USE OF METABOLOMICS IN AGE-RELATED MACULAR DEGENERATION

A thesis submitted to University of Birmingham for the degree of

MD (Inflammation and Ageing)

by

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Abstract

Age-related macular degeneration (AMD) is a major cause of irreversible central sight loss in the elderly. Many factors affect disease onset and progression, and these include age, environmental stressors such as smoking, diet, inflammation and genetic polymorphisms, all of which are likely to influence metabolism. In some complex diseases, metabolomics, which involves the identification of a metabolic fingerprint in a biofluid or tissue, has been shown to discriminate metabolic changes associated with different disease processes and to identify specific phenotypes. We have applied metabolomics, in the first study of its kind, to analyse, using NMR spectroscopy, both serum and urine metabolic characteristics in patients with dry and wet AMD (n=104). NMR spectral analysis showed good clustering as well as separation among the serum and urine from dry and wet AMD patients. The results show that metabolite profiles can distinguish dry and wet AMD, but that the pathways involved, glycolysis, urea cycle and Kreb's cycle, are involved in both forms of the disease. It is likely that the pathogenesis of dry and wet AMD is similar and that the severity of ocular damage and systemic inflammation would account for the distinguishing profiles. These data support the use of metabolomics in identifying biological pathways involved in pathogenesis of AMD, but not in the diagnosis or prognosis of disease.

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AGE	Advanced glycation end products
AMD	Age related macular degeneration
AREDS2	Age-Related Eye Disease Study 2
ARMS2	Age-related maculopathy susceptibility protein 2
ATP	Adenosine triphosphate
BrM	Bruch's membrane
CEC	Choroidal endothelial cells
CEP	Carboxyethylpyrrole
CFH	Complement factor H
CNV	Choroidal neovascularisation
CRP	C- reactive protein
CSF	Cerebrospinal fluid
CU	Chronic uveitis
DESG	Dry eye syndrome group
DHA	Docosahexaenoic acid
DR	Diabetic retinopathy
FA	Fluorescein angiography

GA	Geographic atrophy
GAG	Glycosaminoglycans
GCL	Layer of ganglion cells
GSH	Glutathione
GSSH	Oxidised glutathione
GWAS	Genome wide association studies
hESC	Human embryonic stem cell
HMDB	Human Metabolome Database
HTRA1	High temperature required factor A1
ICAM-1	Intercellular adhesion molecule-1
ICG	Indocyanine green
IIH	Intracranial hypertension
IL	Interleukin
INL	Inner nuclear layer
IPL	Inner plexiform layer
LIU	Lens-induced uveitis
MAC	Membrane attack complex
MS	Multiple sclerosis
NMR	Nuclear Magnetic Resonance

NOS	Nitric oxide synthase
OCT	Optical Coherence Tomography
ONL	Outer nuclear layer
OPL	Outer plexiform layer
O-PLS-DA	Orthogonal signal correction PLS-DA
PAS	Periodic acid–Schiff
PC	Principal components
PCA	Principal component analysis
pCRP	Pentameric CRP
PEDF	Pigment epithelium-derived factor
PLS-DA	Partial least squares discriminant analysis
POAG	Primary open angle glaucoma
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
RAGE	Advanced glycation end products receptor
RAP	Retinal angiomatous proliferation
RNAseq	RNA sequencing
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RPE	Retinal pigment epithelium
SIMCA	Soft Independent Modelling of Class Analogy
SNP	Single nucleotide polymorphism
TCA	Tricarboxylic acid
TGF-β	Transforming growth factor-β
TIMP3	Tissue Inhibitor of Metalloproteinases 3
TMSP	Trimethylsilylpropanoic acid
TSP-1	Thrombospondin-1
TUDCA	Tauroursodeoxycholic acid
VEGF	Vascular endothelial growth factor
WES	Whole exome sequencing
WGS	Whole genome sequencing

Chapter 1

MACULAR DEGENERATION AND METABOLOMICS

A. MACULAR DEGENERATION: AN OVERVIEW

Age-related macular degeneration (AMD) is a major cause of ocular morbidity in the elderly in developed countries, causing irreversible central sight loss. It is thought to affect more than 3 million people in the United States by 2020 (Eye Diseases Prevalence Research Group, 2004). The prevalence study of late AMD in the United Kingdom by Owen et al showed overall prevalence of late AMD to be 2.4%, equivalent to 513 000 cases, estimated to increase to 679 000 cases by 2020 (Owen CG et al., 2012).

In this chapter, I have briefly outlined the following:

1. Anatomy of macula and choroid
2. Macular degeneration: types and new classification
3. Pathophysiology of AMD
4. Role of risk factors in the pathogenesis of AMD
5. Diagnosis of AMD
6. Treatment of AMD

1. Anatomy of macula and choroid

The eye consists of three layers: an outer layer of sclera and cornea; a middle layer consisting of iris, ciliary body and choroid (uvea); and an inner layer, the retina, the light sensitive layer of the eye. Near the centre of the retina is the macula, a 5-6 mm wide area, rich in xanthophyll pigments, lutein and zeaxanthin (Provis J et al., 2005). The macula is responsible for photopic and colour vision and provides the greatest

resolving power of the eye. The macula is located inside the temporal vascular arcades, 2 disc-diameters temporal to the optic disc; the centre of macula is called fovea (Figure 1.1).

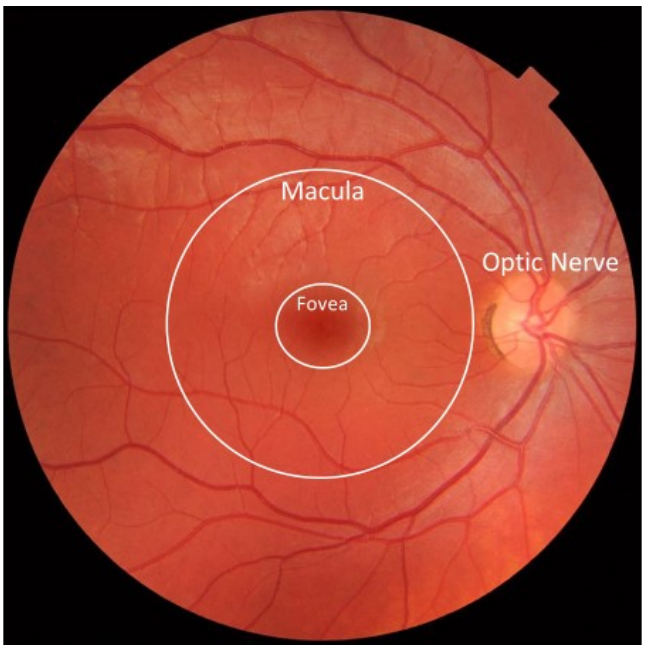

Figure 1.1: Anatomy of retina

The retina consists of an outer retinal pigment epithelial layer (RPE) and an inner neuro sensory layer. The primary layers of the retina, from outermost surface to innermost surface, are as follows (Figure 1.2):

- Retinal pigment epithelium (RPE): these are the supporting cells for the neural portion of the retina
- Rod and cone layer or Layer of photoreceptor cells: this contains the outer segments and inner segments of the rod and cone photoreceptors
- Outer limiting membrane

- Outer nuclear layer (ONL): contains cell bodies of rods and cones
- Outer plexiform layer (OPL): contains rod and cone axons, horizontal cell dendrites and bipolar dendrites
- Inner nuclear layer (INL): Nuclei of horizontal, bipolar and amacrine cells
- Inner plexiform layer (IPL): axons of bipolar (and amacrines), dendrites of ganglion cells
- Layer of ganglion cells (GCL): Nuclei of the ganglion cells and displaced amacrine cells
- Layer of optic nerve fibres: contains fibres from ganglion cells traversing the retina to leave the eyeball at the optic disc

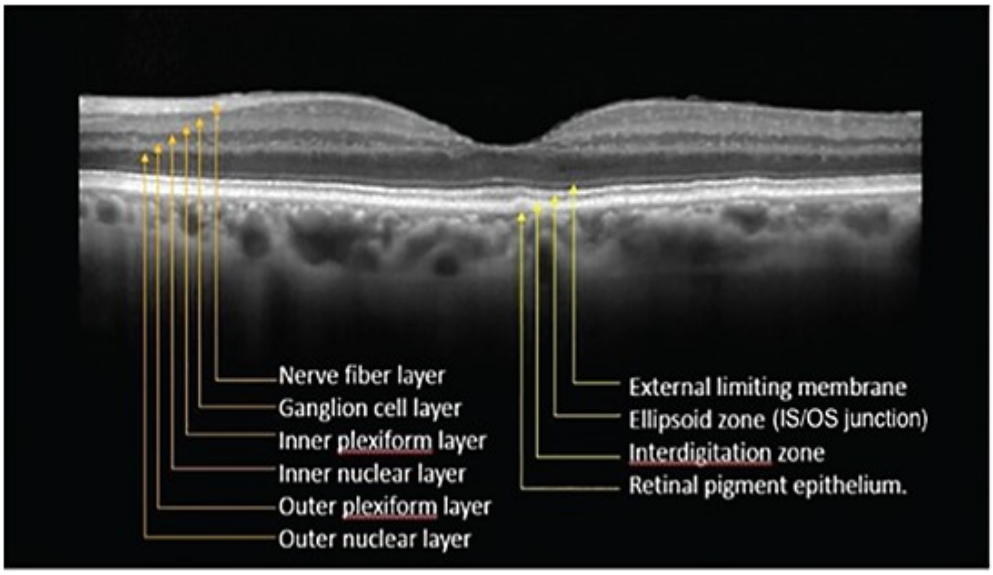

Figure 1.2: Layers of retina as seen in optical coherence tomography (OCT) (Image courtesy of Jessica G. Lee, MD and the Photography Department at New York Eye and Ear Infirmary of Mount Sinai, July 2015)

Photoreceptor cells, RPE cells, Bruch's membrane (BrM), and choriocapillaris are interdependent layers of the outer retina, which contribute to normal vision, and are affected in ageing process and macular degeneration (Chirco KR et al., 2017).

Photoreceptor cells are highly metabolically active cells converting photons to electrical impulses, which requires constant regeneration of their outer segments. Photoreceptor cells are normally lost with ageing in healthy retina, however this happens at an accelerated rate in the central 1mm of macula in macular degeneration (Chirco KR et al., 2017; Curcio C, 2001).

The retinal pigment epithelium (RPE) is a single layer of hexagonal epithelial cells with densely packed pigment granules. They are tall and narrow columnar epithelial cells in the macula, becoming more cuboidal outside the macula. The microvilli on the apical surface of the RPE cover the photoreceptor outer segment and support in their turnover. The hemidesmosomes in the basal aspect of the RPE anchor them to the basal laminae; this forms the innermost layer of the Bruch's membrane (Chirco KR et al., 2017).

RPE cells phagocytose and recycle the shed photoreceptor outer segments. The RPE absorbs excess light, converting all-trans retinol to 11-cis retinal as part of the visual cycle, and secretes growth factors to maintain photoreceptor cells and the choroidal vasculature. Further, RPE cells transport nutrients and waste material between photoreceptor cells and choriocapillaris and form the outer blood–retinal barrier (Bok, 1993).

With ageing, pigmentation in the RPE cells is altered through loss of melanosomes, increase in lipofuscin granules and increase in toxic by-products of the visual cycle.

Bruch’s membrane

Bruch’s membrane (BrM) is a 2-4 μm-thick extracellular matrix complex that separates the RPE and choroid. It is composed primarily of elastin and collagen. BrM consists of the following five distinct layers: (1) the RPE basement membrane (innermost layer), (2) the inner collagenous layer, (3) the elastic layer, (4) the outer collagenous layer, and (5) the choriocapillary endothelial cell basement membrane (outermost layer) (Nakaizumi Y,1964).

The BrM contains a reservoir of anti-angiogenic molecules such as thrombospondin-1 (TSP-1), angiostatin, endostatin, and pigment epithelium-derived factor (PEDF); this reservoir acts both as a barrier that prevents cell migration from retina or choroid, and as a filter that controls passage of various molecules between RPE and choroid. BrM also provides a surface for RPE cell adhesion, growth, and physical support (Prea S et al., 2015).

Choroid

The choroid is the vascular bed that lies just beneath the RPE, between BrM and the sclera. It is composed of blood vessels, melanocytes, fibroblasts, immunocompetent cells and supporting collagenous and elastic connective tissue. The vascular region of the choroid consists of the outer Haller's layer of large blood vessels and the inner Sattler's layer of medium and small arteries and arterioles that feed the capillary network and veins. The stroma (extravascular tissue) contains collagen and elastic fibres, fibroblasts, non-vascular smooth muscle cells and melanocytes. The capillary bed (the choriocapillaris) which makes up the innermost layer of the choroid provides

the principal blood supply, thus delivering oxygen to the outer retina (RPE and photoreceptor cells). Other functions of the choroid include light absorption, thermoregulation via heat dissipation, and modulation of intraocular pressure (IOP) via vasomotor control of blood flow. The choroid also plays an important role in the drainage of the aqueous humour from the anterior chamber, via the uveoscleral pathway. The loss of choriocapillary endothelial cells is a key contributor to the development of AMD.

2. Macular degeneration

Age-related macular degeneration (AMD) is a painless, irreversible, degenerative disease of the macula, resulting in loss of central vision, which is essential for our daily activities including reading and driving. Early AMD often is asymptomatic, however most eyes with choroidal neovascularisation (CNV) due to AMD under the fovea become legally blind within two years of diagnosis, many within months.

Socioeconomic impact of sight impairment

In 2013, there were an estimated 1.93 (1.58 to 2.31) million people with sight loss and blindness in the UK (2.5% to 3.6% of the population) (Pezzullo L, 2018), AMD being the leading cause of blindness (Owen C, 2003). From 2013 to 2050, the share of sight loss and blindness from AMD is projected to change from 23.1% to 29.7%, more than doubling from 445,809 (363,900 to 532,800) people to 1.23 (1.01 to 1.47) million people (Pezzullo L, 2018). The cost of detection, treatment and provision of state and family social care for everyone with AMD was more than £1.6bn in 2010. The

estimated cumulative cost over the decade from 2010 to 2020, is expected to rise to £16.5bn due to demographic change: while the healthcare treatment component amounts to 18% of the total (more than £2.9bn), the personal and social costs make up 76% (more than £12.5bn) (Minassian D, 2009).

Types of AMD

Previously, AMD was described to be either 'dry' with no neovascularisation, or 'wet' in which new vessels from the choroid disrupt retinal tissue. Dry AMD is associated with gradual visual loss, while wet AMD is associated with sudden and severe visual loss.

New classification of AMD

In 2013, the Macular Research Classification Committee proposed a new clinical classification for macular degeneration, based on fundus lesions assessed within 2 disc-diameters of fovea in patients over 55 years of age. The committee agreed that a single term, age-related macular degeneration, should be used for the disease. Using a modified Delphi technique, the committee developed a 5-stage AMD classification scale: no apparent ageing changes, normal ageing changes, early AMD, intermediate AMD or late AMD (Table 1.1) (Ferris FL et al., 2013).

Persons with no visible drusen or pigmentary abnormalities should be considered to have no signs of AMD. Persons with small drusen (<63 μm), also termed drupelets, should be considered to have normal ageing changes with no clinically relevant increased risk of developing late AMD. Persons with medium drusen (≥ 63 - <125 μm), but without pigmentary abnormalities thought to be related to AMD, should be

considered to have early AMD. Persons with large drusen or with pigmentary abnormalities associated with at least medium drusen should be considered to have intermediate AMD. Persons with lesions associated with neovascular AMD (choroidal neovascularisation (CNV)) or geographic atrophy (GA) should be considered to have late AMD (Ferris FL et al., 2013).

Classification of AMD	**Definition** (lesions assessed within 2 disc-diameters of fovea in either eye)
No apparent ageing changes	No drusen; and No AMD pigmentary abnormalities
Normal ageing changes	Only drupelets (small drusen ≤63 μm); and No AMD pigmentary abnormalities
Early AMD	Medium drusen>63 μm and ≤125 μm; and No AMD pigmentary abnormalities
Intermediate AMD	Large drusen> 125 μm; and/or Any AMD pigmentary abnormalities
Late AMD	Neovascular AMD; and/or Any geographic atrophy

Table 1.1: Proposed new classification of age related macular degeneration. (Classification published by Ferris FL et al., 2013)

Depending on the location of the CNV, it can be of 3 types. Type 1 or occult CNV is where CNV remains beneath the RPE monolayer but does not invade the photoreceptors and neural retina, whereas CNV that proliferates within the sensory retina is termed classic or type 2 CNV (Wang H, 2016; Grossniklaus HE, 2004). Vascular lesions

arising from the retinal vasculature in the deep retina is known as retinal angiomatous proliferation (RAP), or type 3 neovascularization (Wang H and Hartnett ME, 2016; Yannuzzi L, 2001).

Drusen

A characteristic feature of AMD is the presence in the retina of drusen, yellow deposits of lipids and proteins that accumulate with age. Increased size and number of drusen are associated with increased severity of disease (Oishi et al., 2017; Mullins et al., 2000). Drusen is composed of degradation products for photoreceptor cells, and RPE cells and immune processes, including serum amyloid P, immunoglobulin light chains and complement proteins (Anderson D et al., 2002). Drusen has been identified as inflammatory in the retina via infiltration and activation of macrophages a significant feature (Cruz-Guilloty F et al., 2013).

Geographic atrophy

Geographic atrophy (GA) is a manifestation of late-stage AMD, characterized by localized atrophy of retinal pigment epithelium, the overlying photoreceptors and the choriocapillaris, leading to central scotomas and permanent loss of visual acuity. In the early stages, when distance visual acuity (VA) is marginally reduced or indeed unaffected, patients with GA have trouble with reading and seeing in low-light conditions, problems that may not be reflected in VA measurements. Currently, there are no approved treatments to prevent GA or limit its progression when already present, nor are there any treatments that can reverse the pathology. Geographic atrophy is usually bilateral, with major impact on vision-related quality of life.

*In our research we have categorised patients into two groups: those with choroidal neovasclarisation as **wet AMD group** and all the others (early and intermediate AMD as well as late AMD without neovasclarisation (GA)) as the **dry AMD group**.*

3. Pathophysiology of AMD

One of the changes seen in the macula of patients with early AMD is development of drusen. During ageing process, there is alteration of metabolism in RPE cells causing destruction of BrM and accumulation of extra cellular material between RPE and Bruch's membrane, leading to the development of drusen.

Histopathologic and proteomic analyses have shown increased deposition of compounds such as TIMP3 and SerpinA3, vitronectin, bisretinoid fluorophores, complement, oxidized proteins, lipoprotein derived debris, cholesterol esters and 7-ketocholesterol, beneath the RPE cells and within Bruch's membrane (Nita M et al., 2014; Hageman GS and Mullins RF, 1999).

Drusen can be nodular or diffuse. Nodular drusen consists of a focal thickening of the RPE basement membrane. In fluorescein angiography (FA), they cause early hyperfluorescence and late staining. Histologically, nodular drusen have an eosinophilic, PAS-positive appearance and are located externally or replace the thin basement membrane of the RPE (Guymer R et al., 1999).

In diffuse drusen, materials get deposited between the basement membrane of the RPE and the inner collagen layer of the Bruch's membrane. In FA, they are hypofluorescent due to lipid accumulation and later show staining (Guymer R et al., 1999).

Drusen and other extracellular deposits accumulate toxic substances which obstruct the passage of nutrients and oxygen from the choriocapillaris to the overlying RPE and retina, putting the RPE cells under stress (Guymer R et al., 1999).

In the natural history of disease progression, the drusen accumulation is followed by hyperpigmentation and drusen regression, resulting in atrophy of RPE and choriocapillaris. This leads to photoreceptor cell loss and development of geographic atrophy (GA) (Klein ML et al, 2008).GA can remain stable over time, or progress quicker in volume and area before CNV develops.

Some deposits located above the RPE, called reticular pseudodrusen or drusenoid subretinal deposits (Zweifel et al., 2010), do not fluoresce with fluorescein or indocyanine green in angiography. These can also precede GA due to the damage to the RPE caused by the underlying atrophy and fibrosis of the choroid (Klein ML et al, 2010).

In neovascular AMD, hypoxia of the overlying RPE cells contributed by the loss of choriocapillaris, results in the release of angiogenic factors leading to the development of CNV (Bhutto I and Lutty G, 2012). The choroidal endothelial cells (CECs) migrate to and across the RPE monolayer and into the sensory retina where they proliferate and develop into CNV, anywhere between the choriocapillaris and neural retina. These new vessels leak blood and serum, causing an irreversible damage and progressive vision loss.

4. Role of risk factors in the pathogenesis of AMD

AMD is a multifactorial disease, with several factors attributed to the development and progression of AMD. Non-modifiable risk factors include age, family history and genetic factors including complement pathway and immunological factors. The modifiable risk factors are smoking, dietary factors, obesity, and underlying vascular risk factors such as high blood pressure and cholesterol. Recently, inflammation has also been suggested to play a role in the pathogenesis of AMD.

Age

AMD is a disease of the elderly, a cumulative outcome of several metabolic and cellular changes, likely to occur in individuals over the age of 60 years. The risk of developing AMD increases dramatically with age, the prevalence increasing to almost three-fold in those over 80 years of age (Owen CG et al, 2012). With ageing, gradual loss of retinal neuron cells, photoreceptors and RPE cells occur (Gao H and Hollyfield JG, 1992). In addition to cell loss, an increase in intra and extra cellular deposition of material has also been seen with advancing age. Lipofuscin, intracellular deposit in RPE, when exposed to oxygen and light, generate reactive oxygen species (ROS) (Boulton et al, 1993), which is implicated in the pathogenesis of AMD. Lipofuscin has also been implicated in immune dysregulation via monocyte and microglial activation (Ma et al, 2013). Another change noted with ageing is the transformation of classically activated M1 macrophages (anti-angiogenic) to M2 macrophages which contributes to aberrant inflammation, losing its ability to prevent angiogenesis (Kelly et al, 2007). Cholesterol intermediates activate alternative complement pathway, inciting inflammation and contributing to the pathogenesis of atherosclerosis, AMD and Alzheimer's disease

(Sene A et al, 2013; Sharma et al 2014). These changes are not seen in the younger age group, further strengthening the theory that AMD is an ageing disease and not a developmental disease.

Ethnicity

Ethnicity is associated with disease risk, with Caucasians at a significantly higher risk of AMD than African-Americans in the United States, especially with regard to wet AMD. Global AMD prevalence by ethnic group is estimated as 12.3% for European ancestry, 10.4% for Hispanics, 7.5% for African ancestry, and 7.4% for Asians (Eye Diseases Prevalence Research Group, 2004; Owen CG et al, 2012).

Smoking

The risk of developing AMD in current smokers is two- or three-times the risk for never-smokers. There is a greater than four-fold increased risk for neovascular AMD in smokers (Velilla et al., 2013). Choroidal blood flow is reduced in AMD (Friedman E et al., 1995; Ciulla et al., 2002). Grunwald *et al* (2003) discovered an association between decreased circulation within the choroid and increased risk for wet AMD implicating ischemia in CNV formation. Smoking may also reduce choroidal blood flow in the eye, and promote ischaemia and hypoxia, further increasing the susceptibility of the macula to degenerative changes (Steigerwalt RD Jr et al., 2000). Nicotine administration appeared to increase severity of experimental choroidal neovascularization in a mouse model (Suñer IJ et al., 2004). Smoking has also been shown to reduce macular pigment optical density, which reflects levels of the protective carotenoids, lutein, and zeaxanthin in the macular retina (Kirby ML et al, 2010).

Cigarette smoke contains high quantities of pro-oxidant benzene derivative hydroquinone. Incubation of RPE cells with hydroquinone induced an injury response similar to sub-RPE deposit formation. Exposure to hydroquinone also resulted in decreased levels of matrix metalloproteinase-2 and increased levels of collagen IV resulting in a net decrease in extracellular matrix turnover, causing thickening of Bruch's membrane or formation of sub-RPE deposits (Kirby ML et al., 2010).

In an experimental mice model with laser induced CNV, there was a statistically significant increase in the size of CNV in those exposed to cigarette smoke compared to control models (Suñer IJ et al, 2004).

Cigarette smoking may increase the risk of AMD through enhancement of the immune cascade. Smoking leads to low levels of carotenoids in both blood and the retina, probably through a free radical dependent mechanism. The exposure to constituents of cigarette smoke increases the presence of proinflammatory cytokines like C- reactive protein (CRP), interleukin-6, soluble tumour necrosis factor alpha receptor 2, soluble intercellular adhesion molecule-1 (ICAM-1), and apolipoprotein B (ApoB) (Nita M and Grzybowski, 2016). Cigarette smoking has been shown to trigger the alternative pathway of complement activation and increase leukocyte recruitment in both endothelial and epithelial tissues (Kew RR et al., 1985).

Role of inflammation

Recent studies have shown a strong link between inflammation and diseases of ageing. The presence of activated microglia around the lesions in the chronic neurodegenerative disorders like Alzheimer’s and Parkinson’s diseases supported the role of inflammation

in these diseases. It was suggested that long-term use of non-steroidal anti-inflammatory drugs may be protective or may slow the onset of conditions such as Alzheimer's disease and AMD (Wang J et al., 2015).

Histopathological evaluation of AMD lesions has illustrated the presence of inflammatory cells including macrophages, mast cells, and lymphocytes. Anderson et al (2002) proposed that immune factors, with activation of the complement cascade and release of inflammatory mediators, contribute to the development of changes in the RPE and Bruch's membrane, which in turn predispose to the development of AMD. They noticed an accumulation of C- reactive protein (CRP) and complement (C5) in the cytoplasm in a group of dying RPE cells. Their study also showed drusen containing both C3 and C3 activation fragments and complement reactivity in sub-RPE deposits associated with the collagen layers of the Bruch membrane (Anderson DH et al., 2002).

Pentameric CRP (pCRP) is an acute-phase protein, whose level increases from <1 up to >500 μg/ml, with onset of inflammation. As a result, the serum-associated pCRP is commonly used as a biomarker for infection or inflammation, regardless of the cause. pCRP, when bound to activated platelets or apoptotic cells, dissociate into monomers (mCRP), loses its anti-inflammatory activity and adopt potent pro-inflammatory properties (Mitta VP et al., 2013).

Macrophages are thought to play an important role in controlling the events in AMD. Macrophages are recruited to remove debris, thus preventing inhibition of RPE cell function and oxidative stress and to restore homeostasis in people with normal functioning immune system (Skeie JM and Mullins RF, 2009). However, in immunosenescence, when the normal regulatory control breaks down, parainflammation

ensues, resulting in suboptimal or altered function of the macrophages. It can result in either progressive RPE cell death with minimal inflammation (dry AMD) or enhanced inflammation by recruiting proinflammatory macrophages resulting in angiogenesis (wet AMD). The type of macrophages recruited depends on the cytokines released in response to the tissue injury: interleukins (IL) 1 and 18 are proinflammatory, while IL 10 is anti-inflammatory.

Elevated oxidative stress has been linked to several aging-related illnesses, including macular degeneration. The ability of a cell to resist oxidative damage is determined by the balance between the generation of reactive oxygen species and the defensive capacity to produce antioxidants. Glutathione is the most abundant endogenous antioxidant found within cells, playing a central defensive role against oxidative stress. When cells are unable to maintain intracellular glutathione concentrations, irreversible cell damage occurs. It has been noted that blood levels of glutathione are greatly diminished in ageing blood, indicating altered metabolic activity (Forrester JV, 2013; Skeie JM and Mullins RF, 2009). Glutathione deficiency in aging results in an increased pro-oxidizing shift and elevated oxidative stress, but underlying mechanisms are not well understood.

It is believed that lutein, zeaxanthin and meso-zeaxanthin in the macula block blue light from reaching the underlying structures in the retina, thereby reducing the risk of light-induced oxidative damage that could lead to AMD. A nutritional supplement containing lutein, zeaxanthin and meso-zeaxanthin effectively increased the optical density of the macular pigment in eyes of most human subjects (Ma L et al., 2016). Another study concluded that lutein, zeaxanthin and meso-zeaxanthin filter short-wavelength light and

prevent or reduce the generation of free radicals in the retinal pigment epithelium and choroid. The study also suggested that a mixture of these carotenoids is more effective than any individual one at the same total concentration (Li B et al., 2010).

Gene expression profiling of dermal fibroblasts from AMD patients showed no difference from controls until an oxidative or inflammatory insult was given (Strunnikova N et al., 2005). This gives an important insight into the possible interaction between environment and disease. This decline in biochemical protective mechanisms may relate to the low-grade inflammation, resulting in raised CRP, and such a decline is more profound in the apparently healthy elderly. This could result from metabolic changes in the liver, the major source of glutathione and CRP, or in the immune cell activity driving this process (Mitta V et al., 2013). Oxidative damage to docosahexanoic acid (DHA), which is found in abundance in the retina, leads to the production of carboxyethylpyrrole (CEP), which can bind to other self-proteins and act as a hapten. CEP modified proteins were shown to be more prevalent in eyes from donor with AMD compared to age-matched controls. Moreover, CEP altered proteins and anti-CEP antibodies are more abundant in patients with AMD. (Hollyfield JK et al., 2008).

Case control studies have shown 50% increase in AMD in age matched groups adjusted for cigarette smoking, when the measured high-sensitivity CRP (hsCRP) level was > 3 mg/L. The study also showed a nearly 2-fold increased risk of neovascular AMD. This finding further supports the theory that low-grade systemic inflammation contributes to AMD development in the general population (Forrester JV, 2013; Hollyfield JG, 2008).

Advanced glycation end products (AGEs) are proteins or lipids that are covalently bound with sugar molecules. The AGEs are formed extracellularly via a non-enzymatic process. They accumulate in extracellular matrices, such as BrM, and disrupt normal protein function. Once AGE accumulation reaches a certain level, the AGE receptor (RAGE) can be engaged and activated. RAGE is present on many cell types, including endothelial cells and leucocytes, and its activation induces an inflammatory response that could help trigger the conversion from acute inflammation to chronic inflammation and tissue damage, and may have a role in angiogenesis (Howes KA et al., 2004). RAGE may also contribute to the activation of immune cells in laser-induced CNV lesion in a mouse model of AMD (Chen M et al., 2014).

Intracellular adhesion molecule 1 (ICAM-1) is a glycoprotein expressed on the endothelial cells of the healthy choriocapillaris, especially within the macula. This is in contrast to other vascular beds where ICAM-1 is only expressed during an immune response. Elevated ICAM-1 in AMD could promote the recruitment of leukocytes into the choroid to evoke or propagate an immune response (Skeie JM et al., 2010).

Complement system

The complement system is a proteolytic cascade comprising approximately 40 plasma and cell surface-associated proteins involved in the elimination of pathogenic cells, dead cells, and other dangerous cellular debris from the body.

The complement pathway can be activated by three routes: antigen-antibody complexes activate the classical pathway, lectins activate the lectin pathway, and foreign organisms activate the alternate pathway. These three pathways have distinct mechanisms of initiation, but they all utilize the same terminal pathway involving C5 cleavage. Once

C3 is cleaved and the levels of C3b begin to increase, C5 convertases are formed (C4b2a3b and C3bBb3b). These enzymes cleave C5 to generate C5a and C5b, C5b then interacts with C6, C7, C8, and many copies of C9 to form the membrane attack complex (MAC). MAC formation is the final step in complement activation and it results in target cell lysis. The complement cascade also produces potent anaphylatoxins (C3a and C5a), resulting in phagocyte recruitment and leukocyte and endothelial cell activation causing efficient clearance of lysed cells (Figure 1.3) (Chirco KR et al., 2017). Bora and associates (2006) reported the activation of alternate pathway in the development of CNV secondary to laser burn in a murine model (Bora NS et al., 2006).

In proteomic studies of donor macula, TIMP3, the complement proteins C7, C5, and C9 were found to be more abundant in BrM/ choroid tissue from early- or mid-stage AMD eyes compared with controls. In advanced wet AMD, however, complement C3 protein levels were elevated compared with controls. These suggests inflammatory processes, including complement activation, as early factors in disease progression with the potential to be triggers for AMD development (Chirco KR et al., 2017; Yuan X et al., 2010).

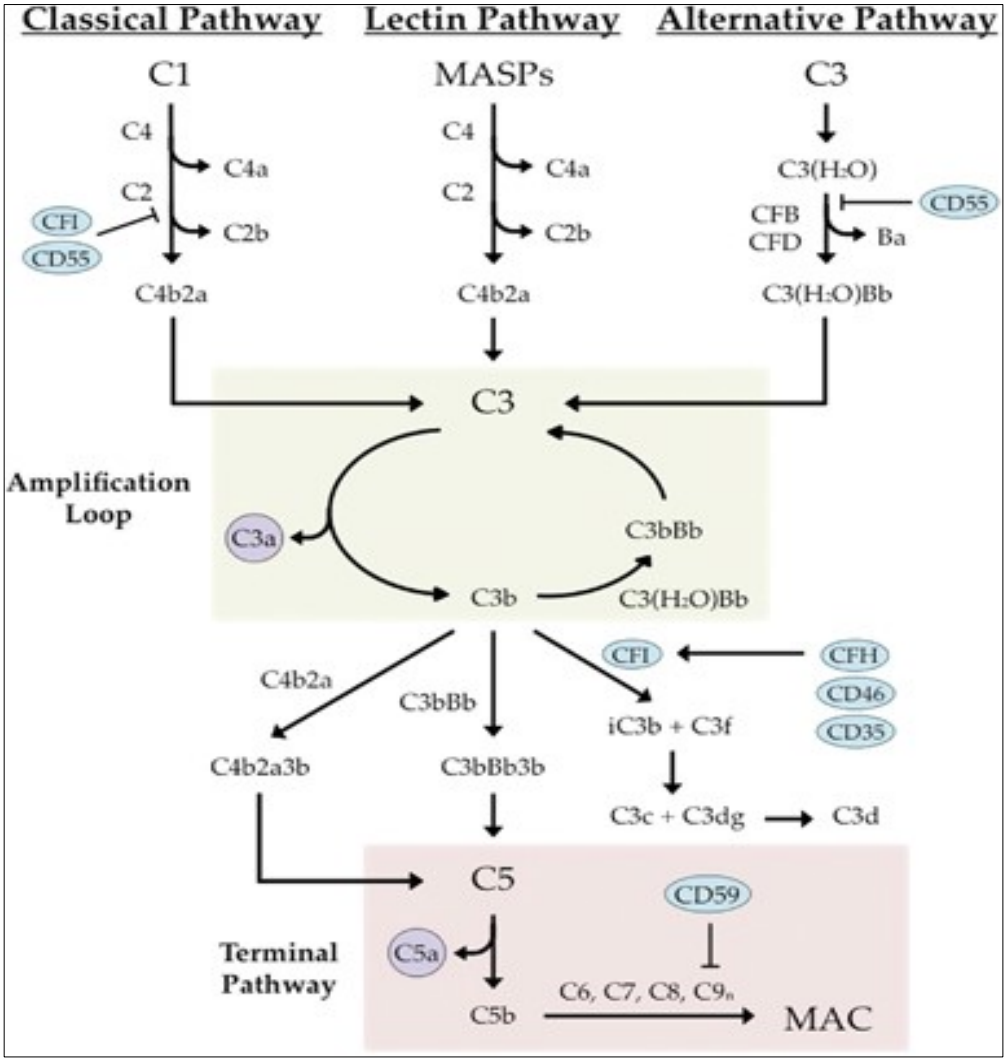

Figure 1.3: Overview of complement system. (Figure adapted from Chirco KR et al., 2017)

Genetic factors controlling immune response in AMD

Complement factor H (CFH) recognizes polyanionic structures such as sulfated glycosaminoglycans (GAG) found on host tissues, and thereby inactivates any C3b that becomes deposited. A single nucleotide polymorphism (SNP) in CFH (Y402H) has been strongly associated with an increased risk of AMD (Hageman GS et al., 2005). This polymorphism, involving a tyrosine-to-histidine coding change, alters the binding

of CFH to sulfated GAGs, as well as to other ligands including C-reactive protein, necrotic cells and bacterial coat proteins. Compared with 402Y allotype, the 402H allotype interacts less well with binding sites within the macula (e.g. Bruch's membrane). Reduced binding of the 402H allotype results in impaired regulation of complement leading to chronic local inflammation, accumulation of drusen and development of AMD. A single copy of the risk-associated haplotype increases the risk of AMD by two- to four-fold, whereas those with a dual copy may increase their lifetime risk by five- to seven-fold (Klein RJ et al., 2005).

Donors homozygous for the high-risk CFHY402H polymorphism were found to have choroidal MAC levels 69% higher than that of the low-risk donors. The choroids of eyes homozygous for the high-risk CFHY402H allele have elevated MAC deposition in the choriocapillaris and are almost 24% thinner compared with low-risk eyes. This pointed to an association between the high-risk CFH genotype and increased choroidal atrophy, due to increase in MAC accumulation around the vessels in high-risk donor eyes (Mullins RF et al., 2011).

The binding affinity between mCRP and mutant CFH (402H) is as much as 45% lower than that of mCRP and wild-type CFH (402Y) as the Y402H variation occurs in the CCP7 domain, which is one of the two CCP regions that interact with mCRP (Lauer N et al., 2011).

Transforming growth factor-β (TGF-β) is a product of both immune cells and RPE cells. TGF-β plays a central role in the normal immunosuppressive intraocular microenvironment mediating the immune response and inhibiting proinflammatory responses in the eye (Stein-Streilein J, (2008). The *HTRA1* gene, a member of the high

temperature requirement A serine protease family, has been shown to play an important role as an antagonist of the TGF-β family proteins. DeWan and associates reported that a SNP in the promoter region of *HTRA1* predisposes patients of Asian descent to AMD, with a 10-times greater risk compared to individuals with wild genotype (Dewan A et al., 2006). Of interest is the fact that the wet type of AMD, albeit more frequently seen without drusen or as a polypoidal variant, is more prevalent in the Asian than in the White population, and the complement factor H Y402H variant is seen less frequently in people with Japanese and Chinese ancestry (less than 5% compared with approximately 35% in White population) (Nussenblatt R and Ferris F, 2007; Ng TK et al., 2008).

Age-related maculopathy susceptibility protein 2 (ARMS2) is a mitochondrial protein, the SNP of which is associated with wet AMD and the frequency of progression from dry to wet forms of the disease but are less associated with dry AMD. In a Spanish population, ARMS2 has been reported to be one of the principal risk factors for AMD (Fuse N et al., 2011).

Weeks et al hypothesised that the effect of smoking on the risk of developing AMD is accentuated by a gene in the 10q26 region of the genome (Weeks DE et al., 2004).

5. Diagnosis of AMD

Clinical evaluation with slit lamp biomicroscopy with 90D or 78D lens, Optical Coherence Tomography (OCT), Fundus Fluorescein Angiography (FFA) and Indocyanine green angiography (ICG) are the main tools in the diagnosis of AMD.

Fluorescein Angiography

H.R. Novotny and D.L. Alvis, two medical students, described the technique of retinal fluorescein angiography (FA) in 1961. Fluorescein dye is injected intravenously, usually through an antecubital vein. White light from a flash is passed through a blue excitation filter. Blue light (wavelength 465-490nm) is then absorbed by unbound fluorescein molecules, and the molecules fluoresce, emitting light with a longer wavelength in the yellow-green spectrum (520-530nm). A barrier filter blocks any reflected light so that the images capture only light emitted from the fluorescein. Images are acquired immediately after injection and continued for ten minutes depending on the pathology being imaged (Singerman L,1986).

Based on the pattern of leakage seen in FA images, CNV can be classified as Classic showing intensely bright fluorescence in early phases of the angiogram and leaks in late phases and occult, in which leakage is less intense and appears in the late phases of disease (Macular Photocoagulation Study Group, 1991).

Indocyanine green angiography

Indocyanine green (ICG), a water-soluble, protein-bound dye, is retained in the choroidal circulation due to its low permeability. This makes ICG angiography ideal for imaging choroidal circulation. One advantage of ICG is its ability to fluoresce better through pigment, fluid, lipid, and haemorrhage than fluorescein dye. This increases the possibility of detecting abnormalities such as CNV that may be blocked by an overlying thin, sub-retinal haemorrhage or hyperplastic RPE on a fluorescein angiogram. This allows enhanced imaging in occult CNV and pigment epithelial detachments (Yannuzzi L et al., 1993).

Optical Coherence Tomography

Developed in 1991, Optical Coherence Tomography (OCT) is the most valuable advance in retinal diagnostic imaging. It is a non-invasive imaging technique relying on low coherence interferometry to generate in vivo, cross-sectional imagery of ocular tissues.

Here, the light source is split between that entering the eye and a reference path. The light reflected back from the two paths forms an interference pattern when the distance in the two paths matches to within the coherence length of the light source. Due to the coherent detection, OCT allows measurements of weakly reflecting retinal layers. This may be measured in the Time domain (taking into account the position of a reference mirror or optical delay line in the reference channel), or in the Spectral domain using a spectrometer or sweeping the light source wavelength and calculating the inverse Fourier transform as a function of wavelength. The high resolution of OCT makes it is possible to obtain images in the living eye that previously were only possible by removing the tissue and examining it under the microscope (Huang D et al, 1991; Gong J et al., 2016).

OCT is very useful in the assessment and diagnosis of dry and wet AMD. It enables clinician to evaluate and measure response to treatment of wet AMD. The high-resolution images have reduced the necessity to perform invasive diagnostic tests like FA and ICG (Gong J et al., 2016).

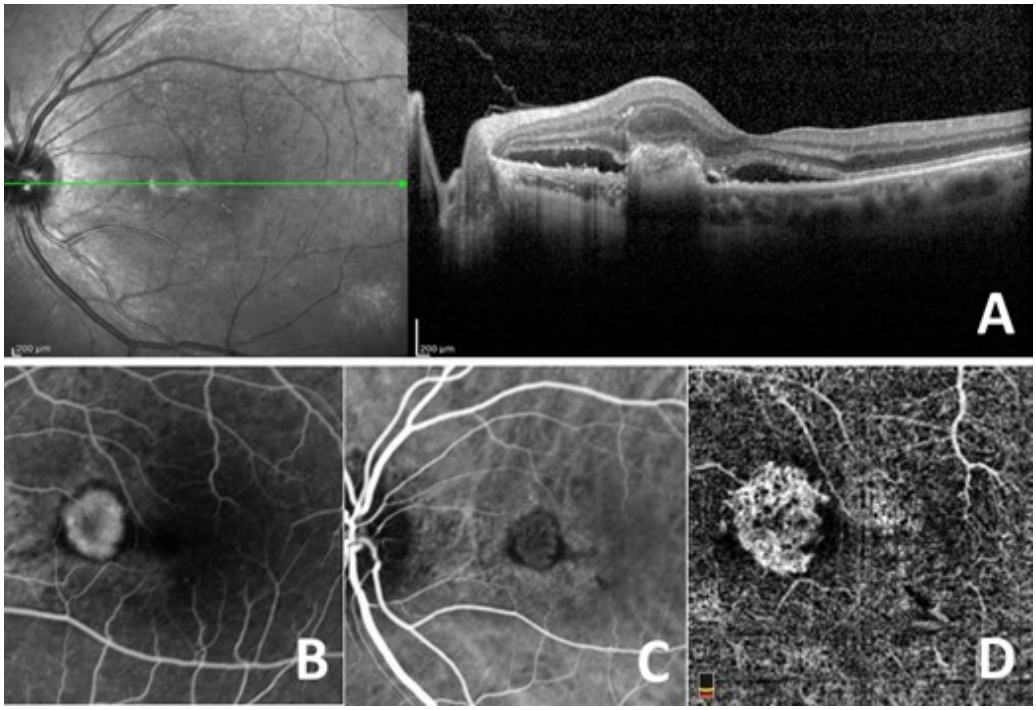

Figure 1.4: Neovascular AMD as seen in (A) OCT B-scan (B) FA (C) ICG (D) OCTA (Figure adapted from Eandi CM et al 2017): (A) OCT B-scan shows a juxtafoveal hyper-reflective area and adjacent subretinal fluid, (B) FA shows a juxtafoveal CNV with well-defined borders, (C) ICG confirms the presence of the well-defined CNV, (D) OCTA demonstrates the capillary CNV with well-defined borders

Optical Coherence Tomography Angiography

Optical coherence tomography angiography (OCTA) is a new imaging technology which allows for non-invasive visualization of normal and pathologic vascularization. OCTA allows for the visualization of both the inner and outer retinal vascular plexi, as well as the choriocapillaris layer, without the need for dye injection and provides a segmentation of the different layers using the en face OCT modality. This imaging technique detects erythrocyte movement by comparing sequential OCT B-scans at a

given cross section. OCT angiograms are co-registered with OCT B-scans from the same area, allowing for simultaneous visualization of structure and blood flow (Eandi CM et al., 2017).

6. Treatment of AMD

Dry AMD

The dry type of AMD affects approximately 80- 90% of individuals with AMD. Initial stages of the disease are asymptomatic. It can cause gradual deterioration of vision as disease progresses. Unfortunately, there is no treatment currently available for dry AMD. Several different treatment strategies are being investigated, including complement inhibition, neuroprotection, and visual cycle inhibitors. Recently, at the American Academy of Ophthalmology (AAO) 2017 Annual Meeting, researchers reported negative findings for two pivotal phase-3 clinical trials of Lampalizumab, exploring complement inhibition as a means of halting the progression of geographic atrophy.

There is some evidence that antioxidants and high doses of vitamin A, C, E, the minerals zinc and copper, and the micronutrient lutein when taken together may help slow down the progression of dry AMD, particularly if AMD has already caused vision changes in one eye.

Age-Related Eye Disease Study (AREDS) was designed to gain better understanding of the natural history and the risk factors of age-related macular degeneration (AMD) and cataract, and to evaluate the effect of high doses of vitamin C, vitamin E, beta-carotene and zinc on the progression of AMD and cataract. The study showed that high levels of antioxidants and zinc significantly reduce the risk of advanced AMD and its associated vision loss (AREDS Research Group Report No. 9. 2001).

Out of the 3640 enrolled participants in AREDS study, data was available from 3597 participants to review the effect of interventions on visual acuity. In patients with extensive intermediate drusen, large drusen, or noncentral GA in one or both eyes, or advanced AMD or visual acuity <20/32 attributable to AMD in one eye (classified as category 3 and 4 patients), treatment with zinc alone or in combination with antioxidants reduced the risk of progression to advanced AMD. Compared with the placebo group, only those participants in Categories 3 and 4 assigned to antioxidants plus zinc had a statistically significant reduction in the odds of a 15-letter or greater visual acuity decrease (P=.008). Findings of AREDS suggest that the estimated 27% odds reduction in the visual acuity outcome for the combination arm may be the combined benefit of the zinc component (odds reduction of 17%) and the antioxidant component (odds reduction of 15%). The visual acuity benefit observed for the combination arm remains consistent for other, more severe, visual acuity outcomes, such as visual acuity worse than 20/100 or a decrease in visual acuity of 6 lines or more. These same nutrients had no significant effect on the development or progression of cataract (AREDS Research Group Report No. 9. 2001).

AREDS 2 studied the effect of omega-3 fatty acids, as well as the antioxidants lutein and zeaxanthin, which are in the same family of nutrients as beta-carotene. Researchers also substituted lutein and zeaxanthin for beta-carotene, which prior studies had associated with an increased risk of lung cancer in smokers. The study found that while omega-3 fatty acids had no effect on the formulation, lutein and zeaxanthin together appeared to be a safe and effective alternative to beta-carotene (AREDS2 Research Group, 2013).

Wet AMD

Wet AMD is responsible for severe visual loss if left untreated. Initially, verteporfin photodynamic therapy was introduced to treat classic or predominantly classic CNV. This was found to prevent severe visual loss in patients with wet AMD (Blinder KJ et al., 2003). In photodynamic therapy, a light-sensitive medicine called verteporfin (Visudyne; Novartis) is injected into the bloodstream, and collects in the abnormal blood vessels under the macula. Laser light is then shone into the eye, which activates the medicine and causes it to create blood clots that block the abnormal blood vessels. By sealing the leaky blood vessels, photodynamic therapy slows down the build-up of fluid under the retina.

Vascular endothelial growth factor (VEGF) is widely considered the main growth factor leading to the increased angiogenesis causing choroidal neovascular membrane. The first breakthrough in anti-VEGF therapy for the treatment of wet AMD treatment was pegaptanib (Macugen; Eyetech Pharmaceuticals/Pfizer) in 2004 (Kourlas H and Schiller

DS, 2006). However, visual decline has been found to continue in AMD patients who were treated with pegaptanib.

Bevacizumab (Avastin; Genentech), first used off-label in 2005 (Yoganathan P et al., 2006), and Ranibizumab (Lucentis; Novartis (FDA approved in June 2006)), (Rosenfeld PJ et al., 2006; Brown, DM et al., 2009), are other two anti-VEGF agents with similar efficacy in treating wet AMD when injected monthly into the eye.

The two pivotal phase III trials (ANCHOR and MARINA) investigated the benefits of Ranibizumab in the treatment of wet AMD. 'Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization (CNV) in Age-related Macular Degeneration' (ANCHOR) trial compared Ranibizumab with Verteporfin photodynamic therapy (PDT) in treating predominantly classic CNV. At 2 years, the visual acuity benefit from ranibizumab was statistically significant (P<0.0001 vs. PDT): 90.0% of ranibizumab-treated patients had lost <15 letters from baseline (vs. 65.7% of PDT patients); 34% to 41.0% had gained >15 letters (vs. 6.3% of PDT group) (Brown, DM et al., 2009)

The 'Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration' (MARINA) study investigated the response of neovascular AMD patients with minimally classic or occult CNVs to Ranibizumab (Rosenfeld PJ et al., 2006). After 24 months 90% of the Ranibizumab-treated patients had lost fewer than 15 letters compared with 53% in the control group. Moreover, 26.1% of patients receiving 0.3 mg Ranibizumab gained at least 15 letters of visual acuity versus only 3.8% in the sham-injected patients.

Bevacizumab, showed promising results in a published series for treating neovascular AMD with similar visual outcomes and reduction of retinal thickness, when compared with the licensed intravitreal Ranibizumab (IVR). However, Ranibizumab was noted to be more effective in reducing the lesion size (Comparison of Age-related Macular Degeneration Treatments Trials (CATT) Research Group; IVAN Study Investigators, 2012).

Aflibercept (VEGF Trap-eye, Eylea, (Bayer)) was approved in November 2011 by FDA for the treatment for wet AMD (Heier JS et al., 2012). The binding affinity and long half-life of this agent present the possibility of cost savings and decrease in frequency of use.

The 'VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD' (VIEW 1 and VIEW 2) studies demonstrated that intravitreal Aflibercept, dosed every 2 months after three initial monthly doses, was non-inferior and clinically equivalent to monthly Ranibizumab injection. Furthermore, patients in the intravitreal Aflibercept (IVA) 8-weekly group achieved visual and anatomical improvements similar to the IVR group with a mean of five fewer injections over 96 weeks (Heier JS et al., 2012).

Late AMD is characterized by irreversible cell loss, initially of RPE cells and subsequently of neuroretinal and choroidal cells. da Cruz et al recently published results of phase 1 study of successful delivery and survival of the Retinal Pigment Epithelial (RPE) patch on two patients with advanced wet macular degeneration. This work supports the feasibility and safety of human embryonic stem cell (hESC)- RPE patch transplantation as a regenerative strategy for AMD (da Cruz L et al., 2018).

B. METABOLOMICS: AN OVERVIEW

Eye diseases are often multifactorial in origin, with several environmental and genetic risk factors at play. In most cases, pathogenetic mechanisms remain poorly understood. 'Omics', with the use of DNA sequencing, mass spectrometry and bioinformatic analyses, attempts large-scale characterization and quantitation of biological molecules in whole systems (Lauwen S et al, 2017). This is particularly useful in human diseases where the aetiopathology is poorly understood and can provide novel insights to enhance our understanding of disease processes.

Omics technique (Figure 1.4) include:

- **Genomics**
    - Genome wide association studies (GWAS) (Bush WS and Moore JH, 2012).: DNA samples of large case-control cohorts are genotyped using microarrays, targeting a large number of single nucleotide polymorphisms (SNPs) distributed over the genome. Subsequent statistical analysis determines, for each variant, whether the frequency is significantly different in cases compared to controls. This helps to detect genetic variants that are mostly common in the population. To identify rare variants, whole exome sequencing (WES), or whole genome sequencing (WGS) is more effective. WES identifies and sequences the DNA that codes for the proteins (exons) while WGS determines the complete DNA sequence of the genome at a single time (Fritsche LG, Fariss RN, 2014).

- **Epigenomics**
  - Refers to the analysis of functionally relevant changes to the genome that do not involve a change in the DNA sequence, such as DNA methylation and different types of histone modifications (Dirks RA et al., 2016).
- **Transcriptomics**
  - Refers to the analysis of all the mRNA molecules transcribed from the genome, either using microarrays or RNA sequencing (RNAseq). RNAseq is preferred as it uses next generation sequencing (NGS) to identify the presence and quantify RNA in a biologic sample (Mantione KJ et al., 2014).
- **Proteomics**
  - Refers to the study of proteins and their modifications.
- **Metabolomics**
  - Refers to the study of types and quantities of various metabolites in biological fluids or tissues. It is a powerful discipline that attempts to characterize the complex time-dependent metabolite profiles within biofluids and tissues.

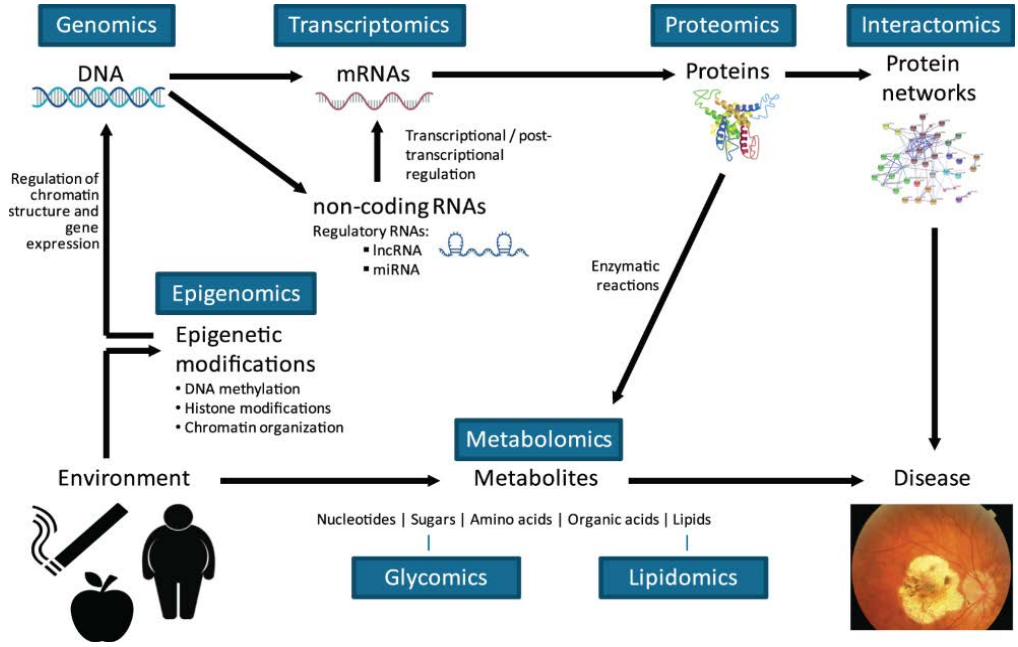

Figure 1.5: Overview of the different layers within a biological system contributing to multifactorial diseases and their relation to each other. (Figure adapted from Lauwen S et al., 2017)

While all of these approaches can offer novel insights into the genetics and/or aetiopathology of human disease, metabolomics has some advantages since it measures products derived from the function of genes and proteins and thus gives an overall assessment of the potential changes suggested by gene and protein expression profiling. In other words, it analyses the overall metabolic activity of an organism, and so metabolomics and its application to diseases will be described further.

Metabolomic analysis

Metabolism underpins the function of all cells and tissues and the metabolites used and produced by particular cell types reflect their function. A metabolic profile of a tissue or biofluid can be influenced by genetic factors, age, lifestyle, environmental factors,

nutritional status, drugs and metabolites from gut microflora. Once the noise from the day-to-day variation is isolated, it gives a stable, natural and invariable profile of that subject. Assfalg et al in a study of healthy donors generated metabolic fingerprints using one-dimensional ^{1}H-Nuclear Magnetic Resonance (NMR) spectroscopy that identified strong characteristics of each donor sample making it possible to identify individual donors from their metabolic profile with 100% probability (Assfalg M et al., 2008). This demonstrates the ability of the approach to discriminate specific phenotypes and so suggests that perturbation of these in disease might be identifiable. Although this study used NMR spectroscopy to derive the metabolic profiles, these can also be obtained by other analytical techniques, in particular, mass spectrometry.

NMR spectroscopy and Mass spectrometry

The nuclear magnetic resonance phenomenon in solids and liquids was discovered by Bloch and Purcell in 1945. When radiofrequency energy is applied to nuclei within a sample, the energy can be absorbed and re-emitted, and the frequency of the transmitted signal reflects the molecular environment of the nuclei. NMR spectra arise from transitions made by atomic nuclei between different energy states. When pulses of electromagnetic radiation are applied at frequencies that precisely match energy gaps, a stronger, coherent signal is emitted by nuclei within the sample and these emitted signals allow identification of molecular structures. Nuclei in different chemical environments (e.g. the different ^{1}H nuclei in a protein) will resonate at different frequencies, producing a plot of intensity against resonance frequency known as a 1D NMR spectrum. The 2D ^{15}N-HSQC (heteronuclear single-quantum coherence) spectrum, shows a signal for each covalently bonded ^{1}H-^{15}N group. Each signal in this

spectrum has an intensity and two chemical shifts (one for the ^{1}H and another for the ^{15}N nucleus) and the spectrum plotted provides a topographic map. Further analysis allows identification of the molecular structures within a sample (Beckonert O et al., 2007).

In mass spectrometry, molecular structure is probed by bombarding molecules with a beam of high energy electrons, causing ionization of proteins or metabolites, followed by mass spectrometric analysis of the mass/charge (m/z) ratios of a peptide or metabolite. The metabolite can then be identified from its precise mass and charge from public databases (Mishur R et al., 2012; Bowrey H et al., 2016).

While mass spectrometry is a chemical process, NMR spectroscopy is a physical process. Mass spectrometry is highly specific and sensitive, however, it measures mass and charge, so it cannot always delineate between two molecules of similar mass. NMR spectroscopy is somewhat less sensitive but the signal derived is directly related to the concentration of a molecule in a sample and it can identify molecules by their structure. Thus, each approach has advantages and disadvantages.

Metabolomics in systemic diseases

Metabolomic analysis of biofluids has been applied to successfully discriminate patients in terms of prognosis and diagnosis of human diseases such as diabetes, high blood pressure and cancer. Identifying metabolomic profiles may also help to predict a response to treatment.

Changes have been noted in the urinary metabolic profile of those patients with rheumatoid (RA) and psoriatic arthritis (PsA) who received anti-TNF therapy; different metabolic profiles were also observed with different medications. Baseline urine metabolic profiles histamine, glutamine, xanthurenic acid, and ethanolamine discriminated between RA patients who did or did not have a good response to anti-TNF therapy with a sensitivity of 88.9% and a specificity of 85.7%. In both RA and PsA, urinary metabolic profiles changed between baseline and 12 weeks of anti-TNF therapy. Within the responders, urinary metabolite changes distinguished between etanercept and infliximab treatment. These authors suggested that TNF-driven cachexia was driving the production of muscle degradation products in those patients who responded to anti-TNF, thus providing and explanation for the predictive value of the metabolite profiles (Kapoor SR et al., 2013).

The serum metabolic profile of patients with established rheumatoid arthritis was found to be different from that of healthy controls. Inflammation appeared to be driving these differences since the researchers were able to stratify patients according to the level of inflammation, with the metabolic profile correlating with their C-reactive protein levels (Young S et al., 2013). An increased concentration of metabolites such as low-density lipoprotein lipids, lactate, glucose, taurine, acetylglycine, and methylguanidine seen in RA patients correlated well with the measured CRP level. Another metabolite of interest was 3-hydroxybutyrate which was elevated in the patients with established RA compared to controls, suggesting an increased level of lipolysis in RA patients. A noticeable change was low level of lipid signals in patients with established RA. Indeed, changes in lipid levels have been noted previously in patients in the period before they developed full RA suggesting that the lipid changes may be important in the

development of the disease (Myasoedova E et al., 2010; Kapoor SR et al., 2013; Young SP et al., 2013).

In a study of coronary heart disease, analysis of serum NMR spectra successfully differentiated between varying degrees of coronary artery stenosis that ordinary blood tests failed to recognise. The change in the circulating level of metabolites from immediately before exercise to immediately after exercise was calculated separately in cases (patients with inducible ischaemia) and controls (with no ischaemia). Concordant decrease in levels of lactate, and increase in levels of hypoxanthine, inosine and alanine was noted in both cases and controls. However, γ-aminobutyric acid, oxaloacetate, citrulline and argininosuccinate was noted to decrease in cases but not in controls. Further, oxaloacetate and citrulline levels correlated with the extension of perfusion defect. These metabolites belonging to the citric acid pathway showed statistically significant relation to the probability of ischemia. The citric acid cycle plays a central role in oxidative phosphorylation in the myocardium. In normal cardiomyocytes, there is a constant rate of mitochondrial efflux of citric acid cycle intermediates. This stresses the need for preservation of citric acid cycle intermediates to defend ATP production in acute ischaemia (Sabatine M, 2005).

In NMR spectroscopy of cerebrospinal fluid (CSF) (Sinclair et al., 2009), patients with multiple sclerosis (MS) showed higher CSF levels of lactate, creatinine and fructose, compared to control patients. Concentration of α-hydroxyisobutyrate, a partial degradation product of branched chain amino acids, was also found to be high in MS patients. This is thought to be secondary to respiratory-chain deficiency, leading to impaired oxidation of NADH. In a second study (Reinke et al), mean levels of choline,

myo-inositol and threonate were found to be increased in CSF, whereas 3-hydroxybutyrate, citrate, phenylalanine, 2-hydroxyisovalerate and mannose were decreased in MS-derived CSF ($p<0.05$), suggesting alterations to energy and phospholipid metabolism.

Patients with idiopathic intracranial hypertension (IIH) showed high levels of lactate, glucose, acetate, 3-hydroxybutyrate and oxaloacetate, and low levels of citrate, urea, creatinine and 3-hydroxy isovalerate in their CSF. Predominant lactate peaks suggest a possible inflammatory aetiology in IIH. It could also indicate anaerobic metabolism due to reduced blood supply resulting from compressed vasculature in IIH patients. The interpretation of CSF metabolites is complex as the metabolites may reflect systemic metabolism and subsequent transfer across the blood brain barrier or local CNS generation (Sinclair et al., 2010).

Metabolomics and the eye

In preliminary experiments, research team at the University of Birmingham investigated the use of NMR-based metabolomics to compare serum samples from healthy elderly and young individuals and showed that a clear stratification is possible. In a longitudinal study of metabolites in the blood and urine of healthy controls, little variability was noted between subjects and study days, suggesting that metabolomic data have acceptable variability and may highlight biomarkers of disease (Lenz EM et al., 2003).

Metabolomics has been employed to explore the various pathways involved in the pathogenesis of specific ocular conditions. Some of the pathways thus identified include

pathways of urea metabolism, inflammation, and lipid metabolism; these are some of the pathways explored in the pathogenesis of AMD.

- **Uveitis**

The metabolomic profiling of samples of vitreous humour from patients with ocular inflammatory conditions demonstrated its ability to differentiate between lens-induced uveitis (LIU) and chronic uveitis (CU), with a sensitivity of 78% and specificity of 85%, with urea, oxaloacetate and glucose all being raised in LIU samples compared to CU samples. Both urea and oxaloacetate are part of urea cycle, urea being produced during conversion of arginine to ornithine. This process is prominent in activated macrophages and endothelial cells, suggesting more active inflammation in the LIU patients (Young SP et al., 2009).

- **Glaucoma**

In primary open angle glaucoma (POAG) metabolic analysis of sera showed altered lipid metabolism, separating them from control samples. The metabolites palmitoyl carnitine, sphingolipids, vitamin D-related compounds, and steroid precursors were identified. These metabolites suggested involvement of galactose metabolism, fructose and mannose metabolism, and steroid hormone biosynthesis pathways in the pathogenesis of POAG (Burgess L et al., 2015).

- **Myopia**

The heritability for refractive error has been calculated to be larger than 50%. GWAS and WES studies performed in families have suggested some of the possible myopia-associated pathways; however, still only 3.4% of all genetic variability of refractive error can be explained. Metabolomic analysis identified 29 metabolites in aqueous

humour of myopes compared to controls. More than half of the changed metabolites were highly and positively associated, giving insight into important roles of pathways involved in the metabolism of these metabolites in relation to high myopia. Among the metabolites, four amino acids glutamine, N-alpha-acetyl-L-ornithine, nicotinoylglycine, and o-hydroxyhippuric acid were significantly increased. This indicates that metabolism of amino acids played a vital role in the development of high myopia. Glutamine 1 was reported to be associated with the generation of reactive oxygen species (ROS), which may indicate an increased oxidative stress in patients with high myopia (Ji Yet al., 2017).

- **Dry eye syndrome**

Analysis of reflex tears from patients with dry eye disorders using ^{1}H NMR spectroscopy showed different metabolites in those with dry eyes compared to controls. The most abundant molecules in the control tears were glycoprotein, lipids, cholesterol, leucine, glycerol, and glutamate. The discriminating analysis of the dry eye syndrome group (DESG) revealed a significant increase in glucose and lactate, whereas formate and N-acetylglucosamine noticeably decreased in the moderate DESG compared to the mild DESG. The presence of lactate in human fluids is a consequence of anaerobic and aerobic metabolism of glucose. It also indicates a deficiency in the mitochondrial respiratory chain arising from the oxidative stress, causing decreased oxygenation and increased lactate production (Galbis-Estrada C et al., 2015).

Further analysis following administration of antioxidant and essential polyunsaturated fatty acids supplements in DESG showed difference in metabolites before and after supplementation. H cholesterol, N-acetylglucosamine, glutamate, amino-n-butyrate,

choline, glucose, and formate were detected before supplementation while choline/acetylcholine were seen in samples tested after supplementation (Galbis-Estrada C et al., 2015).

In another metabolome-wide association study of dry eye syndrome patients, dryness and irritation symptoms were found to be particularly strongly associated with decreased androgen steroid metabolites, especially epiandrosterone sulfate, further strengthening the hypothesis on the links between androgen and ocular surface diseases (Vehof J et al., 2017).

- **Diabetic eye disease**

Diabetes-induced corneal pathologies are known to delay corneal wound healing. Researchers studied the effects of diabetes on extracellular matrix, lipid transport, and cellular metabolism by defining the entire metabolome and lipidome of Type 1 and Type 2 human diabetic corneal stroma. Metabolomics analysis identified elevated tryptophan metabolites, independent of glucose metabolism, which correlated with upregulation of the Kynurenine pathway in diabetic corneas. A significant upregulation of novel biomarkers aminoadipic acid, D,L-pipecolic acid, and dihydroorotate was also noted, thus linking aberrant tryptophan metabolism to end-stage pathologies associated with diabetes indicating the potential of the Kynurenine pathway as a therapeutic target for inhibiting diabetes-associated defects in the eye (Priyadarsini S et al., 2016) .

In another study, plasma metabolites were identified that distinguished patients with diabetes and retinopathy from those without retinopathy. Two novel biomarkers were identified, 2-Deoxyribonic acid and 3,4-dihydroxybutyric acid. The researchers also identified pentose phosphate pathway as a key metabolic dysregulation associated with

DR, demonstrating the involvement of oxidative stress in disease pathogenesis (Chen L et al., 2016).

- **Leber's hereditary optic neuropathy**

In a study of one of the commonest mitochondrial DNA-related disease, Leber's hereditary optic neuropathy, metabolomics identified thirty-eight metabolites: decreased concentrations of all proteinogenic amino acids, spermidine, putrescine, isovaleryl-carnitine, propionyl-carnitine and five sphingomyelin species, together with increased concentrations of 10 phosphatidylcholine species. This suggested an involvement of the endoplasmic reticulum. All these changes could be reversed by the endoplasmic reticulum stress inhibitor, tauroursodeoxycholic acid (TUDCA) (Chao de la Barca JM et al., 2016).

- **Macular degeneration**

Metabolomics is also being employed to understand the pathways involved in age related macular degeneration (AMD). Initial studies of sera with mass spectrometry and then with NMR spectroscopy have shown promising results in separating AMD patients from controls. The metabolites identified included di- and tripeptides, covalently modified amino acids, bile acids, and vitamin D-related metabolites. The pathway analysis has shown broader changes in tyrosine metabolism, sulphur amino acid metabolism, and amino acids related to urea metabolism (Osborn M et al., 2013; Laíns I et al., 2017).

Previous works assessing the metabolite profile of patients with systemic and ocular diseases showed a strong segregation of different disease groups and demonstrated a relationship between serum markers and the metabolite profile. It seems that the

multiplexed analysis inherent to metabolomics, which considers all metabolite signals regardless of whether they have been specifically identified, is able to provide information not available by other means. This strongly suggests that metabolomics may provide important insights into the role of changing metabolites in the AMD pathological process. This provides the basis of our research hypothesis that the metabolic characteristics of patients with dry and wet AMD are different and can identify pathways involved.

Chapter 2

METHODOLOGY

Methods

Ethics approval was obtained from NRES committee North East- Sunderland (REC reference number- 11/NE/0162).

Patients with dry and wet macular degeneration were recruited from the medical retina clinics at Royal Victoria Infirmary, Newcastle upon Tyne. 104 consecutive patients who had AMD confirmed by clinical evaluation and investigations were included in the study. Informed consent was obtained. Detailed history including duration of symptoms, smoking habits, general medical history and systemic treatment, if any, were noted. Patients with diabetes mellitus were not excluded, and BMI was not measured for the participants. Blood and urine samples were collected from a total of 104 patients; 73 patients had wet and 31 had dry macular degeneration. Patients in both groups were matched in every respect including age, sex and systemic illnesses. Being matched in all respects, except the type of macular degeneration, we inferred that any changes we may see will be due to the type of the disease and as such a healthy control group was not deemed necessary.

Inclusion criteria

Patients over 55 years of age with confirmed diagnosis of age related macular degeneration.

Exclusion criteria

Patients who were unable to give consent and those with known history of cancer or severe inflammatory disease, e.g. rheumatoid arthritis, were excluded from the study.

Sample collection

20ml of a "random" urine sample (i.e. the timing of the collection was not controlled) provided by the patient was collected into a universal container and was frozen immediately in a -80 ^{o}C freezer.

Non-fasting blood was collected from patients in a red top vacutainer serum tube (with clot accelerator). The tube was left to stand for an hour in a refrigerator, before centrifugation in the cold at 3000 rpm for 10 minutes. Then, 2 ml serum was transferred into a cryovial using a Pasteur pipette, marked with the patient's ID and date, then immediately frozen in a -80 ^{o}C freezer. The samples were transported on dry ice to the academic unit at University of Birmingham. The samples were then stored at -80 ^{o}C in a freezer in the Institute of Biomedical Research, at University of Birmingham until analysis.

Laboratory analysis: sample preparation and analysis

After thawing, serum was centrifuged for 5 mins at 15,000 x g(av) and an aliquot (200 microL) was removed and filtered by centrifuging at 10,000 x g(av) for 10 mins at 4 ^{o}C through a centrifugal 3kD MW cut-off filter to remove proteins. The filters had been previously washed 6x by centrifuging through warm water (0.5ml at 3,000 x g(av)) to remove preservative glycerol. The protein-free filtrate was made with 10% in D_2O, 20mM phosphate 0.5mM TMSP and pH 7.00 by the addition of a 4x concentrated "NMR buffer".

Urine samples were defrosted on ice, filtered to remove any cells or bacteria. 500 microL of the sample was transferred into a fresh microcentrifuge tube, and centrifuged at high

speed (13,000xg) for 5 minutes. Then D_20, phosphate buffer and TMSP was added NMR standard. The filtrate was made 10% in D_2O, 20mM phosphate 0.5mM TMSP and pH 7.00 by the addition of the 4x NMR buffer. The pH of urine can be variable and so each sample was carefully taken to pH 7.00 by the addition of NaOH or HCl. The samples were left for 30 mins at room temperature and the pH adjusted again to 7.00.

Samples of urine or serum (50 microL) were transferred to small conical glass vials, capped and frozen at -80 ^oC until analysis. For analysis, samples were defrosted and a sample (35 microL) was transferred to a 1.7mm glass NMR tube using an Anachem robotic sample handler. The tubes were capped, wiped and placed in rack in the spectrometer.

One-dimensional ^1H spectra were acquired using a NOESY sequence with water saturation with pre-sat on a Bruker DRX 600 MHz NMR spectrometer equipped with a 1.7 mm cryoprobe. 2D JRes spectra also was acquired to aid metabolite identification by comparison with the Birmingham Metabolite Library.

NMR spectroscopy requires samples to contain concentrations of target molecules in the high micro molar range for it to be detectable due to its low sensitivity. However, the cryoprobe in the NMR spectroscope enabled us to minimize electronic noise and maximize its sensitivity to the small NMR signals from dilute samples. This made it possible to acquire well-resolved spectra from the samples in a short span of time.

Spectra were segmented into 0.005 ppm (2.5 Hz) chemical shift ‘bins’ between 0.2 and 10.0 ppm, the spectral area within each bin integrated. This turns each spectrum from a continuous analogue signal into an array of data points. Each spectrum is segmented into

approximately 2,000 regions and the area under each segment integrated. The total spectrum of each sample is normalised. The binned data describing each spectrum is compiled into a matrix with each row representing an individual sample. The spectra contain both dominant and less intense peaks. Once log-transformed, the small and large peaks will have equal weighting.

Multivariate analysis of samples

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) of the processed data were conducted using PLS Toolbox (Eigenvector Research) within Matlab.

One of the difficulties inherent in multivariate statistics is visualizing data that has many variables. In data sets with many variables, groups of variables often move together. The multiple variables are simplified by replacing a group of variables with a single new variable. PCA is the most widely used multivariate analysis method for achieving this simplification. The method generates a new set of variables, called principal components (PC). Each principal component is a linear combination of the original variables. All the principal components are orthogonal to each other, so there is no redundant information. The principal components as a whole form an orthogonal basis for the space of the data. PCA of the transformed datasets assesses the covariance around the mean of the data in each region of the spectrum and assesses how they relate to each other. The first principal component is a single axis in space. Each observation projected on that axis form a new variable. And the variance of this variable is the maximum among all possible choices of the first axis. The second principal component

is another axis in space, perpendicular to the first. Projecting the observations on this axis generates another new variable. The variance of this variable is the maximum among all possible choices of this second axis. The PCs describe the direction of the greatest variation in the data, with 2 or 3 PCs explaining the major covariance trends within the data. In effect, PCA identifies the most highly variable metabolites in the NMR spectra (Cho H et al., 2008).

The separation seen in the PCA is further enhanced in the clustering analysis, PLS-DA. While PCA is an unsupervised model, PLS-DA is a supervised analysis. This technique builds a model which identifies the metabolites which vary between diagnostic groups, potentially enabling the identification of diagnostic groups.

A venetian blind is used to cross validate the PLS-DA model, a method which re-assigns randomly selected spectral data to the PLS-DA model to determine the sensitivity and specificity of the model in correctly assigning diagnostic groups. This helps to determine the accuracy of the model in correctly assigning class membership. PLS-DA allows a predictive model to be generated and the validated model segregated the dry and wet AMD with a sensitivity and selectivity of 73.3% and 87.5%.

Statistical analysis

Multivariate analysis methods help to identify biologically relevant spectral features for further targeted analyses. The two methods commonly used are principal component analysis (PCA) and partial least squares projection to latent structures (PLS).

The strength of metabolomic analysis is not based strictly on numbers, as it is the total profile that is being measured. There will be differences between normal individuals based on lifestyle, but the disease specific profile stands out from this noise, as has been shown in previous studies (Assfalg et al., 2008; Young S et al., 2009 and 2013).

The techniques utilised were (a) Principal components analysis (PCA) using the PLS Toolbox (version 5.8) (Eigenvector Research) in Matlab (release 2010a) (Mathworks), and (b) Partial least square discriminant analysis (PLS-DA) using PLS Toolbox (details given above).

Principal Components Analysis (PCA) is an unsupervised method that facilitate the extraction of implicit patterns and elicit the natural groupings of the spectral dataset without prior information about the sample class (Jensen et al., 2004; Beckonert et al., 2003). The PCA helps to arrive at a linear transformation preserving the variance in the original data as possible. The unsupervised nature of the PCA algorithm reveals group structure when 'within-group' variation is sufficiently less than 'between-group' variation. The results of PCA analyses was used to formulate an initial biological conclusion, which was then verified with the supervised forms of discriminant analysis such as Partial Least Squares (PLS-DA) that rely on the class membership of each observation (Cho H et al., 2008).

PLS-DA is used to predict a continuous or discrete/categorical variable. It helps to optimise separation between different groups of samples, by linking two data matrices X (raw data) and Y (groups, class membership etc.). Orthogonal signal correction PLS-DA or O-PLS-DA is an extension of PLS-DA which seeks to maximize the explained variance between groups in a single dimension or the first latent variable (LV), and separate the

within group variance (orthogonal to classification goal) into orthogonal LVs (Wold S et al., 1998). Therefore, orthogonal PLS-DA compared to predictive analysis utilises information related to structured variance removing residual variance or noise. Orthogonal data is therefore based on many fewer variables than in predictive analysis (Johnson C et al., 2016).

The PCA helps to arrive at a linear transformation preserving the variance in the original data as possible. The unsupervised nature of the PCA algorithm reveals group structure when 'within-group' variation is sufficiently less than 'between-group' variation. The results of PCA analyses was used to formulate an initial biological conclusion, which was then verified with the supervised forms of discriminant analysis such as Partial Least Squares (PLS-DA) that rely on the class membership of each observation. Once classification is done in PLS-DA, further classification is performed in the SIMCA (Soft Independent Modelling of Class Analogy).

The quantitative NMR spectral profiling using Human Metabolome Database, BAYESIL and Chenomx was utilised to identify the metabolites.

The Human Metabolome Database (HMDB) is an electronic database containing detailed information about small molecule metabolites found in the human body. The database contains or link three kinds of data: 1) chemical data, 2) clinical data, and 3) molecular biology/biochemistry data. The database contains 74,462 metabolite entries including both water-soluble and lipid soluble metabolites as well as metabolites that would be regarded as either abundant (> 1 uM) or relatively rare (< 1 nM). Additionally, 5,701 protein sequences are linked to these metabolite entries (Wishart D et al., 2007; 2013).

BAYESIL is a web system that automatically identifies and quantifies metabolites using 1D ^{1}H NMR spectra of ultra-filtered plasma, serum or cerebrospinal fluid. It can autonomously find the concentration of NMR-detectable metabolites accurately (~ 90% correct identification and ~ 10% quantification error), in less than 5 minutes on a single CPU (Ravanbakhsh S et al., 2015).

CHENOMX on the other hand, offers a method of simplifying the measurement of the concentration of biological mixture components as measured by NMR instruments. The method is based on software and an extensive spectral signature library of relevant metabolites. It consists of both theoretical and empirical data within a model which adjusts to the NMR field strength and sample conditions to closely match that of the experiment spectrum. Then the software, through a combination of automated and supervised operation, uses the models to de-convolute the spectral signatures of the various components in the mixture being measured. Overlapping spectral areas are simplified by the subtraction of the identified peaks of known components from the spectrum as they are fitted into the spectrum. Then the 'left over' spectral peaks are made visible to be compared to other compound signatures (Ellinger J et al., 2013).

Chapter 3

RESULTS

A total of 104 consecutive patients, aged between 55 and 94 years, who attended medical retina clinics and met the inclusion criteria were enrolled into the study. All 104 patients provided blood samples; random urine sample was given by 103 patients. Fourteen patients with wet AMD and 4 patients with Dry AMD were diabetic. Ethnicity data was not collected. The demographic detail of our sample group is given in Table 3.1 below.

Total number of patients	104	
Patients with Dry AMD	31	
Dry AMD patients with GA	8	
Patients with Wet AMD	73	
Wet AMD patients on anti VEGF treatment (Ranibizumab)	45	
Serum samples	104	
Urine samples	103	
	Wet AMD	Dry AMD
Males	27	11
Females	46	20
Smokers	31	4
Non-smokers	42	27

Table 3.1: Demographic details of patients in our cohort

Analysis of all serum samples

All 104 serum samples were processed and analysed using NMR spectroscopy. The NMR spectra were then further analysed using human metabolome database (HMDB), Bayesil spectral analysis (Bayesil.ca) and Chenomx to identify the significant individual metabolites.

The samples were first analysed using PCA to identify any trends or clustering in the standardised data, and the maximum variation of the data. PC1 (30.02%) showed clustering either side of median, while PC2 (19.34%) showed a potential separation of two populations within the cohort of samples. There were a couple of outliers that could not be explained by the demographic data (Figure 3.1) and may have arisen from technical issues with the spectra.

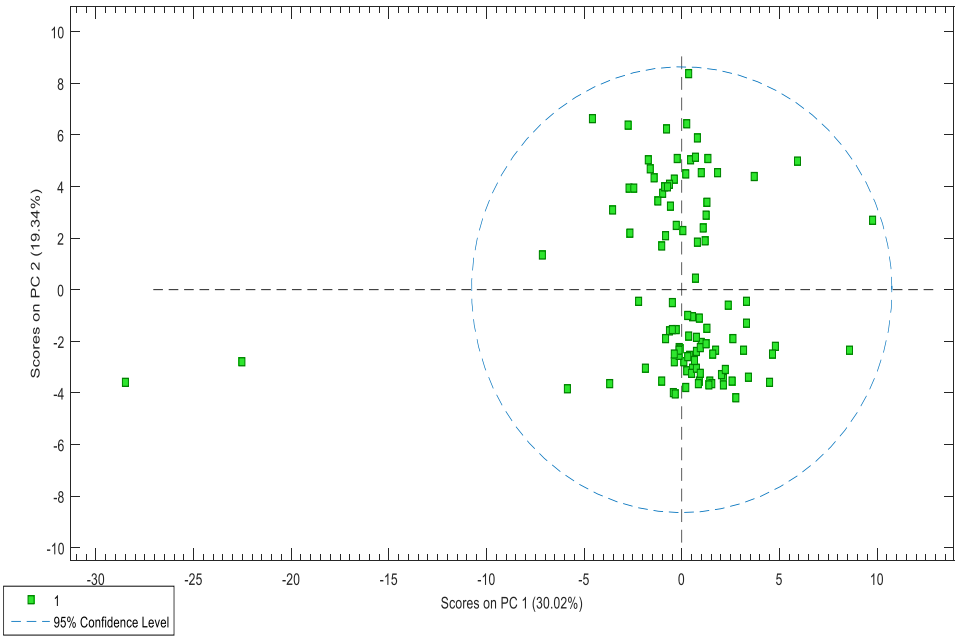

Figure 3.1: Principal Component Analysis of all serum samples (N= 104)

When the samples were analysed on the basis of dry or wet AMD diagnosis, PCA analysis showed no obvious clustering in either component in serum samples from patients with dry (dark blue) and wet (light blue) in each quadrant (Figure 3.2).

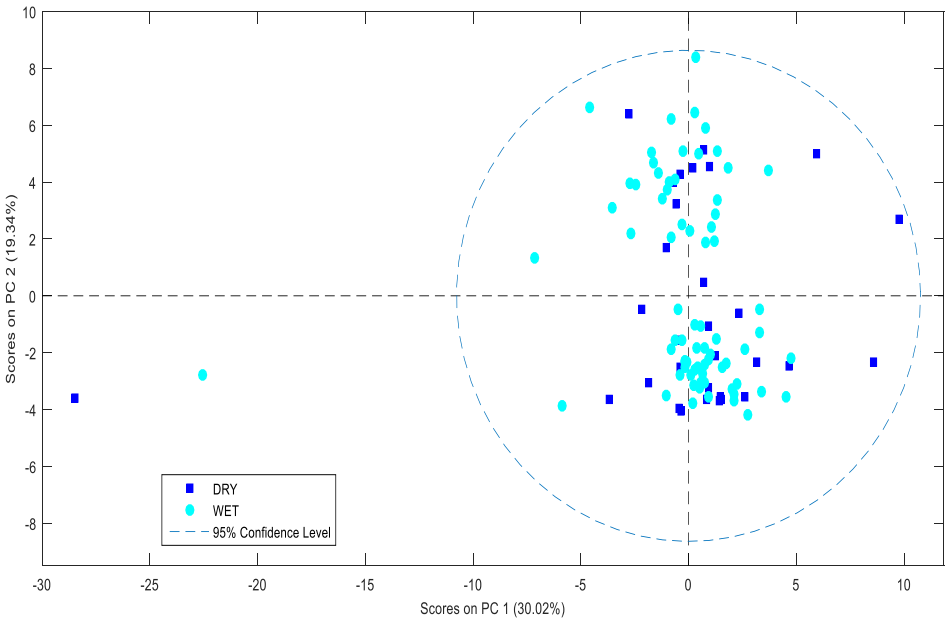

Figure 3.2: Principal Component Analysis of all serum samples (N= 104) showing wet and dry AMD samples

To further investigate any segregation of the data, a supervised PLS-DA analysis of the NMR spectra of the serum samples was done and this showed clustering of the majority of samples of patients with wet AMD (Figures 3.3 and 3.4), and this difference was augmented after orthogonal signal correction was applied (Figure 3.4). The cross-validated model segregated the dry and the wet AMD with a sensitivity and specificity of 73.3% and 87.5% respectively. However, it also showed overlap of samples of patients with wet and dry AMD samples.

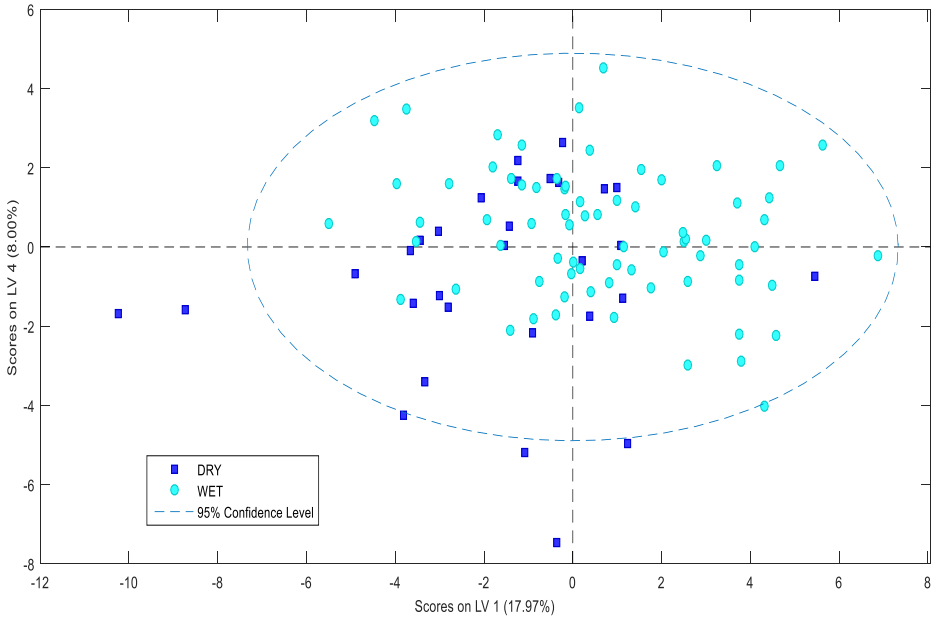

Figure 3.3: Partial Least Squares Discriminant Analysis (PLS-DA) of all serum samples (N= 104) showing clustering and separation of wet and dry AMD samples

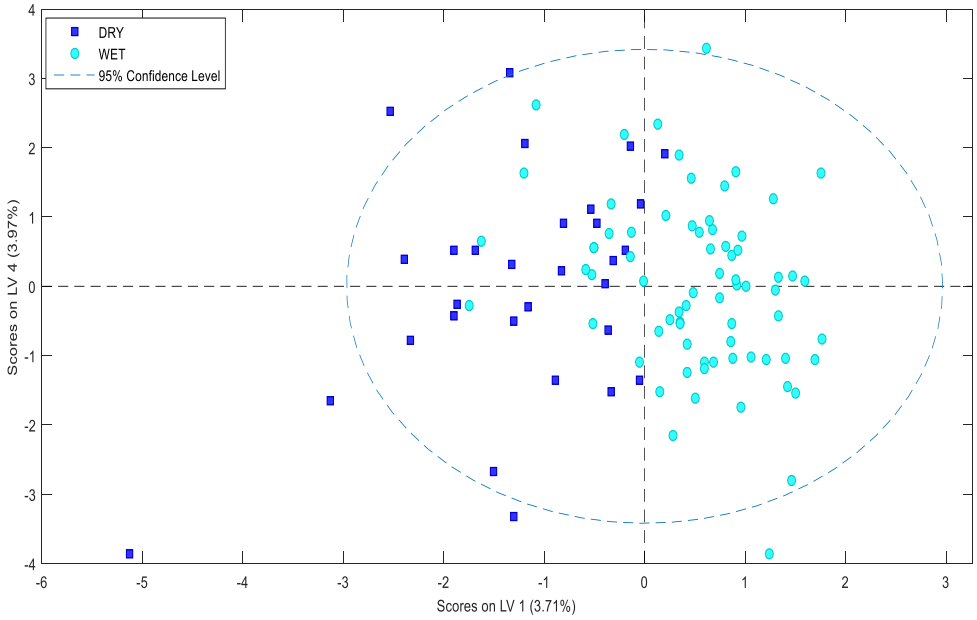

Figure 3.4: Partial Least Squares Discriminant Analysis (PLS-DA) of all serum samples (N= 104) after orthogonal signal correction was applied showing more clustering and separation of wet and dry AMD samples

Metabolites in wet and dry AMD serum

The loading plots from the PLSDA models identified which regions of the NMR spectra were contributing to the discrimination between the wet and dry sample groups. These loading plots were further analysed using human metabolome database (HMDB), Bayesil spectral analysis (Bayesil.ca) and the Chenomx library on NMR spectra, to identify significant individual metabolites associated with such regions of the spectra.

The metabolites identified in the wet AMD serum samples which dominated in the loading plot indicated increases in lactate, xanthine, gallic acid, pyridoxal, glycine and succinate, while the dry AMD sample showed increases in arginine, methyl guanine, 2,5 dimethyl furan, uracil, gallic acid, succinic acid and ornithine (Fig 3.5, 3.6; Table 3. 2).

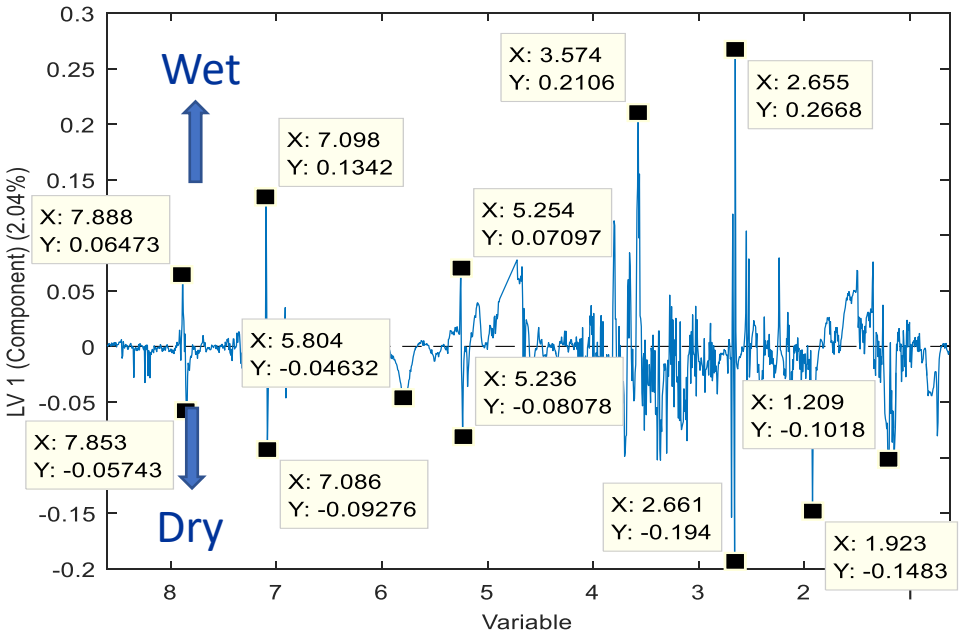

Figure 3.5: NMR spectra of serum samples showing various metabolite peaks in wet and dry AMD patients

Wet AMD	Dry AMD
Lactate	Arginine
Xanthine	Methyl guanine
Gallic acid	Gallic acid
Pyridoxal	Uracil
Glycine	2,5 dimethyl furan
Succinate	Succinic acid
	Ornithine

Table 3.2: List of metabolites which showed an increase in serum analysis of wet and dry AMD patients.

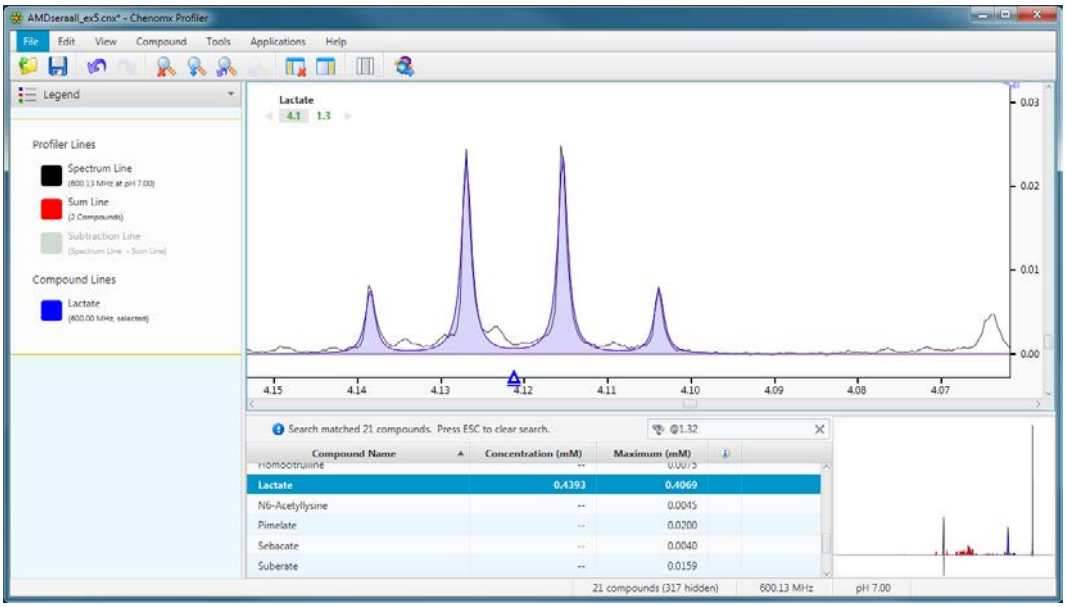

Figure 3. 6: Chenomx analysis of the serum NMR spectrum: metabolite identified is lactate

Lactate and succinate are increased in inflammatory sites where cells switch ATP production from oxidative phosphorylation to glycolysis regardless of oxygen levels. Succinate has been shown to increase IL-1β stabilization of the enzyme hypoxia-inducible factor-1α, particularly in myeloid cells such as macrophages (Tannahill et al., 2013). The metabolites data is supported by an increase in macrophages in human retina with AMD compared to aged healthy controls (McLeod et al., 2016). Moreover, these cells are more active than cells from controls. Increased levels of IL-1β have also been reported in the vitreous of patients with AMD (Lechner et al., 2017; Zhao et al., 2015).

Xanthine is a metabolite in the pathway that converts ATP to uric acid via adenosine and hypoxanthine. Serum uric acid levels have been associated with neovascular AMD in a Malaysian cohort of patients (Subramani et al., 2010). However, this was not confirmed in a European cohort, although there were inverse correlations with antioxidants (superoxide dismutase) and cytokines (IL-6) in patients with wet AMD (Colak E et al., 2017).

In patients with dry AMD, arginine and ornithine are components of the urea cycle that converts toxic ammonia to urea for excretion. The urea cycle has previously been associated with AMD in a liquid chromatography mass spectrometry analysis of plasma samples from patients with neovascular AMD (Osborn M et al., 2013).

2, 5- dimethylfuran has been identified as one of the components of cigar smoke and blood concentration can be used as a biomarker for smoking. As stated, smoking is a risk factor for AMD but there have been no previous association of 2,5 dimethyl furan and AMD.

Taken together, metabolomic analysis of serum samples does not show any obviously different pathways involved in the two clinical forms of AMD. The increase in lactate and succinate in wet AMD suggests increased inflammation, possibly due to increased macrophage infiltration or increased activation of these cells.

Analysis of all urine samples

All 103 urine samples (one patient did not provide urine sample) were processed and analysed with NMR spectroscopy.

The initial analysis of urine samples was done using PCA to identify any trends or clustering in the standardised data, and the maximum variation of the data. PC1 (33.95%) showed clustering either side of median, while PC2 (10.31%) showed a potential separation of two populations within the cohort of samples. As seen in serum samples, there were a few outliers in the urine samples as well that could not be explained by the demographic data (Figure 3.7).

When the samples were analysed on the basis of dry or wet AMD diagnosis, PCA analysis showed no obvious clustering in either component in urine samples from patients with dry AMD (dark blue) and wet AMD (light blue) in each quadrant. (Figure 3.8).

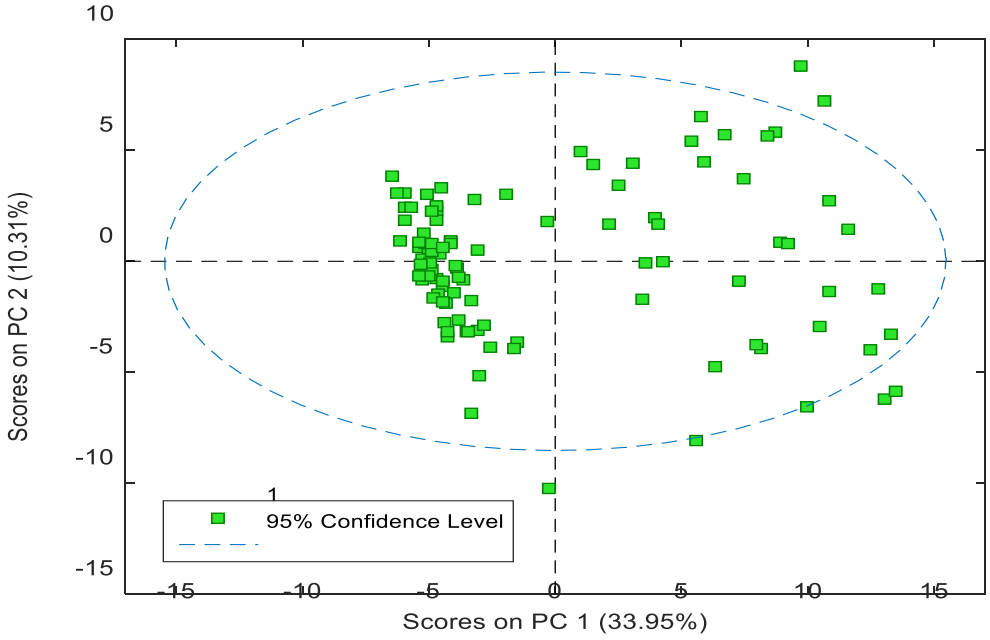

Figure 3.7: Principal Component Analysis of all urine samples (N= 103)

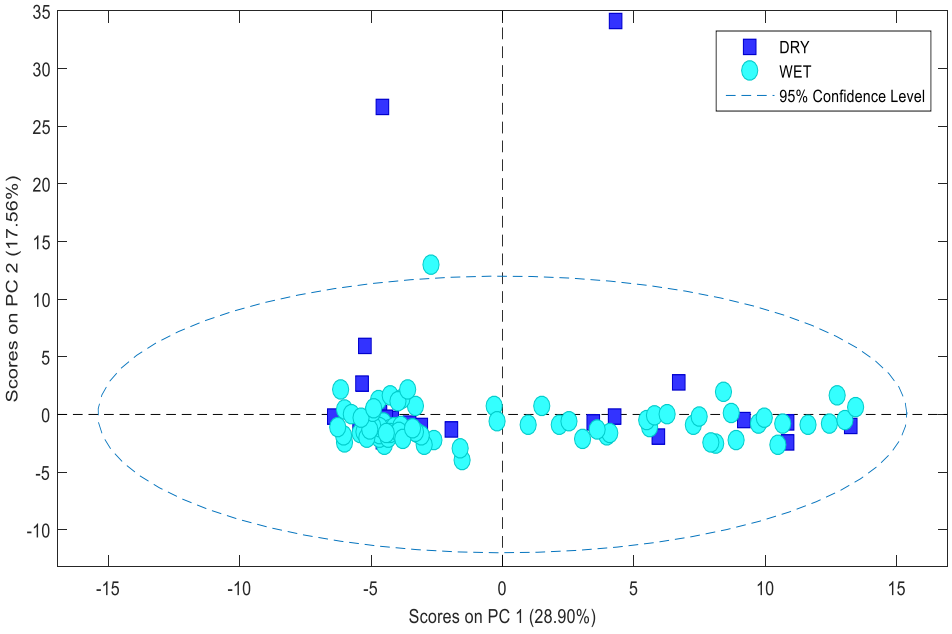

Figure 3.8: Principal Component Analysis of all urine samples (N= 103) showing wet and dry AMD samples

It was interesting to see that the PLS-DA analysis (Figures 3.9, 3.10) of the urine samples showed better clustering, separating the wet from the dry AMD samples, especially after orthogonal signal correction (Figure 3.10). The validated model segregated the dry and wet AMD with a sensitivity and specificity of 35.7% and 84.9% respectively. As noted in the pilot analysis, some of dry samples were noted to cluster with wet AMD samples, the significance of which needs to be explored further.

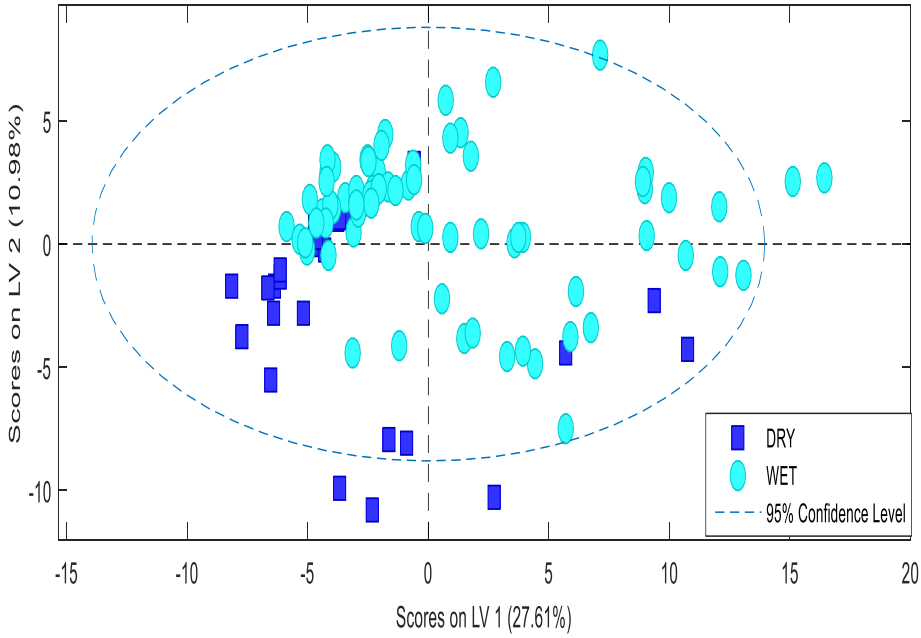

Figure 3.9: Partial Least Squares Discriminant Analysis (PLS-DA) of all urine samples (N= 103) showing wet and dry AMD samples

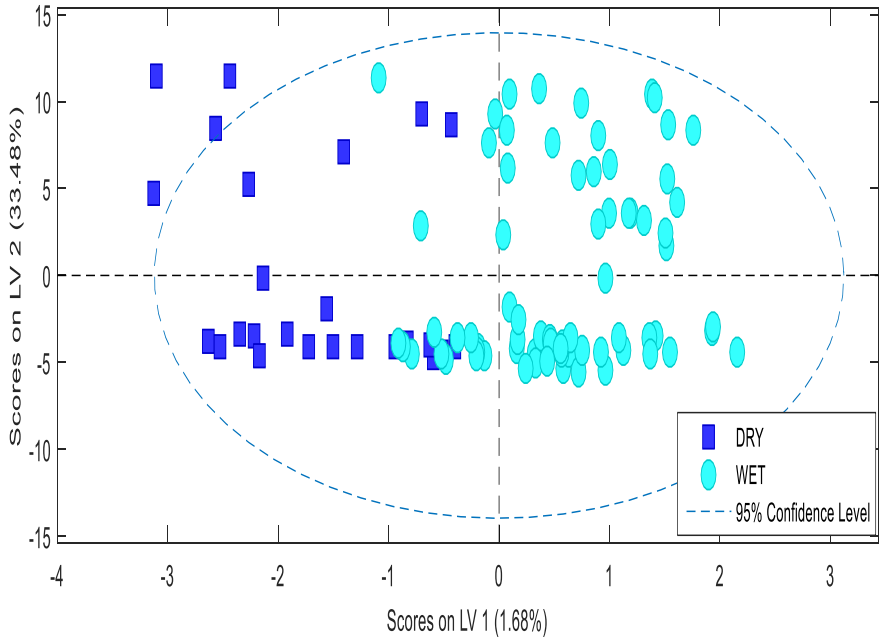

Figure 3.10: Partial Least Squares Discriminant Analysis (PLS-DA) of all urine samples (N= 103) after orthogonal signal correction showing better clustering and separation of wet and dry samples

Metabolites in wet and dry AMD urine

In the urine samples from wet AMD patients, NMR spectroscopy showed peaks of alanine, carnitine, ornithine, citrate, trimethylamine-N-oxide, creatinine and hippurate, while samples from dry AMD showed peaks for cholate, succinate, syringic acid, isovaleroglycine, methanol, acetyl salicylate and N-phenyl acetyl glycine (Figure 3.11, 3.12; Table 3.3).

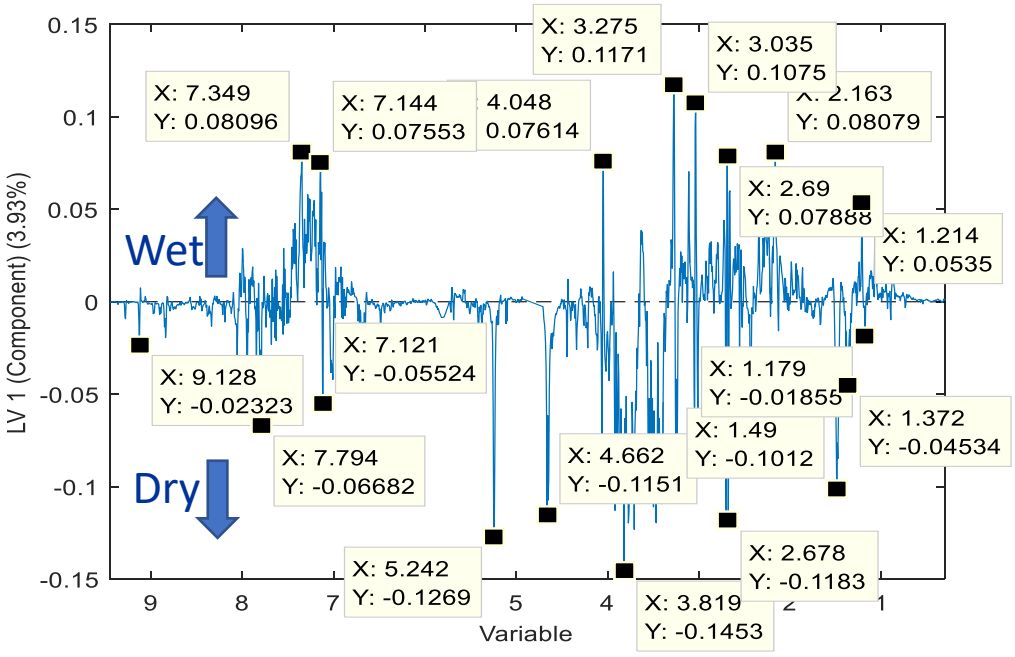

Figure 3.11: NMR spectra of urine samples showing various metabolite peaks in wet and dry AMD patients

Wet AMD	Dry AMD
Ornithine	Cholate
Alanine	Succinate
Citrate	Syringic acid
Trimethylamine-N-oxide	Isovaleroglycine
Creatinine	Acetyl salicylate
Hippurate	N-phenyl acetyl glycine

Table 3.3: List of metabolites which showed an increase in urinalysis of wet and dry AMD patients

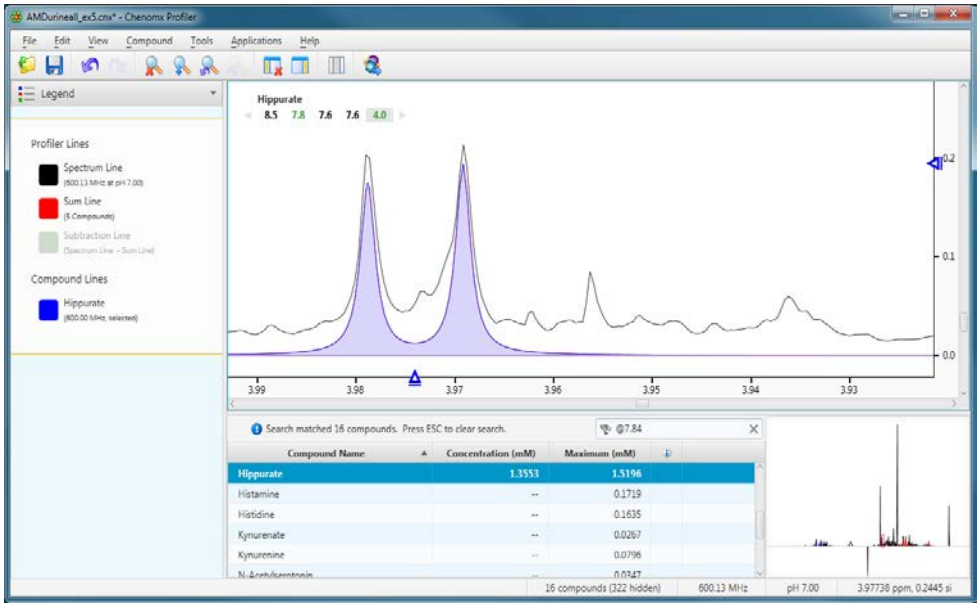

Figure 3.12: Chenomx analysis of the urine NMR spectrum: metabolite identified is hippurate

The results show that certain metabolites such as ornithine and succinate are raised in urine from patients with AMD, although not necessarily in the same form of disease. Creatinine is formed in the muscle and degraded by the kidneys and is a normal component of urine. Raised levels have been reported in patients with type II diabetes with retinopathy and correlated with severity of ocular damage (Saxena et al., 2017).

Syringic acid is a naturally occurring phenolic compound found in various plants such as mushrooms and in wine. It has an antioxidant activity and has been shown to inhibit osteoporosis in mice fed a high syringic diet (Tanaka et al., 2017). While inhibition of oxidative stress would be useful in mediating AMD, this metabolite is dietary and therefore its relevance is unclear.

Male and female: Serum

We analysed the serum samples and found that the male and female samples separated well, with some female samples showing clustering within the male samples (Figure 3.13). The validated model segregated the male and female patients with AMD with a sensitivity and specificity of 89.6% and 82.9% respectively.

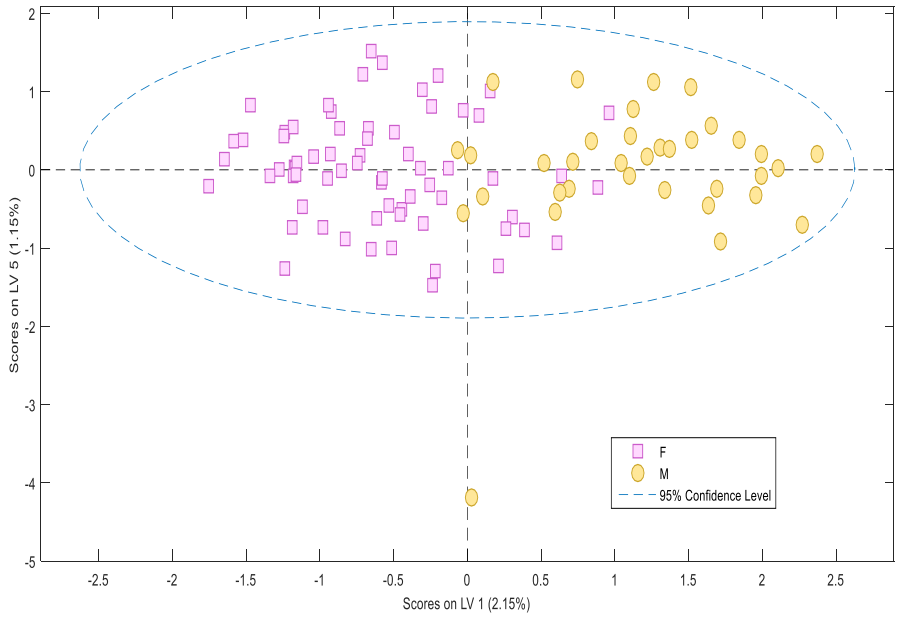

Figure 3.13: Partial Least Squares Discriminant Analysis (PLS-DA) of all serum samples (N= 104) showing male and female patients

The analysis of the NMR spectra revealed the following metabolites: male samples showed peaks of succinate, syringic acid, gallic acid and creatinine; the female serum samples on the other hand showed peaks of thymine, syringic acid, glycolic acid, acetone and methyl malonic acid (Figure 3.14; Table 3.4).

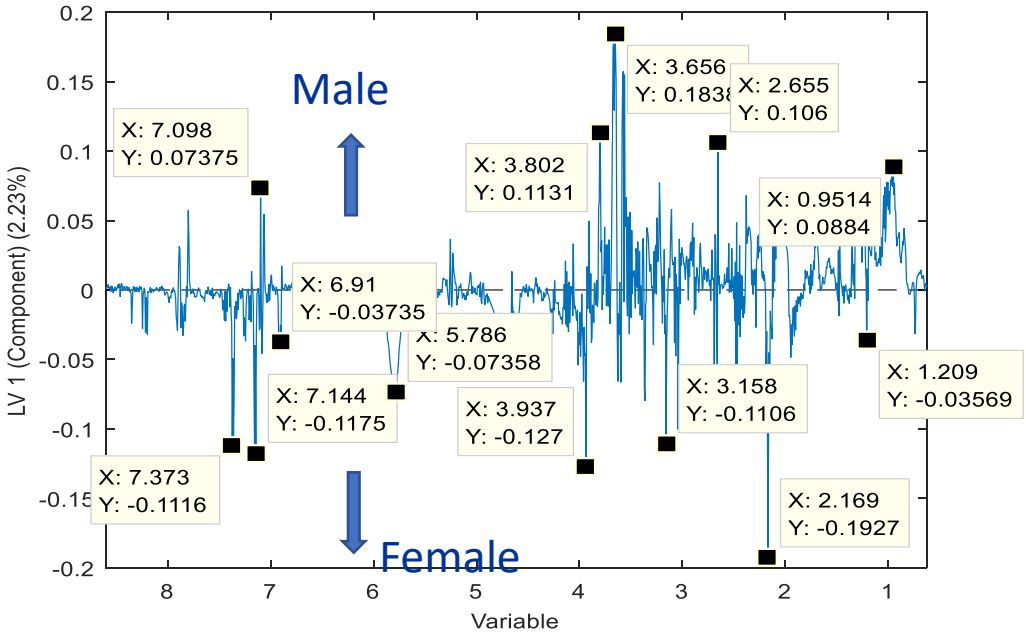

Figure 3.14: NMR spectra of serum samples showing various metabolite peaks in male and female AMD patients

Male	Female
Succinate	Thymine
Syringic acid	Syringic acid
Gallic acid	Glycolic acid
Creatinine	Acetone
	Methyl malonic acid

Table 3.4: List of metabolites which showed an increase in serum analysis of male and female AMD patients

While there was good separation between sera from males and females, there was no specific pathway that distinguished between the two cohorts. Increased succinate could indicate more inflammation in males, while the increased creatinine may be indicative of greater muscle mass in males. Interestingly, similar to syringic acid, gallic acid is an antioxidant found in wine (Yildirim et al., 2004).

Male and female: Urine

Sub-analysis of the urine samples showed that it was possible to separate male and female urine sample reasonably, with some overlap (Figure 3.15). The validated model segregated the male and female patients with AMD with a sensitivity and specificity of 65.1% and 64.6% respectively.

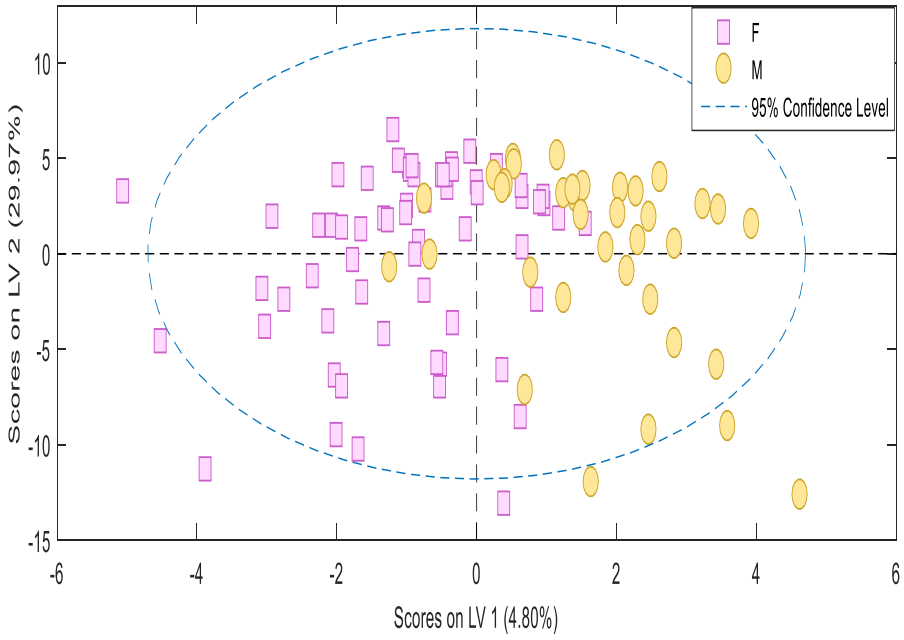

Figure 3.15: Partial Least Squares Discriminant Analysis (PLS-DA) of all urine samples (N= 103) showing male and female patients

Both male and female samples showed creatinine, and 7-methyl adenine. In addition, the male urine showed peaks for succinate, citrate and purine while female samples showed ornithine and syringic acid (Figure 3.16; Table 3.5).

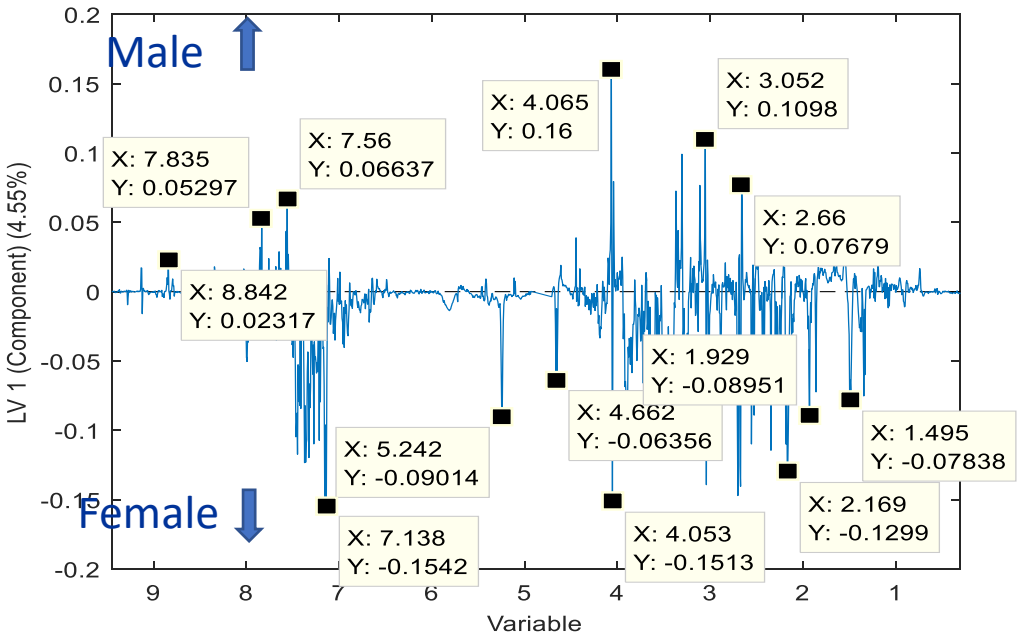

Figure 3.16: NMR spectra of urine samples showing various metabolite peaks in male and female AMD patients

Analysis of urine based on gender showed good discrimination, but similar to serum, there was no specific pathway that suggested a different pathogenesis related to gender.

Separate analysis: male and female serum and urine: wet vs. dry AMD

To investigate the role of gender in AMD, we further reassessed male and female serum and urine samples separately (Figures 3.17, 3.18, 3.19 and 3.20) from patients with wet and dry disease.

Male	Female
Creatinine	Creatinine
7-methyl adenine	7-methyl adenine
Succinate	Ornithine
Citrate	Syringic acid
Purine	

Table 3.5: List of metabolites which showed an increase in urinalysis of male and female AMD patients

Female patients wet vs. dry AMD: serum samples

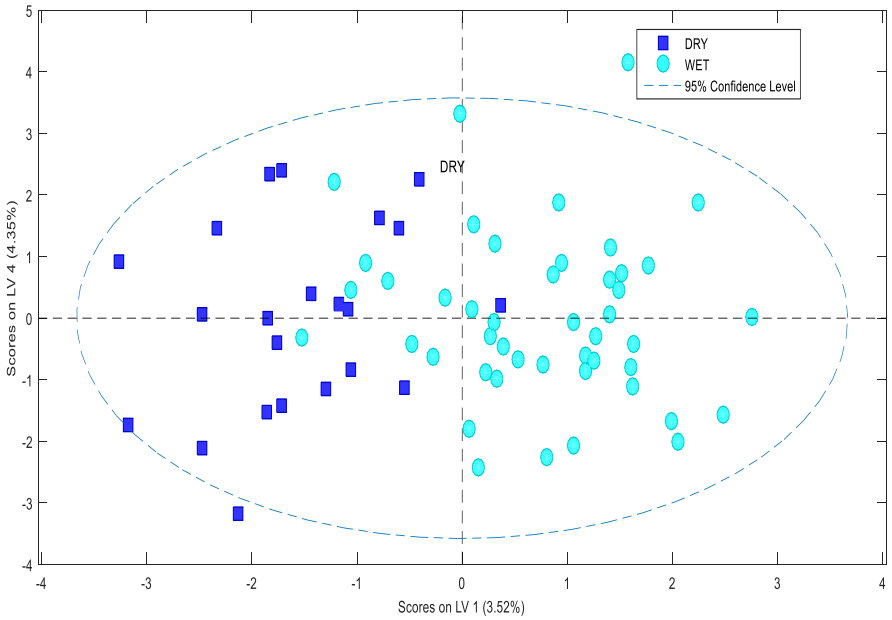

Figure 3.17: Partial Least Squares Discriminant Analysis (PLS-DA) of serum samples of female patients (N= 66) showing separation of wet and dry AMD samples

Male patients wet versus dry: serum samples

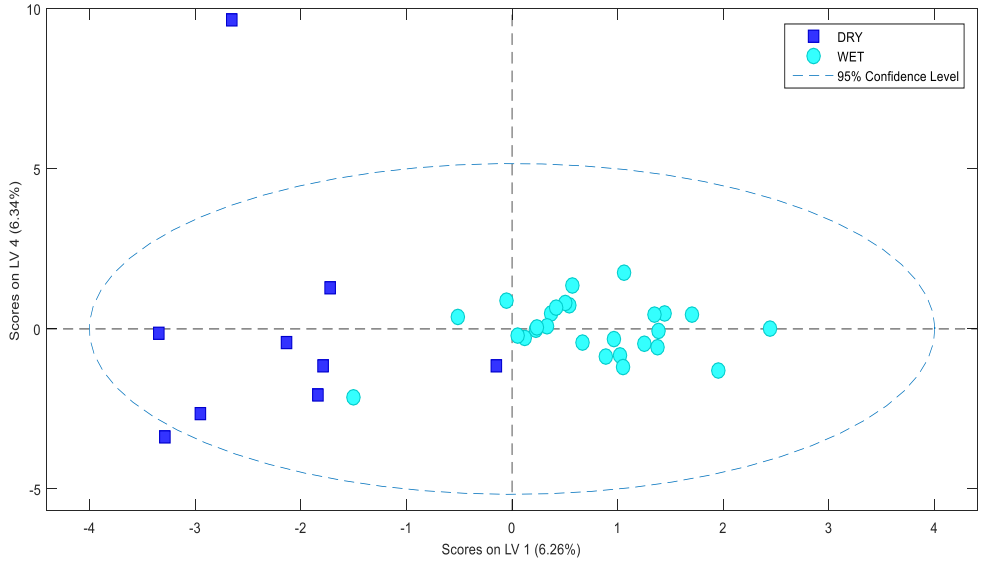

Figure 3.18: Partial Least Squares Discriminant Analysis (PLS-DA) of serum samples of male patients (N= 38) showing separation of wet and dry AMD samples.

Female patients wet versus dry: urine samples

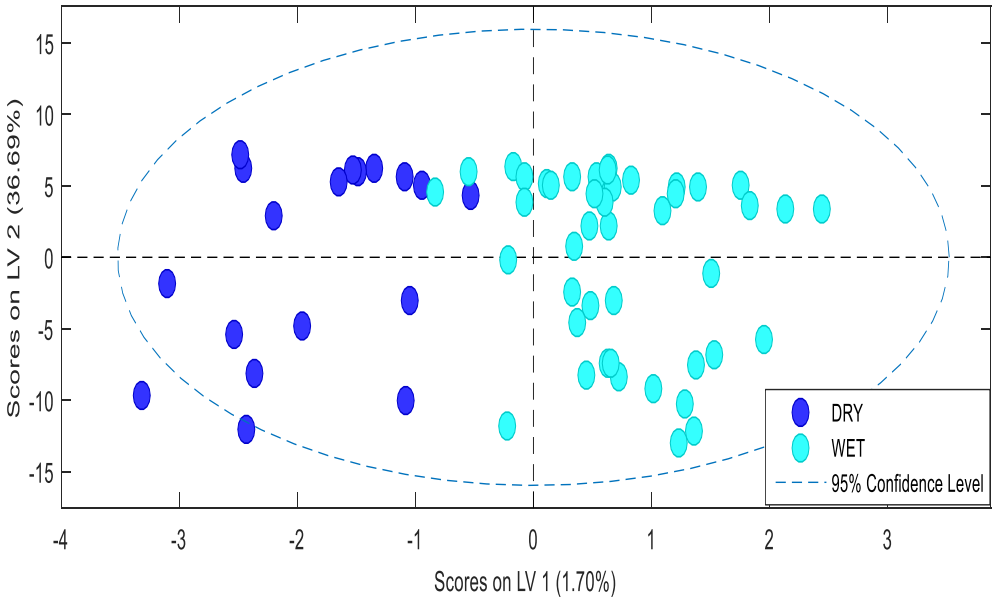

Figure 3.19: Partial Least Squares Discriminant Analysis (PLS-DA) of urine samples of female patients (N= 65) showing separation of wet and dry AMD samples

Male patients wet versus dry: urine samples

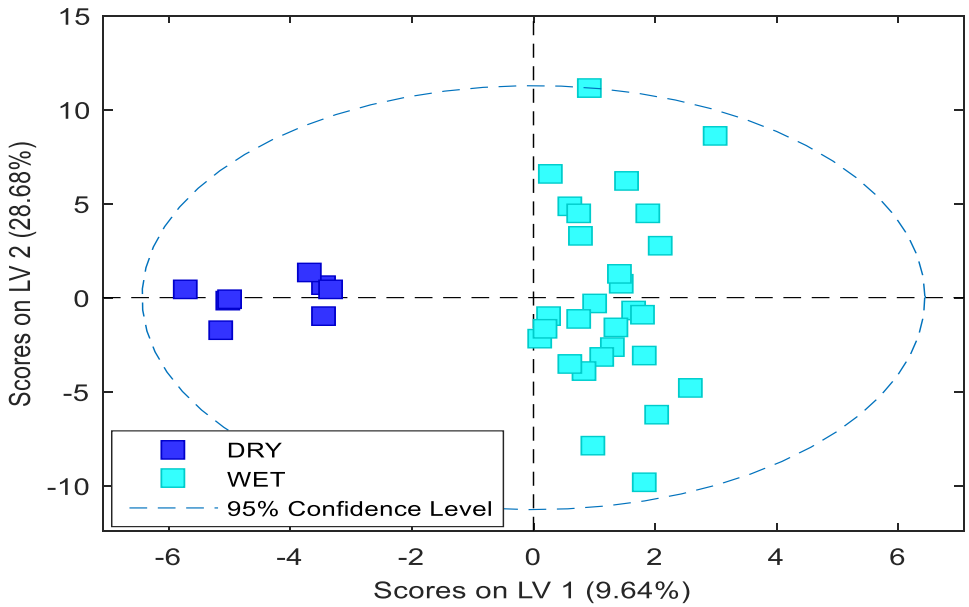

Figure 3.20: Partial Least Squares Discriminant Analysis (PLS-DA) of urine samples of male patients (N= 38) showing separation of wet and dry AMD samples

It was interesting to note that the male urine samples separated the two disease groups better than the female samples (Figures 3.19, 3.20).

Smokers versus non-smokers: Serum

As smoking is a risk factor for AMD, we separately analysed serum samples to see if smoking induced any specific pathways related to the disease. The serum samples from non-smokers separated well from smokers in both female and male patients (Figure 3.21 and 3.22). The validated model segregated the female smokers and non-smokers with AMD with a sensitivity and specificity of 77.4% and 78.1% respectively while for male smokers and non-smokers it was 77.4% and 83.6%.

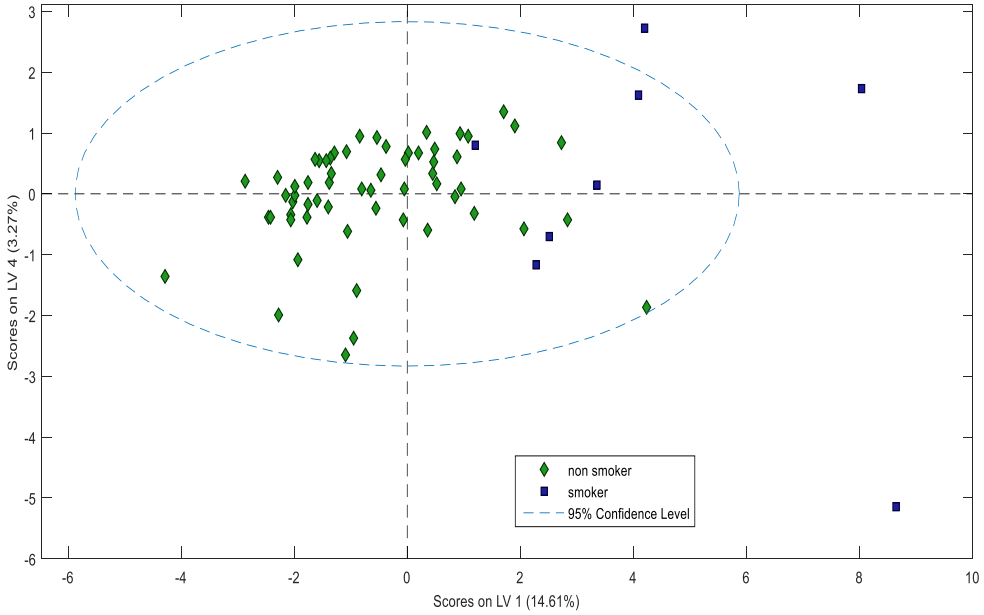

Figure 3.21: Partial Least Squares Discriminant Analysis (PLS-DA) of serum samples of female patients (N= 66) showing separation of samples of smokers from non-smokers

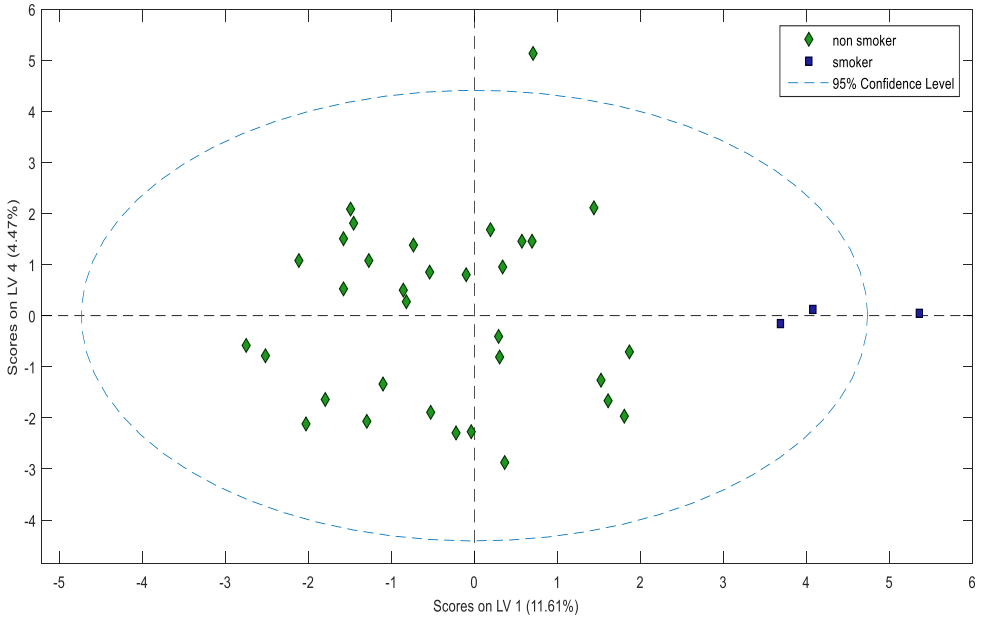

Figure 3.22: Partial Least Squares Discriminant Analysis (PLS-DA) of serum samples of male patients (N= 38) showing separation of samples of smokers from non-smokers

Smokers versus non-smokers

We sub-analysed male and female patients with wet AMD to see the influence of smoking. There were very few smokers in the dry AMD group, hence separate sub analysis for dry AMD patients was not possible. The samples from non-smokers separated well from smokers in both female and male patients with wet AMD (Figure 3.23 and 3.24).

Metabolic analysis of wet AMD serum: smokers versus non-smokers

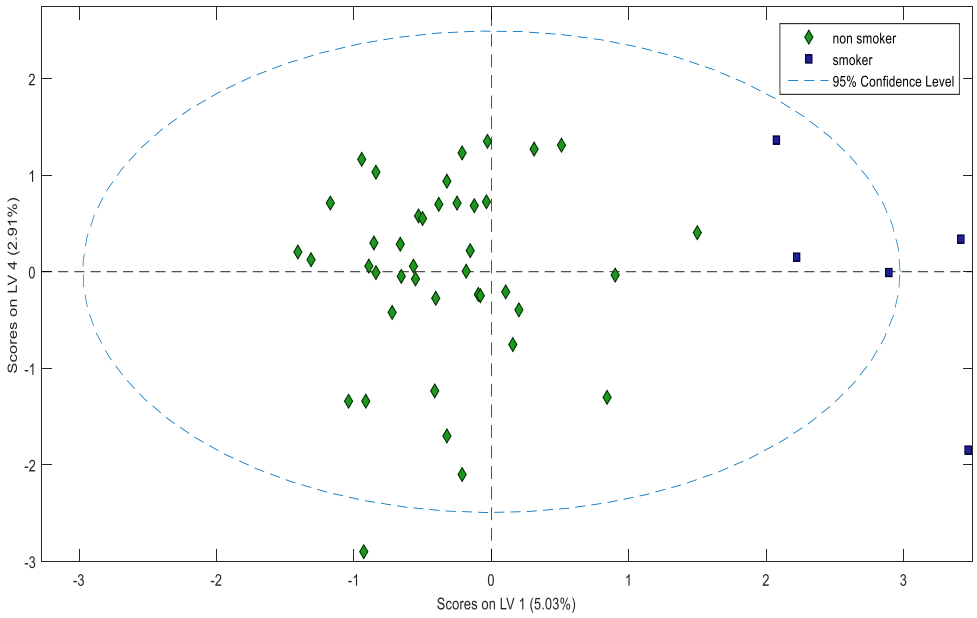

Figure 3.23: Partial Least Squares Discriminant Analysis (PLS-DA) of serum samples of female patients with wet AMD (N= 46) showing separation of samples of smokers from non-smokers.

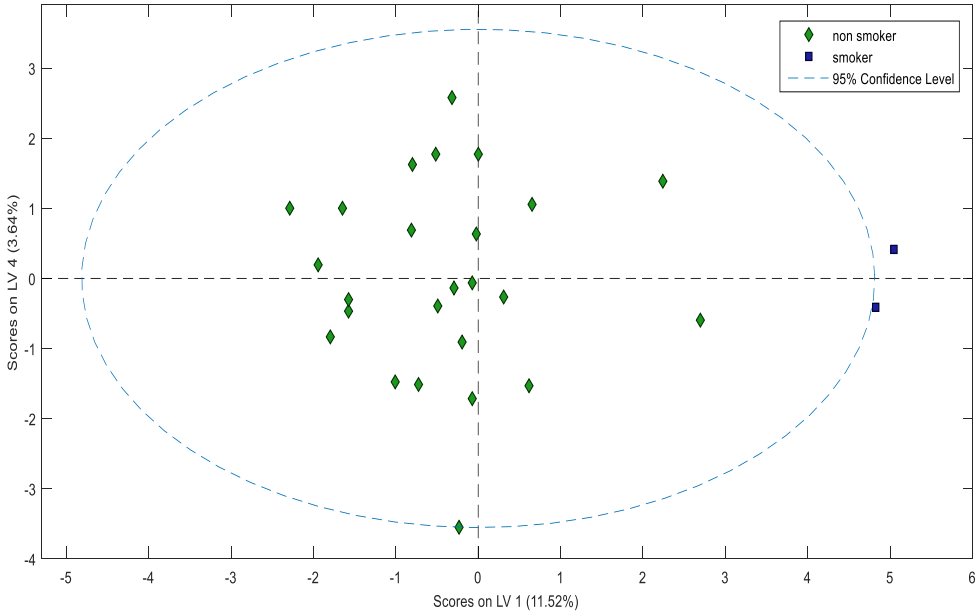

Figure 3.24: Partial Least Squares Discriminant Analysis (PLS-DA) of serum samples of male patients with wet AMD (N= 27) showing separation of samples of smokers from non-smokers

The NMR spectra of the subset of wet AMD serum sample sub-analysis identified the metabolites that were predominant in smokers and non-smokers (Figure 3.24).

The metabolites that predominated in smokers were hypoxanthine, glycine, acetone, 1,2 dibromoethane and α-hydroxyisobutyric acid, while non-smokers had gallic acid, deoxyguanosine, pyruvate, succinate and citric acid (Figure 3.25; Table 3.6).

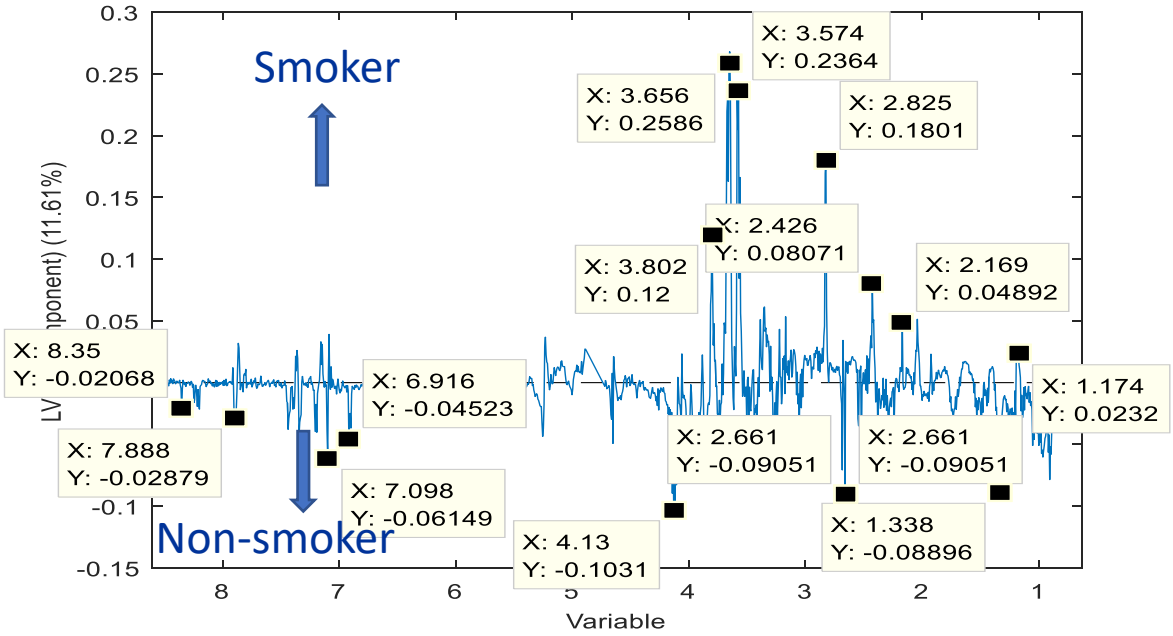

Figure 3.25: NMR spectra of serum samples showing various metabolite peaks in smokers and non-smokers

Smokers	Non-smokers
Hypoxanthine	Gallic acid
Glycine	Deoxyguanosine
1,2 dibromoethane	Pyruvate
α-hydroxy isobutyric acid	Succinate
	Citric acid

Table 3.6: List of metabolites which showed an increase in serum analysis of smokers and non-smokers among AMD patients

In smokers, hypoxanthine was increased, suggesting that increased activity of the urea cycle seen in previous analyses may be driven by smoking. α-hydroxybutyrate is derived from α-ketobutyrate following amino acid catabolism and is metabolized to propionyl-CoA for entry into the Kreb's cycle.

Smokers versus non-smokers: Urine

The urine samples from non-smokers separated well from smokers in both male and female patients with wet AMD (Figure 3.26, 3.27). The validated model segregated the female smokers and non-smokers with AMD with a sensitivity and specificity of 32.5% and 82.3% respectively, while for male smokers and non-smokers it was 92.3% and 50.0%.

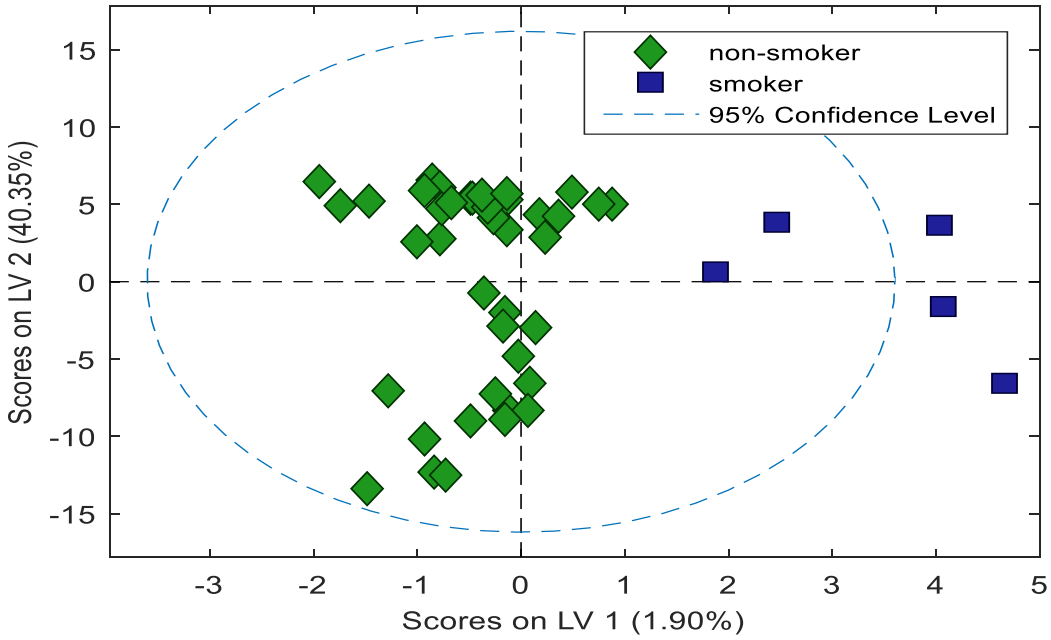

Figure 3.26: Partial Least Squares Discriminant Analysis (PLS-DA) of urine samples of female patients with wet AMD (N= 46) showing separation of samples of smokers from non-smokers

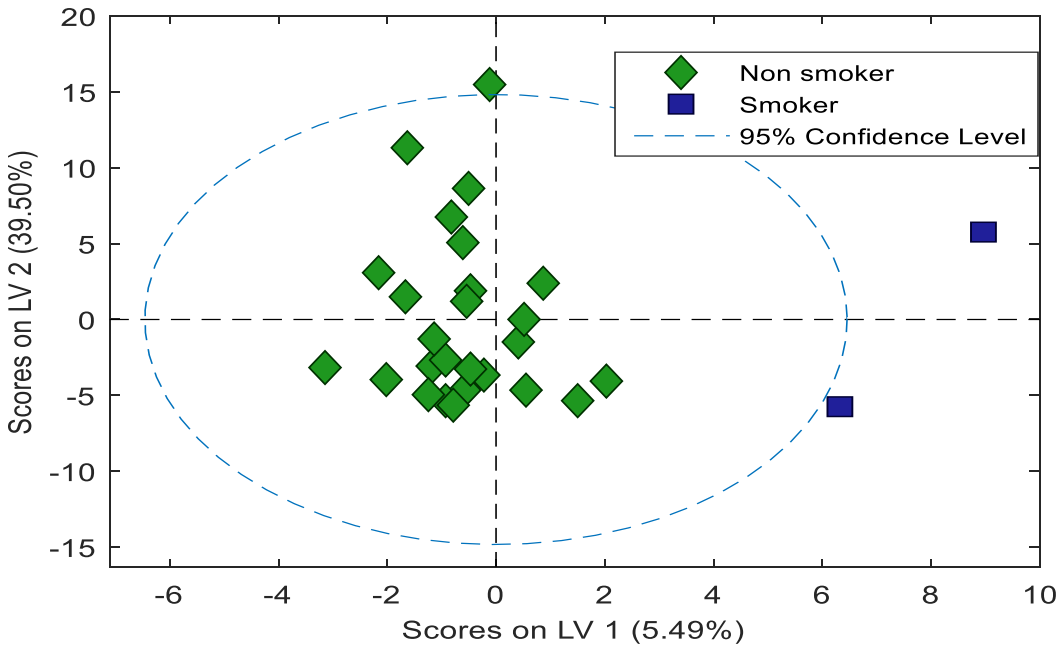

Figure 3.27: Partial Least Squares Discriminant Analysis (PLS-DA) of urine samples of male patients with wet AMD (N= 27) showing separation of samples of smokers from non-smokers

The analysis of the NMR spectra of the smokers and non-smokers urine samples showed peaks of creatinine and succinate; while smokers in addition showed 7-methyl adenine, acetone and purine, non-smokers had ornithine and gallic acid in their urine samples (Figure 3.28; Table 3.7).

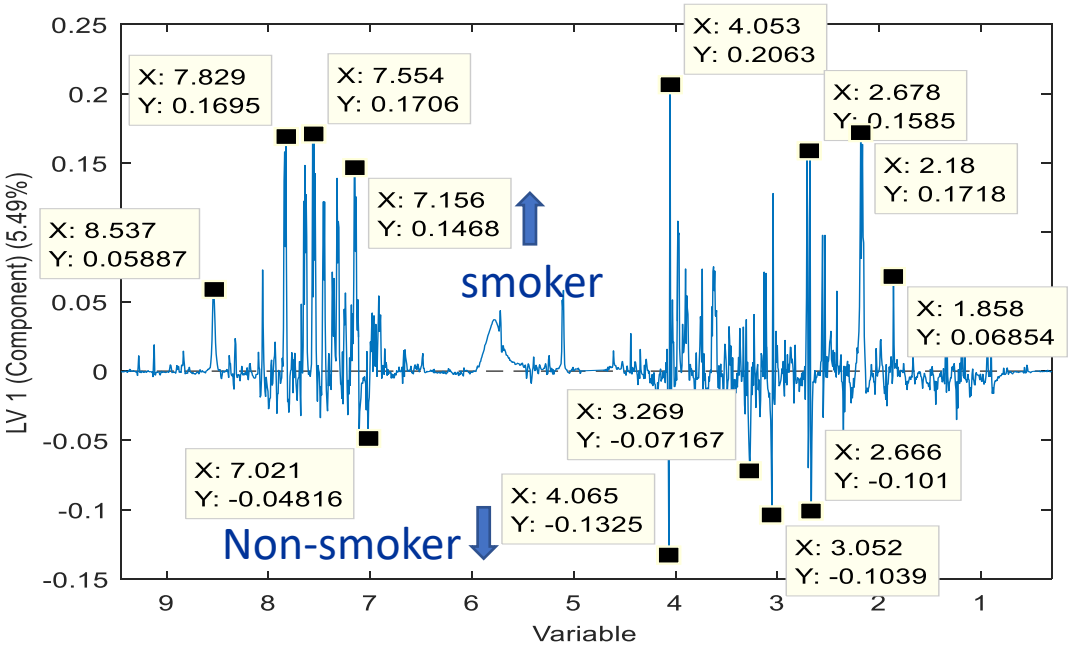

Figure 3.28: NMR spectra of urine samples showing various metabolite peaks in smokers and non-smokers

Smoker	Non-smoker
Creatinine	Creatinine
7 methyl adenine	Gallic acid
Acetone	Ornithine
Succinate	Succinate
Purine	

Table 3.7: List of metabolites which showed an increase in urinalysis of smokers and non-smokers among AMD patients

Chapter 4

DISCUSSION

This thesis reports on a cross sectional study that investigated the metabolic characteristics of serum and urine from patients with dry and wet macular degeneration. Until recently this was the largest cohort study in AMD to explore the feasibility of separating the two disease entities based on their metabolic profiles and is the first to analyse these body fluids. The results show that both serum and urine samples from dry and wet AMD can be distinguished by NMR metabolomics. The results may however indicate production of different metabolites from the same biological pathways, or an increased inflammatory response. The study also showed that samples could be separated based on gender and smoking, but these analyses did not suggest any further pathways to distinguish them, and therefore would appear to have a minimal role in the development of AMD.

As stated, age-related macular degeneration can present in two forms: wet, characterised by choroidal neovascularisation and dry, without choroidal neovascularisation. Although there has been extensive research into AMD, the aetiology is still unclear. A build-up of drusen is associated with AMD but can also be found in age-matched individuals who do not have the disease. Several environment factors have been linked to AMD, including smoking, UV light and diet, but the strongest links are genetic.

Polymorphisms in genes encoding complement factor H, high temperature required factor A1 (HTRA1), age-related maculopathy susceptibility protein 2 (ARMS2) and proteins involved in lipid metabolism have all been associated with AMD (Shaw et al., 2016). Activity of Complement Factor H (CFH), an inhibitor of the complement pathways, is increased in the retina, linking to the inflammageing response to altered self-proteins (Thakkinstain, 2006). The fact that different polymorphisms in CFH gene

are associated with susceptibility to AMD in Caucasian and Asian populations, is suggestive of the central role of CFH in the pathogenesis of AMD (Horie-Inoue et al., 2014). HTRA1, is a serine protease that disrupts matrix and can increase VEGF production.

One of the questions commonly raised by clinicians is about the likelihood of a patient with dry AMD progressing on to wet AMD. This is significant as time is of essence in the management of wet AMD; early identification and prompt intervention with pharmacologic treatments can greatly improve visual outcomes in wet AMD. Hence researchers have always been interested in identifying the clinical parameters that might predict the conversion of dry AMD to wet AMD. The main features such as the area/size of drusen, presence of retinal pigment epithelial hypertrophy etc. have already been looked into, in an attempt to identify any signs that may predict the risk of conversion. The most important risk factor identified so far is the presence of wet AMD in the fellow eye: the rate of development of wet AMD in the fellow eye has been estimated to be around 40% (Silva R et al., 2011).

Metabolomics, with its inherent multiplexed analysis, has enabled us to generate fingerprints of changes in blood and urine metabolites in patients with AMD. NMR spectral analysis showed good clustering as well as separation among the serum and urine samples from dry and wet AMD patients (Figures 3.3, 3.4, 3.9 and 3.10). The variance was more pronounced when orthogonal signal correction was applied as it utilises information related to structured variance, removing residual variance or noise. This was more so in urine samples than the serum, the significance of which is not clear at this stage. It was interesting to note the presence of some of the dry serum samples

clustering along with the wet samples. This could be indicative of the initial common pathway for dry and wet AMD, or an overlap in the pathways involved due to the ongoing degenerative process, at least during part of their progression (Table 4.1; Fig. 4.1a and 4.1b). It would be interesting to carry out a longitudinal analysis of these dry AMD patients, and to look for any changes in metabolites associated with the conversion to wet AMD.

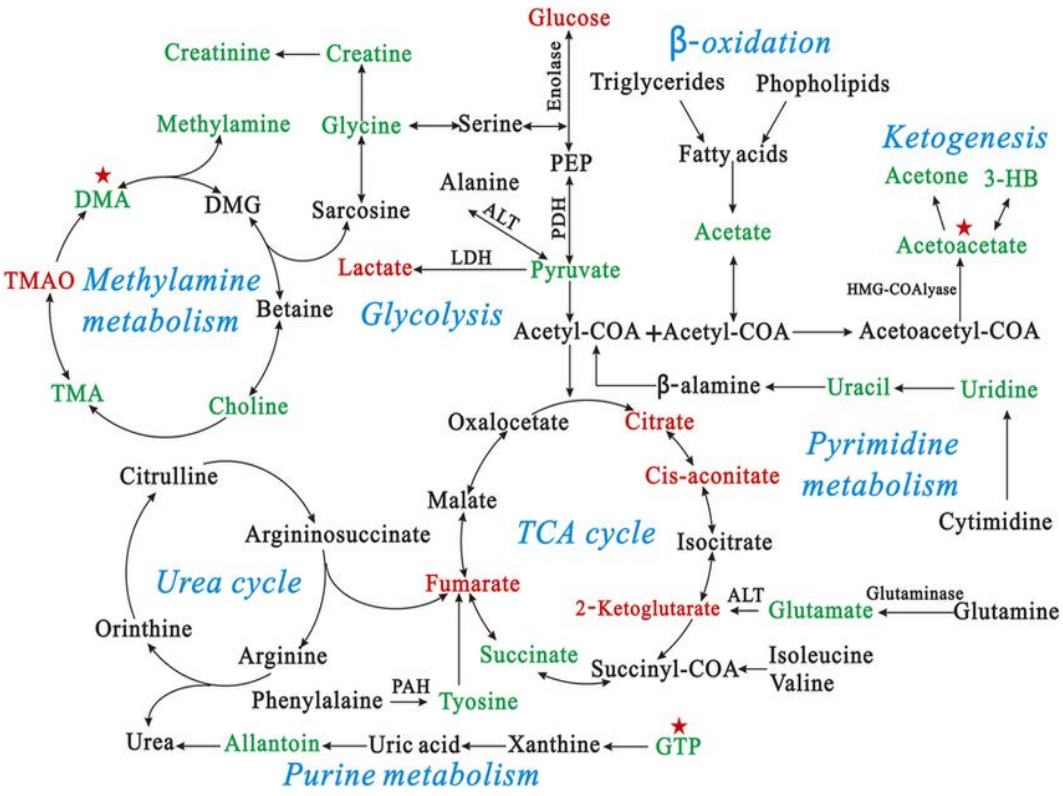

Fig: 4.1a: Metabolic Pathway analysis [image from Wei T et al. (2015)]

Wet AMD serum		Dry AMD serum	
Lactate	Glycolysis	Arginine	Urea cycle
Xanthine	Purine/Urea cycle	Methyl guanine	Inflammation
Galic acid	? Dietary	Gallic acid	? Dietary
Pyridoxal	Amino acid metabolism	Uracil	Pyrimidine
Glycine	Creatine	Ornithine	Urea cycle
Succinate	TCA cycle	Succinic acid	TCA cycle
Wet AMD Urine		**Dry AMD Urine**	
Ornithine	Urea cycle	Cholate	Bile acid
Alanine	Glycolysis	Succinate	TCA cycle
Citrate	Glycolysis	Acetylsalicylate	Aspirin
Creatinine	Creatine	Syringic acid	Diet/microbiome
Hippurate	Diet/microbiome	Isovaleroglycine	Fatty acids
Trimethyl-N-oxide	Diet/microbiome	N-phenylacetylglycine	Fatty acids

Table 4.1: Metabolites and the associated metabolic pathways

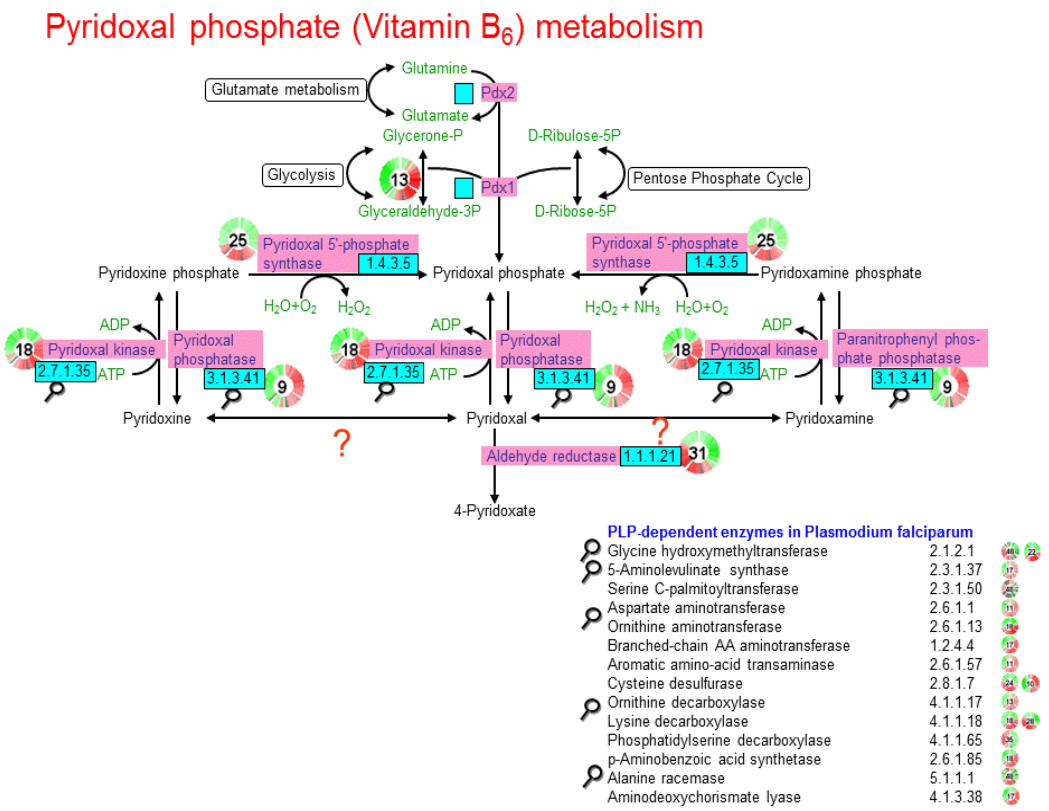

Fig. 4.1b: Vitamin B6 metabolism

[image from http://mpmp.huji.ac.il/maps/vitaminB6metpath.html]

Despite the clustering, the samples also showed peaks of different metabolites in the dry and wet AMD samples. This could perhaps indicate a different aspect of the disease process or that similar pathways were involved but levels of metabolites were greater in one form or the other. This could also be reflective of the influence of systemic inflammation or oxidative stress or alternative complement pathway alteration affecting the ageing process.

The NMR spectra of serum samples showed increase in lactate in patients with wet AMD. Retinal adenosine triphosphate (ATP) is mainly generated by glycolysis

(Yokosako et al., 2014). Pyruvate, the end-product of glycolysis, is usually converted to acetyl-CoA and CO_2 in the mitochondria via the tricarboxylic acid (TCA) cycle in aerobic conditions; however, in the anaerobic state, it is converted to lactate. It has been suggested that inhibition of glycolysis may promote the development and progression of AMD. High lactate/pyruvate ratio is indicative of mitochondrial impairment and poor oxidative function in AMD. Yokosako et al (2014) hypothesized that decreased pyruvate kinase activity in glycolysis would promote the development and progression of AMD. The urinalysis by Yokosako et al (2014) showed high lactate/pyruvate ratio in wet AMD patient group compared to those without AMD. In our cohort, serum analysis of wet AMD patients, but not dry AMD, showed high levels of lactate; this was however not seen in the urine samples. The absence of lactate peaks in the dry AMD cohort is intriguing, the implication of which needs further investigation. One possibility is the increased oxidative stress leading to wet AMD, with macrophage activation and infiltration responsible in part for lactate production.

α-hydroxy butyrate was found elevated in the serum samples of smokers with wet AMD. α-hydroxy butyrate is derived from α-keto butyrate, a product of amino acid catabolism. It is thought to be an early marker for both insulin resistance and impaired glucose regulation, due to increased lipid oxidation and oxidative stress. This further supports the observation by Yokosako et al on the role of glycolysis in the pathogenesis of AMD. It may be further indicative of the added contribution of smoking to the already existing oxidative stress.

In our cohort, a high level of succinate was noted in both wet and dry serum samples, and in urine samples from dry AMD patients. Succinate is an intermediate in the TCA

cycle. An imbalance between energy demand and supply of oxygen has been thought to cause accumulation of succinate in the mitochondria and extracellular environment, which is indicative of mitochondrial stress (Sadagopan N et al., 2007). In response to activation, many cells including macrophages alter their metabolic pathway, to support production of cytokines and other products, characterised by TCA cycle intermediates such as succinate, citrate and fumarate. As these metabolites are found in both serum and urine in dry and wet AMD, this would support a systemic inflammageing phenotype that both drives and is exacerbated by AMD. In healthy individuals M2 macrophages protect the retina, while in AMD proinflammatory M1 macrophages drive inflammation and M2 macrophages produce increased VEGF that increase neovascularisation. (Wu et al 2010).

Another molecule observed to be at high concentration in the serum samples from dry AMD patients was arginine. This high arginine level may be indicative of high nitric oxide (NO) production. NO is an important intercellular signalling molecule; it is synthesized from arginine by NO synthase (NOS) in vascular endothelium. One of the main roles of NO is as a vasoactive mediator. Nitric Oxide is also a chief component of cigarette smoke (CS), a risk factor implicated in the pathogenesis of AMD. CS and NO induce RPE apoptosis, vascular permeability, and angiogenesis unless neutralized. NO from CS or neuronal nitric oxide synthase (nNOS), the predominant isoform in the RPE, could injure the RPE by impairing the Nrf2 (cytoprotective transcription factor) response. Both in vitro and in vivo studies suggest that NO is involved in the control of retinal blood flow (Veriac S et al., 1993). Intriguingly, the link between AMD and UV light has been well researched but a causative mechanism has not been found. Recent studies have shown that, in addition to inducing vitamin D, UV is a potent inducer of

NO. UV light has been shown to be protective in cardiovascular disease in a vitamin D independent mechanism due to NO production (Weller et al., 2016). It is possible that while being cardioprotective via maintaining vascular growth and health, increased NO may be deleterious in patients with AMD. Seizure disorders are observed two to three-fold more in the elderly over the age of 75 years (Ramsay RE et al., 2004). Oxidative damage increases with age, one of the suggested mechanism being mitochondrial impairment. Excessive NO (as reactive nitrogen species (RNS)) causes impairment of mitochondrial functions which can result in seizures and neurotoxicity (Shin EJ et al, 2011; Gupta et al, 2004).

As stated, oxidative stress has a major role in AMD and other diseases of ageing. Glutathione (GSH) is a major water-soluble antioxidant. GSH acts as a reductant of peroxides either by a non-enzymatic reaction or by a reaction catalysed by glutathione peroxidase (Rathbun et al., 1989). Our serum samples showed decrease in GSH levels in dry AMD patients. This reinforces the findings of Samiec et al (1998) whose studies showed decrease in GSH levels with ageing along with a concomitant increase in oxidised glutathione (GSSH) levels (Samiec PS et al., 1998). They also found a significantly lower level of plasma GSH in AMD patients, compared to younger patients. A closer look into the GSH levels in our serum samples from wet AMD patients would be beneficial in understanding the significance of this finding in dry AMD samples.

In the urine sample analysis, there was clear separation of dry and wet AMD, particularly on orthogonal analysis. However, the peaks represented different

metabolites compared to the serum samples. Alanine was the major metabolite peak identified in wet AMD urine. In experimental models, it has been proposed that glucose in the retinal glial cells is converted to alanine, which maintains the redox potential, contributing to NH_3 homeostasis. Similar activity was noted in the purified photoreceptor suspensions as well. Further study is needed to assess the role of NH_3 homeostasis in AMD (Tsacopoulos et al., 1994). However, even though the metabolites may differ, similar pathways are indicated, such as the TCA cycle, urea cycle and those linked to oxidative stress.

The current data on NMR analysis of serum and urine samples can be compared to previous studies of metabolomics in AMD. Osborn et al (2013) analysed the plasma samples of 26 patients with wet AMD against 19 control sample with no AMD using mass spectrometry. The specific metabolites that distinguished wet AMD patients from controls were di- and tripeptides, covalently modified amino acids, bile acids, and vitamin D-related metabolites. In their cohort, bile acid was found to be of lower level in the AMD group (Osborn M et al., 2013). In our cohort, bile acid (cholate) was noted to be elevated in the urine samples of dry AMD patients. Osborn identified metabolites such as phenylalanine, tyrosine, glutamine, and aspartate, and described the urea cycle as a pathway involved in AMD; this supports our findings of arginine and ornithine in urine samples from wet AMD. Osborn et al also identified vitamin D pathway metabolites; while these were not identified in the current study, possibly due to the different analysis methods, our finding of a potential increase in NO would similarly link to UV light exposure.

A second study analysed the metabolites in plasma samples from patients with AMD, from two different geographic area, Boston, U.S (113 patients and 40 controls) and Coimbra, Central Portugal (201 patients 42 controls) by NMR spectroscopy (Laíns I et al., 2017). Interestingly the results show distinct metabolic profile of AMD patients from the two sampling areas compared to their own controls. The authors suggest that the differences are due to the influence of nutrition, life style habits and environment on the development of AMD. The patients were classified as having early, intermediate or late (wet) AMD. In dry AMD (early and intermediate AMD) patients, acetate, creatine, dimethyl sulfone, cholesterol, HDL, choline and unsaturated fatty acids were found to be elevated in Coimbra subjects, while glutamine, histidine, unsaturated fatty acids and albumin, in Boston subjects. No such difference was described in wet AMD cohort (late AMD). In our cohort, high level of creatinine was seen in urine samples of wet AMD patients, but not in serum samples or in dry AMD patients. In support of the current study, where increase in succinate and creatinine was found in smokers and non-smokers, Lains et al found that smoking, gender and age had little influence in either cohort. Future studies could include whether non-smokers had ever smoked and for how long. Moreover, as smoking history is a volunteered information and passive smoking among non-smoker cannot be ruled out, we cannot attribute much weightage to this finding alone.

It is unlikely that the metabolites identified in all three studies were only derived from the eye. Rather the profiles might define an underlying systemic response such as inflammageing or atherosclerosis that in susceptible individuals could lead to the retinal damage characteristic of AMD.

Limitations of the study

The study cohort reflected real-life AMD patients attending the medical retina clinic; as such, the number of patients belonging to the various sub-types of AMD may still be too small to enable us to do sub-analysis on the differences in metabolites in these categories of patients. With the other published studies, it should be able to provide power calculations for future studies. Secondly, the effects of treatment were not analysed in this study as only patients with wet AMD would be on some form of treatment. Moreover, the influence of anti-VEGF treatment in the metabolomic status of our patients was complicated as various patients were on different stages of their treatment, having received different numbers of injection. It would be beneficial to do a longitudinal analysis, looking into the changes in metabolites before and during treatment. Finally, the influence of genetics was not analysed in this study.

Conclusion

The results of our study show that metabolomics can separate serum and urine samples from patients with dry AMD from those with wet AMD. Certain biological pathways were identified, some of which have been previously reported and some which are novel. However, the pathways identified may be present in both dry and wet AMD, and the concentration of the metabolite or the extent of the damage in the retina may have led to the difference. A second and not mutually exclusive possibility is that the genetic polymorphisms associated with AMD are influencing the metabolomic data. This may also have a role to play in the different profiles identified by Lains et al (2017) in their two cohorts. The results from studies by Osborn and Lains together show the potential

for metabolomics in the investigation of AMD. The data at the moment does not support the potential identification of patients with dry AMD who will progress to wet AMD.

Future work

Our study as well as published literature have shown that metabolomics has a role in differentiating dry and wet macular degeneration. These studies have helped us identify some of the metabolic pathways involved in the pathogenesis of this complex multifactorial disease entity. However, it has also raised several pertinent questions. Our future work is intended to try and answer some of these questions.

- Would metabolomic profiling be able to predict progression of dry AMD to wet AMD? Long term follow-up and longitudinal sampling of dry AMD patients could give us further insight into the changes in their metabolomic profile and disease progression.
- Would metabolomic studies help to develop new treatment options? Better understanding of the difference in metabolomic profile at various stages of the disease process may enable further studies to explore specific interventions at the relevant metabolic pathways that might alter the course of the disease. This could also help to identify possible influences that might trigger the conversion of dry AMD to wet AMD, and to investigate the effect of potential interventions that would modify those influences.
- Would metabolomic studies help to identify population at risk of developing a disease? Patients would require to be genetically typed for known polymorphisms to determine the influence of genes on metabolic pathways. If specific metabolites can be identified from a urine or blood sample that is unique

to the genetic polymorphisms known to influence the onset, progression and long-term outcome, it could pave way for early identification of population at risk for developing the disease.

- How to improve the efficiency of metabolomic screening tools? Different methodologies such as mass spectroscopy and NMR could be carried out on the same samples to improve the metabolite detection rate. As more metabolites are identified, we will be able to understand more about the various pathways involved and their influences in the manifestation of the disease process.
- How to explain the difference in metabolomic profiles observed in Spanish and Boston populations? In response to the data from Lains et al, more detailed demographic data beyond gender and smoking would be necessary to separate biological from environmental influences on AMD. Deep phenotyping with detailed demographic data including ethnicity, body mass index (BMI) and dietary habits could shine light on some of the variable findings and overlaps seen for the two disease types in different study populations. As the Spanish and Boston population in the Lains et al study showed different metabolites, analysing gut microbiome would be another interesting line of work.

Metabolomics-genome-wide association studies (mGWAS) have uncovered many metabolic quantitative trait loci (mQTLs) influencing human metabolic individuality. Linking metabolites to known genetic variants associated with AMD, and the potential pathways involved, may enable us to address many of the unanswered questions.

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