples.

What will happen to the blood sample?

The blood sample will be processed in the laboratory to extract the genetic material (DNA) from the cells. This DNA can then by analysed to look for genes that we think may have an effect on the risk of developing AMD. In addition, some of the sample may be processed in a special way to create what is called a cell line. This means that the cells from the blood can be kept growing in the laboratory to increase the amount of DNA that can be obtained from the sample. This is done in the hope of ensuring that we will have enough DNA for the many experiments that will need to be done before the genetics of AMD is completely understood. Certain specific components of the blood sample will be analysed to look for specific compound in the blood that may have an effect on the risk of developing AMD.

What will happen to the results of the study?

Any results that emerge from the study will be published in scientific and medical journals. In addition, the information collected will be stored on computer and may also be used by pharmaceutical companies in an attempt to develop a therapy for people with AMD. The results from the study will be anonymous and the identity of individuals taking part will not be declared. It will not be possible for you to learn of the results of tests on your blood sample in this study.

Who is funding the research?

The Medical Research Council (MRC) is funding the collection of DNA samples from persons with AMD and control persons over a three year period. Future studies on the blood samples from this collection will be undertaken by various other groups and funded separately.

Who has reviewed the study?

The study has been reviewed by scientific and medical experts recruited by the MRC. The research has also been reviewed by the Ethics committee at King's College Hospital and Moorfields Eye Hospital. Any future studies on the blood samples will have also been carefully reviewed by a panel of experts, and this fact will be ensured by the investigators

and curators of the blood sample collection at King's College Hospital and Moorfields Eye Hospital.

How long will the research last?

Although the collection of blood samples will last three years, it is not known how long the research on those samples will last. It is intended that large number of blood samples generated from this collection will be available for many years afterwards to doctors and scientists as more genes that might cause AMD are discovered. However, the identity of the people providing the blood samples would remain unknown to them.

How many times do I need to visit the hospital for the study?

You only need to be seen once for the study. However, we are asking each participant if they would agree to be contacted for a further examination should this be useful in the future for the research. There is no obligation for you to choose to do this. Also, if you do choose to agree to be contacted there will still be no obligation for you to be examined again in the future should you change your mind.

Contact for further information

Please ask any questions you have about the study before deciding to take part. If you wish to contact someone once you have left the hospital about the study please contact the doctor/genetic research co-ordinator below who will be able to answer any questions you may have.

Victor Chong
Consultant Ophthalmic Surgeon
Tel: 020 7346 4548

PIS: Version 2 Date: 3rd September 2002

King's College Hospital NHS
NHS Trust

King's College Hospital NHS Trust
King's College Hospital
Denmark Hill London SE5 9RS

Direct tel: 020 7346 4548
Direct fax: 020 7346 3738

Consent Form

Title: Human DNA and serum sample collection and phenotypic characterisation of age-related macular degeneration

Lead Researcher: Victor Chong

King's protocol number: 02-156
Patient Trial Number:

I confirm that I have read and understand the information sheet dated 3rd September (version 2) for the above study and have had the opportunity to ask questions. ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

I understand that sections of any of my medical notes may be looked at by responsible individuals authorised by the lead researcher or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

I consent for my blood sample to be studied by other researchers at other Institutions for research into age-related macular degeneration. ☐

Print Name……. Signature……
Date…./……../……../

Ethics Committee Approval II

Cranfield University
Silsoe
Bedfordshire
MK 45 4DT
England
Tel: +44 (0) 1525 863000
Fax: +44 (0) 1525 863001
www. silsoe.cranfield.ac.uk

Institute of Bioscience and Technology

28th June 2004

Dear Sobha

Re: Human serum sample collection and phenotypic characterisation of age-related macular degeneration

I am pleased to confirm that the Cranfield University at Silsoe Clinical Research Ethics committee is satisfied that you have taken into account all the appropriate data protection and ethical considerations which affect Cranfield University as a result of you undertaking the aforementioned study, and are therefore happy for you to proceed subject to the following terms and conditions.

1 wish to remind you that should you make any subsequent alterations to the protocol of the aforementioned study which is likely to affect matters concerning data protection and/or the ethical position; the committee must be informed immediately.

Approval to proceed is limited solely to the recruitment of volunteers who are members of staff or currently registered for a higher degree at Cranfield University at Silsoe. Barton Road, Silsoe. MK.45 4DT. It is your responsibility to obtain appropriate approval to undertake any research related to this project in or at any external organisation. Approval is limited to providing "control" samples as part the study: Human DNA and serum sample collection and phenotypic

characterisation of age-related macular degeneration - Kings College Hospital Study number: 02-1 56. All conditions imposed under this approval must be adhered to as part of this approval.

It is your responsibility to ensure that the University is indemnified against legal action incurred as a result of you undertaking this research.

I wish you well in the project

Dr. Anthony Woodman BSc (Hons), MSc, PhD
Head, Cranfield Biomedical Centre.

Volunteer information sheet

Study Title – Estimation of serum elastin derived peptides in patients with age related macular degeneration and controls.

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled "Medical Research and You". This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for reading this.

What is the purpose of the study?

Age related macular degeneration is the commonest cause of blindness in the elderly in the western world. In spite of its public health impact, the cause of the disease remains largely unknown. Several theories suggest age related changes in the Bruch's membrane of the choroid layer of the eye to be related to the pathogenesis of the disease.
The Bruch's membrane is a penta-laminar structure that separates the retinal epithelium from its nutrition. It has a central elastic layer that is shown to fragment with age. Fragmentation of elastin releases elastin derived peptides in the circulation. This project aims to estimate serum elastin derived peptides in patients with age related macular degeneration and an age matched control group to estimate and compare elastin turnover in the two groups.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time without giving a reason.

What will happen to me if I take part?

You will be asked to give one 10ml blood sample, which will be taken by the Medical Centre Nurse.

What are the possible disadvantages and risks of taking part?

The risks associated with this are minimal and are those normally associated with taking a blood sample – you may suffer some discomfort and slight bruising in the area from which the blood was taken.

What happens when the research study stops?

Any samples remaining at the end of the study will be destroyed. The results will be analysed and will be used to form the basis of a publication.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of this research will be kept strictly confidential.

Who is organising and funding the research?

The research is being run and funded by Cranfield Biomedical Centre.

Who has reviewed this study?

The Cranfield Biomedical Ethics Committee.

Contact for further information

Should you have any queries about this study, please contact:

Dr. Sobha Sivaprasad
s.sivaprasad.s03@cranfield.ac.uk.
Or
Dr. Tracey Bailey
t.a. bailey@cranfield.ac.uk

Institute of
BioScience and Technology

Cranfield
UNIVERSITY
Silsoe

Volunteer Consent Form

Study Title – Estimation of serum elastin derived peptides in patients with age related macular degeneration and controls.

Please initial all boxes:

1 I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions ☐

2 I understand that my participation is voluntary and that I am free to withdraw at any time. ☐

3 I agree to take part in the above study. ☐

- Name of Volunteer…….

  Signature……..Date……..

- Name of person taking consent…….

  Signature……..Date……..

Ethics Committee Approval III

King's College Hospital Research Ethics Committee
Camberwell Building
King's College Hospital
Denmark Hill
London SE5 9RS
Email: Janet.Browning@kingsch.nhs.uk
Tel: 020 7346 3923 Fax 020 7346 5085

26 November 2004

Mr Victor Chong
King's College Hospital
Consultant Ophthalmic Surgeon
King's College Hospital
Denmark Hill
London
SE5 9RS

Dear Mr Chong

Full title of study:	**Age-related macular degeneration in patients with abdominal aortic aneurysm**
REC reference number:	**04/Q0703/121**

Thank you for your letter, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

The further information was considered at the meeting of the Sub-Committee of the REC held on 24 November 2004.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

The favourable opinion applies to King's College Hospital.

Conditions of approval
The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents
The final list of documents reviewed and approved by the Committee is as follows:

Document Type:	Version:	Date Received:
Application	Version 2 dated 17 November 2004	17/11/2004
Investigator CV		27/09/2004
Protocol	version 1 dated 23/09/2004	27/09/2004
GP/Consultant Information Sheets	GP Letter Version 2 dated 01.11.04	17/11/2004
Participant Information Sheet	Version 2 dated 01.11.04	17/11/2004
Participant Consent Form	version 1 dated 23/09/2004	27/09/2004

Management approval
The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Notification of other bodies
The Committee Administrator will notify the research sponsor that the study has a favourable ethical opinion.

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Reference: 04/Q0703/121 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely,

Dr David Jewitt
Chair King's College Hospital Research Ethics Committee

Patient information sheet

Study Title – Age-related macular degeneration in patients with abdominal aortic aneurysm

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled "Medical Research and You". This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for reading this.

What is the purpose of the study?

Atherosclerosis is the main cause of abdominal aortic aneurysm (AAA). Recent epidemiological studies have reported a link between atherosclerosis and age-related macular degeneration (AMD). Both the aorta and the vascular coat of the eye share similar age-related changes. Studies in both structures suggest that they may be a link between the eye findings and the growth of the AAA. We plan to investigate whether changes in the retina and choroids suggestive of these structural changes may be a predictive rupture risk parameter for AAA.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time without giving a reason.

What will happen to me if I take part?

You will be asked to come to the Retinal Research Unit in Normandy building with a list of medications you are on. A brief medical history will be noted. You will then have an eye test. Your pupils will be dilated with drops for examination of the back of the eye and photographs will be taken.

What are the possible disadvantages and risks of taking part?

The risks associated with this are minimal. We dilate your pupils and so you are required to avoid driving for a few hours after the test.

What happens when the research study stops?

The results will be collated and analysed and any significant findings will be published.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of this research will be kept strictly confidential.

Who is organising and funding the research?

The research is being run and funded by the Ophthalmic Fund of Kings College Hospital.

Will information from the research be passed to your GP?

Your GP will be informed of your eye test and the findings

Contact for further information

Should you have any queries about this study, please contact:

Mr. Victor Chong
Consultant Ophthalmic Surgeon
Retinal Research unit
Kings College Hospital
Tel: 020 7346 4548

Patient Consent Form

Age-related macular degeneration in patients with abdominal aortic aneurysm

Please initial all boxes:

1 I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions ☐

2 I understand that my participation is voluntary and that I am free to withdraw at any time. ☐

3 I agree to take part in the above study. ☐

- Name of Patient…….

  Signature……..Date……..

- Name of person taking consent…….

  Signature……..Date……..

Ethics Committee Approval IV

King's College Hospital Research Ethics Committee
Camberwell Building
King's College Hospital
Denmark Hill
London SE5 9RS
Email: Janet.Browning@kingsch.nhs.uk
Tel: 020 7346 3923 Fax 020 7346 5085

18 March 2005

Mr Victor Chong
King's College Hospital
Consultant Ophthalmic Surgeon
King's College Hospital
Denmark Hill
London SE5 9RS

Dear Mr Chong

Full title of study:	***Pulsatile Ocular Blood flow in Asymmetric Age-Related Macular Degeneration***
REC reference number:	***04/Q0703/140***

Thank you for your letter of 10th March 2005 responding to the Committee's request for further information on the above research and submitting revised documentation.
The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.
However, the Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the research site(s) taking part in this study. The favourable opinion does not therefore apply to any site at present. I will write to you again as soon as one Local Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at sites requiring SSA.
Conditions of approval
The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.
Approved documents
The final list of documents reviewed and approved by the Committee is as follows:

Document Type:	Version:	Date Received:
Application	Version 2 dated 10th March 2005	10/03/2005
Investigator CV		26/10/2004
Protocol	Version 1 dated 26th October 2004	26/10/2004
Covering Letter	dated 25th September 2004	26/10/2004
GP/Consultant Information Sheets	GP Letter Version 1 dated 26th October 2004	26/10/2004
Participant Information Sheet	Version 1 dated 26th October 2004	26/10/2004
Participant Consent Form	Version 1 dated 26th October 2004	26/10/2004
Response to Request for Further Information		10/03/2005

R&D approval
The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Membership of the Committee
The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Notification of other bodies
The Committee Administrator will notify the research sponsor and the R&D Department for NHS care organisation that the study has a favourable ethical opinion.

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

04/Q0703/140 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely,

Dr David Jewitt
Chair King's College Hospital Research Ethics Committee

Patient information sheet

Study Title – Pulsatile ocular blood flow in asymmetric age-related macular degeneration

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled "Medical Research and You". This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for reading this.

What is the purpose of the study?

Age related macular degeneration is the most common cause of blindness in the elderly. Despite this, the cause of the disease remains largely unknown. Recent evidence has suggested that decrease blood flow to the eyes as a result of hardening of the blood vessels may result in the disease. We plan to investigate whether changes in the retina and choroids blood flow is related to the stage of the disease

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time without giving a reason.

What will happen to me if I take part?

You will be asked to come to the Retinal Research Unit in Normandy building with a list of medications you are on. A brief medical history and your blood pressure and pulse rate will be recorded. You will then have an eye test and the blood flow of the eye will be assessed using computer software. Your pupils will be dilated with drops for examination of the back of the eye and photographs will be taken.

What are the possible disadvantages and risks of taking part?

The risks associated with this are minimal. We dilate your pupils and so you are required to avoid driving for a few hours after the test.

What happens when the research study stops?

The results will be collated and analysed and any significant findings will be published.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of this research will be kept strictly confidential.

Who is organising and funding the research?

The research is being run and funded by the Ophthalmic Fund of Kings College Hospital.

Will information from the research be passed to your GP?

Your GP will be informed of your eye test and the findings

Contact for further information

Should you have any queries about this study, please contact:

Mr. Victor Chong
Consultant Ophthalmic Surgeon
Retinal Research unit
Kings College Hospital
Tel: 020 7346 4548

Patient Consent Form

Pulsatile ocular blood flow in asymmetric Age-related macular degeneration.

Please initial all boxes:

1 I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions ☐

2 I understand that my participation is voluntary and that I am free to withdraw at any time. ☐

3 I agree to take part in the above study. ☐

- Name of Patient…….

  Signature……..Date……..

- Name of person taking consent…….

  Signature……..Date……..

APPENDIX – 2

Formulations

ABTS buffer
One ABTS tablet -50mg
Dissolved in 50ml of ABTS buffer

0.1 M Sodium Carbonate buffer
$NaCO_3$ 2.12 g
Distilled water 200ml
pH 9.0

Phosphate Saline Buffer (PBS)
One tablet of PBS (Invitogen ^{TM}, Paisley)
Distilled water 500ml

PBST
0.5 ml of 0.05% (v/v) Tween is added to 1 litre PBS

PBST-BSA
70g of 7% (w/v) Bovine serum albumin (BSA) is added to 1 litre PBST

Tris Saline Buffer (TBS)
Firstly a 20x solution of Tris saline buffer is made
Tris 121.1grams
NaCl 175.3grams
Distilled water 1 litre
pH 7.5
then 50ml of the 20x is diluted with 1 litre of distilled water so a 1xTBS solution is obtained.

TBST
0.5 ml of 0.05% v/v Tween is added to 1 litre 1X TBS

MMP-2 ELISA protocol for the R&D Quantikine total human MMP-2 immunoassay kits (DMP200)

1. Standard (rMMP-2) preparation:
    a. Add 1ml of sterile dH2O to dissolve the dried MMP-2 powder at RT for 15 mins.
    b. Prepare 7 Epp-tube each contains 120ul of Calibrator Diluent RD5-32.
    c. Add 120ul of undiluted MMP-2 to Epp-tube1, mix and transfer 120ul to Epp-tube2. Finish the series dilution until Epp-tube7.
    d. The preparation is sufficient for 2 sets of standards of 7 serial dilutions.
    e. Undiluted MMP-2 could be stored frozen at -80'C.
2. Sample preparation:
    a. Thaw the frozen plasma samples (at -80 ^{0}C) on ice and mix content before dispensing.
    b. Prepare 3 Epp-tubes; the 1st one contains 230µl of RD5-32, whereas the 2nd and 3rd ones contain 120 µl of RD5-32.
    c. Add 10.5 µl of plasma samples to tube1, mix and transfer 120 µl to tube2. From tube2 transfer 120 µl to tube3. Thereby, dilutions of tubes 1-3 are 1:45, 1:90 and 1:180 respectively.
    d. The preparation is sufficient for 2 sets of samples of 3 serial dilutions.
3. Plates:
    a. Add 100 µl of Assay Diluent RD1-74 (blue in colour) into each well.
    b. Standards: add 50ul each of the serial diluted MMP-2 into wells along the first and second columns (S1 to S7). To the last well of the first 2 columns add 50 µl of RD5-32 (as Blank).
    c. Add 50 µl of the diluted samples into the corresponding wells.
4. Cover the plate and shake at 250rpm at RT for 2 hrs.
5. Dilute the Stock Wash Buffer 25 times. Discard contents of the plate and add to each well 400 µl of diluted Wash Buffer. Repeat the washing 1 more time.
6. To each well as 200 µl of the MMP-2 conjugate (colourless). Cover the plate and shake at 250rpm at RT for 2 hrs.
7. Wash the plate 3 times as step5.
8. Mix equal volume of Reagents A and B and use within 15 mins. To each well add 200 µl of the mixture. Cover the plate and incubate at RT for half an hour in darkness.
9. Terminate the reactions by adding 50 µl of Stop Solution. Measure ASAP and within half an hour.
10. ELISA reader setting:
    a. Read absorbance at 450 nm.
    b. Enter the concentrations of the standards accordingly.
    c. Plot the OD values and concentrations of the standards.

MMP-9 ELISA protocol for the R&D Quantikine total human MMP-9 immunoassay kits (DMP900)

1. Standard (rMMP-9) preparation:
    a. Add 1ml of sterile dH2O to dissolve the dried MMP-9 powder at R/T for 15 mins.
    b. Prepare 6 Epp-tube each contains 240 µl of Calibrator Diluent RD5-10.
    c. Add 240 µl of undiluted MMP-9 to Epp-tube1, mix and transfer 240 µl to Epp-tube2. Finish the series dilution until Epp-tube6.
    d. The preparation is sufficient for 2 sets of standards of 6 serial dilutions.
    e. Undiluted MMP-9 could be stored frozen at -80'C.
2. Sample preparation:
    a. Thaw the frozen plasma samples (at -80 ^{0}C) on ice and mix content before dispensing.
    b. Prepare 3 Epp-tubes, the 1st one contains 312 µl of RD5-10, whereas the 2nd and 3rd ones contain 220 µl of RD5-10.
    c. Add 13.2ul of plasma samples to tube1, mix and transfer 110 µl to tube2. From tube2 transfer 110 µl to tube3. Thereby, dilutions of tubes 1-3 are 1:25, 1:75 and 1:225 respectively.
    d. The preparation is sufficient for 2 sets of samples of 3 serial dilutions.
3. Plates:
    a. Add 100 µl of Assay Diluent RD1-34 (blue in colour) into each well.
    b. Standards: add 100 µl of undiluted MMP-9 (S1), and 100 µl each of the serial diluted MMP-9 into wells along the first and second columns (S2 to S7). To the last well of the first 2 columns add 100 µl of RD5-10 (as Blank).
    c. Add 100 µl of the diluted samples into the corresponding wells.
4. Cover the plate and shake at 250rpm at RT for 2 hrs.
5. Dilute the Stock Wash Buffer 25 times. Discard contents of the plate and add to each well 400ul of diluted Wash Buffer. Repeat the washing 1 more time.
6. To each well as 200ul of the MMP-9 Conjugate (pink in colour). Cover the plate and shake at 250rpm at RT for 1 hr.
7. Wash the plate 3 times as step5.
8. Mix equal volume of Reagents A and B and use within 15 mins. To each well add 200ul of the mixture. Cover the plate and incubate at RT for half an hour in darkness.
9. Terminate the reactions by adding 50 µl of Stop Solution. Measure ASAP and within half an hour.
10. ELISA reader setting:
    a. Read absorbance at 450 nm.
    b. Enter the concentrations of the standards accordingly.
    c. Plot the OD values and concentrations of the standards.

Protocol for assay of plasma C3a des Arg

1. Precipitation of whole proteins from plasma

To 225 µl of thawed plasma, add 225 µl Complement reagent A and vortex thoroughly.
Add 50 µl of 10N HCL to the sample and vortex and incubate for an hour,
Spin at 10,000 rpm in a micro-centrifuge at room temperature for 5 mins.
Transfer 180 µl supernatant to fresh tube and add 20 µl of 9N NaOH and vortex.
Add 10.7 µl of assay buffer and vortex to ensure 1:10 dilution.
Dilute 50 µl of each sample with 950 µl of assay buffer and vortex
There is a 1: 200 dilution of plasma samples when the above steps are followed.

2. Prepare standards

Allow the stock standard to warm to room temperature. Label 7 tubes 1-7. In the first tube pipet 1 ml assay buffer and 500 µl to tube 2-7.
Remove 20 µl from tube 1 and add 20 µl of stock standard to it and vortex. Remove 500 µl of tube 1 to tube 2 and vortex. Continue this to tube 7.

3. Assay procedure

Pipet 100 µl of assay buffer into NSB and Blank wells. Pipet standards 1-7 to appropriate wells. Also pipet 100 µl of 1: 200 samples into appropriate wells in duplicate. Pipet 50 µl of assay buffer into the NSB wells. Pipet 50 µl of conjugate into each well except blank well. Then add 50 µl of antibody into each well except blank and NSB.
Incubate at room temperature on a plate shaker at 500 rpm for 2 hours.
Use 400 µl of wash buffer to wash wells three times.
Add 200 µl of the p-Npp substrate solution to each well and incubate for 1 hour at 37^0 C without shaking.
Add 50 µl of stop solution to every well.
The plates are read at 405nm.

4. Calculation of Results

Net OD is equal to average of sample OD – average NSB OD

Percent bound is $\frac{\text{Net OD x 100}}{\text{Net Blank OD}}$

Plot standard curve with % bound in y-axis and log of concentrations of standards (ng/ml) in x-axis.
Determine values of C3a des Arg in samples by interpolation.

Protocol for AVR calculation

1. Each venular and arteriolar diameter is measured five times.
2. The mean of each venular and arteriolar diameter are obtained.
3. The mean is in pixels. So to convert it to micrometers, use a conversion factor of 1.91.
4. Place venular and arteriolar mean diameters in ascending order.
5. Then to calculate CRVE when four venular diameters are available, the formula is: SQRT ((0.72*POWER(x, 2)) + (0.91*POWER(y, 2))+450.05), in which x is the smaller value and y is the larger value of the venular diameter.
6. If there are only 3 venial diameters then the combinations are slightly different: As above, whichever is smaller comes first, thus the formula reads SQRT((0.72*POWER(R50,2))+(0.91*POWER(Q52,2))+450.05)
7. A similar process is used for calculating the central retinal artery equivalents from the arteriole measurements but using a different formula:
   The final formula is now SQRT ((0.87*POWER(x, 2)) + (1.01*POWER(y, 2))-(0.22*x*y)-10.76), where x is the smaller value and y is the larger of the arteriolar diameters
8. The CRAE is then divided by the CRVE to give the AV ratio.

APPENDIX-3

Results of analysis of S-EDP

Control group

Sample no.	age	sex	S-EDPng/ml
72	82	F	30.22
75	74	F	7.17
76	72	F	12.46
77	67	M	39.85
98	86	F	11.9
99	81	F	4.67
102	90	F	18.53
107	63	M	4.14
108	67	M	18.37
109	91	M	6.28
113	65	F	7.17
114	79	F	20.5
123	83	M	15.56
126	82	F	23.84
133	61	M	9.87

Results of analysis of S-EDP

ARM group

Sample no.	age	sex	S-EDPng/ml
29	81	F	22.2
45	70	F	20.9
63	78	F	9.81
65	83	F	19.3
71	65	F	12.96
80	83	M	16.6
90	78	M	39.81
106	80	M	7.9
116	80	M	31.25
117	74	M	28.1
128	73	F	26.6
144	73	F	30.05
1192	85	F	20.92
1266	88	M	17.41
1272	72	F	15.08
1276	74	M	20.08
1280	69	M	38.25
1328	67	F	39.08
1335	68	M	35.08
1356	88	M	19.75
1370	75	F	12.58
1372	85	M	24.58
1373	68	M	21.58
1384	69	F	37.08
1390	84	F	47.75
1394	68	F	31.25
1403	70	F	16.08
1405	88	F	14.75
1406	82	F	14.75
1412	81	F	14.75

Results of analysis of S-EDP

CNV group

Sample no.	AGE	SEX	S-EDP ng/ml
36	92	M	8.07
41	84	F	43.67
73	87	F	42.53
79	86	F	47.35
81	65	F	25.03
82	85	F	88.67
83	88	F	18.42
87	79	M	32.53
88	79	F	40.05
89	81	F	44.36
92	79	M	9.14
103	62	F	59.88
110	85	M	27.89
112	78	M	32.47
115	92	M	17
118	87	F	42.17
127	90	F	40.05
129	66	M	52.47
132	79	F	6.64
134	86	M	50.74
135	79	M	7.8
136	75	M	84.53
140	67	M	55.56
146	75	F	60.56
1269	82	F	14.91
1270	75	F	14.75
1314	69	F	8.916

Results of analysis of Plasma MMP-2 and MMP-9

Group	Sample No.	MMP-2 ng/ml	MMP-9 ng/ml
Control	66	487	372
	53	495	241
	47	686	176
	17	417	122
	58	394	323
	1	426	138
	20	457	418
	3	284	174
	40	583	456
	5	610	199
	6	429	208
	7	378	239
	8	576	57
	9	798	160
	61	417	122
	48	502	329
	54	487	543

Group	Sample No.	MMP-2 ng/ml	MMP-9 ng/ml
ARM	23	427	736
	56	655	1054
	36	917	797
	35	374	667
	8	569	72
	55	327	912
	2	618	483
	73	508	297
	51	496	552
	67	547	559
	29	401	236
	63	452	603
	32	446	706
	18	398	1234
	46	468	978

Results of analysis of Plasma MMP-2 and MMP-9

Group	Sample No.	MMP-2 ng/ml	MMP-9 ng/ml
CNV	50	484	1007
	77	413	393
	24	607	833
	52	507	82
	75	415	159
	30	402	1174
	57	587	769
	76	460	1285
	33	606	906
	20	529	437
	22	486	588
	27	486	249
	19	507	719
	74	583	523
	1	786	2233
	6	538	728
	28	457	703
	70	693	549

Correlation between S-EDP and plasma MMP-2 and MMP-9

CONTROL	age (yrs)	S-EDP ng/ml	MMP-2 ng/ml	MMP-9 ng/ml
1	61	39.85	417	494
2	84	56.25	578	702
3	63	4.1	686	176
4	65	7.7	495	225
5	79	20.5	487	456
6	83	15.6	525	337
7	82	24.36	556	846
8	81	23.84	487	372
9	62	9.8	508	297
10	91	7.9	497	225
ARM		**S-EDP**	**MMP-2**	**MMP-9**
1	83	19.32	587	988
2	96	12.26	432	422
3	83	11.7	427	1256
4	80	31.3	655	1054
5	74	28.2	327	901
6	73	26.6	250	820
7	73	30.05	340	1054
8	79	22.2	430	860
9	70	20.9	460	875
10	79	9.81	328	654
CNV		**S-EDP**	**MMP-2**	**MMP-9**
1	80	47.36	486	588
2	88	25.03	607	833
3	85	88.67	529	437
4	88	18.42	507	719
5	79	32.54	486	249
6	85	40.05	457	703
7	81	44.36	402	1174
8	79	9.1	454	505
9	85	27.9	484	1004
10	78	32.47	507	82
11	92	17	587	769
12	87	42.2	449	889
13	90	40.05	574	742
14	67	52.46	379	372
15	79	6.64	693	549
16	86	50.74	413	401
17	79	6.107	460	1285
18	85	84.53	415	695
19	75	55.56	584	1587
20	75	60.56	497	1003

Analysis of Plasma C3a des Arg

Control group

sample no	age	sex	Plasma C3ades Arg (ng/ml)
899	72	M	30.87
1395	71	M	59.05
491	84	M	48.76
845	85	M	50.60
556	87	M	15.65
1730	80	M	84.23
791	71	M	62.81
863	83	F	58.13
363	81	F	52.90
1676	72	F	61.80
616	71	F	61.20
1635	64	F	49.60
1732	89	F	55.40
1610	86	F	35.20
1646	81	F	58.20
268	79	M	53.80
1114	85	M	18.82
1340	82	F	49.80
1644	83	M	45.40
1218	83	F	6.52
114	79	F	72.12
97	84	F	11.57
108	67	M	7.82
126	82	F	7.10
123	83	F	6.51
72	82	F	168.28
75	74	F	164.44
76	72	F	100.97
77	67	M	81.74
98	86	F	76.93
99K	81	F	76.93
102K	90	F	23.64
105K	81	F	11.95
107	63	M	198.09
109K	91	M	173.09
113	65	F	168.28
125	81	M	164.44
133	61	M	23.64

Analysis of plasma C3a des Arg

ARM group

sample no	age	sex	Plasma C3ades Arg (ng/ml)
1241	84	M	61.00
314	55	F	51.38
453	63	M	64.78
910	69	F	61.00
554	84	M	2.78
672	72	M	49.82
731	94	F	57.26
1049	79	M	45.42
980	65	M	58.14
1030	70	F	49.82
650	73	F	49.82
99	67	M	16.48
441	73	F	22.66
1154	102	F	53.82
1481	71	M	49.82
112	82	F	36.6
569	62	F	51.38
241	73	F	54.66
29	81	F	76.93
106	80	M	23.64
144	73	F	11.95
1192	85	F	23.64
1390	84	F	168.28
1372	85	F	81.74
1403	70	F	76.93
1406	82	F	198.09
1356	88	M	173.09
1384	69	F	11.57
1373	68	M	7.82
1412	81	F	7.10
1405	88	F	6.51
1335	68	M	168.28
1280	69	M	164.44
1370	75	F	100.97
1276	74	M	173.09
1328	67	F	198.09
1266	88	M	100.97
1272	72	F	81.74
1394	68	F	76.93

Analysis of Plasma C3a des Arg

CNV group

sample no	age	sex	Plasma C3ades Arg (ng/ml)
1098	85	M	27.70
1013	83	M	85.90
1521	87	M	56.20
591	79	M	28.87
1542	79	M	43.36
317	78	M	41.31
932	84	M	3.13
1078	81	M	55.50
1021	82	F	25.22
1376	84	M	53.00
1164	87	F	56.80
686	81	M	56.40
1347	79	F	46.20
109	78	F	60.90
787	88	F	16.90
291	85	F	62.80
1268	83	F	66.80
41	84	F	250.02
40	80	M	198.09
112K	78	M	173.09
101	85	M	168.28
134	86	M	164.44
88	79	F	250.02
117	80	M	173.09
89	81	F	76.93
1269	82	F	7.10
1314	69	F	173.09
1270	75	F	168.28
81	65	F	164.44
83	88	F	100.97
82	85	F	81.74
87	79	M	6.51
132	79	F	81.74
135	79	M	100.97
36	92	M	198.09
92	79	M	7.10
110	85	M	250.02
127	90	F	173.09
118	87	F	100.97
140	67	M	7.10
103	62	F	76.93
146	75	F	11.95

Pulsatile Ocular Blood Flow in Asymmetric AMD

First eye

	No	SEX	AGE	diag_eye1	AL-eye1	IOP-eye1	PA-eye1	PV-eye1	PR-eye1	OBF-eye1
GROUP 1	1	F	89	CNV	22.09	14.1	2.9	6.1	72.4	912.0
	2	F	78	CNV	22.15	19.5	3.4	5.7	98.6	1297.2
	3	F	88	CNV	22.78	14.4	3.3	6.8	56.4	766.8
	4	F	89	CNV	22.69	9.0	2.5	7.8	64.0	1087.2
	5	F	90	CNV	21.41	7.3	4.9	16.2	62.4	2053.2
	6	F	81	CNV	25.78	11.4	3.3	8.3	70.2	1198.8
	7	F	78	CNV	22.49	8.1	4.1	13.0	72.4	1926.0
	8	F	87	CNV	23.57	15.1	3.9	7.6	67.2	997.2
	9	M	85	CNV	23.45	25.3	3.7	5.0	60.8	594.0
	10	M	81	CNV	24.75	6.9	4.0	14.2	64.0	1763.5
	11	F	86	CNV	22.73	14.3	4.4	9.0	70.0	1309.8
	12	M	77	CNV	24.32	8.2	2.9	9.5	66.2	1222.0
	13	M	78	CNV	24.14	7.5	1.4	5.4	74.0	850.2
	14	M	80	CNV	22.80	11.4	2.6	6.6	65.4	901.4
	15	F	62	CNV	23.32	17.3	1.7	3.2	86.6	589.2
	16	M	64	CNV	23.25	9.8	3.0	8.7	71.6	1309.2
	17	F	74	CNV	21.53	12.8	4.6	10.0	75.0	1551.6
	18	F	82	CNV	23.14	11.3	2.9	7.3	62.6	937.2
	19	M	68	CNV	24.32	10.2	3.3	9.0	64.0	1124.4
	20	F	84	CNV	21.24	9.6	2.6	7.7	68.0	1062.0
	21	M	79	CNV	23.38	14.7	2.0	4.3	83.6	763.4
GROUP2	1	F	90	Disciform	20.62	7.9	3.1	10.4	85.5	1869.0
	2	F	78	Disciform	21.77	11.8	2.9	7.0	96.0	1466.4
	3	F	80	Disciform	22.49	9.2	4.0	11.6	67.6	1563.6
	4	F	75	Disciform	22.29	9.1	4.1	11.8	69.6	1713.0
	5	M	84	Disciform	21.89	17.6	3.5	6.2	68.2	918.4
	6	M	71	Disciform	24.75	15.9	1.8	3.6	83.2	646.4
	7	M	85	Disciform	22.25	13.0	1.8	4.3	77.8	673.2
	8	F	79	Disciform	22.53	18.9	3.7	6.1	62.4	826.8
	9	M	84	Disciform	23.58	17.2	1.7	3.2	78.2	534.0
	10	F	82	Disciform	23.17	14.3	4.6	9.3	54.6	981.6
	11	M	89	Disciform	23.59	13.5	3.1	6.8	63.0	816.0
	12	F	79	Disciform	21.43	13.8	3.5	7.4	79.8	1232.4
	13	M	66	Disciform	23.03	8.8	3.0	9.3	57.0	1237.2
	14	F	78	Disciform	22.83	11.6	2.1	5.3	75.2	820.8
	15	F	84	Disciform	22.53	9.9	3.3	9.2	75.0	1620.0
	16	F	72	Disciform	23.24	15.5	2.2	4.4	57.4	546.0
	17	F	81	Disciform	20.84	14.2	2.9	6.2	75.6	1004.4
	18	F	84	Disciform	24.59	17.5	2.0	3.6	66.2	466.8

AL-axial length; PA- pulse amplitude; PR-Pulse rate; OBF- ocular blood flow

Pulsatile Ocular Blood Flow in Asymmetric AMD

Second eye

	No	diag_eye2	AL-eye2	IOP-eye2	PA-eye2	PV-eye2	PR-eye2	OBF-eye2
GROUP 1	1	Drusen	21.96	10.6	3.0	7.2	74.4	1224.0
	2	Drusen	22.32	20.4	3.5	5.6	101.6	1220.4
	3	Drusen	22.63	11.4	3.1	7.7	56.5	856.5
	4	Drusen	22.56	9.4	2.7	8.1	63.4	1063.2
	5	Drusen	21.86	8.5	5.0	14.8	64.8	1909.2
	6	Drusen	25.88	10.9	3.3	8.5	71.6	1224.0
	7	Drusen	22.60	9.1	4.3	12.4	71.6	1813.2
	8	Drusen	23.80	15.5	3.8	7.3	63.3	939.0
	9	Drusen	23.56	17.3	4.4	7.7	58.6	922.8
	10	Drusen	24.79	6.8	3.6	13.2	57.6	1673.2
	11	Drusen	22.79	13.6	4.6	9.6	70.8	1441.4
	12	Drusen	23.74	9.6	2.8	8.2	65.4	1039.4
	13	Drusen	24.25	9.1	1.8	5.8	74.0	874.4
	14	Drusen	22.94	10.3	2.2	6.1	65.2	809.4
	15	Drusen	23.49	14.6	1.8	3.9	89.4	760.8
	16	Drusen	23.07	9.4	2.9	8.6	69.0	1245.6
	17	Drusen	21.87	12.4	5.0	11.2	74.6	1752.0
	18	Drusen	24.04	10.9	2.7	7.2	62.0	896.4
	19	Drusen	24.68	14.9	2.9	6.0	66.2	861.6
	20	Drusen	21.55	8.2	1.7	5.9	68.0	894.0
	21	Drusen	23.35	13.7	1.9	4.3	80.4	700.4
GROUP 2	1	Drusen	21.72	10.7	4.1	10.6	83.2	1792.8
	2	Drusen	21.90	16.7	2.5	4.7	94.2	952.8
	3	Drusen	22.78	12.3	3.5	8.3	67.8	1136.2
	4	Drusen	23.00	9.3	3.5	10.1	68.4	1375.2
	5	Drusen	22.08	20.4	3.1	4.8	70.0	712.8
	6	Drusen	24.71	14.1	1.7	3.9	84.6	684.4
	7	Drusen	23.36	12.9	2.3	5.4	74.4	805.6
	8	Drusen	22.59	17.2	3.7	6.6	64.8	812.4
	9	Drusen	23.85	16.6	1.9	3.7	80.8	618.0
	10	Drusen	23.02	11.6	4.4	10.5	55.8	1153.5
	11	Drusen	24.33	8.5	2.7	8.8	63.6	1164.0
	12	Drusen	21.83	11.3	3.1	7.8	82.8	1364.4
	13	Drusen	23.21	11.0	3.3	8.6	68.2	1159.2
	14	Drusen	21.41	13.1	2.8	6.3	75.6	990.0
	15	Drusen	23.24	11.3	3.2	8.1	71.0	1272.0
	16	Drusen	23.14	17.3	2.3	4.2	57.6	500.4
	17	Drusen	22.14	16.0	3.2	6.0	78.6	996.0
	18	Drusen	24.39	17.8	2.0	3.7	70.0	524.4

AL-axial length; PA- pulse amplitude; PR-Pulse rate; OBF- ocular blood flow

Retinal vascular calibre changes in asymmetric AMD

Group 1: ARM-CNV group

				DRUSEN					CNV	
	age	sex	CRVE	CRAE	AVR			CRVE	CRAE	AVR
1	89	F	180.19	133.30	0.74		1	169.53	131.03	0.77
2	78	F	299.21	152.02	0.51		2	295.22	148.22	0.50
3	88	F	207.03	136.43	0.66		3	213.20	130.36	0.61
4	89	F	191.50	167.46	0.87		4	201.98	117.62	0.58
5	90	F	145.51	138.24	0.95		5	144.45	134.15	0.93
6	81	F	214.36	150.83	0.70		6	203.33	151.23	0.74
7	78	F	209.36	113.81	0.54		7	208.39	110.87	0.53
8	87	F	200.83	129.15	0.64		8	177.30	203.24	1.15
9	85	M	242.98	113.60	0.47		9	212.40	111.70	0.53
10	81	M	200.97	175.92	0.88		10	192.56	179.20	0.93
11	86	F	178.35	125.36	0.70		11	196.43	117.85	0.60
12	77	M	220.91	120.09	0.54		12	210.33	108.44	0.52
13	78	M	219.24	120.96	0.55		13	234.36	128.58	0.55
14	80	M	215.34	129.29	0.60		14	203.50	103.04	0.51
15	62	F	207.75	123.72	0.60		15	217.49	115.23	0.53
16	64	M	259.20	146.85	0.57		16	225.78	130.52	0.58
17	74	F	265.34	236.47	0.89		17	270.04	203.44	0.75
18	82	F	246.48	142.34	0.58		18	269.43	141.98	0.53
19	68	M	199.85	128.30	0.64		19	195.95	113.01	0.58
20	84	F	199.03	114.67	0.58		20	203.77	106.63	0.52
Mean	**80**		**215.17**	**139.94**	**0.66**		**Mean**	**212.27**	**134.32**	**0.65**
SD	**8**		**34.31**	**28.43**	**0.14**		**SD**	**34.86**	**29.91**	**0.18**

Retinal vascular calibre changes in asymmetric AMD

Group 2: ARM-Disciform group

				ARM				DISCIFORM SCAR		
	age	**sex**	**CRVE**	**CRAE**	**AVR**			**CRVE**	**CRAE**	**AVR**
1	78	F	246.31	186.68	0.76		1	242.35	180.25	0.74
2	80	F	223.16	135.99	0.61		2	225.32	133.24	0.59
3	75	F	248.98	157.04	0.63		3	250.23	155.36	0.62
4	84	M	223.35	146.32	0.66		4	221.64	142.35	0.64
5	71	M	198.32	116.13	0.59		5	201.37	120.56	0.60
6	85	M	210.37	118.32	0.56		6	215.65	115.64	0.54
7	79	F	208.39	118.32	0.57		7	208.39	117.45	0.56
8	82	F	204.55	131.04	0.64		8	200.53	134.65	0.67
9	89	M	202.12	184.65	0.91		9	205.32	189.32	0.92
10	79	F	215.66	118.96	0.55		10	215.66	123.24	0.57
11	66	M	232.48	186.76	0.80		11	230.25	176.86	0.77
12	78	F	231.76	136.98	0.59		12	235.64	132.65	0.56
13	84	F	206.80	119.98	0.58		13	210.32	117.29	0.56
14	72	F	252.24	190.35	0.75		14	255.13	198.65	0.78
15	81	F	228.63	183.99	0.80		15	225.78	180.38	0.80
mean	**79**		**222.21**	**148.77**	**0.67**		**mean**	**222.91**	**147.86**	**0.66**
SD	**6**		**17.60**	**29.84**	**0.11**		**SD**	**17.20**	**29.52**	**0.11**

REFERENCES

1. Abalain JH, Carre JL, Leglise D, Robinet A, Legall F, Meskar A, Floch HH, Colin J. Is age-related macular degeneration associated with serum lipoprotein and lipoparticle levels? Clin Chim Acta. 2002 Dec; 326 (1-2):97-104.
2. Age-Related Eye Disease Study Research Group (AREDS), Risk factors associated with age-related macular degeneration: A case-control study in the Age-Related Eye Disease study: Age-Related Eye Disease study report number 3. Ophthalmology 2000; 107: 2224–2232
3. Ajani UA, Christen WG, Manson JE, Glynn RJ, Schaumberg D, Buring JE, Hennekens CH.A prospective study of alcohol consumption and the risk of age-related macular degeneration. Ann Epidemiol. 1999; 9(3):172-7.
4. Ambati J, Ambati BK, Yoo SH, Ianchulev S, Adamis AP**.** Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies. Surv Ophthalmol. 2003a; 48(3):257-93.
5. Ambati J, Anand A, Fernandez S, Sakurai E, Lynn BC, Kuziel WA, Rollins BJ, Ambati BK. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. Nat Med. 2003b; 9(11):1390-7.
6. Anderson CR, Ponce AM, Price RJ. Immunohistochemical identification of an extracellular matrix scaffolds that microguides capillary sprouting in vivo. J Histochem Cytochem. 2004; 52(8):1063-72.
7. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol. 2002 Sep; 134(3):411-31.
8. Auge N, Maupas-Schwalm F, Elbaz M, Thiers JC, Waysbort A, Itohara S, Krell HW, Salvayre R, Negre-Salvayre A. Role for matrix metalloproteinase-2 in oxidized low-density lipoprotein-induced activation of the sphingomyelin/ceramide pathway and smooth muscle cell proliferation. Circulation 2004; 110(5):571-8.
9. Baird PN, Guida E, Chu DT, Vu HT, Guymer RH. The epsilon2 and epsilon4 alleles of the apolipoprotein gene are associated with age-related macular degeneration. Invest Ophthalmol Vis Sci. 2004 May; 45(5):1311-5.
10. Baker AH. Zaltsman AB. George SJ. Newby AC. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis. J Clin Invest. 1998; 101(6):1478-87
11. Barnes MJ. Farndale RW. Collagens and atherosclerosis. Experimental Gerontology. 1999; 34(4): 513-25
12. Baydanoff S, Nicoloff G, Alexiev C Age-related changes in the level of circulating elastin-derived peptides in serum from normal and atherosclerotic subjects. Atherosclerosis 1987; 66(1-2):163-8
13. Beatty S, Koh H, Phil M, Henson D, Boulton M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv Ophthalmol. 2000 Sep-Oct;45(2):115-34
14. Beaudeux JL, Giral P, Bruckert E, Foglietti MJ, Chapman MJ. Matrix metalloproteinases, inflammation and atherosclerosis: therapeutic perspectives. Clin Chem Lab Med. 2004; 42(2):121-31
15. Behrendt C. Visual acuity and its decrease in classic neovascular age-related macular degeneration. Ophthalmic Epidemiol. 2004; 11(5):359-67.
16. Belda Sanchis JI, Quijada Gonzalez A, Munoz Ruiz G, Rodriguez-Galietero A, Romero Gomez FJ, Diaz-Llopis M. Are blood lipids a risk factor for age-related macular degeneration? Arch Soc Esp Oftalmol. 2001; 76(1):13-7.

17. Berliner JA. Navab M. Fogelman AM Frank JS. Demer LL. Edwards PA. Watson AD. Lusis AJ. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation. 1995; 91(9): 2488-96

18. Beuche W, Yushchenko M, Mader M, Maliszewska M, Felgenhauer K, Weber F. Matrix metalloproteinase-9 is elevated in serum of patients with amyotrophic lateral sclerosis. Neuroreport. 2000 Nov 9; 11(16):3419-22.

19. Bian ZM, Elner SG, Yoshida A, Elner VM. Differential involvement of phosphoinositide 3-kinase/Akt in human RPE MCP-1 and IL-8 expression. Invest Ophthalmol Vis Sci. 2004 Jun;45(6):1887-96.

20. Bird AC, Bressler NM, Bressler SB, Chisholm IH, Coscas G, Davis MD, de Jong PT, Klaver CC, Klein BE, Klein R, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group.Surv Ophthalmol. 1995 Mar-Apr;39(5):367-74

21. Bird AC. Bruch's membrane change with age. Br J Ophthalmol 1992; 76(3):166-8

22. Bird AC. The Bowman lecture. Towards an understanding of age-related macular disease. Eye. 2003 May;17(4):457-66.

23. Bizbiz L., Alperovitch A., Robert L Aging of the vascular wall: serum concentration of elastin peptides and elastase inhibitors in relation to cardiovascular risk factors. The EVA study.Atherosclerosis 1997; 131(1):73-8

24. Blankenberg S, Rupprecht HJ, Poirier O, Bickel C, Smieja M, Hafner G, Meyer J, Cambien F, Tiret L; AtheroGene Investigators. Plasma concentrations and genetic varioation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. Circulation. 2003; 107: 1579–1585

25. Blumenkranz MS, Russell SR, Robey MG, Kott-Blumenkranz R, Penneys N. Risk factors in age-related maculopathy complicated by choroidal neovascularization. Ophthalmology 1986; 93(5):552-8.

26. Bocan TM. Pleiotropic effects of HMG-CoA reductase inhibitors.Curr Opin Investig Drugs. 2002 Sep;3(9):1312-7.

27. Bora PS, Sohn JH, Cruz JM, Jha P, Nishihori H, Wang Y, Kaliappan S, Kaplan HJ, Bora NS. Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. J Immunol. 2005; 174(1):491-7.

28. Borger PH, van Leeuwen R, Hulsman CA, Wolfs RC, van der Kuip DA, Hofman A, de Jong PT. Is there a direct association between age-related eye diseases and mortality? The Rotterdam Study. Ophthalmology. 2003; 110(7):1292-6.

29. Bouissou H, Pieraggi MT, Julian, M. Aging of elastic tissue in skin and arteries In: Robert, L., Murata, K., Nagai, Y. (Eds.), Degenerative Diseases of Connective Tissue and Aging. Kodansha, Tokyo, 1985; 203–216.

30. Brown LC, Powell JT. Risk factors for aneurysm rupture in patients kept under ultrasound surveillance. UK Small Aneurysm Trial Participants.Ann Surg. 1999;230(3):289-96

31. Burns RP. Feeney-Burns L. Clinico-morphologic correlations of drusen of Bruch's membrane. Transactions of the American Ophthalmological Society. 1980; 78:206-25.

32. Caicedo A, Espinosa-Heidmann DG, Pina Y, Hernandez EP, Cousins SW. Blood-derived macrophages infiltrate the retina and activate Muller glial cells under experimental choroidal neovascularization. Exp Eye Res. 2005 Jul;81(1):38-47.

33. Carmo M, Colombo L, Bruno A, Corsi FR, Roncoroni L, Cuttin MS, Radice F, Mussini E, Settembrini PG. Alteration of elastin, collagen and their cross-links in abdominal aortic aneurysms. Eur J Vasc Endovasc Surg. 2002; 23(6):543-9.

34. Carrell TW. Burnand KG. Wells GM. Clements JM. Smith A. Stromelysin-1 (matrix metalloproteinase-3) and tissue inhibitor of metalloproteinase-3 are overexpressed in the wall of abdominal aortic aneurysms. Circulation. 2002; 105(4):477-82

35. Carreras I, Rich CB, Panchenko MP, Foster JA. Basic fibroblast growth factor decreases elastin gene transcription in aortic smooth muscle cells. J Cell Biochem. 2002; 85(3):592-600

36. Challa JK, Gillies MC, Penfold PL, Gyory JF, Hunyor AB, Billson FA Exudative macular degeneration and intravitreal triamcinolone: 18 month follow up.
Aust N Z J Ophthalmol. 1998 Nov;26(4):277-81.

37. Chen J, Fitzke F, Pauleikhoff D, Bird A. Functional loss in age-related Bruch's membrane change with choroidal perfusion defect. Invest Ophthalmol Vis Sci 1992; 33:334-40.

38. Chen SJ, Cheng CY, Lee AF, Lee FL, Chou JC, Hsu WM, Liu JH. Pulsatile ocular blood flow in asymmetric exudative age related macular degeneration.Br J Ophthalmol. 2001 Dec;85(12):1411-5.

39. Chen SJ, Cheng CY, Lee AF, Lee FL, Hsu WM, Liu JH. Pulsatile ocular blood flow of choroidal neovascularization in asymmetric age-related macular degeneration after transpupillary thermotherapy. Eye. 2004; 18(6):595-9.

40. Cho E, Hankinson SE, Willett WC, Stampfer MJ, Spiegelman D, Speizer FE, Rimm EB, Seddon JM. Prospective study of alcohol consumption and the risk of age-related macular degeneration.Arch Ophthalmol. 2000; 118(5):681-8.

41. Chong NH, Alexander RA, Gin T, Bird AC, Luthert PJ. TIMP-3, collagen, and elastin immunohistochemistry and histopathology of Sorsby's fundus dystrophy Invest Ophthalmol Vis Sci. 2000; 41(3):898-902.

42. Chong NH, Frennesson CI, Keonin J, Luthert PJ, Weingeist DM, Mullins RF, Hageman GS. Decreased thickness and integrity of the macular elastic layer of Bruch's membrane correspond to the distribution of lesions associated with age-related macular degeneration. Am J Path 2005; 166(1):241-51

43. Christen WG, Glynn RJ, Ajani UA, Schaumberg DA, Chew EY, Buring JE, Manson JE, Hennekens CH. Age-related maculopathy in a randomized trial of low-dose aspirin among US physicians. Arch Ophthalmol. 2001; 119(8):1143-9.

44. Chung TW, Kim JR, Suh JI, Lee YC, Chang YC, Chung TH, Kim CH. Correlation between plasma levels of matrix metalloproteinase (MMP)-9 /MMP-2 ratio and alpha-fetoproteins in chronic hepatitis carrying hepatitis B virus. J Gastroenterol Hepatol. 2004; 19(5):565-71.

45. Cianflone, K., Xia, Z. & Chen, L.Y. Critical review of acylation-stimulating protein physiology in humans and rodents. Biochemica Biophysica Acta, 2003; 1609, 127 143

46. Ciulla TA, Harris A, Chung HS, Danis RP, Kagemann L, McNulty L, Pratt LM, Martin BJ.Color Doppler imaging discloses reduced ocular blood flow velocities in nonexudative age-related macular degeneration. Am J Ophthalmol 1999; 128(1):75-80.

47. Ciulla TA, Harris A, Kagemann L, Danis RP, Maturi R, McNulty L, Pratt LM, Xiao M, Criswell MH, Weinberger D.Ciulla TA, Harris A, Kagemann L, et al. Transpupillary thermotherapy for subfoveal occult choroidal neovascularization: effect on ocular perfusion. Invest Ophthalmol Vis Sci 2001; 42(13):3337-40.

48. Ciulla TA, Harris A, Kagemann L, Danis RP, Pratt LM, Chung HS, Weinberger D, Garzozi HJ. Choroidal perfusion perturbations in non-neovascular age related macular degeneration. Br J Ophthalmol 2002;86(2): 209-13

49. Colburn KK, Langga-Shariffi E, Kelly GT, Malto MC, Sandberg LB, Baydanoff S, Green LMAbnormalities of serum antielastin antibodies in connective tissue diseases. J Investig Med. 2003; 51(2):104-9.

50. Cotran RS, Kumar V, Collins T. (Eds) In: Robbins Pathologic basis of inflammation, Saunders, Philadelphia 1999 Ed 6.

51. Cousins SW, Espinosa-Heidmann DG, Csaky KG. Monocyte activation in patients with age-related macular degeneration: a biomarker of risk for choroidal neovascularization?Arch Ophthalmol. 2004 Jul;122(7):1013-8.

52. Crabb JW, Miyagi M, Gu X, Shadrach K, West KA, Sakaguchi H, Kamei M, Hasan A, Yan L, Rayborn ME, Salomon RG, Hollyfield JG. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. Proc Natl Acad Sci U S A. 2002; 99(23):14682-7.

53. Cruickshanks KJ, Hamman RF, Klein R, Nondahl DM, Shetterly SM. The prevalence of age-related maculopathy by geographic region and ethnicity. The Colorado-Wisconsin Study of Age-Related Maculopathy. Arch Ophthalmol. 1997; 115(2):242-50.

54. Curcio CA. Millican CL. Bailey T. Kruth HS. Accumulation of cholesterol with age in human Bruch's membrane. Invest Ophthalmol Vis Sci. 2001: 42(1): 265-74.

55. Dahlen GH, Slunga L, Lindblom B. Importance of Lp(a) lipoprotein and HLA genotypes in atherosclerosis and diabetes. Clin Genet. 1994; 46(1 Spec No):46-51.

56. Danis RP, Ciulla TA, Pratt LM, Anliker W. Intravitreal triamcinolone acetonide in exudative age-related macular degeneration.Retina. 2000;20(3):244-50.

57. Davignon J, Cohn JS, Mabile L, Bernier L. Apolipoprotein E and atherosclerosis: insight from animal and human studies. Clin Chim Acta. 1999; 286(1-2):115-43.

58. Deblack SS. Cigarette smoking as a risk factor for cataract and age-related macular degeneration: a review of the literature. Optometry. 2003; 74(2):99-110.

59. Delaney WV Jr, Oates RP. Senile macular degeneration: a preliminary study.Ann Ophthalmol. 1982; 14(1):21-4.

60. Delcourt C, Michel F, Colvez A, Lacroux A, Delage M, Vernet MH; POLA Study Group. Associations of cardiovascular disease and its risk factors with age-related macular degeneration: the POLA study. Ophthalmic Epidemiol. 2001; 8(4):237-49.

61. Dighiero G, Lymberi P, Guilbert B, Ternynck T, Avrameas S. Natural autoantibodies constitute a substantial part of normal circulating immunoglobulins. Ann N Y Acad Sci. 1986; 475:135-45.

62. Duke-Elder S**.** System of ophthalmology. St Louis: CV Mosby, 1966, 9; 609–13.

63. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. Science. 2005; 308(5720):421-4.

64. El Baba F. Green WR. Fleischmann J. Finkelstein D. de la Cruz ZC. Clinicopathologic correlation of lipidization and detachment of the retinal pigment epithelium Am J of Ophthalmol. 1986; 101(5):576-83

65. Evans JR Risk factors for age-related macular degeneration. Prog Retin Eye Res. 2001; 20(2):227-53.

66. Evans JR. Ginkgo biloba extract for age-related macular degeneration. Cochrane Database Syst Rev. 2000; (2):CD001775.

67. Evereklioglu C, Doganay S, Er H, Cekmen M, Ozerol E, Otlu B. Serum leptin concentrations are decreased and correlated with disease severity in age-related macular degeneration: a preliminary study.Eye. 2003 Apr;17(3):350-5.

68. Farias E, Ranuncolo S, Cresta C, Specterman S, Armanasco E, Varela M, Lastiri J, Pallotta MG, Bal de Kier Joffe E, Puricelli L. Plasma metalloproteinase activity is enhanced in the euglobulin fraction of breast and lung cancer patients. Int J Cancer. 2000; 89(4):389-94.

69. Farkas TG. Sylvester V. Archer D. Altona M. The histochemistry of drusen. Am J Ophthalmol.1971; 71(6):1206-15

70. Fassiadis N, Roidl M, Stannett H, Andrews SM, South LM. Is screening of abdominal aortic aneurysm effective in a general practice setting? Int Angiol. 2005; 24(2):185-8.

71. Figueroa M, Schocket LS, DuPont J, Metelitsina TI, Grunwald JE**.** Effect of laser treatment for dry age related macular degeneration on foveolar choroidal haemodynamics. Br J Ophthalmol. 2004; 88(6):792-5.

72. Flower RW, von Kerczek C, Zhu L, Ernest A, Eggleton C, Topoleski LD.Theoretical investigation of the role of choriocapillaris blood flow in treatment of subfoveal choroidal neovascularization associated with age-related macular degeneration.Am J Ophthalmol. 2001; 132(1):85-93.

73. Forrester JV, Lumsden L, Duncan L, Dick AD. Choroidal dendritic cells require activation to present antigen and resident choroidal macrophages potentiate this response. Br J Ophthalmol. 2005 Mar;89(3):369-77

74. Friedman E, Ivry M, Ebert E, Glynn R, Gragoudas E, Seddon J. Increased scleral rigidity and age-related macular degeneration.Ophthalmology. 1989 Jan;96(1):104-8.

75. Friedman E, Krupsky S, et al. Ocular blood flow velocity in age-related macular degeneration. Ophthalmology 1995; 102:640-6

76. Friedman E, Smith T, Kuwahara T. Senile choroidal vascular patterns and drusen Arch Ophthalmol 1963;69: 220-230

77. Friedman E, The role of the atherosclerotic process in the pathogenesis of age-related macular degeneration. Am J Ophthalmol 2000; 130: 658–663

78. Friedman E. A hemodynamic model of the pathogenesis of age-related macular degeneration Am J Ophthalmol 1997; 124: 677-682

79. Fulop T Jr, Jacob MP, Khalil A, Wallach J, Robert L. Biological effects of elastin peptides. Pathol Biol (Paris). 1998; 46(7):497-506.

80. George SJ Tissue inhibitors of metalloproteinases and metalloproteinases in atherosclerosis Curr Opin Lipidol. 1998; 9(5):413-23.

81. Ghomrasseni S, Dridi M, Gogly B, Bonnefoix M, Vabres P, Venencie PY, Pellat B, Godeau G. Anetoderma: an altered balance between metalloproteinases and tissue inhibitors of metalloproteinases. Am J Dermatopathol. 2002; 24(2):118-29.

82. Girolamo F, Virgintino D, Errede M, Capobianco C, Bernardini N, Bertossi M, Roncali L. Involvement of metalloprotease-2 in the development of human brain microvessels. Histochem Cell Biol. 2004; 122(3):261-70.

83. Goldberg J, Flowerdew G, Smith E, Brody JA, Tso MO**.** Factors associated with age-related macular degeneration. An analysis of data from the first National Health and Nutrition Examination Survey (NHNES). Am J Epidemiol. 1988; 128(4):700-10.

84. Goncalves I. Moses J. Dias N. Pedro LM. Fernandes e Fernandes J. Nilsson J. Ares MP. Changes related to age and cerebrovascular symptoms in the extracellular matrix of human carotid plaques Stroke. 2003; 34(3):616-22.

85. Goverdhan S, W.M. Howell, H. Bacon, I.H. Chisholm, K. Avery, and A.J. Lotery Association of HLA polymorphisms in Age–related macular degeneration Invest. Ophthalmol. Vis. Sci. 2004 45: E-Abstract 1822.

86. Gragoudas ES, Adamis AP, Cunningham ET Jr, Feinsod M, Guyer DR; VEGF Inhibition Study in Ocular Neovascularization Clinical Trial Group. Pegaptanib for neovascular age-related macular degeneration. N Engl J Med. 2004; 351(27):2805-16.

87. Grossniklaus HE Choroidal neovascularization. Am J Ophthalmol. 2004; 137(3):496-503.

88. Grunwald JE, Hariprasad SM, DuPont J, Maguire MG, Fine SL, Brucker AJ,Maguire AM, Ho AC. Foveolar choroidal blood flow in age-related macular degeneration.Invest Ophthalmol Vis Sci. 1998a;39(2):385-90.

89. Grunwald JE, Hariprasad SM, DuPont J. Effect of aging on foveolar choroidal circulation. Arch Ophthalmol. 1998b; 116(2):150-4.

90. Grunwald JE, Metelitsina TI, Dupont JC, Ying GS, Maguire MG Reduced foveolar choroidal blood flow in eyes with increasing AMD severity. Invest Ophthalmol Vis Sci. 2005; 46(3):1033-8.

91. Grunwald JE, Piltz J, Patel N, Bose S, Riva CE. Effect of aging on retinal macular microcirculation: a blue field simulation study. Invest Ophthalmol Vis Sci. 1993 Dec; 34(13):3609-13.

92. Guan K, Hudson C, Flanagan JG. Variability and repeatability of retinal blood flow measurements using the Canon Laser Blood Flowmeter. Microvasc Res. 2003; 65(3):145-51.

93. Guo L, Hussain AA, Limb GA, Marshall J: Age-dependent variation in metalloproteinase activity of isolated human Bruch's membrane and choroid. Invest Ophthalmol Vis Sci 1999; 40:2676-2682.

94. Guymer R, Luthert P, Bird A. Changes in Bruch's membrane and related structures with age. Prog Retin Eye Res. 1999; 18(1):59-90.

95. Guymer RH, Chiu AW, Lim L, Baird PN. HMG CoA reductase inhibitors (statins): do they have a role in age-related macular degeneration? Surv Ophthalmol. 2005; 50(2):194-206.

96. Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, Hageman JL, Stockman HA, Borchardt JD, Gehrs KM, Smith RJ, Silvestri G, Russell SR, Klaver CC, Barbazetto I, Chang S, Yannuzzi LA, Barile GR, Merriam JC, Smith RT, Olsh AK, Bergeron J, Zernant J, Merriam JE, Gold B, Dean M, Allikmets R. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci 2005; 102 (20): 7227-7232

97. Hageman GS. Luthert PJ. Victor Chong NH. Johnson LV. Anderson DH. Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Ret Eye Res. 2001; 20(6): 705-32

98. Haimovici R. Gantz DL. Rumelt S. Freddo TF. Small DM. The lipid composition of drusen, Bruch's membrane, and sclera by hot stage polarizing light microscopy. Invest Ophthalmol Vis Sci. 2001; 42(7):1592-9

99. Haines JL, Hauser MA, Schmidt S, Scott WK, Olson LM, Gallins P, Spencer KL, Kwan SY, Noureddine M, Gilbert JR, Schnetz-Boutaud N, Agarwal A, Postel EA, Pericak-Vance MA. Complement factor H variant increases the risk of age-related macular degeneration. Science. 2005; 308(5720):419-21.

100. Hansson GK. Libby P. Schonbeck U. Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circulation Research. 2002; 91(4):281-91

101. Harris A, Ciulla TA, Pratt LM, Rechtman E, Kagemann L, Piper HC, Garzozi HJ. The effects of dorzolamide on choroidal and retinal perfusion in non-exudative age related macular degeneration**.** Br J Ophthalmol. 2003; 87(6):753-7.

102. Henkind P, Gartner S. The relationship between retinal pigment epithelium and the choriocapillaris.Trans Ophthalmol Soc U K. 1983; 103 (Pt 4):444-7.

103. Heuberger RA, Mares-Perlman JA, Klein R, Klein BE, Millen AE, Palta M. Relationship of dietary fat to age-related maculopathy in the Third National Health and Nutrition Examination Survey. Arch Ophthalmol. 2001; 119(12):1833-8.

104. Hewitt AT. Nakazawa K. Newsome DA. Analysis of newly synthesized Bruch's membrane proteoglycans. Invest Ophthalmol Vis Sci. 1089;30(3):478-86

105. Hirvela H, H. Luukinen H, E. Laara E, Laatikainen L, Risk factors of age-related maculopathy in a population 70 years of age or older. Ophthalmology 1996; 103**:** 871–877.

106. Hogan MJ. Alvarado J. Studies on the human macula. IV. Aging changes in Bruch's membrane. Arch of Ophthalmol. 1967; 77(3): 410-

107. Holz FG, Wolfensberger TJ, Piguet B, Gross-Jendroska M, Wells JA, Minassian DC, Chisholm IH, Bird AC.Bilateral macular drusen in age related macular degeneration: prognosis and risk factors. Ophthalmology 1994; 101:1522–8

108. Holz FG. Pauleikhoff D, Spaide RF, Bird AC Age related macular degeneration. Springer-Verlag 2003 pp 3-8

109. Holz FG. Sheraidah G. Pauleikhoff D. Bird AC. Analysis of lipid deposits extracted from human macular and peripheral Bruch's membrane. Archives of Ophthalmology. 1994;112(3):402-6

110. Hornebeck W, Robert L, Interactions between elastic fibers and cells. In: Labat-RobertJ., Timpl, R., Robert, L. (Eds.), Structural Glycoproteins in Cell–Matrix Interactions. Karger, Basel, 1986; 58–77.

111. Hosal BM, Karakoc G, Gursel E, Camur M**.** Color Doppler imaging of the retrobulbar circulation in age-related macular degeneration.Eur J Ophthalmol. 1998;8(4):234-8.

112. Hubbard LD, Brothers RJ, King WN, Clegg LX, Klein R, Cooper LS, Sharrett AR, Davis MD, Cai J. Methods for evaluation of retinal microvascular abnormalities associated with hypertension/sclerosis in the Atherosclerosis Risk in Communities Study Ophthalmology. 1999 Dec;106(12):2269-80.

113. Hunninghake GW, Davidson JM, Rennard S, Szapiel S, Gadek JE, Crystal RG. Elastin fragments attract macrophage precursors to diseased sites in pulmonary emphysema. Science. 1981; 212 (4497):925-7.

114. Hyman L, Schachat AP, He Q, Leske MC: Hypertension, cardiovascular disease, and age-related macular degeneration. Age-Related Macular Degeneration Risk Factors Study Group Arch Ophthalmol 2000;118:351-358

115. Hyman LG, Lilienfeld AM, Ferris FL 3rd, Fine SL. Senile macular degeneration: a case-control study. Am J Epidemiol. 1983; 118(2):213-27.

116. Hynes RO. Integrins: a family of cell surface receptors. Cell 1987:48; 549–554.

117. Ikeda T, Obayashi H, Hasegawa G, Nakamura N, Yoshikawa T, Imamura Y, Koizumi K, Kinoshita S. Paraoxonase gene polymorphisms and plasma oxidized low-density lipoprotein level as possible risk factors for exudative age-related macular degeneration. Am J Ophthalmol. 2001; 132(2):191-5.

118. Ikram MK, de Jong FJ, Vingerling JR, Witteman JC, Hofman A, Breteler MM, de Jong PT. Are retinal arteriolar or venular diameters associated with markers for cardiovascular disorders? The Rotterdam Study. Invest Ophthalmol Vis Sci. 2004; 45(7):2129-34.

119. Ikram MK, van Leeuwen R, Vingerling JR, Hofman A, de Jong PT Retinal vessel diameters and the risk of incident age-related macular disease: the Rotterdam Study. Ophthalmology. 2005 Apr; 112(4):548-52.

120. Ishibashi T. Sorgente N. Patterson R. Ryan SJ. Aging changes in Bruch's membrane of monkeys: an electron microscopic study. Ophthalmologica. 1986; 192(3):179-90

121. Jarva H, Jokiranta TS, Hellwage J, Zipfel PF, Meri S Regulation of complement activation by C-reactive protein: targeting the complement inhibitory activity of factor H by an interaction with short consensus repeat domains 7 and 8-11.J Immunol. 1999 Oct 1; 163(7):3957-62.

122. Johnson LV, Anderson DH. Age-related macular degeneration and the extracellular matrix N Engl J Med. 2004 22; 351(4):320-2.

123. Johnson LV, Leitner WP, Staples MK, Anderson DH. Complement activation and inflammatory processes in drusen formation and age related macular degeneration. Exp Eye Res. 2001;73(6):887-96.

124. Johnson LV, Ozaki S, Staples MK, Erickson PA, Anderson DH. A potential role for immune complex pathogenesis in drusen formation.Exp Eye Res. 2000; 70(4):441-9.

125. Jonescu-Cuypers CP, Harris A, Wilson R, Kagemann L, Mavroudis LV, Topouzis F, Coleman AL. Reproducibility of the Heidelberg retinal flowmeter in determining low perfusion areas in peripapillary retina.Br J Ophthalmol. 2004; 88(10):1266-9.

126. Kadoglou NP, Liapis CD. Matrix metalloproteinases: contribution to pathogenesis, diagnosis, surveillance and treatment of abdominal aortic aneurysms. Curr Med Res Opin. 2004; 20(4):419-32.

127. Kahn HA, Leibowitz HM, Ganley JP, Kini MM, Colton T, Nickerson RS, Dawber TR The Framingham Eye Study II: Association of ophthalmic pathology with single variables previously measured in the Framingham Heart study. Am J Epidemiol 1977; 106**:** 33–41

128. Kai H, Ikeda H, Yasukawa H, Kai M, Seki Y, Kuwahara F, Ueno T, Sugi K, Imaizumi T. Peripheral blood levels of matrix metalloproteases-2 and -9 are elevated in patients with acute coronary syndromes. J Am Coll Cardiol. 1998; 32(2):368-72.

129. Kalayoglu MV, Galvan C, Mahdi OS, Byrne GI, Mansour S. Serological association between Chlamydia pneumoniae infection and age-related macular degeneration. Arch Ophthalmol. 2003; 121(4):478-82.

130. Kamei M, Hollyfield JG: TIMP-3 in Bruch's membrane: changes during aging and in age-related macular degeneration. Invest Ophthalmol Vis Sci 1999; 40:2367-2375

131. Karwatowski WS. Jeffries TE. Duance VC. Albon J. Bailey AJ. Easty DL. Preparation of Bruch's membrane and analysis of the age-related changes in the structural collagens. Br J Ophthalmol. 1995; 79(10): 944-52

132. Killingsworth MC, Sarks JP, Sarks SH. Macrophages related to Bruch's membrane in age-related macular degeneration. Eye. 1990;4 (Pt 4):613-21.

133. Killingsworth MC. Age-related components of Bruch's membrane in the human eye. Graefes Arch Clin Exp Ophthalmol. 1987; 225(6): 406-12

134. Klaver CC, Assink JJ, van Leeuwen R, Wolfs RC, Vingerling JR, Stijnen T, Hofman A, de Jong PT. Incidence and progression rates of age-related maculopathy: the Rotterdam Study. Invest Ophthalmol Vis Sci. 2001; 42(10):2237-41.

135. Klaver CC. Kliffen M. van Duijn CM. Hofman A. Cruts M. Grobbee DE. van Broeckhoven C. de Jong PT. Genetic association of apolipoprotein E with age-related macular degeneration Am J Hum Genet 1998;63(4):1252

136. Klein BE, Klein R, Jensen SC, Ritter LL. Are sex hormones associated with age-related maculopathy in women? The Beaver Dam Eye Study. Trans Am Ophthalmol Soc. 1994;92:289-95

137. Klein BE, Klein R, Lee KE, Jensen SC. Measures of obesity and age-related eye diseases. Ophthalmic Epidemiol. 2001; 8(4):251-62.

138. Klein BE, Klein R, Lee KE. Reproductive exposures, incident age-related cataracts, and age-related maculopathy in women: the beaver dam eye study.Am J Ophthalmol. 2000; 130(3):322-6.

139. Klein R, Clegg L, Cooper LS, Hubbard LD, Klein BE, King WN, Folsom AR Prevalence of age-related maculopathy in the Atherosclerosis Risk in Communities study. Arch Ophthalmol 1999; 117:1203–1210.

140. Klein R, Klein BE, Jensen SC. The relation of cardiovascular disease and its risk factors to the 5-year incidence of age-related maculopathy: the Beaver Dam Eye Study. Ophthalmology. 1997a; 104(11):1804-12.

141. Klein R, Klein BE, Knudtson MD, Wong TY, Shankar A, Tsai MY. Systemic markers of inflammation, endothelial dysfunction, and age-related maculopathy.Am J Ophthalmol. 2005 Jul; 140(1):35-44.

142. Klein R, Klein BE, Linton KL: Prevalence of age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology 1992a; 99:933-943.

143. Klein R, Klein BE, Marino EK, Kuller LH, Furberg C, Burke GL, Hubbard LD Early age-related maculopathy in the Cardiovascular Health study. Ophthalmology 2003b; 110: 25–33.

144. Klein R, Klein BE, Moss SE. Diabetes, hyperglycemia, and age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology. 1992b; 99(10):1527-34.

145. Klein R, Klein BE, Tomany SC, Cruickshanks KJ. Association of emphysema, gout, and inflammatory markers with long-term incidence of age-related Maculopathy Arch Ophthalmol. 2003a; 121(5):674-8.

146. Klein R, Klein BE, Tomany SC, Moss SE. Ten-year incidence of age-related maculopathy and smoking and drinking: the Beaver Dam Eye Study.Am J Epidemiol. 2002a; 156(7):589-98.

147. Klein R, Klein BE, Tomany SC, Wong TY.The relation of retinal microvascular characteristics to age-related eye disease: the Beaver Dam eye study. Am J Ophthalmol. 2004; 137(3):435-44.

148. Klein R. Klein BE. Jensen SC. Meuer SM. The five-year incidence and progression of age-related maculopathy: the Beaver Dam Eye Study Ophthalmology. 1997b; 104(1): 7-21

149. Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, Sangiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J. Complement factor H polymorphism in age-related macular degeneration. Science. 2005; 308(5720):385-9.

150. Klein, B.E. Klein, S.C. Tomany and K.J. Cruickshanks, The association of cardiovascular disease with the long-term incidence of age-related maculopathy: The Beaver Dam Eye study. Ophthalmology 2003c; 110: 1273–1280.

151. Klein, R, Klein BE and Franke T, The relationship of cardiovascular disease and its risk factors to age-related maculopathy: The Beaver Dam Eye study. Ophthalmology 1993; 100**:** 406–414.

152. Kleiner DE, Stetler-Stevenson WG. Quantitative zymography: detection of picogram quantities of gelatinases.Anal Biochem. 1994 May 1;218(2):325-9.

153. Kliffen M, Mooy CM, Luider TM, Huijmans JG, Kerkvliet S, de Jong PT. Identification of glycosaminoglycans in age-related macular deposits.Arch Ophthalmol. 1996;114(8):1009-14

154. Klingel R, Fassbender C, Fassbender T, Gohlen B. Clinical studies to implement Rheopheresis for age-related macular degeneration guided by evidence-based-medicine.Transfus Apheresis Sci. 2003; 29(1):71-84.

155. Klurfeld DM. Identification of foam cells in human atherosclerotic lesions as macrophages using monoclonal antibodies Archives of Pathology & Laboratory Medicine. 1985; 109(5):445-9.

156. Knupp C, Chong NH, Munro PM, Luthert PJ, Squire JM: Analysis of the Collagen VI Assemblies Associated with Sorsby's Fundus Dystrophy. J Struct Biol 2002; 137:31-40.

157. Kohner EM, Patel V, Rassam SM. Role of blood flow and impaired autoregulation in the pathogenesis of diabetic retinopathy. Diabetes. 1995; 44(6):603-7.

158. Korenzweig AB. Changes in the choriocapillaries associated with senile macular degeneration. Ann Ophthalmol 1977; 9:753–64

159. Krabbe KS, Pedersen M, Bruunsgaard H. Inflammatory mediators in the elderly. Exp Gerontol. 2004 May;39(5):687-99.

160. Krey H. Morphology of the peripheral choriocapillaris in patients of various ages Klinische Monatsblatter fur Augenheilkunde. 1975; 167(2):295-301

161. Kruger A, Matulla B, Wolzt M, Pieh S, Strenn K, Findl O, Eichler HG, Schmetterer L. Short-term oral pentoxifylline use increases choroidal blood flow in patients with age-related macular degeneration. Arch Ophthalmol. 1998; 116(1):27-30.

162. Kuang PP, Goldstein RH. Regulation of elastin gene transcription by interleukin-1 beta-induced C/EBP beta isoforms Am J Physiol Cell Physiol. 2003; 285(6):C1349-55

163. Kucich U, Christner P, Lippmann M, Fein A, Goldberg A, Kimbel P, Weinbaum G, Rosenbloom J. Immunologic measurement of elastin-derived peptides in human serum.Am Rev Respir Dis. 1983;127(2):S28-30.

164. Labat-Robert J. Cell-matrix interactions in aging: role of receptors and matricryptins.Ageing Res Rev. 2004; 3(2):233-47.

165. Lakatta EG. Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a "set up" for vascular disease. Circulation. 2003; 107(1): 139-46

166. Lambert V, Munaut C, Jost M, Noel A, Werb Z, Foidart JM, Rakic JM. Matrix metalloproteinase-9 contributes to choroidal neovascularization.
Am J Pathol. 2002 Oct; 161(4):1247-53.

167. Lambert V, Wielockx B, Munaut C, Galopin C, Jost M, Itoh T, Werb Z, Baker A, Libert C, Krell HW, Foidart JM, Noel A, Rakic JM. MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. FASEB J. 2003; 17(15):2290-2.

168. Laufer J, Katz Y, Passwell JH. Extrahepatic synthesis of complement proteins in inflammation. Mol Immunol. 2001; 38(2-3):221-9.

169. Lee WR, Blass GE, Shaw DC Age-related retinal vasculopathy Eye 1987; 1: 296 303

170. Lengyel I, Tufail A, Hosaini HA, Luthert P, Bird AC, Jeffery G. Association of drusen deposition with choroidal intercapillary pillars in the aging human eye.Invest Ophthalmol Vis Sci. 2004; 45(9):2886-92.

171. Libby P. Vascular biology of atherosclerosis: overview and state of the art. Am J Cardiol. 2003 6; 91(3A):3A-6A.

172. Libby P. Inflammation in atherosclerosis. Nature. 2002; 420(6917):868-74

173. Lim JI Age related macular degeneration Marvel Dekker 2002 pp39-42

174. Lindholt JS, Heickendorff L, Henneberg EW, Fasting H. Serum-elastin-peptides as a predictor of expansion of small abdominal aortic aneurysms. Eur J Vasc Endovasc Surg. 1997; 14(1):12-6.

175. Loeffler KU, Lee WR. Basal linear deposit in the human macula. Graefes Arch Clin Exp Ophthalmol 1986; 224:493-501.

176. Lorenzl S, Albers DS, Relkin N, Ngyuen T, Hilgenberg SL, Chirichigno J, Cudkowicz ME, Beal MF. Increased plasma levels of matrix metalloproteinase-9 in patients with Alzheimer's disease. Neurochem Int. 2003 Aug; 43(3):191-6.

177. Macular Photocoagulation Study Group. Laser photocoagulation of subfoveal neovascular lesions in age-related macular degeneration Arch Ophthalmol 1991; 109:1220–31

178. Macular Photocoagulation Study Group. Risk factors for choroidal neovascularization in the second eye of patients with juxtafoveal or subfoveal choroidal neovascularization secondary to age-related macular degeneration.Arch Ophthalmol. 1997; 115(6):741-7.

179. Mares-Perlman JA, Brady WE, Klein R, VandenLangenberg GM, Klein BE, Palta M. Dietary fat and age-related maculopathy. Arch Ophthalmol. 1995; 113(6):743-8.

180. McCarty CA, Mukesh BN, Fu CL, Mitchell P, Wang JJ, Taylor HR. Risk factors for age-related maculopathy: the Visual Impairment Project. Arch Ophthalmol. 2001; 119(10):1455-62.

181. McGeer PL, McGeer EG. Inflammation, autotoxicity and Alzheimer disease.
Neurobiol Aging. 2001 Nov-Dec; 22(6):799-809.

182. McGeer PL, Sibley J. Sparing of age-related macular degeneration in rheumatoid arthritis. Neurobiol Aging. 2005 Aug-Sep; 26(8):1199-203.

183. McGwin G Jr, Xie A, Owsley C. The use of cholesterol-lowering medications and age-related macular degeneration. Ophthalmology. 2005; 112(3):488-94.

184. McMillan WD, Pearce WH. Increased plasma levels of metalloproteinase-9 are associated with abdominal aortic aneurysms. J Vasc Surg. 1999 Jan;29(1):122-7

185. Mecham RP, Lange G**.** Antibodies to insoluble and solubilized elastin.Methods Enzymol. 1982; 82 Pt A: 744-59.

186. Meschia JF. Gerber TC. Editorial comment--Vascular thickness and calcification as markers of atherosclerotic burden. Stroke. 2003; 34(10): 2372-3

187. Metchnikoff E Lecons sur la pathologie compare de l inflammation, Masson, Paris 1892.

188. Metelitsina TI, Grunwald JE, DuPont JC, Ying GS**.** Effect of niacin on the choroidal circulation of patients with age related macular degeneration. Br J Ophthalmol. 2004; 88(12):1568-72.

189. Miller DM, Espinosa-Heidmann DG, Legra J, Dubovy SR, Suner IJ, Sedmak DD, Dix RD, Cousins SW. The association of prior cytomegalovirus infection with neovascular age-related macular degeneration. Am J Ophthalmol. 2004; 138(3):323-8.

190. Mitchell P. Smith W. Attebo K. Wang JJ. Prevalence of age-related maculopathy in Australia. The Blue Mountains Eye Study. Ophthalmology. 1995; 102(10): 1450-60.

191. Mold C, Gewurz H, Du Clos TW. Regulation of complement activation by C-reactive protein Immunopharmacology 1999; 42(1-3):23-30.

192. Moore DJ, Hussain AA, Marshall J. Age-related variation in the hydraulic conductivity of Bruch's membrane.Invest Ophthalmol Vis Sci. 1995 Jun;36(7):1290-7

193. Mori F, Konno S, Hikichi T, Yamaguchi Y, Ishiko S, Yoshida A. Factors affecting pulsatile ocular blood flow in normal subjects. Br J Ophthalmol 2001a; 85:529-30.

194. Mori F, Konno S, Hikichi T, Yamaguchi Y, Ishiko S, Yoshida A. Pulsatile ocular blood flow study: decreases in exudative age related macular degeneration. Br J Ophthalmol 2001b; 85:531-3.

195. Morris CJ, Bradby GV, Walton KW. Fibrous long-spacing collagen in human atherosclerosis. Atherosclerosis. 1978; 31(3):345-54.

196. Moses MA. Langer R. A metalloproteinase inhibitor as an inhibitor of neovascularization. J Cel Biochem. 1991; 47(3):230-5

197. Moss SE, Klein R, Klein BE, Jensen SC, Meuer SM Alcohol consumption and the 5-year incidence of age-related maculopathy: the Beaver Dam eye study.Ophthalmology. 1998; 105(5):789-94.

198. Mullins RF, Russell SR, Anderson DH, Hageman GS. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. FASEB J. 2000; 14(7):835-46.

199. Nackman GB, Karkowski FJ, Halpern VJ, Gaetz HP, Tilson MD. Elastin degradation products induce adventitial angiogenesis in the Anidjar/ Dobrin rat aneurysm model. Ann N Y Acad Sci. 1996; 800:260-2.

200. Nagel E, Vilser W, Lindloh C, Klein S. [Measuring retinal vascular diameter using the scanning laser ophthalmoscope and computer. Initial results] Ophthalmologe. 1992; 89(5):432-6.

201. Newsome DA, Hewitt AT, Huh W, Robey PG, Hassell JR. Detection of specific extracellular matrix molecules in drusen, Bruch's membrane, and ciliary body.Am J Ophthalmol. 1987; 104(4):373-81.

202. Newsome DA, Swartz M, Leone NC, Hewitt AT, Wolford F, Miller ED. Macular degeneration and elevated serum ceruloplasmin.Invest Ophthalmol Vis Sci. 1986 Dec;27(12):1675-80.

203. Noji Y, Kajinami K, Kawashiri MA, et al. Circulating matrix metalloproteinases and their inhibitors in premature coronary atherosclerosis. Clin Chem Lab Med. 2001; 39: 380–384

204. Ohbayashi H. Matrix metalloproteinases in lung diseases. Curr Protein Pept Sci. 2002 Aug; 3(4):409-21.

205. Okamoto T, Akaike T, Sawa T, Miyamoto Y, van der Vliet A, Maeda H. Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. J Biol Chem. 2001 Aug 3; 276(31):29596-602.

206. Oksjoki R, Jarva H, Kovanen PT, Laine P, Meri S, Pentikainen MO. Association between complement factor H and proteoglycans in early human coronary atherosclerotic lesions: implications for local regulation of complement activation. Arterioscler Thromb Vasc Biol. 2003 Apr 1;23(4):630-6.

207. Ok Okubo A, Rosa RH Jr, Bunce CV, Alexander RA, Fan JT, Bird AC, Luthert PJ.The relationships of age changes in retinal pigment epithelium and Bruch's membrane.Invest Ophthalmol Vis Sci. 1999 Feb;40(2):443-9.

208. Pache M, Nagel E, Flammer J. Reproducibility of measurements with the retinal vessel analyzer under optimal conditions Klin Monatsbl Augenheilkd. 2002 Jul; 219(7):523-7.

209. Pardo A, Selman M, Proteinase-antiproteinase imbalance in the pathogenesis of emphysema: the role of metalloproteinases in lung damage. Histol. Histopathol. 1999:14; 227–233.

210. Parr JC, Spears GF. General caliber of the retinal arteries expressed as the equivalent width of the central retinal artery.Am J Ophthalmol. 1974;77(4):472-7.

211. Partridge SM, Elastin. Adv. Prot. Chem. 1962**;** 17**:** 227–302

212. Paul N. Baird, Elizabeth Guida, Diep T. Chu, Hien T. V. Vu, and Robyn H.Guymer The E4 Alleles of the Apolipoprotein Gene Are Associated with Age-Related Macular Degeneration. Invest. Ophthalmol. Vis. Sci. 2004 45: 1311-1315.

213. Pauleikhoff D, Chen J, et al. Choroidal perfusion abnormality with age-related Bruch's membrane change Am J Ophthalmol 1990; 109:211-7

214. Pawlak K, Pawlak D, Mysliwiec M. Extrinsic coagulation pathway activation and metalloproteinase-2/TIMPs system are related to oxidative stress and atherosclerosis in hemodialysis patients. Thromb Haemost. 2004; 92(3):646-53.

215. Penfold P, Killingsworth M, Sarks S. An ultrastructural study of the role of leucocytes and fibroblasts in the breakdown of Bruch's membrane.Aust J Ophthalmol. 1984 Feb;12(1):23-31.

216. Penfold PL, Gyory JF, Hunyor AB, Billson FA. Exudative macular degeneration and intravitreal triamcinolone. A pilot study.Aust N Z J Ophthalmol. 1995 Nov;23(4):293-8.

217. Penfold PL, Killingsworth MC, Sarks SH. Senile macular degeneration: the involvement of immunocompetent cells.Graefes Arch Clin Exp Ophthalmol. 1985;223(2):69-76.

218. Penfold PL, Madigan MC, Gillies MC, Provis JM. Immunological and aetiological aspects of macular degeneration. Prog Retin Eye Res. 2001; 20(3):385-414.

219. Petersen E. Wagberg F. Angquist KA. Serum concentrations of elastin-derived peptides in patients with specific manifestations of atherosclerotic disease. European Journal of Vascular & Endovascular Surgery. 2002; 24(5):440-4

220. Pickering MC, Cook HT, Warren J, Bygrave AE, Moss J, Walport MJ, Botto M.Uncontrolled C3 activation causes membranoproliferative glomerulonephritis in mice deficient in complement factor H. Nat Genet. 2002 Aug;31(4):424-8

221. Piguet B, Palmvang I, et al. Evolution of age-related macular degeneration with choroidal perfusion abnormality. Am J Ophthalmol 1992; 113:657-63

222. Polak K, Dorner G, Kiss B, Polska E, Findl O, Rainer G, Eichler HG, Schmetterer L. Evaluation of the Zeiss retinal vessel analyser.Br J Ophthalmol. 2000; 84(11):1285-90.

223. Pose-Reino A, Gomez-Ulla F, Hayik B, Rodriguez-Fernandez M, Carreira-Nouche MJ, Mosquera-Gonzalez A, Gonzalez-Penedo M, Gude F. Computerized measurement of retinal blood vessel calibre: description, validation and use to determine the influence of ageing and hypertension.J Hypertens. 2005;23(4):843-50.

224. Pulido J, Sanders D, Winters JL, Klingel R. Clinical outcomes and mechanism of action for rheopheresis treatment of age-related macular degeneration (AMD).J Clin Apheresis. 2005a May 12; [Epub ahead of print]

225. Pulido JS, Sanders D, Klingel R. Rheopheresis for age-related macular degeneration: clinical results and putative mechanism of action.Can J Ophthalmol. 2005b; 40(3):332-40.

226. Ramrattan RS. van der Schaft TL. Mooy CM. de Bruijn WC. Mulder PG. de Jong PT. Morphometric analysis of Bruch's membrane, the choriocapillaris, and the choroid in aging. Investigative Ophthalmology & Visual Science. 1994;35(6):2857-64

227. Reed D, Reed C, Stemmermann G, Hayashi T. Are aortic aneurysms caused by atherosclerosis? Circulation. 1992; 85(1):205-11.

228. Remsch H, Spraul CW, Lang GK, Lang GE. Changes of retinal capillary blood flow in age-related maculopathy. Graefes Arch Clin Exp Ophthalmol. 2000; 238(12):960-4

229. Ritter LL, Klein R, Klein BE, Mares-Perlman JA, Jensen SC. Alcohol use and age-related maculopathy in the Beaver Dam Eye Study.Am J Ophthalmol. 1995 Aug; 120(2):190-6.

230. Riva CE, Grunwald JE, Sinclair SH, Petrig BL Blood velocity and volumetric flow rate in human retinal vessels. Invest. Ophthalmol. Vis. Sci. 1985: 26, 1124 1132

231. Robert L, Robert AM, Jacotot B. Elastin-elastase-atherosclerosis revisited.Atherosclerosis 1998; 140(2):281-95.

232. Ross R. Atherosclerosis - An inflammatory disease. N Engl J Med 1999; 340: 115-26

233. Ross RD, Barofsky JM, Cohen G, Baber WB, Palao SW, Gitter KA Presumed macular choroidal watershed vascular filling, choroidal neovascularization, and systemic vascular disease in patients with age-related macular degeneration.Am J Ophthalmol. 1998 Jan; 125(1):71-80.

234. Ruberti JW. Curcio CA. Millican CL. Menco BP. Huang JD. Johnson M. Quick-freeze/deep-etch visualization of age-related lipid accumulation in Bruch's membrane. Invest Ophthalmol Vis Sci. 2003;44(4):1753-9

235. Rus HG. Niculescu F. Vlaicu R. The relationship between macrophages and C5b-9 complement complexes in human atherosclerosis. Clin Immunol Immunopathol. 1988; 48(3): 307-16

236. Sakalihasan N, Limet R, Defawe OD. Abdominal aortic aneurysm.Lancet. 2005; 365(9470):1577-89.

237. Sanders TA, Haines AP, Wormald R, Wright LA, Obeid O. Essential fatty acids, plasma cholesterol, and fat-soluble vitamins in subjects with age-related maculopathy and matched control subjects.Am J Clin Nutr. 1993; 57(3):428-33.

238. Sarks SH. Ageing and degeneration in the macular region: a clinico-pathological study. Br J Ophthalmol. 1976; 60(5): 324-41

239. Sato E, Feke GT, Menke MN, Wallace McMeel J**.** Retinal haemodynamics in patients with age-related macular degeneration. Eye. 2005 Jun 3

240. Saulnier JM, Hauck M, Fulop T Jr, Wallach JM. Human aortic elastin from normal individuals and atherosclerotic patients: lipid and cation contents; susceptibility to elastolysis. Clin Chim Acta. 1991; 200(2-3):129-36.

241. Schaumberg DA, Christen WG, Hankinson SE, Glynn RJ. Body mass index and the incidence of visually significant age-related maculopathy in men.Arch Ophthalmol. 2001; 119(9):1259-65.

242. Schlingemann RO. Role of growth factors and the wound healing response in age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol. 2004 Jan;242(1):91-101.

243. Schmetterer L, Kruger A, Findl O, Breiteneder H, Eichler HG, Wolzt M. Topical fundus pulsation measurements in age-related macular degeneration.Graefes Arch Clin Exp Ophthalmol. 1998 Mar; 236(3):160-3.

244. Seddon JM, Cote J, Davis N, Rosner B. Progression of age-related macular degeneration: association with body mass index, waist circumference, and waist-hip ratio.Arch Ophthalmol. 2003 Jun; 121(6):785-92.

245. Seddon JM, Gensler G, Milton RC, Klein ML, Rifai N. Association between C-reactive protein and age-related macular degeneration. JAMA. 2004; 291(6):704-10.

246. Seddon JM, George S, Rosner B, Rifai N. Progression of age-related macular degeneration: prospective assessment of C-reactive protein, interleukin 6, and other cardiovascular biomarkers.Arch Ophthalmol. 2005 Jun; 123(6):774-82.

247. Seddon JM, Rosner B, Sperduto RD, Yannuzzi L, Haller JA, Blair NP, Willett W. Dietary fat and risk for advanced age-related macular degeneration.Arch Ophthalmol. 2001; 119(8):1191-9.

248. Seifertl BU, Vilser W. Retinal Vessel Analyzer (RVA)--design and function. Biomed Tech (Berl). 2002; 47 Suppl 1 Pt 2:678-81.

249. Senior RM, Griffin GL, Mecham RP. Chemotactic activity of elastin-derived peptides J Clin Invest. 1980; 66(4):859-62.

250. Sethi CS. Bailey TA. Luthert PJ. Chong NH. Matrix metalloproteinase biology applied to vitreoretinal disorders. British Journal of Ophthalmology.2000; 84(6):654-66.

251. Sheraidah G. Steinmetz R. Maguire J. Pauleikhoff D. Marshall J. Bird AC. Correlation between lipids extracted from Bruch's membrane and age. Ophthalmology.1993;100(1):47-51

252. Silver DM, Farrell RA, Langham ME, O'Brien V, Schilder P. Estimation of pulsatile ocular blood flow from intraocular pressure. Acta Ophthalmol Suppl 1989; 191:25-9

253. Smith W, Assink J, Klein R, Mitchell P, Klaver CC, Klein BE, Hofman A, Jensen S, Wang JJ, de Jong PT. Risk factors for age-related macular degeneration: Pooled findings from three continents. Ophthalmology. 2001;108(4):697-704.

254. Smith W, Mitchell P, Leeder SR and Wang JJ, Plasma fibrinogen, othercardiovascular risk factors, and age-related maculopathy: the Blue Mountains Eye study. Arch Ophthalmol 1998; 116:583–587.

255. Smith W, Mitchell P, Leeder SR Dietary fat and fish intake and age-related maculopathy.Arch Ophthalmol. 2000; 118(3):401-4.

256. Smith W, Mitchell P, Wang JJ. Gender, oestrogen, hormone replacement and age-related macular degeneration: results from the Blue Mountains Eye Study. Aust N Z J Ophthalmol. 1997 May;25

257. Smith W, Mitchell P. Alcohol intake and age-related maculopathy. Am J Ophthalmol. 1996 Nov; 122(5):743-5.

258. Snow KK, Seddon JM. Do age-related macular degeneration and cardiovascular disease share common antecedents? Ophthalmic Epidemiol. 1999; 6(2):125-43.

259. Souied EH, Benlian P, Amouyel P, Feingold J, Lagarde JP, Munnich A, Kaplan J, Coscas G, Soubrane G. The epsilon4 allele of the apolipoprotein E gene as a potential protective factor for exudative age-related macular degeneration.Am J Ophthalmol. 1998; 125(3):353-9.

260. Spaide RF, Sorenson J, Maranan L. Photodynamic therapy with verteporfin combined with intravitreal injection of triamcinolone acetonide for choroidal neovascularization.Ophthalmology. 2005 Feb;112(2):301-4.

261. Spaide RF. Ho-Spaide WC. Browne RW. Armstrong D. Characterization of peroxidized lipids in Bruch's membrane. Retina. 1999; 19(2):141-7

262. Sperduto RD, Hiller R. Systemic hypertension and age-related maculopathy in the Framingham Study. Arch Ophthalmol. 1986;104(2):216-9.

263. Spraul CW, Grossniklaus HE: Characteristics of Drusen and Bruch's membrane in postmortem eyes with age-related macular degeneration. Arch Ophthalmol 1997; 115:267-273.

264. Spraul CW. Lang GE. Grossniklaus HE. Lang GK. Histologic and morphometric analysis of the choroid, Bruch's membrane, and retinal pigment epithelium in postmortem eyes with age-related macular degeneration and histologic examination of surgically excised choroidal neovascular membranes. Surv of Ophthalmol. 1999; 44:S10-32

265. Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W, Rosenfeld ME, Schaffer SA, Swartz CJ, Wagner WD, Wissler RW. Special report: A definition of initial, fatty streak and intermediate lesions in atherosclerosis: A report from the committee on vascular lesions of he council on arteriosclerosis, American heart association. 1994: 89(5); 2462-2478.

266. Steen B. Sejersen S. Berglin L. Seregard S. Kvanta A. Matrix metalloproteinases and metalloproteinase inhibitors in choroidal neovascular membranes. Invest Ophthalmol Vis Sci. 1998; 39(11):2194-200

267. Stehbens WE The pathogenesis of atherosclerosis: A critical evaluation of the evidence. Cardiovasc Pathol 1997; 6:123-153.

268. Stevens RL, Colombo M, Gonzales JJ, Hollander W, Schmid K. The glycosaminoglycans of the human artery and their changes in atherosclerosis. J Clin Invest. 1976; 58: 470-81

269. Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P. Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells.J Clin Invest. 1998;102(3):576-83.

270. Takada Y. Ohkuma H. Ogata N. Matsushima M. Sugasawa K. Uyama M. Proteoglycan in Bruch's membrane of senescence accelerated mouse: localization and age-related changes Nippon Ganka Gakkai Zasshi - Acta Societatis Ophthalmologicae Japonicae. 98(5):469-76, 1994

271. TAP Study group. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials--TAP report. Treatment of age-related macular degeneration with photodynamic therapy (TAP) Study Group Arch Ophthalmol 1999; 117:1329-1345

272. Tayebjee MH, Lip GY, Blann AD, Macfadyen RJ. Effects of age, gender, ethnicity, diurnal variation and exercise on circulating levels of matrix metalloproteinases (MMP)-2 and -9, and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMP)-1 and -2.Thromb Res. 2005;115(3):205-10.

273. Tchetverikov I, Ronday HK, Van El B, Kiers GH, Verzijl N, TeKoppele JM, Huizinga TW, DeGroot J, Hanemaaijer R. MMP profile in paired serum and synovial fluid samples of patients with rheumatoid arthritis. Ann Rheum Dis. 2004; 63(7):881-3.

274. The Anecortave Acetate Clinical Study Group. Anecortave acetate as monotherapy for treatment of subfoveal neovascularization in age-related macular degeneration: twelve-month clinical outcomes. Ophthalmology 2003;110:2372–2382

275. The Eye Disease Case-Control Study Group, Risk factors for neovascular age- related macular degeneration. Arch Ophthalmol 1992; 110: 1701–1708.

276. Thompson MM. Controlling the expansion of abdominal aortic aneurysms.Br J Surg. 2003; 90(8):897-8.

277. Tomany SC, Wang JJ, Van Leeuwen R, Klein R, Mitchell P, Vingerling JR, Klein BE, Smith W, De Jong PT. Risk factors for incident age-related macular degeneration: pooled findingsfrom 3 continents.Ophthalmology. 2004 Jul;111(7):1280-7.

278. Tsutsumi C, Sonoda KH, Egashira K, Qiao H, Hisatomi T, Nakao S, Ishibashi M,Charo IF, Sakamoto T, Murata T, Ishibashi T. The critical role of ocular-infiltrating macrophages in the development of choroidal neovascularization.J Leukoc Biol. 2003 Jul;74(1):25-32.

279. Uretmen O, Akkin C, Erakgun T, Killi R**.** Color Doppler imaging of choroidal circulation in patients with asymmetric age-related macular degeneration. Ophthalmologica. 2003; 217(2):137-42.

280. van der Schaft TL, Mooy CM, de Bruijn WC, Oron FG, Mulder PG, de Jong PT. Histologic features of the early stages of age-related macular degeneration. A statistical analysis Ophthalmology 1992;99(2):278-86.

281. van Leeuwen R, Ikram MK, Vingerling JR, Witteman JC, Hofman A, de Jong PT, Blood pressure, atherosclerosis, and the incidence of age-related maculopathy: The Rotterdam study. Invest Ophthalmol Vis Sci 2003a; 44**:** 3771–3777.

282. van Leeuwen R, J.R. Vingerling, A. Hofman, P.T. de Jong and B.H. Stricker, Cholesterol lowering drugs and risk of age related maculopathy: Prospective cohort study with cumulative exposure measurement. *BMJ* 2003b; 226**:** 255–256.

283. van Leeuwen R, Klaver CC, Vingerling JR, Hofman A, de Jong PT. The risk and natural course of age-related maculopathy: Follow-up at 6 and a half years in the Rotterdam study. Arch Ophthalmol 2003c; 121: 519–526

284. van Leeuwen R, Klaver CC, Vingerling JR, Hofman A, van Duijn CM, Stricker BH, de Jong PT. Cholesterol and age-related macular degeneration: is there a link?Am J Ophthalmol. 2004 a; 137(4):750-2.

285. van Leeuwen R, Tomany SC, Wang JJ, Klein R, Mitchell P, Hofman A, Klein BE,Vingerling JR, Cumming RG, de Jong PT. Is medication use associated with the incidence of early age-related maculopathy? Pooled findings from 3 continents. Ophthalmology. 2004b; 111(6):1169-75.

286. Verhoeff F, Grossman H. Pathogenesis of disciform degeneration of the macula. Arch Ophthalmol. 1937; 18: 561-585

287. Vidaurri JS, Pe'er J, Halfon ST, Halperin G, Zauberman H. Association between drusen and some of the risk factors for coronary artery disease.Ophthalmologica. 1984; 188(4):243-7.

288. Vilser W, Nagel E, Lanzl I. Retinal Vessel Analysis--new possibilities.Biomed Tech (Berl). 2002; 47 Suppl 1 Pt 2:682-5.

289. Vinding T, Appleyard M, Nyboe J, Jensen G Risk factor analysis for atrophic and exudative age-related macular degeneration: An epidemiological study of 1000 aged individuals. Acta Ophthalmol (Copenh) 1992; 70: 66–72.

290. Vine N, Powell JT. Metalloproteinases in degenerative aortic disease.Clin Sci (Lond). 1991; 81(2):233-9.

291. Vingerling JR, Dielemans I, Bots ML, Hofman A, Grobbee DE, de Jong PT, Age-related macular degeneration is associated with atherosclerosis. The Rotterdam study. Am J Epidemiol 1995a; 142: 404– 409

292. Vingerling JR, Dielemans I, Witteman JC, Hofman A, Grobbee DE, de Jong PT. Macular degeneration and early menopause: a case-control study.
BMJ. 1995b; 310(6994):1570-1.

293. Vingerling JR. Dielemans I. Hofman A. Grobbee DE. Hijmering M. Kramer CF. de Jong PT. The prevalence of age-related maculopathy in the Rotterdam Study Ophthalmology. 1995c:102(2): 205-10

294. Virmani R. Kolodgie FD. Burke AP. Farb A. Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol. 2000; 20(5):1262-75

295. Visse R Nagase H Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry.Circ Res. 2003; 92(8):827-39.

296. Wachter A, Sun Y, Dasch B, Krause K, Pauleikhoff D, Hense HW. Munster age- and retina study (MARS). Association between risk factors for arteriosclerosis and age-related macular degeneration Ophthalmologe. 2004; 101(1):50-3.

297. Walker ER. Frederickson RG. Mayes MD. The mineralization of elastic fibers and alterations of extracellular matrix in pseudoxanthoma elasticum. Ultrastructure, immunocytochemistry, and X-ray analysis. Archives of Dermatology. 1989; 125(1):70-6.

298. Wang JJ, Foran S, Smith W, Mitchell P. Risk of age-related macular degeneration in eyes with macular drusen or hyperpigmentation: The Blue Mountains Eye study cohort. Arch Ophthalmol 2003a**;** 121: 658–663.

299. Wang JJ, Mitchell P, Leung H, Rochtchina E, Wong TY, Klein R. Hypertensive retinal vessel wall signs in a general older population: the Blue Mountains Eye Study. Hypertension. 2003 Oct;42(4):534-41

300. Wang JJ, Mitchell P, Rochtchina E, Tan AG, Wong TY, Klein R. Retinal vessel wall signs and the 5 year incidence of age related maculopathy: the Blue Mountains Eye Study.Br J Ophthalmol. 2004 Jan; 88(1):104-9.

301. Wang JJ, Mitchell P, Smith W, Gillies M, Billson F; Blue Mountains Eye Study. Systemic use of anti-inflammatory medications and age-related maculopathy: the Blue Mountains Eye Study. Ophthalmic Epidemiol. 2003b; 10(1):37-48.

302. Wanhainen A, Bergqvist D, Boman K, Nilsson TK, Rutegard J, Bjorck M. Risk factors associated with abdominal aortic aneurysm: a population-based study with historical and current data. J Vasc Surg. 2005; 41(3):390-6.

303. Wei SM, Erdei J, Fulop T Jr, Robert L, Jacob MP. Elastin peptide concentration in human serum: variation with antibodies and elastin peptides used for the enzyme-linked immunosorbent assay.J Immunol Methods. 1993; 164(2):175-87.

304. Williams KJ, Tabas I. The response to retention hypothesis of early atherogenesis. Arterioscler Thromb Vasc Biol. 1995; 15: 551–561

305. Wirostko E, Wirostko WJ, Wirostko BM. Age-related macular degeneration is an inflammatory disease possibly treatable with minocycline. Acta Ophthalmol Scand. 2004; 82(2):243-4.

306. Wong TY, Knudtson MD, Klein BE, Klein R, Hubbard LD. Medication use and retinal vessel diameters.Am J Ophthalmol. 2005; 139(2):373-5.

307. Wong TY, Shankar A, Klein R, Klein BE, Hubbard LD. Prospective cohort study of retinal vessel diameters and risk of hypertension.BMJ. 2004 10; 329(7457):79.

308. Xu XP, Meisel SR, Ong JM, Kaul S, Cercek B, Rajavashisth TB, Sharifi B, Shah PK. Oxidized low-density lipoprotein regulates matrix metalloproteinase-9 and its tissue inhibitor in human monocyte-derived macrophages. Circulation 1999; 99:993–998

309. Xuan B, Xu XR, Chiou GC, Zhang YH, Peng SX**.** Relationship between nitric oxide production and choroidal blood flow. J Ocul Pharmacol Ther. 2003; 19(3):247-53.

310. Xuan B, Zhou YH, Li N, Min ZD, Chiou GC**.** Effects of crocin analogs on ocular blood flow and retinal function. J Ocul Pharmacol Ther. 1999a; 15(2):143-52.

311. Xuan B, Zhou YH, Varma R, Chiou GC**.** Effects of some N-nitropyrazole derivatives on ocular blood flow and retinal function recovery after ischemic insult.J Ocul Pharmacol Ther. 1999b; 15(2):135-42.

312. Yamamoto T. Yamashita H. Scanning electron microscopic observation of Bruch's membrane with the osmium tetroxide treatment. Br J Ophthalmol. 1989; 73(3):162-7

313. Yamamoto Y. Measurement of blood flow velocity in feeder vessels of choroidal neovascularization by a scanning laser ophthalmoscope and image analysis system .Jpn J Ophthalmol. 2003; 47(1):53-8.

314. Yasuda Y, Li Z, Greenbaum D, Bogyo M, Weber E, Bromme D. Cathepsin V, a novel and potent elastolytic activity expressed in activated macrophages. J Biol Chem. 2004; 27; 279(35):36761-70

315. Yeh DC, Bula DV, Miller JW, Gragoudas ES, Arroyo JG. Expression of leukocyte adhesion molecules in human subfoveal choroidal neovascular membranes treated with and without photodynamic therapy.
Invest Ophthalmol Vis Sci. 2004 Jul;45(7):2368-73.

316. Yeow KM. Kishnani NS. Hutton M. Hawkes SP. Murphy G. Edwards DR. Sorsby's fundus dystrophy tissue inhibitor of metalloproteinases-3 (TIMP-3) mutants have unimpaired matrix metalloproteinase inhibitory activities, but affect cell adhesion to the extracellular matrix. Matrix Biology. 2002; 21(1):75-88

317. Yoshida A, Feke GT, Mori F, Nagaoka T, Fujio N, Ogasawara H, Konno S, Mcmeel JW. Reproducibility and clinical application of a newly developed stabilized retinal laser Doppler instrument.Am J Ophthalmol. 2003; 135(3):356-61.

318. Young RW. Pathophysiology of age-related macular degeneration. Survey of Ophthalmology. 1987; 31(5):291-306

319. Zemel PC, Sowers JR. Relation between lipids and atherosclerosis: epidemiologic evidence and clinical implications.Am J Cardiol. 1990; 66(21):7I-12I.

320. Zhao J, Frambach DA, Lee PP, Lee M, Lopez PF Delayed macular choriocapillary circulation in age-related macular degeneration. Int Ophthalmol 1995; 19:1-12

PUBLICATIONS AND POSTERS FROM THIS THESIS

- Bruch's Membrane and the Vascular Intima: Is There a Common Basis for Age-related Changes and Disease? Clin Experiment Ophthalmol. 2005 Oct; 33(5):518-23.
- Serum Elastin Derived Peptides in Age-related Macular Degeneration. Invest Ophthalmol Vis Sci. 2005 Sep; 46(9):3046-51.
- Plasma MMP-2 and MMP-9 in Age-related Macular Degeneration ARVO 2005
- Plasma C3a des Arg in Age-related Macular Degeneration EVER 2005
- Dysregulation of extracellular matrix in Age-related Macular Degeneration EVER 2005
- The Complement system and age related macular degeneration EYE 2005 (in press).
- Pulsatile ocular blood flow in asymmetric age related macular degeneration EYE 2005 (in press)