The Genotype-Phenotype Correlation of the key features of Non-Proliferative Diabetic Retinopathy

UCL PhD Degree

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I, Elizabeth Pearce, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Diabetic Retinopathy (DR) is a leading cause of visual impairment but its pathophysiology is not well understood. Moderate/severe non-proliferative DR (NPDR) is characterised by the presence of three features: deep haemorrhages (DH), venous beading (VB) and intraretinal microvascular abnormalities (IRMA). They are grouped together as risk factors for progression to sight threatening DR. It remains unclear whether these individual features have similar pathophysiologies, and whether they respond equally to anti-VEGF, a new therapy for NPDR.

Optomap images of 504 NPDR eyes were examined to evaluate the distribution and prevalence of these three features. DNA samples from 199 patients with NPDR and 397 diabetic patients with no DR were collected. The genotype of specific candidate genes were evaluated in patients with DR, VB or IRMA vs no DR. Optical coherence tomography angiography (OCTA) images of 30 patients were examined for focal ischemia adjacent to VB and IRMA. The responses of these three features to anti-VEGF treatment were also re-examined in the images from the CLARITY trial.

DH were present in most cases of NPDR. VB and IRMA did not always co-exist in the same eye and when they do, were often in different locations. VEGF, TGFb-1 and ARHGAP22 polymorphisms (ischaemia-related genes) were more common in patients with DR and IRMA, but not VB. Areas of focal ischaemia were more frequently adjacent to IRMA than to VB. DH and IRMA responded to anti-VEGF therapy but VB did not.

These findings suggest that VB and IRMA do not share the same pathophysiology, and that IRMA are more likely to be ischaemic driven. Nonetheless, some IRMA may not be driven by ischaemia as they have no adjacent ischaemia on OCTA, do not carry the specific genotype, and do not respond to anti-VEGF. Furthermore, patients with VB may not benefit from anti-VEGF therapy.

Impact Statement

Diabetic Retinopathy (DR) is one of the leading causes of visual impairment; however, the pathophysiology is poorly understood. The DR severity score (DRSS) was derived based on fundus features and can be used to predict the risk of visual loss. Deep haemorrhages (DH), venous beading (VB) and intraretinal microvascular abnormalities (IRMA) are three key features in the DRSS within the non-proliferative DR (NPDR) range, and their presence is used to predict progression to sight threatening DR (STDR). The DRSS is used as a clinical trial endpoint for new drug approvals. However, patients with the same DRSS level do not always progress in the same manner. Recently, anti-VEGF therapy has become a treatment for NPDR but which patients are likely to benefit from the therapy remains unclear. In this study, the three key features were investigated in more detail, in patients with moderate/ severe non-proliferative DR (NPDR), a key target group for treatment who have the highest risk of progression to STDR.

In this group of NPDR patients, DH were very common, so I focused on the differences between VB and IRMA. The findings that VB and IRMA do not always co-exist in the same eye and when they do, they are often not in the same quadrant, suggests different pathophysiology. Furthermore, our study found that VEGF, TGFb-1 and ARHGAP22 polymorphisms (ischaemia related genes) are more common in IRMA but not VB, suggesting ischaemia may play a role in the

pathophysiology of IRMA. This calls for a detailed investigation into the role of genetics into the progression of NPDR. If these findings are confirmed this could pave the way for personalised treatment of those with NPDR, based on the combination of fundus features and genotype. Genetic testing could help identify at risk patients, allowing for more frequent fundus examination or possibly earlier treatment of at-risk groups.

Our discovery that VB do not respond to anti-VEGF therapy is important. This highlights that due to inadequacies of DRSS changes as a treatment response, patients with the same DRSS score but different features may respond differently. For example, if the therapy is anti-VEGF, a patient with IRMA only and the same DRSS score as one with VB only may improve by one or two steps of the scale whereas one with VB only would not change. If all patients enrolled into the study had VB only, then the benefits of a drug effective against DH and IRMA would be overlooked.

Finally, it appears that not all IRMA are the same. Although IRMA in general seem to be related to ischaemia (genetic predisposition and OCTA findings) and improve with anti-VEGF, this is not always the case. It is unclear whether there are different classes of IRMA with different prognostic value. This, combined with their differences from VB, calls for a re-classification of DR.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACCORD	Action to control cardiovascular risk in diabetes
ARHGAP22	Rho GTPase activating protein 22
AGE	Advanced glycation endproducts
AR	Aldose reductase
AMD	Age related macular degeneration

BMI	Body mass index
C3	Complement component 3
C5	Complement component 5
CFB	Complement factor B
CFH	Complement Factor H
CFP	Colour fundus photography
CSMO	Clinically significant macular oedema
CWS	Cotton wool spot
DCCT	Diabetes control and complications trial
DH	Deep haemorrhage
DM	Diabetes mellitus
DMI	Diabetic macular ischaemia
DMO	Diabetic macular oedema
DNA	Deoxyribonucleic acid
DR	Diabetic retinopathy
DRS	Diabetic Retinopathy Study
DRSS	Diabetic retinopathy severity scale
EDIC	Epidemiology of diabetes interventions and complications
eNOS	endothelial nitric oxide sythase
ETDRS	Early Treatment of Diabetic Retinopathy Study
FIELD	Fenofibrate intervention and event lowering in diabetes

FGF2	Fibroblast growth factor 2
FAZ	Foveal avascular zone
FFA	Fundus fluorescein angiography
GWAS	Genome wide association studies
HFE	Haemochromatosis
HR	Hypertensive retinopathy
IRMA	Intraretinal microvascular abnormalities
ILM	Internal limiting membrane
IVT	Intravitreal
KDR	Kinase domain insert receptor
MA	Microaneurysm
NPDR	Non-proliferative diabetic retinopathy
NVD	New vessels on the disc
NVE	New vessels elsewhere
OCT	Optical coherence tomography
OCTA	Optical coherence tomography angiography
OVC	Optic disc venous collaterals
PDR	Proliferative diabetic retinopathy
RPE	Retinal pigment epithelium
PRH	Preretinal haemorrhage
PRP	Panretinal photocoagulation

RAGE	Receptor for advanced glycation endproducts
ROS	Reactive oxygen species
RR	Radiation retinopathy
rs	Reference snp cluster ID
SD-OCT	Spectral domain optical coherence tomography
SLAMP	Sarcolemma associated protein
SNP	Single nucleotide polymorphism
SP	Standard photograph
STDR	Sight threatening diabetic retinopathy
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF-b	Transforming growth factor-beta
UKPDS	United Kingdom prospective diabetes study
UWF	Ultrawide field
UWFSLO	Ultrawide field scanning laser ophthalmoscope
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VB	Venous beading
VDR	Vitamin D receptor
VH	Vitreous haemorrhage
WESDR	Wisconsin epidemiologic study of diabetic retinopathy

Chapter 1: General Introduction

1.1 Diabetes Mellitus

Diabetes is one of the world's oldest known diseases. It was described by the ancient Egyptians 3000 years ago (Ahmed 2002). Diabetes Mellitus (DM) is the most common form of diabetes and is now regarded as a global epidemic. DM is classified as Type 1 Diabetes Mellitus (T1DM) (previously known as Insulin-dependent diabetes) or Type 2 Diabetes Mellitus (T2DM) (previously known as non -insulin dependent or adult onset). Of those with DM, between 90 and 95% have T2DM (American Diabetes Association). A recent study has described a more precise sub classification of DM into five groups. Each group has differences in progression of the disease and susceptibility to complications which are dependent on the presence of different variables such as age of onset, body mass index, genetic profile and insulin resistance rather than just glucose measurements. (Ahlqvist, Storm et al. 2018).

In 2019, it was estimated that 463 million people worldwide (aged 20–79 years) are affected by diabetes which is approximately one in eleven adults, and this number is expected to rise to 700 million by 2045 (International Diabetes Federation, 9th Diabetes atlas, 2019). The rise is mainly due to the increase of T2DM and is a result of a combination of factors including increasing obesity, aging, urbanisation and sedentary lifestyles (Zimmet, Alberti et al. 2001)(Wild, Roglic et al. 2004).

1.2 Risk Factors for Type 1 Diabetes Mellitus

T1DM is thought to develop as a result of both genetic and environmental factors. More than half of the genetic susceptibility to T1DM is thought to involve the human leukocyte antigen (HLA) genes (Knip and Simell 2012).

Evidence to support an environmental influence on T1DM development:

- Only 13-33% of monozygotic twins are pair wise concordant for T1DM (Kaprio, Tuomilehto et al. 1992).

- T1DM is twenty times more prevalent in Caucasian populations in Finland compared to those in Macedonia which cannot be explained purely by genetic factors (EURODIAB ACE Study, 2000) (Knip 2011).
- The incidence of T1DM increases in migrant populations who move from countries with a low incidence to those with a high incidence, suggesting environmental factors play a significant role (Knip 2011).
- Recently those with T1DM and the protective, low-risk HLA genotypes are increasing in number, whilst the high-risk HLA genotypes are less common (Hermann, Knip et al. 2003) (Gillespie, Bain et al. 2004).

The large increase in T1 DM in recent years cannot be explained purely by increased genetic susceptibility (Knip 2011).

It is thought that Beta cells' (B-cell) destruction in the islets of Langerhans of the pancreas by an autoimmune process leads to T1DM (de Gaetano, McEvoy et al. 2018). B-cell loss leads to an inability to produce insulin and exogenous insulin is required for survival. Recent studies suggest that inflammation precedes diabetes-associated autoimmunity (Oresic, Simell et al. 2008) (Pflueger, Seppanen-Laakso et al. 2011) and may be due to a combination environmental factors such as bacterial and viral infection, changes in the gut microbiota or diet (Knip 2011).

The persistent presence of autoantibodies to pancreatic islet antigens is known as islet autoimmunity (Rewers and Ludvigsson 2016), which frequently occurs in early childhood (Ziegler and Bonifacio 2012) (Krischer, Lynch et al. 2015). The presence of two or more islet antibodies, is thought to lead to a 70% chance of progression to diabetes in children over ten years (Ziegler, Rewers et al. 2013).

The increase in T1DM is thought to be due to environmental factors (de Gaetano, McEvoy et al. 2018) and certain triggers have been implicated in its development. One such trigger is likely to be enteroviral infection, during pregnancy and postnatal (Stene and Rewers 2012). Further evidence of enterovirus' involvement are its detection in the pancreas of those recently diagnosed with T1DM, (Rewers

and Ludvigsson 2016) and enteroviral infection of B-cells in post-mortem samples and pancreatic biopsies (Rewers and Ludvigsson 2016). The mechanisms of viral infections in the onset of T1DM are unclear, they may have a direct cytolytic effect on B-cells or may initiate an autoimmune response resulting in their destruction (Knip and Simell 2012). There is evidence of a direct link between the presence autoantibodies associated with diabetes and enterovirus infections, strengthening the case of enterovirus' involvement in the development of T1DM (Lonnrot, Korpela et al. 2000) (Oikarinen, Martiskainen et al. 2011). Other viruses may also act as environmental triggers for the onset of T1DM, such as cytomegalovirus, rubella, mumps, rotavirus, ljungan and retroviruses (TEDDY Study Group, 2008).

Older maternal age, infant weight gain, dietary factors and psychological stress may also be environmental triggers for T1DM (Rewers and Ludvigsson 2016). Higher birth weight (above 9 lbs) (Harder, Roepke et al. 2009) (Cardwell, Stene et al. 2010) and rapid gains in weight in the first two years of life (Johansson, Samuelsson et al. 1994) (Hypponen, Kenward et al. 1999) have both been associated with the development of Type 1 DM. The mechanism is thought to be increased insulin resistance leading to autoimmunity of B-cells (Rewers and Ludvigsson 2016). However, studies in the USA and Germany have not found similar results. Another hypothesis suggests that increased hygiene may contribute to a higher incidence of T1DM in some countries caused by a decline in herd immunity particularly in pregnant women to enterovirus, increasing the susceptibility of the foetus or newborns to infection (Rewers and Ludvigsson 2016). Furthermore, vaccinations against enterovirus have been suggested as a possible solution to help combat the increasing incidence of T1DM (Lonnrot, Korpela et al. 2000).

1.3 Risk factors for Type 2 Diabetes Mellitus

Peripheral insulin resistance and reduced insulin sensitivity are the hallmarks of T2DM, primarily affecting the liver, adipose tissues and skeletal muscles (Forbes

and Cooper 2013). A relative insulin deficiency or an insulin secretory defect may also be present. Insulin resistance describes the loss of cellular signalling in response to the hormone insulin (Forbes and Cooper 2013).

A combination of genetic, metabolic and environmental risk factors are thought to contribute to the development of T2DM (Fletcher, Gulanick et al. 2002). Obesity, sedentary lifestyles, increasing age, ethnicity, a family history of DM and women with a history of gestational diabetes and their children have been identified as high risks.

1.3.1 Obesity

Reports evaluating a Caucasian population suggest that 65-75% of cases of DM could be avoided if Body Mass Indices (BMI) were lower than 25 (Seidell 2000). In particular, the degree and duration of obesity and weight gain in adulthood are strong and independent risk factors for T2DM. Weight gain of more than 10% has been shown to significantly increase the chances of developing T2DM in middle-aged men compared to those who's weight remains stable (Wannamethee and Shaper 1999). This has been demonstrated in other studies of male and female adults. Despite this, the majority of obese people do not develop T2DM (Eckel, Kahn et al. 2011). The exact mechanisms linking T2DM and obesity remain unclear, but improved glycaemic control can be achieved by weight reduction. Higher levels of abdominal visceral or upper body fat are more strongly associated with T2DM and insulin resistance than subcutaneous fat or peripheral body fat (Bjorntorp 1991). Three mechanisms explaining the association between obesity, insulin-resistance and subsequent T2DM development have been suggested (Eckel, Kahn et al. 2011):

- Increased production of adipokines/cytokines that increases insulin resistance.

- Distribution of ectopic fat particularly in the liver and skeletal muscles, which can lead to insulin resistance early on (Larson-Meyer, Newcomer et al. 2011).
- Mitochondrial dysfunction leading to decreased insulin sensitivity and impairment of B-cell function (Bournat and Brown 2010).

Insulin resistance is present to some extent in all those with obesity, however, T2DM only occurs when levels of insulin secretion are unable to compensate for the increased insulin resistance (Roder, Porte et al. 1998) (Al-Goblan, Al-Alfi et al. 2014). In obese individuals, insulin resistance is increased due to increased production of cytokines, glycerol, pro-inflammatory substances, hormones and non-esterified fatty acid levels (Al-Goblan, Al-Alfi et al. 2014).

1.3.2 Sedentary Lifestyles

Sedentary lifestyles, in particular prolonged television watching has been shown to increase the risk of T2DM and obesity, independent of diet and exercise. One study showed that men who watched 40 hours a week were three times more likely to develop T2DM than if one hour a week or less was watched (Hu 2003). More recent meta-analyses of the effects of prolonged sedentary time found a 91% increased risk of developing T2DM (Biswas, Oh et al. 2015) and similarly a 112% increase in the risk of T2DM when comparing the greatest sedentary time with the lowest (Wilmot, Edwardson et al. 2012).

1.3.3 Increasing Age

Adults over the age of 65 in the USA have a prevalence of T2DM, which is eight times greater than among adults between the ages of 18 and 44 years (Selvin and Parrinello 2013).

Despite increasing insulin resistance being linked to increasing age, (Al-Goblan, Al-Alfi et al. 2014) impaired insulin secretion often leads to T2DM in elderly adults compared to those of a younger age (Gunasekaran and Gannon 2011). A decline

in B-cell proliferation as well as increased B-cell apoptosis have also been suggested as contributing factors to the increased incidence of diabetes in the elderly.

1.3.4 Gestational Diabetes (GDM)

Gestational Diabetes is defined as “any degree of glucose intolerance with onset or first recognition during pregnancy that is clearly not overt diabetes” (American Diabetes Association, 2003). It occurs in 1-14% of pregnancies depending on the population, is a risk factor for the development of T2DM and may persist after the pregnancy. Approximately 10-31% of cases of diabetes in parous women are associated with previous GDM (Cheung and Byth 2003). Women with GDM are seven times more likely to develop T2DM at some point after giving birth compared to those without (Bellamy, Casas et al. 2009). Some studies show an even greater risk, in a study of Sri Lankan women, 28% of those with GDM converted to T2DM within 24 months and when studied for ten years they found those with GDM were ten times more likely to convert to T2DM than those who did not develop GDM (Herath, Herath et al. 2017).

Adaptations to glucose metabolism occur during pregnancy. Increased insulin secretion occurs early in pregnancy followed by a reduction which may be genetic in origin (Metzger, Buchanan et al. 2007). Initial increased insulin sensitivity is followed by increasing insulin resistance in the second and third trimester (Soma-Pillay, Nelson-Piercy et al. 2016). In normal pregnancy, increased insulin secretion compensates for insulin resistance and normal glucose levels are maintained. In GDM, B-cell dysfunction or insulin resistance leads to an inability to maintain insulin levels sufficiently resulting in hyperglycaemia (Kautzky-Willer, Harreiter et al. 2016) (Metzger, Buchanan et al. 2007).

1.3.5 Family History

Family history of T2DM is a strong independent risk factor for developing T2DM, the greatest risk found in those with both parents having the disease. This risk is

further increased if parents (more so the mother) were diagnosed with T2DM before the age of fifty. (Scott, Langenberg et al. 2013). In this European study, they found that adjusting for variables such as BMI, diet, smoking, waist and hip circumference, physical activity, levels of education as well as genetic risk score together only explained 13% of the risk of T2DM associated with family history leaving the majority of the risk is unexplained. Studies of adoptees have found no increased of T2DM from adoptive parents with the condition (Hemminki, Li et al. 2010). Yet studies show a significantly higher risk of glucose intolerance and T2DM in spouses of those with T2DM than in those who's spouses do not have the condition (Khan, Lasker et al. 2003), drawing conflicting conclusions as to the impact of a shared environment and exposure.

1.3.6 Other Risk Factors

Smoking has been shown to increase the chance of developing T2DM, (Manson, Ajani et al. 2000) as has high alcohol intake in men (Cullmann, Hilding et al. 2012).

1.4 Complications of Diabetes Mellitus

Chronic elevations of blood glucose levels cause damage to blood vessels throughout the body in DM, and can be subdivided into Microvascular and Macrovascular complications (Forbes and Cooper 2013). The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR study) showed a strong link between hyperglycaemia and the incidence of microvascular and macrovascular complications (Klein 1995).

1.4.1 Microvascular Complications of Diabetes Mellitus

The microvascular complications of DM include diabetic neuropathy, diabetic nephropathy and diabetic retinopathy.

1.4.1.1 Diabetic neuropathy

Diabetic neuropathy is the most prevalent neuropathy in the developed world (Said 2007) and has a lifetime prevalence of 50% (de Gaetano, McEvoy et al. 2018), making it one of the most common complications of diabetes (Singh, Kishore et al. 2014), (Tesfaye, Boulton et al. 2010). Diabetic neuropathy can manifest as paresthesia, pain and ulceration. It affects the peripheral nervous system and can lead to limb amputations and impaired wound healing due to sensory loss. Those with DM have a 15% lifetime risk of amputations (Forbes and Cooper 2013).

1.4.1.2 Diabetic Nephropathy

The incidence of diabetic nephropathy has been reported to be 25% in the Diabetes Control and Complications Trial (DCCT) and 17% in the Pittsburgh Epidemiology of Diabetic Complications study. Diabetic nephropathy has become the leading cause of end-stage renal disease (Bek 2014). It is characterised by proteinuria which leads to diabetic glomerular lesions and a reduction of glomerular filtration (Lim 2014). Risk factors for diabetic nephropathy include a strong family history, race (more common in African Americans and Mexican Americans), hypertension, hyperglycaemia and dyslipidaemia (Lim 2014) as well as smoking and increasing age (Scott, Warram et al. 2001).

1.4.1.3 Diabetic Retinopathy (DR)

Diabetic Retinopathy is also one of the most common complications of DM (Fong, Aiello et al. 2004). Estimates suggest that 35% of people with diabetes have signs of DR and that 12% of those have signs of sight-threatening retinopathy (International Diabetes Federation, 9th Diabetes atlas, 2019). The individual lifetime risk of DR is up to 90% in those with Type 1 DM and about 50–60% in a person with Type 2 DM (Klein 2007). Vision may be lost from Diabetic Macular Oedema (DMO), Diabetic macular Ischaemia (DMI) and Proliferative Diabetic Retinopathy (PDR). This complication will be discussed in more detail in Chapter 2.

1.4.2 Macrovascular Complications of Diabetes Mellitus

Cardiovascular disease, cerebrovascular disease and peripheral artery disease (PAD) are all macrovascular complications of DM which lead to myocardial infarctions, stroke and amputations. The macrovascular complications of DM are the cause of death of approximately 70% of those with DM (Stitt, Curtis et al. 2016).

1.4.2.1 Cardiovascular Disease

The Framingham Study found that those with diabetes were two to three times more likely to develop atherosclerotic disease than those without (Kannel and McGee 1979). The risk of death from cardiovascular disease in men with DM was three times that of those without DM (Stamler, Vaccaro et al. 1993). Other studies have shown a high mortality rate amongst those with DM from vascular disease (Moss, Klein et al. 1991) (Garcia, McNamara et al. 1974).

1.4.2.2 Cerebrovascular Disease

People with DM have a much greater risk of stroke than those without the disease (Ergul, Kelly-Cobbs et al. 2012) and DM is considered to be the fastest growing risk factor for stroke (Ergul, Kelly-Cobbs et al. 2012). This effect is seen in both T1DM and T2DM. Of further concern, the increased risk for diabetic patients of a stroke may be seen shortly after diagnosis (Ergul, Kelly-Cobbs et al. 2012). Younger patients with T1DM (between the ages of 15 and 34 years) are more than sixteen times more likely to suffer strokes than those without DM (Sundquist and Li 2006).

Cerebrovascular disease in diabetes has been implicated in the development of Alzheimer's Disease and Vascular Cognitive Impairment (Huber 2008) (Sima, 2010).

1.4.2.3 Peripheral artery disease (PAD)

Peripheral artery disease (PAD) is a complication of DM. It is caused by systemic atherosclerosis which narrows the arteries supplying the peripheral extremities, in

particular the legs (American Diabetes Association, 2003). The prevalence in those with DM is difficult to ascertain as many sufferers are asymptomatic particularly in the early stages and symptoms are often not reported (Clark N, 2003). The risk of PAD increases with age, duration of DM and the presence of peripheral neuropathy. Prevalence rates of 20-29% have been reported in those with DM (Elhadd TA 1999) (Hirsch, Criqui et al. 2001).

Diabetic patients with PAD may present later and with more severe disease than those without diabetes. PAD is therefore a significant risk factor for amputations in those with diabetes (Clark N, 2003).

Chapter 2: Introduction

Diabetic retinopathy (DR) is a common microvascular complication of DM. It is a leading cause of acquired and preventable vision loss worldwide in working-aged and recently elderly people due to increasing longevity (Moss, Klein et al. 1998) (Klein 2007) (Cheung, Mitchell et al. 2010).

As the numbers of people with DM increases, the number of those with DR and site-threatening DR (STDR) is also increasing and has been estimated to rise to 245 million and 84 million, respectively by 2045. (International Diabetes Federation. 9th Diabetes atlas, 2019). Severe non-proliferative DR (NPDR), proliferative DR and diabetic macular oedema (DMO) are considered STDR.

Global estimates report a higher incidence of DR in T1DM (77.3%), compared to T2DM (25.2%) and also for STDR (T1DM 32.4% and T2DM 3.0%) (Lee, Wong et al. 2015). As over 90% of DM have T2DM, this translates to larger numbers with diabetic eye disease despite T1DM being more frequently associated with STDR and vision loss (Eppens, Craig et al. 2006) (Klein, Klein et al. 1984).

2.1 Incidence rates of DR and visual impairment in Type 1 DM and Type 2 DM

Reports on the incidence of DR and STDR in T1DM and T2DM vary according to the population studied and the definitions and screening methods used.

- Global Rates: It has been estimated that 35% of people with diabetes have DR (International Diabetes Federation 9th Diabetes atlas, 2019) and that 12% have sight-threatening retinopathy (International Diabetes Federation 9th Diabetes atlas, 2019).
- The World Health Organisation (WHO) estimated that worldwide, 4.2 million are visually impaired due to diabetes (WHO, 2015).
- Analysis of studies in USA, Australia, Europe and Asia between 1980 and 2008 found an overall prevalence of DR of about 35.4% and of sight-threatening DR to be about 7.5% (Lee, Wong et al. 2015).

- The range of DR reported in the USA is: T1DM DR 36.5%-93.6% and STDR 6.7%-34.9% and T2DM DR 28.5%-40.3% and STDR 4.4-8.2%.
- A UK study found the overall prevalence of DR to be 32.4% and 3.4% with STDR. When looking at T1 and T2 individually, the figures are: T1DM 56% DR and 11.2% STDR, T2DM 30.3% DR and 2.9% STDR (Thomas, Dunstan et al. 2015) (Lee, Wong et al. 2015).
- Western populations with T2DM are more likely to have DR and STDR than Asian populations (DR 28.5-40.3% compared to 12.1-23%, STDR 4.4-8.2% compared to 4.3-4.6%) (Lee, Wong et al. 2015).
- In India and China, urbanisation is thought to be behind the increasing prevalence of DR. An Indian study found a DR (Type not specified) rate of 18% in an urban population compared to 10.3% in a rural population. (Raman, Rani et al. 2009) and rates of DR in China have been reported as ranging from 28%-43% in T2DM (Xie, Xu et al. 2008, Wang, Liang et al. 2009).

2.2 Risk factors for Diabetic Retinopathy

The most significant risk factors for the development of DR are duration of diabetes, severity of DR at baseline, hyperglycaemia and hypertension. Obesity, hyperlipidaemia, puberty and pregnancy are also risk factors.

2.2.1 Duration of Diabetes

In both T1DM and T2DM, duration of diabetes is the strongest independent risk factor for the development of DR (Romero-Aroca, Baget-Bernaldiz et al. 2011, Thomas, Dunstan et al. 2012) (Jones, Greenwood et al. 2012) (Xu, Xu et al. 2014). Thomas et al found a cumulative incidence of DR at four years of 36% in a study of those with T2DM and without DR at baseline. They also found that STDR was positively and independently associated with duration of the disease (Thomas, Dunstan et al. 2012).

The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) also found a relationship between onset of retinopathy and duration of diabetes. WESDR followed up those with T1DM for 25 years.

They defined progression as a two-step worsening of the Diabetic Retinopathy Severity Score (DRSS) in the Early Treatment Diabetic Retinopathy Study (ETDRS) (see later). After 16 years, 64.1% had progression of DR and 83.1% had progressed at 25 years. This cohort were followed for the longest time compared to other studies. It also reported a 42% progression of diabetic retinopathy (NPDR) to proliferative diabetic retinopathy (PDR) in 25 years (Klein, Klein et al. 2008). Other studies showed a progression of two DRSS steps in four to six years to be between 24.1% and 38.9% (Lee, Wong et al. 2015).

2.2.2 Baseline Level of DR

WESDR also established that progression of retinopathy was related to the severity of baseline retinopathy. Those with more severe baseline retinopathy were more likely to progress to STDR. Among patients with T2DM, those with no baseline retinopathy had a 7% progression rate to PDR over 10 years, whereas those with moderate/severe NPDR at baseline had an 81% rate of progression to PDR (Klein, Klein et al. 1994). Other studies also show that baseline severity of DR has been shown to predict clinical outcomes: Sato et al (Sato, Lee et al. 2001) reported that 35% of people with moderate NPDR developed PDR within 2 years whereas none of those followed with mild NPDR developed PDR within 2 years. The average time to conversion to PDR for the mild NPDR group was 6 years and 5 months compared to 2 years in the moderate NPDR group (Sato, Lee et al. 2001).

The risk of progression to proliferative diabetic retinopathy (PDR) in those with severe NPDR is high. Fifty percent will develop PDR and 75% of those with very severe NPDR, will develop PDR within one year (ETDRS Report 12, 1991). Another study reported 5-year rates of progression to PDR with respect to baseline

DR levels to be 2.2% for those with no DR, 13.0% for those with mild NPDR, 27.2% for those with moderate NPDR and 45.5% for those with severe NPDR. The same group reported 5-year progression rates to vitreous haemorrhage as no DR 1.1%, mild NPDR 2.9%, moderate NPDR 7.3% and severe NPDR 9.8%. This means that compared to those with no baseline DR, those with mild NPDR were 6.71 times more likely to develop PDR, those with moderate NPDR were 14.6 times and those with severe NPDR 28.19 times more likely to develop PDR (Denniston, Chakravarthy et al. 2017).

2.2.3 Poor diabetic control

Hyperglycaemia has consistently shown to be an important risk factor for DR by the Diabetes Control and Complications Trial (DCCT) in T1DM and UK Prospective Diabetes Study (UKPDS) in T2DM and subsequent studies. These studies found that improving glycaemic control in both Type1 and 2 DM reduces the development and progression of DR (Mohamed, Gillies et al. 2007).

A study evaluating progression to PDR in those with NPDR found a relationship between HbA1c levels and progression. They found that in cases where the mean HbA1c levels were below 8.6% the rates of progression to PDR were 0%,7% and 14% at 2, 5 and 10 years of follow up compared to 5%, 28% and 60% in those with HbA1c of 8.6% or higher (Sato, Lee et al. 2001).

The DCCT studied patients with T1DM and compared DR rates of those who received intensive glycaemic therapy to those receiving conventional therapy. The study found that progression of DR could be cut by 76% by intensive glycaemic therapy (Nathan, Genuth et al. 1993).The DCCT also confirmed a strong association between hyperglycaemia and other diabetic microvascular complications (Nathan, Genuth et al. 1993). They found that the risk of progression of DR could be cut by 54% by lowering HbA1c levels by 10% (DCCT, 1995). The DCCT also found that improving glycaemic control had a more profound effect on

delaying the onset of DR in patients without retinopathy than slowing its progression (Nathan, Genuth et al. 1993).

The UKPDS followed those with a new diagnosis of T2DM, and found that the risk of DR, nephropathy and possibly neuropathy could be reduced by improving glycaemic control (UKPDS 1998). The results were conclusive and microvascular complications were reduced by 25% in those randomised to the intensive versus conventional therapy. The study found that every percentage point decrease in HbA1c (e.g., 10% to 9%) was associated with a reduction in DR development of 40%, along with a reduction in STDR development and the need for laser treatment.

More recently, the ACCORD trial compared intensive versus standard glycaemic treatment in a T2DM population and confirmed that intensive glycaemic control reduced the risk of the development progression of diabetic retinopathy (Chew, Ambrosius et al. 2010).

These three trials (DCCT, UKPDS and ACCORD) found that intensive glycaemic control cannot prevent DR altogether, but it can significantly delay its onset and reduce progression. Long term follow up of participants in these studies demonstrated that the treatment effect of the intensive glycaemic control were sustained for many years after the trial had ended despite the HbA1c levels of the two groups levelling out at similar levels shortly after the termination of the trial. The Epidemiology of Diabetes, Interventions and Complications (EDIC) followed up participants who had been enrolled in the DCCT and is still following this cohort. The EDIC found that those randomized to the intensive glycaemic control continue to have lower rates of DR as well as other vascular complications such as nephropathy, neuropathy and cardiovascular. This concept of ‘glycaemic or metabolic memory’ is poorly understood and the reverse effect has been observed in a study on diabetic dogs (Engerman, Kern, 1987) (Stitt, Curtis et al. 2016) (Duh, Sun et al. 2017). Another beneficial observation was the lower rates of eye surgery

25 years after the DCCT ended in those receiving intensive treatment (Aiello, Sun et al. 2015).

2.2.4 Blood pressure control

Evidence that improving blood pressure to reduce complications from diabetes is not conclusive. Hypertension has been shown to be a modest risk factor for DR. A recent review concluded that controlling blood pressure may help delay the onset of DR for up to 4 to 5 years but there was little evidence to suggest that it slows progression of DR or reduce the need for laser treatment (Do, Wang et al. 2015).

However, randomized control trials have shown benefits of lowering blood pressure in T2DM. The UKPDS found that by lowering systolic blood pressure from 154mmHg to 144mmHg, there was a 37% reduction in microvascular complications, a reduction in the rate of progression of DR (defined as two steps DRSS) by 34%, specifically DMO (78% reduction) over 9 years. The study also found that visual acuity loss (defined as three lines of the ETDRS chart) was reduced by 47% in the group assigned to tight blood pressure control, which is likely to be a result of the lower rates of maculopathy (UKPDS 1998).

However, the more recent ACCORD Eye Study showed that further reduction of systolic blood pressure to 120 mmHg was neither harmful nor beneficial compared to a systolic pressure 140 mmHg (Chew, Ambrosius et al. 2010), concluding that although lowering blood pressure is beneficial in diabetic patients, intensive blood pressure control is unlikely to prevent the progression of DR (Lee, Wong et al. 2015).

2.2.5 Lipid profile

Hyperlipidaemia has been associated with progression of DR in some studies, however results vary and associations between serum lipid levels and DR grades are generally weak (Stitt, Curtis et al. 2016). The DCCT of T1DM showed a positive correlation between triglyceride levels and severity of DR and a negative

association with High Density Lipids (HDL). However, conventional lipid profiles were not associated with DR status (Lyons, Jenkins et al. 2004). The Pittsburgh Epidemiology of Diabetic Complications study of T1DM also found that levels of triglycerides were associated with DR status and its progression to PDR.

Fenofibrate is a peroxisome proliferator-activated receptor alpha (PPARα) agonist which modifies lipids. Two trials investigating the use of fenofibrate to reduce cardiovascular disease by reducing levels of serum triglycerides also looked at the effects on DR. The studies were the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study and the Action to Control Cardiovascular Risk in Diabetes Eye study (ACCORD). The FIELD study compared placebo versus 200mg fenofibrate daily. A positive effect on the progression of DR was observed in the fenofibrate group, but no difference found in the onset of DR between the two groups, progression of DR being judged as a 2 step increase in DRSS score. Fenofibrate reduced the need for laser therapy, both for PDR and macular oedema (5.2% compared to 3.6%, $p = <0.001$) and those in the fenofibrate arm had a lower chance of developing DMO (Keech, Mitchell et al. 2007). The ACCORD study of those with T2DM compared 160 mg fenofibrate daily with simvastatin to placebo and simvastatin. The findings were similar to those of the FIELD study, those with pre-existing DR had a greater reduction of DR progression and were less likely to need laser therapy if they were randomised to the fenofibrate and simvastatin arm. Those in the fenofibrate/simvastatin arm showed a reduced rate of progression of DR (6.5 compared to 10.2 % in the simvastatin and placebo arm) by at least 3 DRSS steps at 4 years (Chew, Ambrosius et al. 2010).

The results of these two trials, FIELD and ACCORD Eye Study, suggest that fenofibrate may be have a positive effect on diabetic retinopathy. However, as fenofibrate failed to show beneficial effects for cardiovascular disease and lipid profiles, fenofibrate is rarely prescribed for the treatment of diabetic retinopathy.

2.2.6 Pregnancy

DR is known to progress during pregnancy. Progression is more common in mothers withType1 DM than in those with Type 2 DM (Egan, McVicker et al. 2015) (Vestgaard, Ringholm et al. 2010) and also more severe (Rasmussen, 2010). Progression is usually temporary and regresses post-partum, not affecting the long-term risk of progression and vision loss. However, more frequent retinal assessments are advised during pregnancy and the year following delivery (DCCT, 2000). The risk of progression to PDR was found to be linked to the retinopathy grade present at the start of the pregnancy. Progression was low for those with no baseline DR compared to those with NPDR, 50% of whom required laser therapy (Stalnikiewicz, Floriot et al. 2010) (Sunness 1988). The progression of DR during pregnancy may be due to increased circulating Insulin-like Growth factor-1 (IGF-1), although the mechanism of action isn't clear (Ringholm, Vestgaard et al. 2011). It has also been suggested that the immune system may play a role (Kastelan, Tomic et al. 2010).

2.2.7 Puberty

As with adults with DM, studies confirm that duration of DM as well as increased HbA1c levels contributes to the development of DR in children with T1DM. Interestingly, the onset of DM during or after puberty increases the risk of developing STDR compared to those developing DM before puberty, particularly in those aged under 5 years. Studies found that all DM complications occurred later in those with longer duration of DM pre-puberty (Olsen, Sjolie et al. 2004) (Donaghue, Fairchild et al. 2003).

2.2.8 Obesity

Investigation into the influence of obesity on DR has produced conflicting results. Some studies, mainly of T1DM, have shown an increase in DR incidence with increasing Body Mass index (BMI) (Henricsson, Nystrom et al. 2003) as well as with increasing waist to hip ratios in both sexes (Chaturvedi, Sjoelie et al. 2001)

(van Hecke, Dekker et al. 2005). The findings of WESDR (both Type 1 DM and Type 2 DM) found a similar result but this was not statistically significant and interestingly, another finding from WESDR was that underweight participants also had an increased incidence of DR (Klein, Klein et al. 1997) . Other studies mainly of T2DM in Asian populations found that obese patients had a lower risk of DR (Lu, Hou et al. 2015) (Rooney, Lye et al. 2015). It has been postulated that those with obesity may have better B-cell function, or more simply, that those with obesity may have a more recent diagnosis of DM, or may have other comorbidities which have been addressed such as reducing lipid levels or reducing hypertension, impacting DR status, but the reasons for these different findings are not understood.

These risk factors help to determine the risk of developing DR, but they do not explain why progression rates vary so much.

2.3 Pathophysiology of Diabetic Retinopathy

Normoglycaemia is maintained by a negative feedback mechanism. The release of glucagon from the pancreas is triggered by low blood glucose levels, which leads to the conversion of glycogen in the liver to glucose (glycolysis). Glucose is subsequently released into the blood stream, elevating its levels, which triggers the release of insulin. Insulin instructs the liver to convert more glucose to glycogen and promotes uptake of glucose from the blood by insulin-dependent tissues, hence reducing blood glucose levels. This feedback mechanism designed to maintain stable blood glucose levels is abnormal in DM (Broadgate, Kiire et al. 2018).

Chronic hyperglycaemia impairs cells' ability to regulate glucose transport leading to high intracellular levels of glucose. As a result, blood vessel and tissue damage in the retina is mediated by several pathways, described below, and lead to microthrombi formation, cell adhesion molecule activation and cytokine and leukocyte activation. These processes lead to over expression of growth factors and cytokines (Wang and Lo 2018),(Fong 2004).

The vascular pathogenic pathways of DR are driven by hyperglycaemia which leads to basement membrane thickening, endothelial injury and endothelial cell death, followed by the loss of pericytes and disruption of tight junctions of the capillary walls (Frank 2004) (Duh, Sun et al. 2017). This is mediated by elevated levels of growth factors and cytokines (Broadgate, Kiire et al. 2018) and leads to the formation of microaneurysms and haemorrhages and breakdown of the blood retinal barrier (BRB) leading to DMO. The damage to capillary vessel walls eventually leads to non-perfusion of the retinal vascular bed which results in occlusion of capillaries and ultimately ischaemia and impaired oxygenation of retinal neurons (Duh, Sun et al. 2017).

Later stages of DR, namely severe-non-proliferative DR are characterised by intraretinal microvascular abnormalities (IRMA) and venous beading as well as cotton wool spots and haemorrhage. Extensive endothelial damage of retinal capillaries at this stage leads to the production of vasoconstrictor agents such as endothelin and thromboxane A and Nitric oxide is also upregulated. These processes cause vasoconstriction, capillary occlusion and ultimately hypoxia. Further endothelial loss and thickened basement membranes promote vessel occlusion and further ischemia (Wong, Cheung et al. 2016).

Proliferative diabetic retinopathy (PDR) develops as a consequence of severe hypoxia. This leads to an imbalance of angiogenic mediators like VEGF and anti-angiogenic mediators, the end result being neovascularisation (Wong, Cheung et al. 2016). Neovascularisation is considered a response to an immune injury or to

ischaemia. Neovascularisation is the body's response to ischaemic injury (Sapieha P et al. 2008) caused by an interruption to a tissue's vascular supply e.g.to a skin cut and is thought to be mediated by VEGF and other growth factors produced by endothelial and muller cells. New vessels elsewhere (NVE) are often observed adjacent to areas of ischemia but are prevented from revascularising the ischaemic tissue. The new vessels are redirected to the vitreous where they often bleed leading to vision loss. The mechanism is unclear but is thought to involve semaphorin3A (Joyal JS et al. 2011). Inflammation is thought play an important role in DR (Tang J et al. 2011) and neovacularisation of the disc (NVD) may present without local ischaemia. The driver for these new vessels may be VEGF or other growth factors produced by immune cells, and a reduction in IVT phagocytes levels leads to reduced neovascularisation (Tang J et al. 2011).

Proteolytic enzymes digest the basement membrane of retinal vessels and fragments of the basement membrane produced, along with VEGF and other angiogenic factors triggered by hypoxia, promote new vessel formation by stimulating migration and replication of endothelial cells. The endothelial cells form solid buds which subsequently grow into new vessels by forming tubes (Beránek, Kanková et al. 2002) (Wong, Cheung et al. 2016) (Broadgate, Kiire et al. 2018).

Although DR is considered a microvascular complication of DM, retinal neurodegeneration occurs early in the disease, before any signs of DR are clinically visible. Evidence suggests that DR is a disease of the retinal neurovascular system (Duh, Sun et al. 2017). Before visible signs of DR appear, neurodegeneration and neuroinflammation are evident (Abcouwer and Gardner 2014).

Evidence that the neuroretina is adversely affected by DM early on in the disease has been gathered by histology studies. Neuroretinal function can be measured by various means, namely colour vision testing, contrast sensitivity measurement, dark adaptation, microperimetry and multifocal electroretinogram (mfERG).

(Verbraak 2014). All tests mentioned have demonstrated functional loss early in DM before vascular lesions are visible (Verbraak 2014). There is however a large variability in the outcomes of functional tests (colour vision, contrast sensitivity, microperimetry and dark adaptation) (Verbraak 2014). Patient's ability to understand and perform the tests reliably mean they are not accurate enough to be used surrogate markers of DR (Duh, Sun et al. 2017).

Electroretinogram tests have measured a reduction in the oscillatory potential before any other detectable functional changes or clinical signs are found in patients with DM. Changes in neuronal function may therefore contribute to the pathology of DR in the early stages of DM (Lieth, Gardner et al. 2000). Retinal structural changes have also been detected early in the disease by means of spectral domain optical coherence tomography (SD-OCT). Decreased thickness of the ganglion cell layer and nerve fibre layer in those with T2DM with low levels of DR were found when compared to those with no DR (van Dijk, Verbraak et al. 2012).

2.3.1 Metabolic Pathways

Several metabolic pathways are induced by hyperglycaemia and lead to microvascular impairment, neurodegeneration and neurovascular unit impairment in DR. These include interrelated pathways: the polyol pathway, the hexosamine pathway, oxidative stress, the activation of protein kinase C, the upregulation of Kinin B1 and B2 (Wong, Cheung et al. 2016) (Brownlee 2001) (Nentwich and Ulbig 2015). Such pathways lead to the production and activation of a variety of proteins and growth factors including vascular endothelial growth factor (VEGF), free radicals (oxidative stress), advanced glycosylation end products (AGE's), angiopoietin2 and protein kinase C (Wong, Cheung et al. 2016) (Fong, Aiello et al. 2004).

Damage to the retinal vessels via these pathways occurs by the following mechanisms. The Polyol pathway metabolises excess glucose into metabolites

which damage cells (Brownlee 2001). Oxidative stress can lead to cell death due to the effects of reactive oxygen species. Aldose reductase is part of the polyol pathway and converts glucose to sorbitol. Sorbitol accumulates in insulin-dependent tissues, drawing water into the tissue producing osmotic stress and damaging endothelial cells and pericytes (Wong, Cheung et al. 2016) (Hampton, Schwartz et al. 2015). AGE's are thought to promote inflammation as well as oxidative stress leading to pericyte loss and microaneurysm formation (Fong, Aiello et al. 2004). Higher levels of protein kinase C in retinal blood vessels causes damage by increasing vascular permeability, basement membrane thickening, leukocyte adhesion, vasodilation, and growth factor signalling (Nonaka 2000) (Nagpala 1996) (Broadgate, Kiire et al. 2018). Kinin B1and B2 are thought to increase inflammation, vascular permeability and leukocyte infiltration (Nentwich and Ulbig 2015). Activation of the hexosamine pathway by hyperglycaemia can lead to loss or migration of pericytes (Stitt, Curtis et al. 2016).

2.3.2 Microthrombin Formation

Microthrombosis is thought to contribute to occlusion of retinal capillaries in DR (Boeri et al. 2001). Oxidative stress leads to apoptosis of retinal vessel endothelial cells and the subsequent development of platelet microthrombi. The endothelial cell surface is normally antithrombotic and blood clots do not form, however endothelial injury causes platelet and leukocyte adherence resulting in the release of cytokines which impact on the proliferative activities of smooth muscle cells (Yamashiro, Tsujikawa et al. 2003) (Broadgate, Kiire et al. 2018).

2.3.3 Leukostasis

An increase in circulating inflammatory mediators are thought to be the trigger for retinal leukostasis, the adhesion of leukocytes to the capillary endothelium (Joussen et al. 2001) (Wong, Cheung et al. 2016) (Wang and Lo 2018). Leukostasis increases in the diabetic eye due to the sensitization of endothelial cells to the effects of cytokines and appears in the early stages of the disease.

Intracellular adhesion molecules are thought to promote leukostasis (Joussen et al. 2001). A slowing of retinal blood flow and capillary occlusion follows clumping in the retinal vessels by leukocyte adhesion. This leads to the breakdown of the BRB, inflammation, loss of endothelial cells, increased cytokine activation and possibly the creation of vascular malformations (Broadgate, Kiire et al. 2018).

2.3.4 Capillary Endothelial cell death

The death of vascular endothelial cells in DR is widespread and leads to ischaemia and capillary closure. Degenerative retinal vessels are always found in diabetic animal models and in post-mortem examinations of those with DR (Gardiner, Archer et al. 2007). The exact mechanism of endothelial cell death is yet to be elucidated, however the end result is acellular retinal capillaries with corresponding areas of non-perfused microvasculature which can be observed with fluorescein angiography. IRMA (which contain large numbers of endothelial-like cells) may be associated with closure of retinal capillaries, (Stitt, Curtis et al. 2016).

2.3.5 Pericyte death

Pericytes are specialised contractile cells present in capillaries, which have a similar function to smooth muscle cells found in larger vessels. (Beltramo and Porta 2013). VEGF, angiopoetin-2, transforming growth factor-B (TGF-B) and platelet derived growth factor beta (PDGF-B) are thought to be involved in the survival and differentiation of pericytes. VEGF produced by pericytes stimulates endothelial cell proliferation and the interaction of endothelial cells and pericytes leads to the differentiation of pericytes by activating TGF-B (Beltramo and Porta 2013). Pericytes have contractile properties and are likely to help control blood flow. They are found in the basement membrane of blood vessels where they help maintain tight junctions and vessel integrity (Gardiner, Archer et al. 2007). The loss of pericytes is thought to begin before clinical signs of DR appear and may lead to vascular leakage, although the mechanism of pericyte loss is unclear. Inflammation, advanced glycation end products (AGE) formation, production of

reactive oxygen species (ROS) as well as Protein Kinase C and Ang-2 related pathways have been implicated. (Stitt, Curtis et al. 2016). Pericyte loss also leads to dysregulation of vascular tone and calibre and the proliferation of endothelial cells, ultimately resulting in microaneurysm and haemorrhage development (Wong, Cheung et al. 2016).

2.3.6 Basement Membrane Thickening

Also implicated in capillary leakage is the thickening of their basement membranes. The thickened basement membranes are a result of increased collagen and other cells, they are dysfunctional and allow leakage of lipids, proteins and inflammatory mediators into the interstitial space (Wong, Cheung et al. 2016). Capillary basement membrane thickening also occurs early in the disease process and leads to a loss of vessel elasticity (Stitt, Curtis et al. 2016).

2.3.7 Retinal blood vessel calibre, oxygen saturation and blood flow

The Airlie House classification described retinal venule widening as a feature of DR, however accurate measurements of vessel calibre at that time were difficult so venous beading was used as a measure (Wu, Fernandez-Loaiza et al. 2013) (Stitt, Curtis et al. 2016). With improvements in digital imaging, such measurements are now reliable and accurate and further studies have found venular widening in DR. (Tsai, Wong et al. 2011). Studies of arteriolar width have found contradictory results with some finding narrower, (Klein, Klein et al. 2003) and some wider widths in the vessels of those with DR. Klein et al found that larger arteriolar and venule calibre was related to progression of DR (Klein, Klein et al. 2004). A recent study found that wider venule diameters and smaller arteriolar diameters were both predictive of PDR development (Broe, Rasmussen et al. 2014) and a study of African Americans with Type 1DM also found that a larger retinal venule diameter can predict progression to PDR (Roy, Klein et al. 2011).

Oxygen levels in retinal blood vessels can now be measured non-invasively by an imaging technique known as oximetry (Hammer, Vilser et al. 2009, Hardarson

2013). In a number of studies, oxygen levels have been found to be higher in retinal arterioles and venules of those with DR compared to controls without DM (Hardarson and Stefansson 2012), and in some studies the levels of oxygen found in retinal venules alone increased rates of DR progression (Hammer, Vilser et al. 2009) (Jorgensen, Hardarson et al. 2014). The reasons for these findings are unclear, they may be due to the shunting of blood through preferred channels such as IRMA which would alter the distribution of oxygen in the retina (Hardarson and Stefansson 2012) (Stitt, Curtis et al. 2016). Alternatively, as the capillary bed declines, less oxygen may be derived from the retinal vessels, and cell loss may reduce the oxygen requirement of retinal tissue. (Stitt, Curtis et al. 2016).

Abnormalities of retinal blood flow in those with DM was reported as long ago as the 1930's (Wagner H.P 1934). More recently haemodynamic changes in the retinal vessels have been shown before the onset of visible retinopathy (Kohner, Hamilton et al. 1975) (Kohner 1993) (Lockhart, McCann et al. 2014) (Stitt, Curtis et al. 2016). Initially, arteriolar vasoconstriction results in decreased retinal blood flow (Ciulla, Harris et al. 2002, Klein, Klein et al. 2003) and as the disease progresses, arteriolar dilatation has been reported. (Schmetterer and Wolzt 1999) (Ciulla, Harris et al. 2002). This enhances blood flow and may contribute to the progression of DR to DMO and PDR (Curtis, Gardiner et al. 2009) (Grunwald, Riva et al. 1992) (Klein, Myers et al. 2012).

2.4 Classification of Diabetic Retinopathy

Evidence suggests that initial retinal damage and significant neuroglial dysfunction due to hyperglycaemia precedes the appearance of visible microvascular abnormalities (Curtis, Gardiner et al. 2009). This is confirmed by changes to colour vision (Roy, Gunkel et al. 1986), contrast sensitivity (Sokol, Moskowitz et al. 1985) (Ewing, Deary et al. 1998) and electroretinogram measurements (Hancock and Kraft 2004) which also precede changes in visual acuity.

Optometrists, ophthalmologists and retinal specialists classify DR and identify people at risk of developing sight-threatening DR based on the lesions identified, including microaneurysms (MA), haemorrhages (including pre-retinal and vitreous), hard exudates (HE), cotton wool spots (CWS), venous abnormalities and intraretinal microvascular abnormalities (IRMA).

The severity of DR is determined using semi-quantitative grading systems whereby the morphological appearance of the fundus is matched with the retinopathy grade as observed on a set of standard fundus photographs. Lesions may be graded differentially depending on whether they are detected in single or multiple fields (Davis, Norton et al. 1968).

It was widely recognised that a uniform qualitative and quantitative analysis approach is needed to critically evaluate DR and, consequently, a reproducible grading system has evolved over the past 50 years.

Table 2.1 Evolution of the DR classification systems by year of development

Airlie House, 1968 (Davis, Norton et al. 1968) (level) Definition	Early Treatment of Diabetic Retinopathy Study (ETDRS) 1991 (ETDRS Report 12) (level) Definition	International Clinical Diabetic Retinopathy Disease severity scale, 2003 (Wilkinson, Ferris et al. 2003) (level) Definition	National Screening Committee (NSC)-UK 2003(Scanlon 2017) (level) Definition	Scottish Diabetic Retinopathy Grading Scheme, 2003 (Scotland, McKeigue et al. 2016) (level) Definition	DR Deep Learning Algorithm, 2016 (Gulshan, Peng et al. 2016) Definition
(0) None	(10) DR absent	No apparent retinopathy	(R0) None	(R0) None	Referable DR
(1) Mild to moderate	(20) MA only	Mild NPDR	(R1) Mild Background DR	(R1) Mild background DR	
	(35) Mild NPDR	Moderate NPDR	(R2) Moderate background DR		Referable maculopathy
	(43) Moderate NPDR			(R2) Moderate Background DR	
(2)	(47)				

Moderate to severe	Moderately severe NPDR		(R3) Proliferative DR		
	(53 A-D) Severe NPDR	Severe NPDR		(R3) Proliferative DR	
	(53E) Very severe NPDR	PDR			

The approximately mapped Airlie House, ETDRS, International, UK and Scottish classification systems. DR, diabetic retinopathy; MA, microaneurysms; NA, not applicable; NPDR, non-proliferative diabetic retinopathy.

2.4.1 Airlie House Classification

The conference held at Airlie House, Virginia, in 1968 evaluated the knowledge of the natural history of DR at the time and, after consensus, the attendees devised a formal and comprehensive standard classification of DR (Davis, Norton et al. 1968). This classification used standard stereoscopic fundus photography in four or five predetermined fields for the grading of lesions, whereby changes considered less severe than the standard example were classified as grade 1, and changes that were equal or worse than the standard example as grade 2. Written materials documenting the number/size and type of lesions were also utilised to evaluate findings, such as new vessels and fibrous proliferation (Davis, Norton et al. 1968).

2.4.2 ETDRS and International DR Classifications

The subsequent Early Treatment for Diabetic Retinopathy Study (ETDRS) disease severity scale is based on a modified version of the Airlie House classification

system (Table 1). The ETDRS used stereoscopic colour fundus photography in seven standard fields (30 degrees) and became the reference standard against which screening tests were judged (ETDRS Report 10 1991). Of these seven standard fields, field one is centred on the optic disc, field two is centred on the macula, field three is temporal to the macula and fields four to seven surround fields one to three (ETDRS Report 10 1991). More recently, digital evaluations have been found to be comparable to both the ETDRS severity levels and design outcomes from the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study (DCCT/EDIC), and have therefore largely replaced stereoscopic colour photographs (Hubbard, Sun et al. 2011). The ETDRS classification system allowed therapeutic endpoints and techniques for measuring endpoints to be defined in clinical trials, and provided a reliable assessment of treatments whereby the efficacy of a treatment could be assessed on stepwise changes in DR severity levels (ETDRS Report 12 1991).

While the ETDRS classification is widely recognised as appropriate for publications, research and communications between retinal specialists, it was considered overly complicated for everyday clinical practice, requiring comparisons with standard photographs and includes too many levels, so an alternative simplified scale was developed: The International Clinical Diabetic Retinopathy Disease severity scale (Wilkinson, Ferris et al. 2003). This simplified scale allows disease severity to be easily communicated to primary healthcare providers who may not be experts in the field to ensure appropriate clinical recommendations for follow up or treatment. The scale is illustrated below. It starts with ‘no apparent retinopathy’, ‘mild’, ‘moderate’ and ‘severe’ NPDR, with a final level of PDR.

Table 2.2 Early Treatment of Diabetic Retinopathy Final Diabetic Retinopathy Severity Scale

ETDRS Report 12 (1981)

Level	Severity	Definition
10	No Retinopathy	Diabetic retinopathy absent
20	Very mild NPDR	Microaneurysms only
35	Mild NPDR	Hard exudates, cotton-wool spots, and/or mild retinal haemorrhages
43	Moderate NPDR	43A retinal haemorrhages moderate (>photograph 1) in four quadrants or severe (≥ photograph 2A in one quadrant. 43B Mild IRMA (<photograph 8A) in one to three quadrants
47	Moderate NPDR	47A Both level 43 characteristics 47B Mild IRMA in four quadrants 47C Severe retinal haemorrhages in two to three quadrants

		47D Venous beading in one quadrant
53 A-D	Severe NPDR	53A ≥2 level 47 characteristics 53B Severe retinal haemorrhages in all four quadrants 53C Moderate to severe IRMA (≥photograph 8A) in at least one quadrant 53D Venous beading in at least two quadrants
53E	Very severe NPDR	≥2 level 53A-D characteristics
61	Mild PDR	NVE <0.5 disc area in one or more quadrants
65	Moderate PDR	65A NVE ≥0.5 disc area in one or more quadrants 65B NVD< photograph 10A (<0.25-0.33 disc area)
71,75	High-risk PDR	NVD ≥ photograph 10A, or NVD < photograph 10A or NVE ≥ 0.5 disc area plus VH or PRH, or VH or

		PRH obscuring ≥ 1 disc area
81,85	Advanced PDR	Fundus partially obscured by VH and either new vessels ungradable or retina detached at the centre of the macula

NPDR (Non-proliferative diabetic retinopathy), IRMA (Intra retinal microvascular abnormalities), NVE (new vessels elsewhere), NVD (new vessels at the disc), PRH (pre-retinal haemorrhage), VH (vitreous haemorrhage)

Figure 2.1 Standard photographs 2A, 6A and 8A. ETDRS Report 10 1991

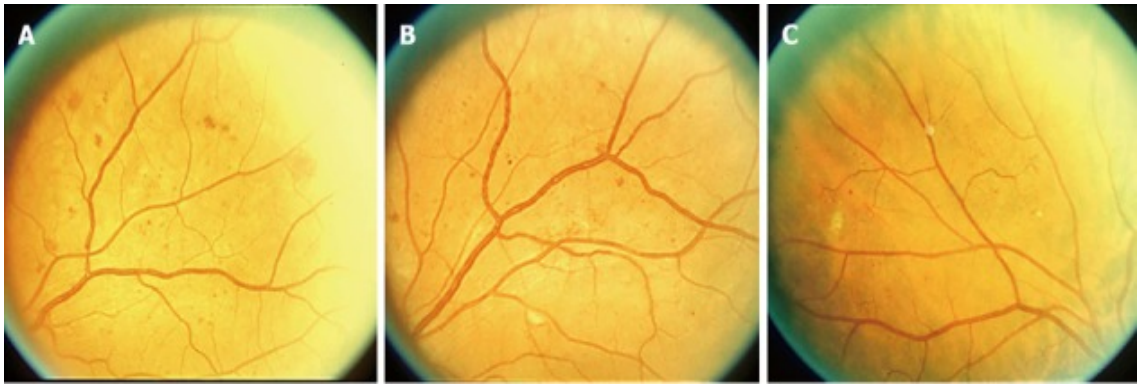


Table 2.3. International Clinical Diabetic Retinopathy Disease Severity Scale (Wilkinson, Ferris et al. 2003)

Proposed Disease Severity Level	Findings Observable Upon Dilated Ophthalmoscopy
No apparent retinopathy	No abnormalities
Mild non-proliferative diabetic retinopathy	Microaneurysms only
Moderate non-proliferative diabetic retinopathy	More than just microaneurysms but less than severe non-proliferative diabetic retinopathy
Severe non-proliferative diabetic retinopathy (4-2-1 Rule)	Any of the following: • More than 20 intraretinal haemorrhages in each of 4 quadrants • Definite venous beading in 2 or more quadrants • Prominent IRMA in 1 or more quadrants and no sign of proliferative diabetic retinopathy
Proliferative diabetic retinopathy	One or both of the following: • Neovascularisation • Vitreous/Preretinal haemorrhage

2.4.3 UK-based Classifications: England and Scotland

In the UK, screening protocols have also simplified the ETDRS classification and applied it to one-field (Scotland) and two-field (England and Wales) fundus

photography (Scanlon 2017). These simplified approaches are based on DR features that a non-ophthalmologist/accredited photographic grader can recognise and assess, and provides guidelines for severity levels which merit patient referral for expert opinion from an ophthalmologist and possible treatment.

2.4.4 Artificial intelligence and automated DR detection

The most transformative advance in DR screening of this century is automated DR detection. Software for automated detection of DR lesions from fundus photographs have been developed with the potential to reduce the workload of retinal graders by providing an automated real-time evaluation that can expedite referral and diagnosis, thus improving efficiency and cost-effectiveness (Sim, Keane et al. 2015). In addition, integrating digital DR images with electronic medical records may improve individual patient prognosis and provide an opportunity for predictive modelling of medical risk factors based on broad population data. When fully implemented, this approach has the potential to substantially improve the way diabetes eye care is delivered (Sim, Keane et al. 2015).

Using retinal images from the EyePACS database and other sources, Google Research, Inc. has developed and tested an automated deep learning system for the detection of DR. This system has demonstrated consistent interpretation, high sensitivity and specificity and rapid reporting of results (Gulshan, Peng et al. 2016). Detection accuracy can be further improved by using an adjudicated tuning dataset and higher resolution images (Krause, Gulshan et al. 2018). Other algorithms for automated grading of retinal images, capable of recognising MA and other DR lesions, have been developed, including the FDA-approved IDx-DR system (van der Heijden, Walraven et al. 2014). This is the first autonomous artificial intelligence diagnostic device approved for detection of DR in primary care, and is viewed as a promising system for modernising healthcare delivery. In terms of real-world application, automated DR detection has already been trialled in Scotland

using a two-tier strategy, replacing initial manual grading to assess image quality and to detect the presence of any retinopathy. Automated grading in this trial proved to be similarly effective and less costly than manual grading (Scotland, McNamee et al. 2007).

2.5 Classification of DMO

Increased vascular permeability may result in thickening of the retina (oedema) and lipid deposits (exudates). The degree and area of thickening were considered in The Early Treatment of Diabetic retinopathy Study (ETDRS). The classification came about as part of the trial to elucidate the most beneficial time to treat with laser.

The ETDRS defined Clinically Significant Macular Oedema (CSMO) by three criteria:

1. Retinal thickening within 500 μm of the centre of the macular.

2. Hard exudates within 500 μm of the centre of the macular adjacent to an area of retinal thickening.

3. one-disc diameter of retinal thickening part of which is within one disc diameter of the centre of the macular (ETDRS Report 1, 1985)

The term CSMO was chosen to define macular oedema affecting the centre of the macular, which has the most detrimental effect on vision.

The International Clinical Diabetic Retinopathy Disease severity scale simplified the grading of DMO. They classified DMO as Apparently Absent and Apparently Present. If present it was classified as mild, moderate and severe.

Table 2.4 Diabetic Macular Oedema Disease Severity Scale (Wilkinson, Ferris et al. 2003)

Proposed Disease Severity Level	**Findings Observable on Dilated Ophthalmoscopy**
Diabetic macular oedema apparently absent	No apparent retinal thickening or hard exudates in posterior pole
Diabetic macular oedema apparently present	Some apparent retinal thickening or hard exudates in posterior pole

If diabetic macular oedema is present, it can be categorized as follows:

Proposed Disease Severity Level	**Findings Observable on Dilated Ophthalmoscopy***
Diabetic macular oedema present	Mild diabetic macular oedema: Some retinal thickening or hard exudates in posterior pole but distant from the centre of the macula Moderate diabetic macular oedema: Retinal thickening or hard exudates approaching the centre of the macula but not involving the centre

	Severe diabetic macular oedema: Retinal thickening or hard exudates involving the centre of the macula

*Hard exudates are a sign of current or previous macular oedema. Diabetic macular oedema is defined as retinal thickening, and this requires a three-dimensional assessment that is best performed by a dilated examination using slit-lamp biomicroscopy and/or stereo fundus photography.

More recently DMO has been referred to as Centre-Involving or Non-Centre-Involving. This sub classifying of DMO describes whether the centre of the macular is involved based on optical coherence topography (OCT).

2.6 Proliferative Diabetic Retinopathy (PDR)

Proliferative diabetic retinopathy (PDR) is the most severe stage of DR. The hallmark of PDR is the growth of new vessels (neovascularization) in the retina and iris, followed by vitreous haemorrhage and tractional retinal detachment. New vessels form as a result of hypoxia which increases VEGF levels. They mostly appear at the interface of perfused and ischaemic areas of retina. New vessels usually grow from post-capillary venules. As new vessels are fragile, they frequently bleed into the vitreous cavity and grow into the vitreous gel. In time, the new vessels may become surrounded by fibrovascular tissue which when contracts, can detach the retina leading to irreversible vision loss (Wong, Cheung et al. 2016) (Simo, Hernandez 2008). New vessel growth in the anterior segment of the eye, on the iris or in the anterior chamber angle may lead to obstruction of the trabecular meshwork. This leads to a rise in intra-ocular pressure and neovascular glaucoma (Wong, Cheung et al. 2016) leading to severe visual loss.

2.7 Non-Proliferative Diabetic Retinopathy Features

2.7.1 Microaneurysms

Retinal microaneurysms (MA) are present in a number of conditions affecting the retinal vasculature, for example hypertension and retinal vein occlusions but appear most commonly in DR (Curtis, Gardiner et al. 2009). The first detectable clinical signs of DR are microaneurysms and dot haemorrhages. MA appear as small round red dots of varying size (10-125 μm in diameter) with sharp margins (ETDRS Report 10,1991) and may leak leading to dot haemorrhages, oedema and exudates (Reznicek, Kernt et al. 2011).

They may appear white with time as the lumen becomes occluded with hyaline. Any red dot larger than 125 μm is considered to be a haemorrhage, unless it has smooth margins, a round shape and a central light reflex in which case it is likely to be a MA. MA can occur in isolation or in groups. MA are thought to form as a result of capillary basement membrane thickening and endothelial cell damage which leads to loss of pericytes and tight junctions (Wong, Cheung et al. 2016). The subsequent dilatation of the capillary wall gives a saccular appearance to MA. They are mainly located in the inner nuclear layer of the retina and develop principally from the retinal venous capillaries (Reznicek, Kernt et al. 2011). MA can disappear with time due to thrombosis and subsequent reabsorption of haemorrhage as well as any oedema and exudate. Once absorbed they are often replaced by new MA. Recent studies have shown that high rates of MA turnover is associated with a greater risk of developing macular oedema (Cunha-Vaz, Ribeiro et al. 2017). MA are often seen temporal to the fovea and at areas of capillary non-perfusion (Infeld and O'Shea 1998). Published studies have reported that the presence, size and shape of MA may be critical in determining the development of (DR. High numbers of MA (up to 7) in people with NPDR was found to increase the risk of long-term development of sight-threatening DR (both PDR and DMO), without predicting the stepwise progression of DR (Rasmussen, Broe et al. 2015).

The size of MA may be important, as owing to a greater MA radius-to-vessel-diameter ratio, smaller MA may be more prone to leaking and rupture than larger MA (Dubow, Pinhas et al. 2014). As this group also identified different shapes of MA, it would be advantageous to know which MA confer a higher risk given the option of laser-targeted treatment for preventing microaneurysm rupture. As MA can rupture forming haemorrhages their presence may be significantly associated with developing vitreous haemorrhages in later life (Lee, Lee et al. 2017).

2.7.2 Cotton Wool Spots

Cotton wool spots (CWS) are retinal features that have the appearance of cotton wool. They have been described as focal nerve fibre layer infarcts caused by ischaemia which lead to interruptions of axoplasmic flow in the nerve fibre layer (Wong, Cheung et al. 2016), (Infeld and O'Shea 1998), (Patel et al 2012) and are a sign of local ischaemia. They appear as pale yellow to white, superficial inner retinal lesions with ill-defined feathery margins, resulting from coagulative necrosis of isolated retinal foci (Feman 1994). CWS are detected where the retinal capillary bed already showed marked abnormalities, therefore, while CWS may be a hallmark of DR, wide areas of subclinical retinal microvascular disease are likely to predate their onset/detection, and as a result, the role of CWS as predictors of future progression to sight-threatening DR is not clear (Feman 1994) (Naqvi, Zafar et al. 2018). There is significant overlap between DR and hypertensive retinopathy (HR), whereby severely elevated blood pressure may cause similar pathology to DR, including CWS, flame-shaped haemorrhages, arteriovenous nicking and optic disc oedema (Infeld and O'Shea 1998). Indeed, some lesion patterns have characteristics of both DR and HR, and could be interpreted as either DR worsened by hypertension or HR developing in people with diabetes (Bek and Helgesen 2001). Matthews et al showed that the level of hypertension in people with diabetes was related to the presence and number of CWS (Matthews, Stratton et al. 2004). The pathogenesis and overlap of CWS in these diseases are not fully understood, but impaired retinal blood flow autoregulation in people with diabetes

may be involved (Rassam, Patel et al. 1995) in addition to the well-documented disturbed microcirculation. CWS are also commonly associated with IRMA (see later) but on their own they are not an independent predictor of DR progression.

2.7.3 Hard Exudates

Hard exudates, found principally in the posterior pole of the macular region in the outer layers of the retina, are one of the hallmark signs of DR and can severely compromise sight (Infeld and O'Shea 1998). HE are yellowish-white in colour and composed largely of extracellular lipid that has leaked from abnormal retinal capillaries. They can appear alone or in groups or may form a ring pattern around the leaking vessels (Feman 1994) or areas of oedema (ETDRS Report 10 1991). Quantitative measurement of HE in people with diabetes is associated with triglyceride and lipid levels, and a higher risk of central involvement (Sasaki, Kawasaki et al. 2013).

2.7.4 Deep Haemorrhages

Intraretinal haemorrhages can occur in different layers of the retina. The shape of a haemorrhage is indicative of its location, reflecting the architecture of the retinal layer in which they occur. Haemorrhages in the nerve fibre cause blood to dissect among the axons in the superficial retina which run parallel to the retinal surface, resulting in flame-shaped haemorrhages (Infeld and O'Shea 1998). Flame-shaped haemorrhages may appear in DR but are more usually associated with hypertensive retinopathy, retinal vein occlusions, low tension glaucoma and other conditions affecting the vasculature of the superficial and peripapillary capillary beds (Hayreh, Zimmerman et al. 1994, Liou, Sugiyama et al. 2001).

Deep haemorrhages (DH) are described as blot-shaped or dot haemorrhages, and emanate from the deep capillary plexus in the outer layers of the retina. They are found within the inner nuclear or outer plexiform retinal layers and are more common in DR than flame-shaped haemorrhages. They appear slightly darker than other retinal haemorrhages.

DH can appear at all stages of DR. When more numerous and present in all four quadrants of the retina they are considered a sign of severe NPDR as described in the 4-2-1 rule of the ETDRS (ETDRS Report 12 1991). In this category, DR is considered severe if there are DH in all four quadrants of the retina, venous beading (VB) in two quadrants or IRMA in one quadrant (or more than one of these patterns). Findings from the large, multicentre ETDRS study reported that in severe NPDR, dot/blot-shaped haemorrhages in four quadrants were indicative of future development of PDR (ETDRS Report 12, 1991).

2.7.5 Venous Changes

Venous beading, venous loops and venous reduplications are features of more severe NPDR.

A venous loop is defined in ETDRS Report 12 as ‘an abrupt, curving deviation of a vein from its normal path’. Reduplication of a vein is described as the dilatation of an existing vessel or proliferation of a new channel adjacent to and of the same calibre as the originating vein. Venous loops and reduplications are not commonly found in those with DR and ETDRS did not find a strong correlation between their baseline severity and the development of PDR but they are more commonly found in the later stages of NPDR and in PDR (Sato, Kamata et al. 1993)

2.7.5.1 Venous Beading in DR

Diffuse dilations of retinal veins are observed during the early stages of DR which may be due to retinal hyperperfusion (Bek 2002). VB describes localized changes in retinal vessel calibre which sometimes resemble a string of beads (ETDRS Report 10 1991). Venous beading in DR has traditionally been linked to future progression of NPDR to PDR (ETDRS Report 12, 1991) (Wong 2011) . This has been recently disputed in a Chinese population where the presence of VB was found to correlate with proliferative diabetic retinopathy (Chen, Zhang et al. 2018). This study found VB in two or more quadrants to be present in only 2.1% of those with severe NPDR and that 95% of eyes with this stage of VB already had PDR.

This group therefore hypothesise that VB, in this Chinese population is much more prevalent in PDR and suggest that less than one quadrant of VB may be a more sensitive predictor of progression from NPDR to PDR as once 2 quadrants are present, the DR has already become proliferative.

The 4-2-1 rule derived by the ETDRS for diagnosis of NPDR states that VB in two quadrants is considered severe NPDR (ETDRS Report 12 1991), with a high risk of progression to PDR. The ETDRS study found that VB was the most powerful predictor of subsequent development of PDR. (ETDRS Report 12, 1991). A recent study of a Chinese population found that those with NPDR and VB were approximately 7.5 times more likely to progress to PDR (Chen, Zhang et al. 2018). This study of those with Type 2 DM found VB to be more prevalent in PDR than NPDR and that VB was strongly associated with a longer duration of DM, a younger age of onset of DM and with capillary non-perfusion. They reported a lower rate of VB in NPDR than other studies (5.9%).

In a study of Japanese eyes, most (42%) VB appeared in the second branch of veins (Sato, Kamata et al. 1993)

2.7.5.2 Venous Beading in Radiation Retinopathy (RR)

Radiation retinopathy (RR) is a complication of treatment for malignancies of the head, neck, globe and orbit and thought to result from endothelial cell damage. Radiation retinopathy usually presents 6-12 months after completion of radiotherapy. It is irreversible and dose related (Archer, Amoaku et al. 1991) although radiation induced macular oedema has been successfully treated with anti-VEGF therapy (Reichstein 2015). Bilateral venous beading has been described as part of a spectrum of retinal morbidities caused by radiation including haemorrhages, optic neuropathy, telangiectasia, neovascularisation, macular oedema and macular degeneration. (Uzun, Toyran et al. 2016) The pathogenesis of RR is considered to be a result of endothelial cell loss and capillary closure, with pericytes less affected early on (Archer, Amoaku et al. 1991). The posterior pole

and macula of those with RR show severe ischaemia (Gupta, Dhawahir-Scala et al. 2007). Patients with DM are considered more at risk of RR and the appearance of VB in DR and RR are similar in severity and distribution.

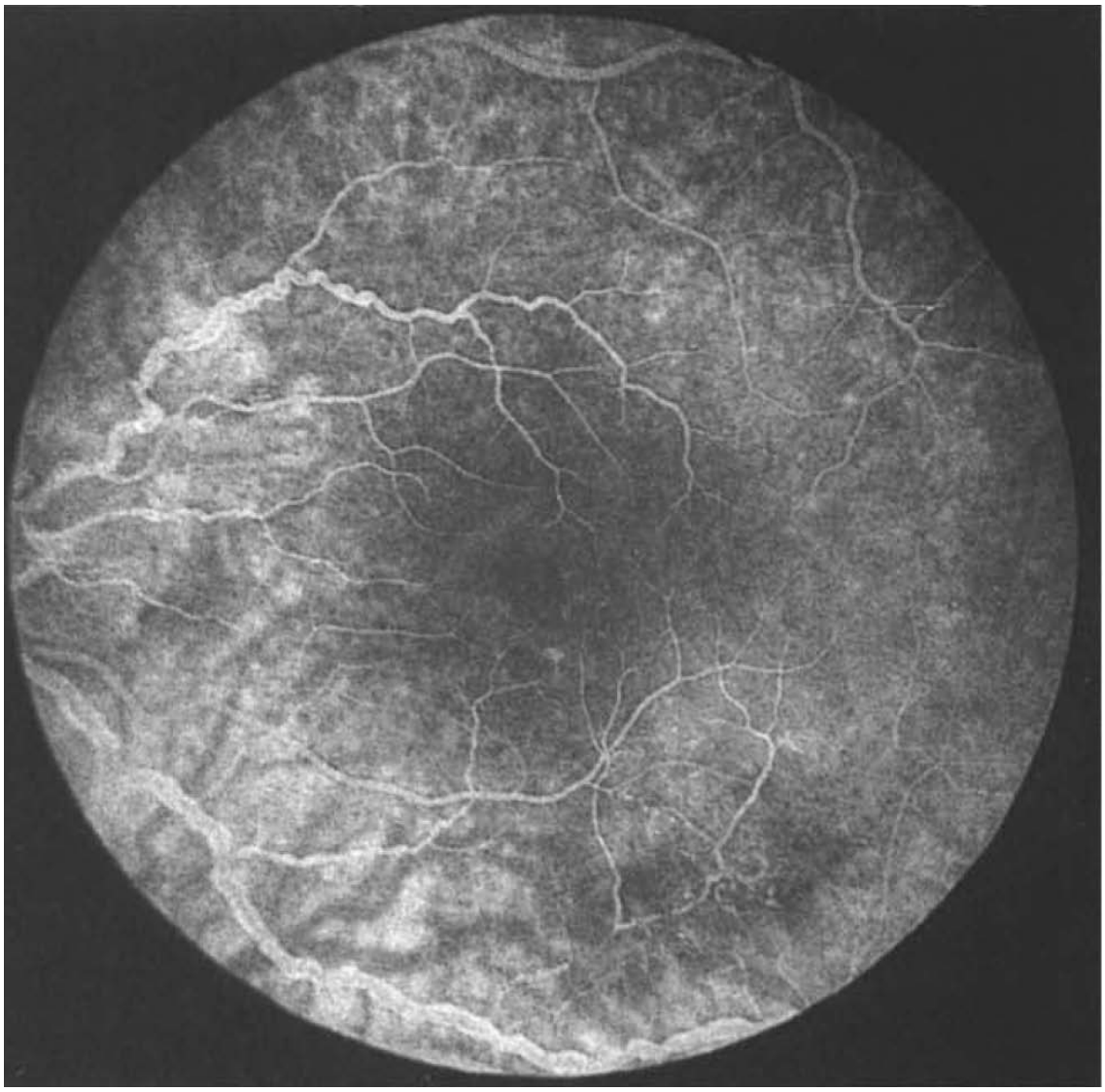
2.7.5.3 Idiopathic Venous Beading

Venous beading has been associated with recurrent branch vein occlusion in a rare case; the aetiology of the beading remained unexplained but is thought to have caused the vein occlusions, possibly due to the increased stasis causing damage to the vascular endothelium promoting thrombosis (Fonseca and Dantas 2002. The appearance of the VB in this case was more severe than that found in DR or radiation retinopathy. Another case of VB with recurrent pre-retinal haemorrhage was reported by Keyser et al (Keyser, 1997). The haemorrhage was considered a consequence of structural weakness in the beaded veins. This case showed very severe VB in all four quadrants of one eye which was more severe than VB usually found in those with DR. The other eye had mild VB. No systemic or ocular disorders explained the beading and haemorrhage and the patient's family were not affected.

2.7.5.4 Inherited Venous Beading

Inherited Venous Beading is a very rare autosomal condition first reported by Meredith in 1987 (Meredith 1987) and again in 1988 by Stewart et al (Stewart and Gitter 1988) and Piguet et al (Piguet, Gross-Jendroska et al. 1994).Those affected may have other features of retinopathy: microaneurysms, oedema, retinal neovascularisation, vitreous haemorrhage and conjunctival vascular abnormalities (Meredith 1987). Inherited venous beading is not associated with DR (Sato, Kamata et al. 1993) and its pathogenesis remains unclear although some affected had lower than normal leukocyte and neutrophil counts. The condition may be asymmetric between eyes, and the VB irregular and segmental (Piguet, Gross-Jendroska et al. 1994). As in the idiopathic cases reported, the beading is very prominent (see figure 2.2).

Fig 2.2 Fluorescein angiogram showing prominent beading in the left eye of a 26 year old (Piguet, Gross-Jendroska et al. 1994)



2.7.5.5 Venous Beading: Hypotheses of aetiology

The pathophysiology of venous beading is not understood. In a healthy retinal vein, the diameter decreases monotonically but retinal veins exhibiting beading have periodic constrictions which have been likened to a string of beads. VB has been described as an expansion of the vein in response to ischemia or other

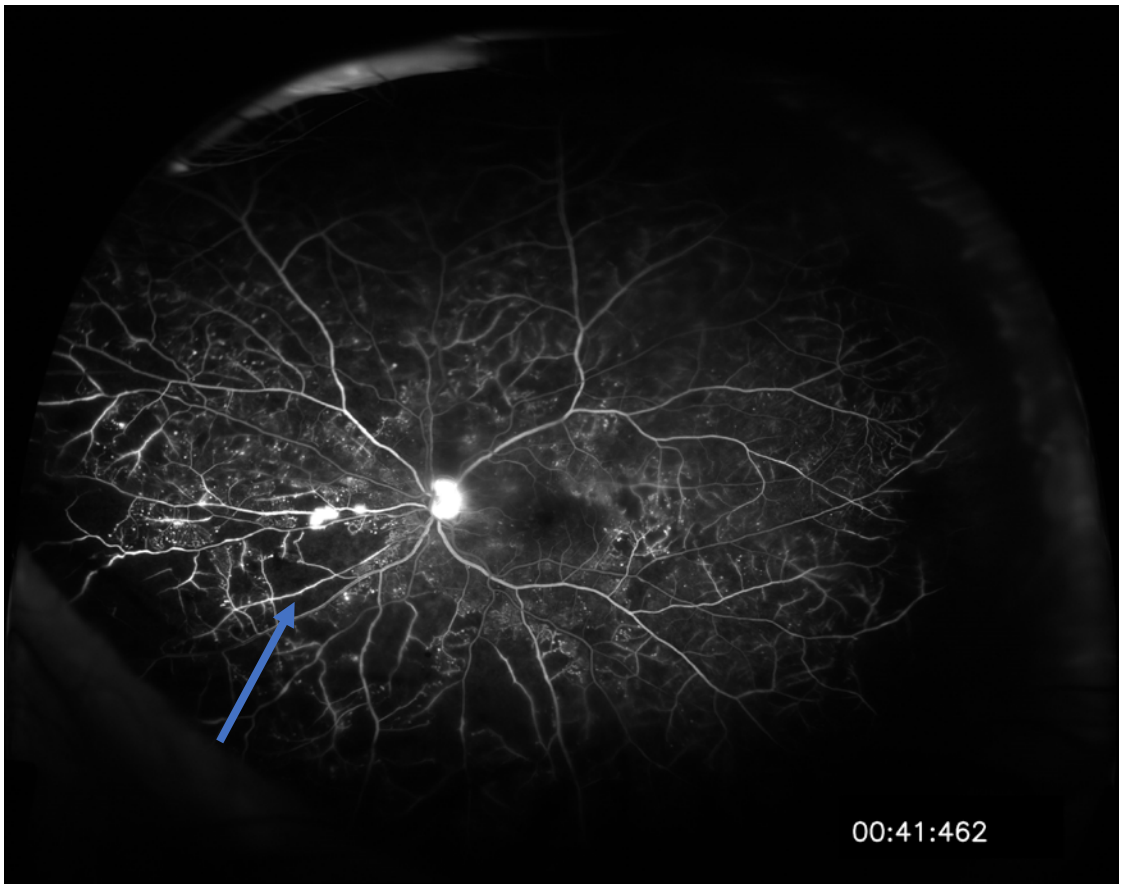
abnormalities (Chen, Zhang et al. 2018) and is often found adjacent to areas of non-perfusion (Chen, Zhang et al. 2018).

Conflicting information exists as to whether the walls of retinal veins contain muscle. Hogan et al in 1963 found that the walls of the veins do not contain elastic tissue or smooth muscle (Hogan and Feeney 1963). More recent studies have found that larger venules contain smooth muscle and the walls of retinal veins contain smooth muscle cells (Yu, Su et al. 2016).

Kur et al described retinal venular muscle cells being similar to that of pericytes (Kur, Newman et al. 2012), which are thought to play a key role in diabetic retinopathy.

Sympathetic vasomotor innervation is not present in retinal veins, therefore changes in vessel calibre may be caused by responses of the vessel wall to local stimuli, possibly increased ischaemia. Alternatively, VB could be a response to vasoactive agents within the circulation (Piguet, Gross-Jendroska et al. 1994). As retinal vessels have no autonomic innervation, local vascular control mechanisms are likely to regulate retinal blood flow but these mechanisms remain to be elucidated (Yu, Su et al. 2016). Changes in vessel calibre as seen in VB may be caused by metabolic disturbances affecting the vascular smooth muscle, resulting in focal narrowing of the vessel (Gregson, Shen et al. 1995).

Fig 2.3 Shows a Fundus Fluorescein Angiogram picture of venous beading near an area of ischaemia (Blue arrow)



2.7.6 Intraretinal Microvascular Abnormalities (IRMA)

IRMA were first described in the 1986 Airlie House Classification. IRMA have been described as dilated, tortuous intraretinal vascular segments, varying in calibre from barely visible to 31 μm (ETDRS Report 10 1991) and appear as abnormally branching and dilated capillaries and shunts between arteries and veins (Wong, Cheung et al. 2016) that may supply areas of non-perfusion. Alternative descriptions include ‘dilated, tortuous intraretinal vascular loops that are not consistent with the natural distribution of normal capillaries and do not breach the internal limiting membrane’ (Sorour, Mehta et al. 2020). IRMA typically develop

next to areas of non-perfused capillaries or CWS (Stitt, Curtis et al. 2016) which suggests that they are either vascular shunt vessels or early new vessels which are yet to penetrate the internal limiting membrane (Infeld and O'Shea 1998) . Different appearances of IRMA have recently been described, namely dilated trunk, looping, twisted loop, sea-fan and net shaped, although the significance of the different morphological appearance has not been ascertained (Sorour, Mehta et al. 2020).

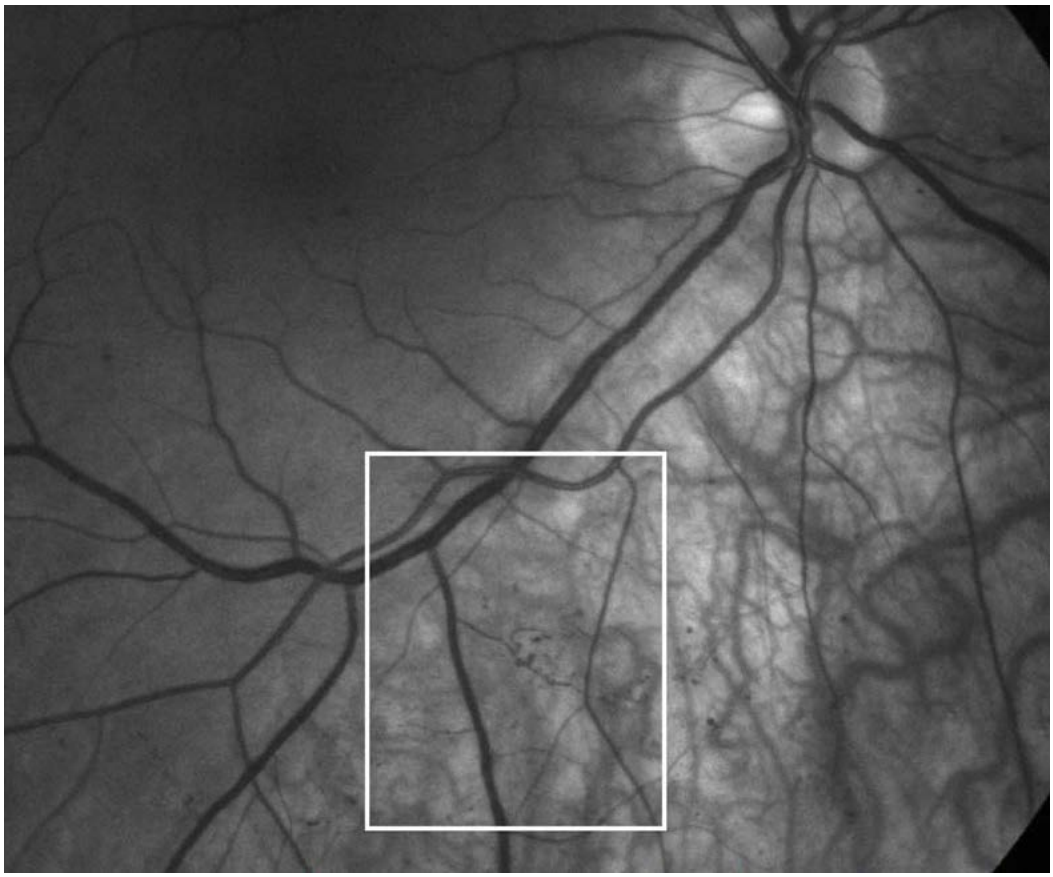
A post mortem study of eyes with IRMA identified by fundus photography 3-20 months before death were evaluated by light and electron microscopy by Imesch et al. The morphology of those IRMA examined included features usually seen in new vessels supporting the theory that they have the potential to develop into new vessels, and have been observed doing so (Lee 2015), or are new vessels within the retina (Imesch, Bindley et al. 1997). The examination found that the IRMA were located in the inner retina, close to the internal limiting membrane and were made up of multiple, closely spaced thin walled vascular lumina, with a calibre measuring 20-70 μm. The lamina of IRMA were enveloped in a perivascular cuff and comprised of tightly spaced thick endothelial cells. They also found pericyte degeneration and numerous endothelial cell nuclei in the IRMA tissue. This study found evidence that IRMA may resolve, as some lesions previously identified as IRMA showed features of atrophy. The cuff surrounding IRMA contained collagen fibrils resembling the extracellular matrix of preretinal new vessels, supporting the theory that IRMA may be new vessels that are yet to break through the ILM. Further similarities with pathological new vessels were a similar locality in the retina and the presence of endothelial fenestrations and short but tight junctions. The presence of these fenestrations may explain the observation that IRMA occasionally leak when viewed with fluorescein angiography, although not to the same extent as new vessels. (Imesch, Bindley et al. 1997). This minimal fluorescein leakage from IRMA has long been used to differentiate IRMA from new vessels. Other post-mortem studies of IRMA describe wide-calibre multicellular

channels within the capillary bed. (Gardiner, Archer et al. 2007). Another group observed that IRMA contain high numbers of endothelial cells and appear near acellular capillaries close to the arterial circulation. Their traversing of ischemic retina linking pre-capillary arterioles and post-capillary venules suggests their role as shunt vessels attempting to re-vascularise ischemic retinal tissue. (Stitt, O'Neill et al. 2011, Stitt, Curtis et al. 2016). This group argues that as IRMA are predominantly found on the arterial side of the circulation, they are unlikely to develop into new vessels which generally arise from venules. More recent studies have however observed IRMA emerging from venules, as discussed below.

Information gathered from these examinations are limited however, as they are looking at a single point in the lesion's evolution (Lee, Lee et al. 2015). A study by Chang et al investigated the potential of IRMA to develop into new vessels by following their natural history. They found that 70% of new vessels had their origin near pre-existing IRMA, providing strong support that IRMA may become pathological new vessels (Chang 1995).

Arteriovenous IRMA have been observed, which connect an arteriole with a venule and also those emerging from a venule and connecting to another part of the same venule, veno-venous connections (Petersen and Bek 2019). This group evaluated oxygen saturation levels in IRMA, new vessels and venous loops and found that IRMA and new vessels both bypass vascular segments with capillary occlusion by connecting arterioles and venules. Based on their oximetry measurements, the group hypothesised that IRMA may progress to neovascularization in order to increase the amount of shunting required to bypass more extensive areas of capillary occlusion (Petersen and Bek 2019).

Figure 2.4 Example of arteriovenous IRMA, courtesy of Peterson and Bek (Petersen and Bek 2019)



Arrows: arteriole and venule

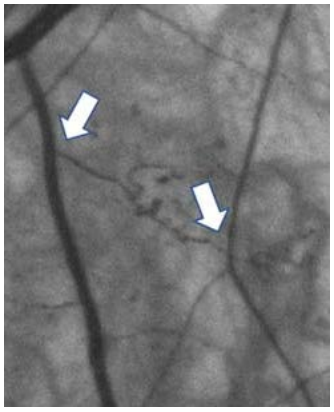
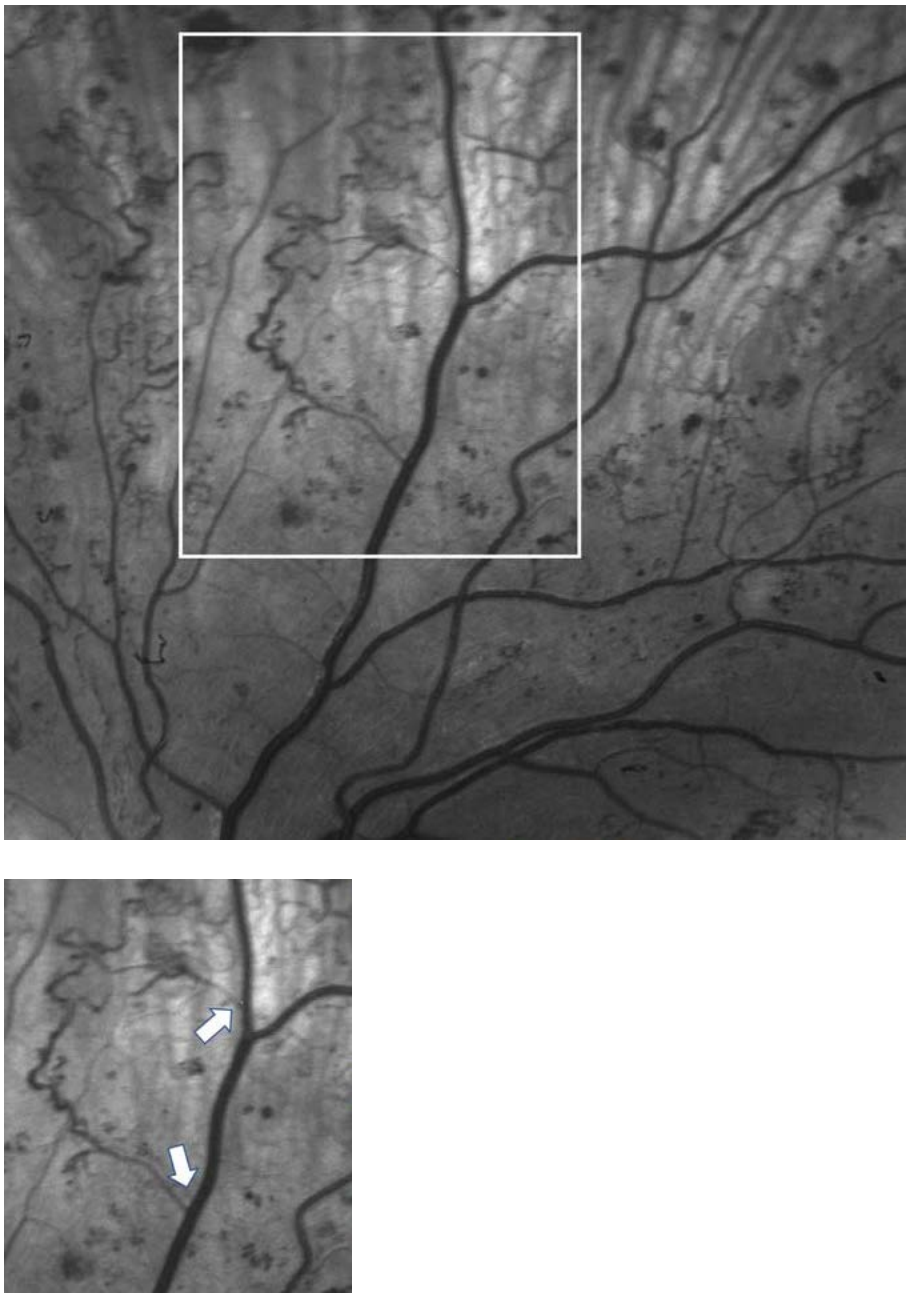


Fig 2.5 Example of IRMA connecting two sections of the same venule (from Peterson, Bek 2019) arrows pointing to the two entry points on venule



Lee et al examined patients with both IRMA and new vessels elsewhere (NVE) using colour and red free fundus photographs, fluorescein angiography images and

Spectral Domain Optical Coherence Tomography (SD-OCT). The study looked at examples of clinical diagnoses of IRMA aided by red-free fundus photographs (Lee, Lee et al. 2015). Some of these lesions had broken through the ILM when examined with SD-OCT thus being reclassified as NVE. IRMA were observed with SD-OCT as outpouchings of the ILM without penetrating the posterior hyaloid. Hyperreflective spots were identified in the inner retina where IRMA and NVEs were present. They may be microglia-activated cells that promote inflammation (Vujosevic, Bini et al. 2013) and may be a sign of activity as they were absent in fibrosed, inactive NVE so patients identified with these spots may warrant close follow up. The study identified three IRMA that progressed to NVE and findings suggest SD-OCT is a useful and non-invasive tool to differentiate IRMA from NVE.

In a similar finding to Chang et al, a more recent study with OCTA found that 50% of eyes with IRMA were found next to preretinal neovascularisation, which suggests that IRMA may be new vessels yet to break through the ILM and become new vessels (de Carlo, Bonini Filho et al. 2016).

A recently published study using an algorithm in addition to OCT Angiography that detects relative blood flow speeds found that IRMA and new vessels appeared to originate from areas of relatively slow blood speeds, adding further support to their having similar origins. They found that IRMA showed turbulent intermediate to slow flow, new vessels from venous origin had lower flow speeds and those from the arterial side of the retinal circulation demonstrated higher flow speeds (Arya, Filho et al. 2020).

A recently published study by this group found that anti-VEGF therapy reduces IRMA in a PDR population, although some regression was found if sixteen weeks elapsed between treatments (Pearce, Chong et al. 2020). This will be discussed in more detail in Chapter 6.

In summary, it is thought that IRMA may originate via angiogenesis and may be precursors to the earliest stages of neovascular growth in the retina (Muraoka and

Shimizu 1984). IRMA represent either new vessel growth within the retina or remodelling and growth of pre-existing vessels through endothelial cell proliferation stimulated by hypoxia, often bordering areas of capillary nonperfusion. IRMA may possibly have two distinct aetiologies, angiogenesis and vascular remodelling.

In the ETDRS longitudinal case evaluation, IRMA were found to be predictive of future development of PDR (ETDRS Report 12, 1991) and therefore to significant sight loss. According to the 4:2:1 rule of the ETDRS, severe NPDR is diagnosed with ≥1 prominent IRMA in ≥1 quadrant, by comparing fundus findings with ETDRS standard photograph 8A (ETDRS Report 10, 1991).

A more recent study found IRMA to be the most significant predictor of progression to PDR, being associated with a 1.77-fold higher chance of developing PDR than VB in two quadrants (Lee, Lee et al. 2017).

The pathophysiology of DH, VB and IRMA, the features of moderate/severe NPDR most predictive of progression to PDR are likely to involve endothelial cell proliferation, ischaemia, VEGF, angiogenesis and pericyte loss. Changes in the vessel walls observed in the development of IRMA and similarities to the structure of NV as well as their location near areas of ischaemia directed my selection of SNPs to be investigated towards those related to ischaemia and VEGF. The pathophysiology of VB remains elusive but may be due to structural changes in the vessel wall hence my interest in SNPs that may affect retinal vessel wall integrity or function.

Table 2.5 Types of lesions detected in NPDR and potential relevance in NPDR diagnosis and characterization

Lesion type	Description	Relevance in NPDR diagnosis and characterisation
Microaneurysms (MA) and haemorrhages	• Occur secondary to capillary wall outpouching due to pericyte loss • Earliest clinical sign of DR • Rupture of MA results in haemorrhages	• Number, size, distribution and turnover of MA and haemorrhages are important for diagnosis and may help to determine progression rates to sight-threatening DR
Intraretinal microvascular abnormalities (IRMAs)	• Characterise remodelling of pre-existing vessels or growth of new vessels • IRMA are distinctive from the neovascularisation observed in PDR in their larger size and broader arrangement	• Presence of IRMA is necessary for the diagnosis of moderate-to-severe NPDR • Unclear whether the distribution of IRMA is important in assessing severity • IRMA originating via angiogenesis may be important for the

	• Found adjacent to or surrounding areas of occluded capillaries • Visible as telangiectatic, dilated capillaries within the retina	development of PDR
Venous beading/ loops/reduplications	• Venous beading is characterised by irregular constriction and dilation of venules in the retina • Venous loops and reduplications are rarer than venous beading and might result from accentuation of a bead, traction from vitreoretinal adhesions or may be shunt vessels	• Evidence linking venous beading to PDR development is unequivocal • Venous loops/reduplications are observed more frequently in advanced stages of DR and do not appear to lead to sight-threatening changes in the diseased retina
Cotton wool spots (CWS)	• Areas of nerve fibre ischaemia or infarction and axonal swelling induced by	• The early appearance of CWS helps in the early diagnosis of NPDR but may lack

	areas of retinal capillary closure • Signs of poor retinal perfusion and are easily visualised • Associated with systemic hypertension diabetes and are common in DR and hypertensive retinopathy	predictive value for determining retinopathy progression
Hard exudates (HE)	• Lipid and lipoprotein deposits, usually found in the outer layers of the retina • HE have a 'waxy' appearance with sharply defined borders and result from leakage from abnormally permeable MA or capillaries in the retina	• The presence of HE plays a vital role in grading DR into different stages but their appearance was not found to be associated with DR progression

After examining the aetiology of microaneurysms, deep haemorrhage, cotton wool spots, venous beading, IRMA, hard exudates and venous loops/ reduplications, I decided to focus my investigations of DH, VB and IRMA. This is because the

ETDRS found no correlation between the presence of hard exudates and progression to PDR (ETDRS Report 12). Venous loops and reduplications in the ETDRS study were rare and were not present in 25% of eyes studied. Cotton wool spots were not shown to be predictive of progression to PDR in the ETDRS, the risk of progression of eyes with CWS but no VB, IRMA or significant DH was low. Microaneurysms were analysed together with DH in the ETDRS and these combined features were found to be significant predictors of progression. In my analysis, I decided to focus on DH alone and not include MA's as they are less easy to detect than DH (ETDRS Report 12)

2.8 Genetics of DR

Complications of DM are likely to arise from a combination of genetic and environmental factors (McCarthy 2004).

The role of genetic factors in the development of diabetic complications has been widely studied (Doria 2010). Although duration of diabetes and glycaemic control are known risk factors for developing DR, progression in individuals varies enormously. Some patients with poor diabetic control do not develop significant eye disease after many years and others develop STDR shortly after diagnosis despite good glycaemic control. Observations from large clinical trials highlighted the absence of DR and other microvascular complications in approximately 25% of those studied, however, glycaemic control must vary in this large group (Strowig and Raskin 1992). The different phenotypes may therefore be explained by a genetic predisposition to the vascular phenotypes and will be discussed in more detail in Chapter 4.

2.8.1 Candidate Gene Studies

One approach to the identification of genes that might be involved in the pathogenesis of a disease process is to select and evaluate genes that code for

proteins considered most likely to play a role in the development of that disease. This will be discussed further in Chapter 4.

2.8.2 Genome-Wide Studies

Genome-wide association studies enable the entire genome to be analysed for genes affecting susceptibility to DR (Doria 2010). This will be discussed further in Chapter 4.

2.8.3 Twin and Family studies in DR

Twin studies have described a strong link between retinopathy levels in T2DM but not so in twins with T1DM suggesting genetic factors may determine the extent and severity of DR in T2DM but environmental factors may have more influence on DR in T1DM (Leslie and Pyke 1982). In this study, 35 of 37 sets of twins with T2DM with similar durations of DM, shared the same DR grade, whereas of the 31 sets of T1DM twins, 10 had markedly differing DR severities, despite each twin sets also having very similar durations of DM. Most twins had been living apart throughout the duration of their diabetes, implying that environmental factors are not responsible for the similarities and adding weight to the argument that DR in T2DM may be genetically driven.

Another study compared DR severity amongst Mexican-American siblings with T2DM The study found, after adjusting for other risk factors, familial aggregation of severe retinopathy (Hallman et al 2005). However, purely the presence of DR alone, i.e. looking at all grades, showed no familial aggregation. This was also observed in the DCCT when examining families with DR. A study of Indian families with two or more siblings with T2DM found that after adjusting for covariates, the siblings of those with T2DM were at higher risk of DR than the siblings of unaffected probands, contradicting previous studies like DCCT (Rema et al 2002). The differences found in this study could be explained by the Indian study recruiting attendees to a single diabetic clinic, which may introduce bias compared to the general population. Although not conclusive, these studies suggest that

genetics or a shared familial environment may have a greater influence on advanced microvascular complications than on early disease (Hallman et al 2005).

2.9 Treatment of Diabetic Retinopathy

2.9.1 Optimise glycaemic control

As previously discussed, optimizing glycaemic control has been shown to reduce all microvascular complications of DM. As previously described in chapter 2.2.3, improving glycaemic control reduces the progression of DR.

2.9.2 Optimise Blood pressure control

As previously discussed in chapter 2.2.4, studies have shown that hypertension may increase the risk and progression of DR.

2.9.3 Laser for PDR

Laser photocoagulation, or pan-retinal photocoagulation (PRP) was first developed in the 1960's. It has been the standard treatment for PDR since the Diabetic Retinopathy Study (DRS) and Early Treatment of Diabetic Retinopathy Study (ETDRS) demonstrated unequivocally that it significantly reduces the rate of severe visual loss, particularly in those with high-risk PDR characteristics (ETDRS Report 9, 1991). The mechanism of action is however still not understood. Retinal thermal burns produced by the laser form visible scars. The focal retinal destruction is thought to reduce the oxygen demand of the retina, thereby relieving hypoxia, reducing VEGF levels and promoting regression of new vessels (Wong, Cheung et al. 2016) (Duh, Sun et al. 2017). As described, the treatment is destructive and frequent side effects of laser treatment are pain during administration, vitreous haemorrhage, secondary choroidal neovascularisation, macular burns, macular oedema (Bressler, Beck et al. 2011) and reduction of peripheral vision which can impair the ability to drive (Diabetic Retinopathy Study Research Group, Report 2, 1978). Macular oedema development or progression after PRP is usually transient. Colour vision changes have been reported,

particularly in the blue spectrum (Fong, Girach et al. 2007) as well as reductions in contrast sensitivity (Canning, Polkinghorne et al. 1991). Laser burns inadvertently applied to the fovea cause permanent reduction in vision. Problems with night vision, glare and photophobia are also permanent side effects (Fong, Girach et al. 2007). Less common side effects are loss of some of the nerve fibre layer which also leads to vision reduction and poor pupillary dilation, making future fundal examinations and surgery difficult. Tractional retinal detachment and vitreoretinal traction may also follow laser treatment, requiring surgery. (Kaiser, Maguire et al. 2000). PRP is ineffective in some patients.

2.9.4 Vitrectomy for PDR

Vitrectomy surgery is the mainstay of treatment for clearing vitreous haemorrhage and tractional retinal detachment (Fong, Aiello et al. 2004). Vitrectomy surgery involves removal of the vitreous gel, vitreous haemorrhage, and fibrovascular tissue from the eye. During the procedure PRP can be completed with an endolaser, retinal traction relieved and the retina reattached. Anti-VEGF agents may also be administered to treat neovascularisation during the surgery. Early vitrectomy for severe vitreous haemorrhage in T1DM was associated with better visual outcomes, but not for those with T2DM, possibly because those with T2DM generally had less severe retinopathy (Diabetic Retinopathy Vitrectomy Study Report 5, 1990). In conclusion, the ETDRS study recommended early vitrectomy for eyes with very severe PDR in order to preserve or regain vision (ETDRS Report 5, 1990).

2.9.5 Anti-VEGF for PDR

Vascular endothelial growth factors (VEGF) are proteins produced in the eye by RPE cells, pericytes, astrocytes, muller cells, glial cells, retinal neurones and vascular endothelial cells in response to hypoxia or ischemia and act as triggers for angiogenesis and vasculogenesis as well as increasing vascular permeability (Gupta, Mansoor et al. 2013). VEGF is a heparin-binding growth factor which

specifically acts on endothelial cells. VEGF can induce breakdown of the blood-retinal barrier as well as playing a central role in retinal neovascularization (Leung, Cachianes et al. 1989) (Tarr, Kaul et al. 2013).

VEGF levels in the vitreous and retina are elevated in those with DR (Cunningham, Adamis et al. 2005) (Aiello, Avery et al. 1994). Recent clinical trials comparing macular laser for DMO and anti-VEGF have also noted an improvement of DRSS. The RIDE and RISE trials showed that participants receiving Anti-VEGF injections for DME showed a significantly lower rate of progression of DR (worsening of ≥2 or ≥ 3 steps DRSS). More recently, the Clarity study found that anti-VEGF (aflibercept) treatment of those with PDR achieved regression of new vessels more quickly and effectively compared to PRP (Nicholson, Crosby-Nwaobi et al. 2018) as well as achieving better visual acuity improvements than those in the PRP arm.

2.9.6 Laser for DMO

Diabetic macular oedema (DMO) is caused by break down of the blood-retinal-barrier leading to vascular leakage in the macular (Wong, Cheung et al. 2016). The resultant accumulation of fluid leads to reduced vision and sometimes distortion of vision. Laser treatment for DMO can stabilize though not improve visual acuity (Wang and Lo 2018) The ETDRS found that focal laser photocoagulation reduced the risk of vision loss from DMO from 24% to 12% at 3 years but improvement in visual acuity was found in very few participants (3%) (ETDRS Report1 1985) (ETDRS Report 9, 1991). Side effects of laser treatment include formation of paracentral blind spots which may cause difficulty with reading (Pearce, Sivaprasad et al. 2014).

2.9.7 Intravitreal (IVT) steroids for DMO

Corticosteroids have been shown to inhibit the expression of the VEGF gene (Nauck, Karakiulakis et al. 1998) and suppress inflammatory pathways. They are sometimes used as treatment for DMO, particularly in refractory cases and those not responding to anti-VEGF, either as intravitreal injections or as sustained-

release implants. Frequently occurring side effects are cataract and intra-ocular pressure rise (Elman, Aiello et al. 2010) (Boyer, Yoon et al. 2014).

2.9.8 Anti-VEGF for DMO

As previously discussed, the DRSS in most cases progresses with time and does not generally improve by improving blood glucose levels or lowering blood pressure.

Intravitreal injections of anti-vascular endothelial growth factor (VEGF) agents are effective for treating centre-involving diabetic macular oedema (CI-DMO) and are an alternative therapy for PDR. They are now the first-choice treatment for (CI-DMO).

Trials evaluating the effect of anti-VEGF on DMO also described improvements in DRSS. The READ-2 study of patients with DMO compared anti-VEGF alone with focal or grid laser photocoagulation and a combination of anti-VEGF and laser. The study found that intravitreal anti-VEGF injections (of ranibizumab) had a greater effect on reduction of oedema when combined with laser treatment or without laser treatment compared to when treating with laser alone (Nguyen, Shah et al. 2010). A recent study compared the effect of intravitreal aflibercept, bevacizumab and ranibizumab on DMO. The results showed that all three anti-VEGF agents improved CI-DMO equally when baseline vision loss was mild. When baseline vision levels were lower, aflibercept proved more effective and vision gains were greater (Wells, Glassman et al. 2015). The DA VINCI trial for CI-DMO also found aflibercept demonstrated a superior effect of on visual acuity when compared to laser treatment (Do, Nguyen et al. 2012).

Recent clinical trials comparing the effect of anti-VEGF on Diabetic Macular Oedema (DMO) to sham (RISE and RIDE, NCT00473382 and NCT00473330) (Ip, Domalpally et al. 2012) and Anti-VEGF compared to Laser (VIVID, VISTA) (Staurenghi, Feltgen et al. 2018) also looked at the effect of anti-VEGF on the participant's DRSS score.

The RIDE and RISE trials showed that participants receiving anti-VEGF injections for DME showed a significantly lower rate of progression of DR (worsening of ≥2 or ≥ 3 steps DRSS) compared to no treatment. Those receiving sham had a 33.8% chance of progression in 2 years compared to 11.2%-11.5% in those receiving anti-VEGF injections. They also showed that participants in the Anti-VEGF arm had a greater likelihood of improving their DRSS score by 2 or more steps than those in the sham arm.

In those randomised to the sham arm, 15% had mild NPDR (DRSS 35) at baseline, rising to 24% at month 24, compared to baseline levels of DRSS 35 of 16% in the 0.3mg and 17% in 0.5mg ranibizumab arms which rose to 44% and 37% respectively at month 24.

2.9.9 Anti-VEGF for DR

In the VIVID and VISTA trials, retinopathy improved by two or more DRSS steps in those treated with IVT aflibercept compared to those treated with laser. When looking at those with a baseline DRSS of 43 or lower, 5.9% of those treated with laser, compared with 13% of those treated with aflibercept had a 2 or more step improvement in DRSS. For those with a baseline DR score of DRSS 47, 4.5% of those treated with laser compare to 25.8% of those treated with aflibercept improved by 2 or more DRSS steps and for those with a baseline DRSS of ≥53, the improvements were 28.4% for those treated with laser and 64.5% for those treated with aflibercept (Staurenghi 2018).

The VIVID, VISTA, RISE and RIDE trials reported the overall DRSS score changes but not changes to the individual features of NPDR, namely DH, VB and IRMA. These features are also present in eyes with PDR and the effect of anti-VEGF on DH, VB and IRMA will be discussed in more detail in Chapter 6.

The PANORAMA study (NCT02718326) is an ongoing trial looking at the Efficacy and safety of intravitreal aflibercept (Bayer) injections in patients with Moderate or Severe NPDR compared to sham. Again, the study looks at the overall DRSS

score, not individual feature grading. First results showed on the primary endpoint at one year, 80% and 65% of patients receiving intravitreal aflibercept on an every 8- and every 16-week interval (after an initial monthly dosing period), respectively, experienced a two-step or greater improvement from baseline on the Diabetic Retinopathy Severity Scale, compared to 15% of patients receiving sham injection ($p<0.0001$). At week 100, the same level was achieved by 50% and 62% of 8 week and 16 week vs 13% of sham eyes. (Lim 2020). In the second year, those in the 8 week treatment arm received injections as needed.

Despite the positive effect on vison and DRSS, repeated intra-vitreal anti-VEGF injections have risks. A serious but fortunately rare side effect of intra-vitreal anti-VEGF is the risk of infectious endophthalmitis. A study of 818,588 procedures found an endophthalmitis rate of 0.061% (Kiss, Dugel et al. 2018). Post intravitreal rates of sterile inflammation are also rare (0.16%) in aflibercept (Kiss, Dugel et al. 2018).

Further disadvantages of treating DMO with anti-VEGF are the need for multiple monthly or bi-monthly injections (12-15 in the first three years of treatment and slightly lower in the following years) and the burden of these numerous clinic visits (Diabetic Retinopathy Clinical Research, Elman et al. 2010). Oedema does not always resolve and some patients receiving anti-VEGF may also need laser. In some patients visual acuity does not improve, approximately 40% of those treated with anti-VEGF gained less than 10 ETDRS letters (Elman, Aiello et al. 2010) . Of interest, animal studies have reported that anti-VEGF could lead to a loss of retinal ganglion cells (Nishijima, Ng et al. 2007) and may impact negatively on the mature blood vessels of the choriocapillaris (Saint-Geniez, Maharaj et al. 2008) as well as causing apoptosis of cells in the inner and outer nuclear retinal layers (Saint-Geniez, Maharaj et al. 2008), though these effects have not been observed in humans undergoing anti-VEGF therapy.

2.10 Optical Coherence Tomography Angiography (OCTA)

OCTA is a relatively new technique, which provides high-resolution images of retinal and choroidal blood flow and structure using motion contrast (de Carlo, Bonini Filho et al. 2016). OCTA uses rapid, sequential B-scans from a specific location to detect changes in red blood cell movement and thereby create a three dimensional map of blood flow and microvascular structure with the ability to detect changes in vessel structure and flow (Takase, Nozaki et al. 2015) (Matsunaga, Yi et al. 2015) (de Carlo, Bonini Filho et al. 2016). By segmentation of captured images, OCTA is able to view both superficial and deep retinal layers.

Vascular changes in NPDR can therefore be clearly visualized. It has advantages over fundus fluorescein angiography (FFA), being non-invasive and quicker to perform as well as being able to visualize vessels in all layers of the retina and choriocapillaris. Resolution of retinal vessels is superior to FFA and both eyes are equally well imaged at one capture unlike with FFA. OCTA is however unable to detect leakage, staining or pooling but in some ways this is an advantage as dye leakage can obscure the image, as with FFA. Another drawback is that imaging with OCTA is very sensitive to patient movement and poor fixation which can adversely affect image quality (Lee and Rosen 2016). OCTA is likely to become a valuable tool for early and frequent assessment of vascular changes in DR as it is non-invasive and can therefore be performed at every clinic visit unlike FFA (Matsunaga, Yi et al. 2015).

Various OCTA measurements have been described for assessing vascular perfusion at the macular, specifically for those with DR. These include capillary perfusion density (CPD), vessel density (VD), flow index (FI), skeleton density (SD), vessel area density (VAD), vessel length density (VLD) and capillary density index (CDI) (Gildea 2018). OCTA can also measure the foveal avascular zone (FAZ) clearly in different retinal layers. However, at present there is no

corresponding functional data so which of these measurements are important is yet to be elucidated.

2.10.1 OCTA in DR

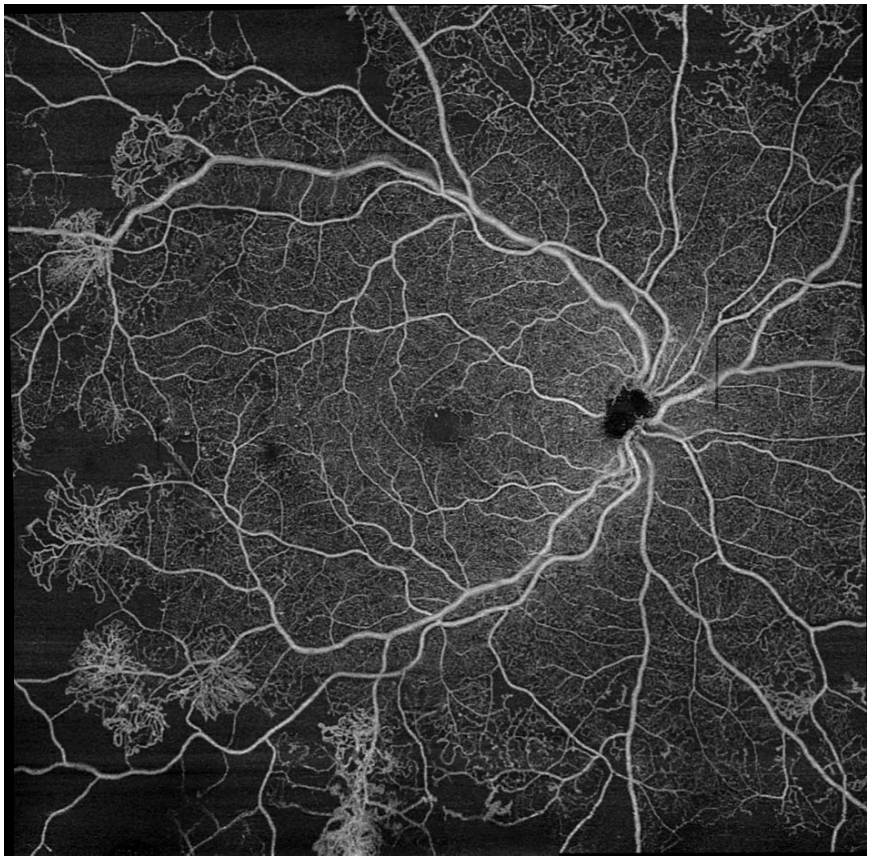
2.10.1.1 Neovascularisation

OCTA is able to identify preretinal neovascularisation in PDR and differentiate between new vessels on the disc (NVD) and optic disc venous collaterals (OVC's) by accurately locating the lesions' position in the retinal layers or in the vitreous and identifying differences in shape. NVD appear as abnormal large vessels extending into the vitreous cavity. OVC's appeared as thin and meandering vessels in the radial capillary layers (Singh, Agarwal et al. 2017). New vessels have been differentiated from IRMA by segmentation of the OCTA image above the ILM where new vessels are observed (de Carlo, Bonini Filho et al. 2016).

2.10.1.2 Diabetic macular ischaemia (DMI)

OCTA has facilitated a better understanding of the relationship between capillary non-perfusion and diabetic macular ischaemia (DMI) by enabling a more detailed and accurate assessment of the foveal avascular zone (FAZ) and the surrounding vasculature than FFA. The FAZ is an area of the macular centred on the fovea which is free from blood vessels. The FAZ receives nutrients by diffusion from the underlying choroidal circulation rather than the retinal circulation (Delaey and Van De Voorde 2000). In DR, increased foveal capillary drop-out and subsequent enlargement of the FAZ is caused by loss of capillaries and capillary closure in the adjacent vessels (Bresnick, Condit et al. 1984). Areas of capillary non-perfusion appear as dark areas in OCTA images as no or very little flow is present (de Carlo, Bonini Filho et al. 2016). It has been reported that areas of non-perfusion are more distinct on OCTA when compared to FFA images (Matsunaga, Yi et al. 2015).

Fig 2.6 Ultra-Widefield OCTA Image of PDR (Arya, Sorour et al. 2019)



2.10.1.3 IRMA

OCTA is able to image IRMA with higher resolution and therefore greater detail than with colour fundus photography (Arya, Rashad et al. 2018). With OCTA imaging, IRMA appear as abnormal branching, dilated retinal vessels that have not broken through the ILM. IRMA have been observed as looping vessels which often appear adjacent to areas of capillary non-perfusion. The loops appear significantly greater in calibre than those of capillaries (Matsunaga, Yi et al. 2015). IRMA have been observed to have different morphological appearances. Sorour et al described five differently shaped categories of IRMA with OCTA. These are ‘Dilated Trunk’ which have an increased calibre compared to surrounding

capillaries and end with a straight or slightly curved shape, ‘Looped IRMA’ which appear as circular looped vascular channels, their origin being the same vessel into which they drain, ‘Twisted Loop’, appearing as a self-rolling loop resembling a pigtail, ‘Sea-fan shaped’ IRMA, with a branching pattern and ‘Net-shaped IRMA’, with a rectangular or irregular outline (Sorour, Mehta et al. 2020).

OCTA studies have confirmed that IRMA are frequently located adjacent to areas of capillary drop out, these areas being easy to identify with this imaging modality (Sorour, Mehta et al. 2020) (Schaal, Munk et al. 2019). OCTA also has a role in differentiating IRMA from neovascularisation (see above), along with the ability to accurately locate the lesions in the retina, OCTA may be superior to that of fluorescein angiography because of the absence of fluorescein dye leakage that can obscure detail. Longitudinal studies of IRMA using OCTA may shed light on the hypothesis of IRMA becoming new vessels with time (Arya, Sorour et al. 2019).

Figure 2.7 Different shaped IRMA, OCTA images from Sorour, Mehta et al. 2020

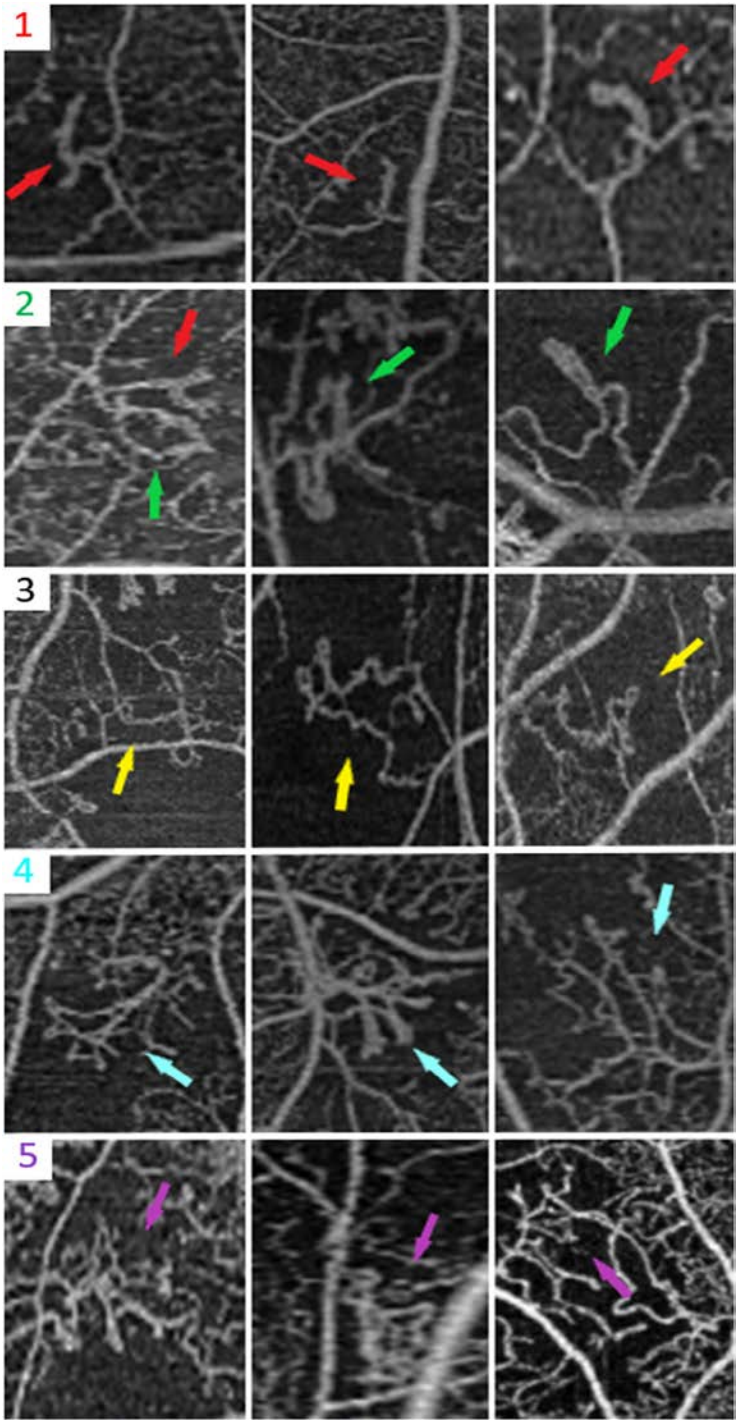


Fig 2.8 OCTA image of IRMA (Arya, Sorour et al. 2019)

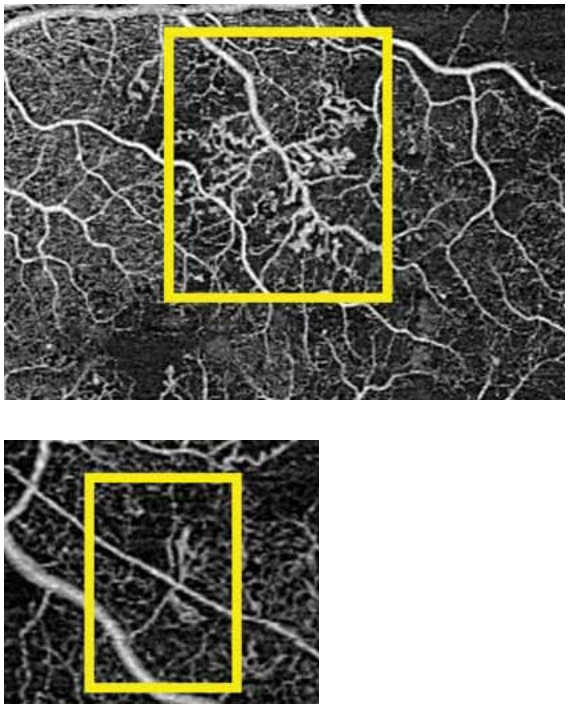
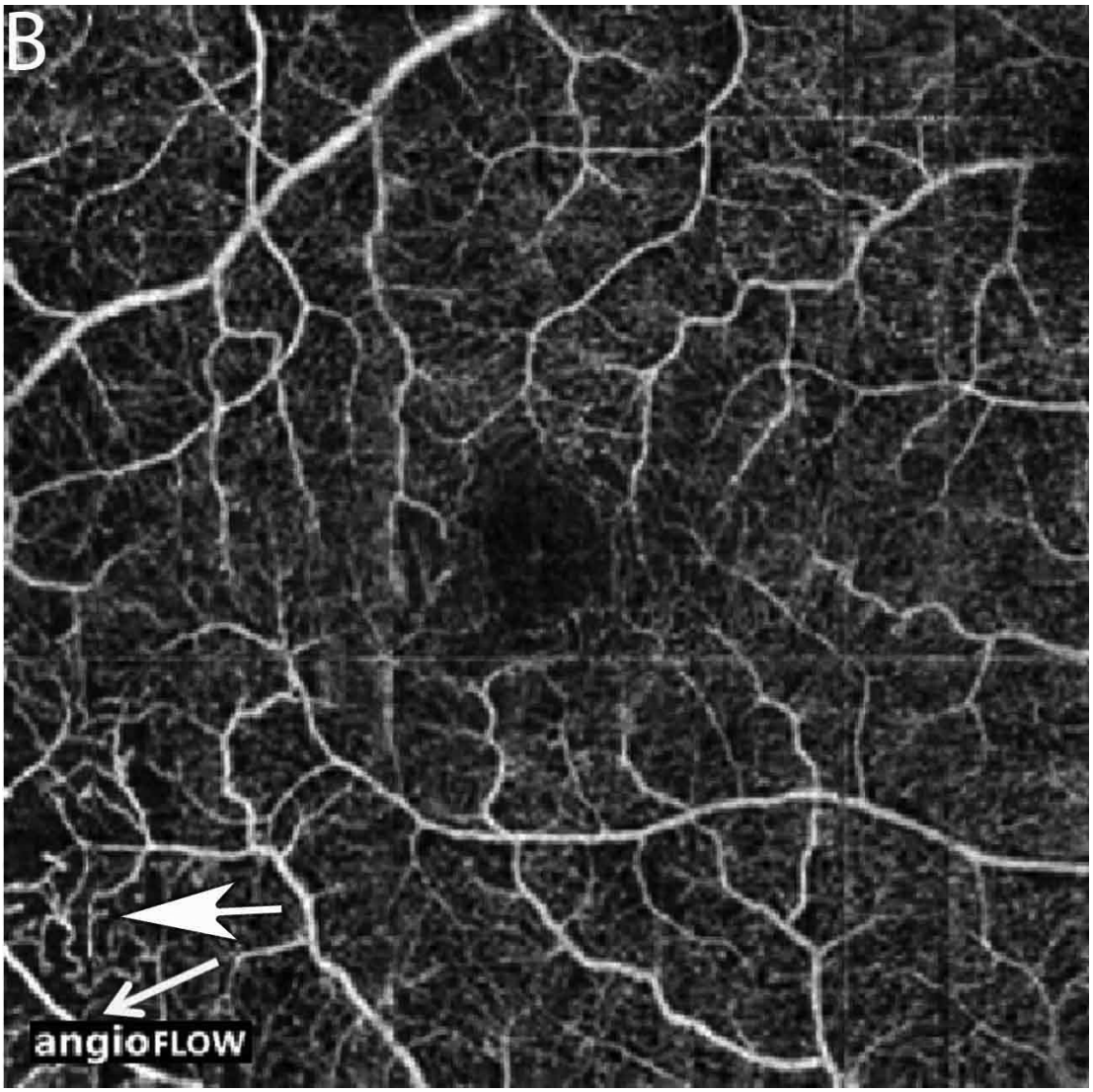


Fig 2.9 Image of IRMA (large arrow) with OCTA (de Carlo, Bonini Filho et al. 2016)



2.11 Summary

Diabetes and its complications, including DR are increasing and constitute a major health burden. Genetic and environmental factors influence the development and progression of DR yet despite extensive investigation, the pathophysiology of DR is still poorly understood. DR is classified by the presence and severity of its different features and their risk of progression to vision loss. Despite improved and more widespread DR screening services leading to earlier detection and treatment as well as improved treatment options, DR is still a leading cause of visual impairment. Treatment options for DR have improved and have less destructive side effects but are invasive. Moderate and severe NPDR are characterized by the presence of three features of DR, namely deep haemorrhages (DH), venous beading (VB) and intraretinal microvascular abnormalities (IRMA). The '4-2-1' rule of DR is generally unidirectional, based on the number, location and type of distinct microvascular lesions, meaning that the chronological appearance and disappearance of fundus lesions are not considered. However, it has been demonstrated that lesions in NPDR, such as CWS and MA, do not simply accumulate to reveal disease progression as previously thought. Instead, the regenerative activity of the retina results in the gradual reabsorption of some lesions, meaning that they can disappear from fundus photographs (Leicht, Kernt et al. 2014). This may also be true of the three features of severe NPDR, or they may progress in chronological order.

DH, VB and IRMA have not previously been examined individually but have been grouped together as risk factors for progression to sight threatening PDR. Investigating the individual features of DR in more detail may help us better understand the pathways and mechanisms important in the development of PDR and ultimately visual loss.

Chapter 3: Background, Hypothesis and Research Questions

Diabetic Retinopathy is a microvascular retinal complication of Diabetes Mellitus (DM). It is classified as mild, moderate and severe non-proliferative (NPDR) and proliferative disease based on the presence of specific clinical features. However, the course of development of these lesions varies between individuals within the same DR severity group. In most cases, DR develops symmetrically over a number of years (Dogru 1998) with only 5-10% of those with PDR in one eye having no or mild NPDR for more than 2 years after the onset of PDR in the fellow eye. Grossly asymmetrical DR is likely to be caused by other pathology (local and systemic) such as carotid artery disease or vein occlusion. Grading of DR between eyes is usually within 2 DRSS steps, only 8.2% showing asymmetry (i.e. more than 2 DRSS steps difference) in one study (Lino et al. 1993), which would reflect the same effect of glycaemic control (Dogru 1998).

Moderate/Severe NPDR is characterised by the presence of deep haemorrhages (DH), venous beading (VB) and/or intraretinal microvascular abnormalities (IRMA). These are the features of interest in this study as they are important risk factors for progression to PDR and subsequent visual loss (ETDRS Report 12, 1991). Since the ETDRS, very few studies have examined the individual components, just the stages of the disease. It remains unclear whether the different components have the same aetiology or pathophysiology. Furthermore, although it is known that anti-VEGF can improve the DRSS, it is unclear whether the different components respond equally. VB and IRMA are distinct anatomical features but it is unclear whether they are induced by the same trigger such as ischaemia or if they have different pathophysiologies.

Despite improvements in therapies for DR, it is still a leading cause of visual loss. Originally developed for the treatment of age-related macular degeneration (AMD) and diabetic macular oedema (DMO), anti-VEGF therapy in recent years has gained popularity in the treatment of PDR and to a lesser extent NPDR. Oral

therapies for NPDR are also under development. The targeting of treatment for moderate/severe NPDR seems appropriate as milder grades may not progress and once DR becomes proliferative it is more established and treatment outcomes are poorer.

DR often manifests gradually, over a period of 10 years from no retinopathy to PDR but progression rates vary enormously. As previously discussed, patients with many years of poor diabetic control may never develop STDR but in some with good control, severe diabetic eye disease can develop quickly (Strowig and Raskin 1992). This suggests that known risk factors do not fully explain the onset and progression of DR, we therefore hypothesise that genetic predisposition may also contribute to the different vascular phenotypes. As DH is very common in moderate and severe NPDR, this study's focus was VB and IRMA.

Currently there is no data describing the prevalence of the three features of moderate-severe NPDR within this group, Since the landmark trials of 1980's, DRS and ETDRS there has been no further investigation of the individual features of DR. A better understanding of these features may help identify those more at risk of progression to sight-threatening complications of diabetes and target treatment to specific phenotypes, as well as stratifying those more likely to benefit from current and future therapies.

The susceptibility to DR is thought to be mediated by a large number of allelic variants and their interaction with each other as well as with environmental factors. Each allelic variant may increase the chances of developing DR by a small degree. (Priščáková, Minárik et al. 2016)

Discovering genetic associations of DR may help identify those at high risk of developing sight-threatening complications and stratifying patients' appointment intervals based on these risks, thereby more efficiently utilising resources. Identifying candidate genes involved in the pathogenesis of DR may also help

develop targeted therapeutic strategies (Simó-Servat, Hernández et al. 2013) and inform investigations of future drug targets.

Optical Coherence Tomography Angiography (OCTA) is a relatively new imaging technique that can image the retinal vasculature at different layers of the retina. It is a non-invasive technique which can identify individual features of DR as well as ischaemic areas. Investigating the relationship between VB and IRMA with ischaemia visualised with this technique may add to our understanding of their aetiology.

3.1 Research Questions:

1. What is the prevalence of these features in patients with moderate/severe NPDR?
2. Do VB and IRMA co-localise?
3. Is there a genetic predisposition to the development of DR and specifically VB and IRMA?
4. Is there a difference in the response of these different features to anti-VEGF therapies?
5. Do VB and IRMA always coexist with areas of ischaemia?

Chapter 4: Genotype Comparison of NPDR and No DR and of the presence of venous beading and IRMA

4.1 Background and Introduction

As previously discussed, the onset of diabetic eye disease is highly variable. Studies have found that diabetic retinopathy (DR) is driven by multiple genes (Doria 2010). However, different studies show different findings. A study of Finnish adults with T1DM found a heritability (h^2) of 0.52 ($p< 0.05$) amongst siblings for PDR and previous studies suggest genetic risk factors could account for 50% of the risk of developing PDR (Hietala, Forsblom et al. 2008). However, another study of siblings found the heretabilty of DR in T2DM to be approximately 25% (Arar, Freedman et al. 2008).

The genetic profile of DR has previously been studied without investigating the genetic risk factors for the individual features of NPDR, namely, deep haemorrhages (DH), venous beading (VB) and intraretinal microvascular abnormalities (IRMA).

4.1.1 Candidate Genes

As previously mentioned, one approach to the identification of genes that might be involved in the pathogenesis of a disease process is to select and evaluate genes that encode proteins considered most likely to play a role in the development of that disease. The frequency of these genes in those with DR are compared to the frequency in those without DR. For example, in DR, SNPs involved in various biochemical pathways implicated in the production of toxins in response to hyperglycaemia can be examined. These include the reactive oxygen species, sorbitol, aldose reductase (ALR2), advanced glycation end products and have been studied along with genes involved in pathways causing retinal damage following production of these agents (Doria 2010). Genes involved in other biochemical pathways thought to lead to DR have also been examined e.g. VEGF, transforming growth factor beta (Mishra, Swaroop et al. 2016).

This study's interest is in the three features of NPDR, in particular VB and IRMA, therefore examination of genes thought to be involved in the development of these features would be of interest.

A disadvantage of candidate gene studies is that they focus on genes already suspected of involvement in the disease of interest and do not look for new ones (Doria 2010).

4.1.2 Genome Wide Association Studies (GWAS)

The largest genome-wide association study (GWAS) of DR to date found no genome wide significant polymorphisms (Pollack, Igo et al. 2019). However, this study examined all types of DR, not specific phenotypes such as DMO, PDR, and NPDR.

A recent review article described 65 genes found to be associated with DR, identified by either linkage analysis, but mostly by candidate gene studies and GWAS (Sharma, Valle et al. 2019). This review then examined the metabolic pathways involved in the pathophysiology of DR and found that most genes implicated belong to pathways known to contribute to DR pathology, for example insulin signalling, inflammation, VEGF, protein kinase signalling, angiogenesis, neurogenesis and the disruption of endothelial cells linked to leukocyte production (Sharma, Valle et al. 2019).

Candidate gene studies and genome wide association studies (GWAS) are preferred when investigating DR (Mishra, Swaroop et al. 2016) and after considering the advantages and disadvantages of both approaches, it was decided to use a candidate gene approach for this study. The deciding factors being cost, the small number of subjects involved and that the study's focus is the vascular changes in the later stages of DR.

Table 4.1 Advantages and Disadvantages of Candidate Genes and GWAS

Candidate Genes	GWAS
Focuses on genes already suspected of involvement in the disease process	The entire genome is investigated and therefore may be more informative
	Very large number of subjects needed (studies with less than 20,000 participants may yield few or no genome-wide significant loci)(Duncan, Ostacher et al. 2019)
Genes with no known association with the pathways involved unlikely be selected	Possibility of identifying new genes of interest
Can have a high false-positive rate and yield inconsistent results. Meta analyses help identify genes for which there is accumulative evidence for associations (Simó-Servat, Hernández et al. 2013)	
Often have insufficient statistical power	Results are highly reproducible and reliable and more statistically robust (Duncan, Ostacher et al. 2019)
Effective in the study of the genetic makeup of complex diseases (Kim, Lee et al. 2012)	

	Difficult to assess the significance of data, a p value of $5x10^{-8}$ is required to correct for multiple testing (Pe'er, Yelensky et al. 2008)

4.1 3 Rationale for selection of the SNPs in the study

This study aimed to compare the genetic profile of those with moderate-severe NPDR and those with no DR at least 5 years after diagnosis in a Caucasian population. As a sub-analysis the study examined the genetic profile of those with IRMA only and those with VB (with or without IRMA). If a gene is found that is significant for those with IRMA or VB then a pathway to block the gene may be discovered. A panel of SNPs which could potentially show a difference between moderate NPDR / severe NPDR and no DR, based on the understanding of the pathophysiology of the individual features was selected. Analysis of DH was not undertaken as most of those with moderate-severe NPDR have DH.

Previously this group performed a literature search of all studies examining gene associations with DR (DMO) in those with T2DM (Broadgate, Kiire et al. 2018). This collation of references was enhanced by a search from 2017-2019 by the author, with the primary interest being severe NPDR. Numerous studies have reported positive associations between a variety of SNPs and DR but many results are conflicting for many reasons, such as different populations studied, varying sample sizes and different endpoints (Hampton, Schwartz et al. 2015).

For the analysis, the study focused on three areas of interest:

- Vascular endothelial growth factor (VEGF)
- Ischaemic-mediated growth factors and cytokines
- Complement

These three groups were chosen for further literature review because anti-VEGF is used as a therapy for diabetic retinopathy and therefore likely to be relevant to the development of DH, VB and IRMA. Cytokines and growth factors associated with ischaemia may also be of interest as both VB and IRMA are believed to be driven by ischaemia. Complement factor was selected as this group has conducted previous research into complement and AMD and is therefore of interest as it has been implicated in other retinal vascular conditions.

4.1.4 VEGF

4.1.4.1 VEGF Pathway

Vascular endothelial growth factors (VEGF) are produced in the eye by RPE cells, pericytes, astrocytes, Muller cells, glial cells, retinal neurones and vascular endothelial cells in response to hypoxia or ischemia but is also necessary for the survival of endothelial cells in normoxic conditions (Gupta, Kenney 2013). Types of VEGF include VEGF-A (also referred to as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PGF). They promote vasculogenesis and angiogenesis by binding to tyrosine kinase receptors on the surface of endothelial cells, stimulating endothelial cell proliferation. Increasing vascular permeability and breakdown of the blood-retinal barrier is facilitated by the binding of VEGF to receptors that activate either a calcium influx channel or a protein kinase signalling pathway. (Tarr, Kaul et al. 2013). VEGF plays a central role in retinal neovascularisation as evidenced by the measurement of elevated levels of VEGF in the vitreous of patients with PDR which reduce after PRP (Aiello, Park 1994). VEGF is therefore has a key role in the development and progression of DR and DMO (Simo and Hernandez 2008).

4.1.4.2 VEGFA and VEGF Receptor

VEGF-A was the first member of the VEGF family to be discovered and was originally called VEGF. VEGF-A is the most important angiogenesis mediator in the retina and choroid and the target of anti-VEGF therapies used to treat DMO, PDR and AMD. VEGF-A has two receptors, VEGFR-1 and VEGFR-2. VEGFR-2 has been implicated in DR and is responsible for most of the vascular endothelial cellular responses to VEGF-A, promoting proliferation and migration of endothelial cells (Gupta, Mansoor et al. 2013). VEGFR-2 is also responsible for endothelial vascular permeability and is a kinase insert domain receptor (KDR) encoded by the KDR gene (Holmes, Roberts et al. 2007). Anti-VEGF therapies, such as ranibizumab, bevacizumab, neutralize VEGF-A isoforms. Aflibercept also inhibits VEGF-B and Placental Growth Factor (PLGF-1) (Amadio, Govoni et al. 2016).

Previous VEGF studies Genetics and DR

Twenty-three VEGF polymorphisms have been found to have a potential association with DR. Ten of these are included in our study and are listed in the following table 4.2

Table 4.2 Previous studies investigating VEGFA polymorphisms

Gene	SNP	Population	Type	Disease	Association	Reference
VEGFA	rs699947	Korean	T2	DR vs NDR	Yes	(Chen, Liou et al. 2016)
VEGFA	rs699947	Spanish	T2	DR vs NDR	Yes	(Bleda, De Haro et al. 2012)
VEGFA	rs699947	Meta-Analysis	T2	DR vs NDR	Yes	(Lu, Ge et al. 2013)
VEGFA	rs699947	Chinese	T2	DR vs NDR	Yes	(Yang, Deng et al. 2014)

VEGFA	rs833061	Caucasian	T1 and T2	PDR Vs NPDR/NDR	Yes	(Ray, Mishra et al. 2004)
VEGFA	rs833061	Indian	T2	PDR Vs no DR	Yes	(Paine, Basu et al. 2012)
VEGFA	rs833061	European	T1/T2	T1/T2DM PDR vs NDR	Yes	(Churchill, Carter et al. 2008)
VEGFA	rs833061	Chinese	T2	DR vs NDR	Yes	(Yang, Deng et al. 2011)
VEGFA	rs833061	Han Chinese	T2	DR vs NDR	Yes	(Yuan, Wen et al. 2014)
VEGFA	rs13207351	Chinese	T2	DR vs NDR	Yes	(Yang, Deng et al. 2014)Yang 2011 and 2014
VEGFA	rs1570360	Korean	T2	DR vs NDR	Yes	(Chun, Hwang et al. 2010)
VEGFA	rs2010963	Japanese	T2	DR vs NDR	Yes	(Awata, Inoue et al. 2002, Awata, Kurihara et al. 2005)
VEGFA	rs2010963	Indian	T2	DR vs NDR	Yes	(Suganthalakshmi, Anand et al. 2006)
VEGFA	rs2010963	European	T2	PDR vs NDR	Yes	(Errera, Canani et al. 2007)
VEGFA	rs2010963	Korean	T2	DR vs NDR	Yes	(Chun, Hwang et al. 2010)
VEGFA	rs2010963	Han Chinese	T2	DR vs Healthy	Yes	(Yang, Deng et al. 2011)
VEGFA	rs2010963	Egyptian	T2	DR vs NDR	Yes	(El-Shazly, El-Bradey et al. 2014)
VEGFA	rs2010963	Taiwanese	T2	DR vs NDR	Yes	(Chen, Liou et al. 2016)

VEGFA	rs2010963	Polish	T2	DR vs NDR	Yes	(Szaflik, Wysocki et al. 2008)
VEGFA	rs2146323	Chinese	T2	DR vs NDR	Yes	(Yang, Deng et al. 2011, Yang, Deng et al. 2014)
VEGFA	rs3025030	Caucasian	T!/T2	DR vs NDR	Ye	(Abhary, Burdon et al. 2009)
VEGFA	rs3025039	Japanese	T2	DR vs NDR	Yes	(Awata, Inoue et al. 2002)
VEGFA	rs10434	Caucasian	T1/T2	DR vs NDR	Yes	(Abhary, Burdon et al. 2009)
VEGFA	rs10738760	Slovenian	T2	PDR vs NPDR	Yes	(Sajovic, Cilenšek et al. 2019)

4.1.4.3 Anti-VEGF in DR, treatment, see chapter 2.9

As previously discussed, IVT anti-VEGF agents effectively treat DMO, improve the DRSS score in DR and promote complete regression of new vessels more quickly than PRP.

VEGF has been implicated in the development and pathogenesis of DR so a selection of SNPs that have already shown an association with DR development were selected for the panel.

SNPs of Interest in VEGF

- rs2010963 the most extensively studied
- rs699947
- rs833061
- rs13207351
- rs1570360
- rs2146323

- rs3025030
- rs3025039
- rs10434
- rs10738760

4.1.5 Ischaemia mediated growth factors and cytokines

The following pathways were considered of interest in DR development due to their mechanism of action.

4.1.5.1 Fibroblast Growth factor 2 (FGF2)

FGF2, also known as Basic fibroblast growth factor (bFGF) is a member of a family of cell signalling proteins that mediate cell proliferation and migration. It is upregulated in diabetic retinopathy and may play a role in neovascularisation by stimulating endothelial and smooth muscle proliferation (Teshima-Kondo, Kondo et al. 2004), thereby implicating FGF2 in diabetic vascular dysfunction. Another study found FGF2 levels to be significantly correlated with HbA1c levels and consequently may contribute to vascular pathology in DM by promoting endothelial cell proliferation (Zimering and Eng 1996). Elevated levels of FGF2 have been measured in the vitreous of patients with PDR (Sivalingam, Kenney et al. 1990) as well as in the serum of those with DR (Stephan, Chang et al. 1998) and in a separate study, levels of FGF2 mRNA were found to be three times higher in the eyes of diabetic rats (Lowe, Florkiewicz et al. 1995). Of particular interest were FGF2's effect on vessel walls and smooth muscle proliferation, which may play a role in IRMA and VB formation therefore SNPs associated with FGF2 were selected for the panel.

Previous studies in FGF2

Petrovic (Petrovic, Krkovic et al. 2008) found an association for rs308398 in T2 with PDR Caucasian, but no association for rs41456044 and rs308395). Beranek

(Beranek, Kolar et al. 2008) found a positive association with rs373341357 in a Caucasian T2 population but no association with rs308398.

Table 4.3 Previous studies investigating FGF2 polymorphisms

Gene	SNP	Population	Type	Disease	Association	Reference
FGF2	rs308398	Caucasian	T2	PDR vs NPDR	Yes	(Petrovic, Krkovic et al. 2008)
FGF2	rs373341357	Caucasian	T2	PDR vs NPDR	Yes	(Beranek, Kolar et al. 2008)

SNPs of Interest in FGF2

- rs308398
- rs373341357

4.1.5.2 Haemochromatosis (HFE)

Systemic glucose levels influence several iron metabolism pathways and the effects of insulin are influenced by changes in iron levels. Abnormalities in iron metabolism has been linked to insulin resistance and iron deposits in pancreatic B-cells have led to their apoptosis causing diabetes. Excess deposits of iron in the liver disturbs glucose metabolism, by altering insulin signalling, resulting in high circulating insulin levels (Fernández-Real, McClain et al. 2015) (Niederau et al. 1984). Iron metabolism has been associated with angiogenesis, extracellular matrix remodelling and fibrosis (Simonart, Degraef et al. 2001) (Gardi, Arezzini et al. 2002), and may play a role in the pathogenesis of T2DM and DR (Peterlin, Globočnik Petrovič et al. 2003) . Patients with hereditary heamochromatosis were first reported as having insulin resistance a century ago (Fernández-Real, McClain et al. 2015).

SNPs for HFE were considered as candidates for the panel in view of the interesting observations of iron's involvement with glucose metabolism and possibly angiogenesis.

Previous studies in HFE

Peterlin (Peterlin, Globočnik Petrovič et al. 2003) found a positive association with PDR progression, for rs1800562 in a Caucasian population of T2 PDR vs No DR. However, Balasubbu (Balasubbu, Sundaresan et al. 2010), in an Indian population found no association between DR vs NDR.

Table 4.4 Previous studies investigating HFE polymorphisms

Gene	SNP	Population	Type	Disease	Association	Reference
HFE	rs1800562	Caucasian	T2	PDR progression	Yes	(Peterlin, Globočnik Petrovič et al. 2003)
HFE	rs1800562	Indian	T2	DR vs NDR	No	(Balasubbu, Sundaresan et al. 2010)

SNPs of interest in HFE

- rs1800562

4.1.5.3 Kinase domain insert receptor (KDR)

KDR is also known as VEGF receptor 2, as previously discussed, VEGF and hence KDR play an important role in angiogenesis, endothelial cell proliferation and vascular permeability. KDR receptor was therefore included in the panel of SNPs to be examined.

Previous Studies in KDR

A study of a Chinese population of T2DR vs no DR, found a positive association with rs2071559 (Yang, Deng et al. 2014).

Table 4.5 Previous studies investigating KDR polymorphisms

Gene	SNP	Population	Type	Disease	Association	Reference
KDR	rs2071559	Chinese	T2	DR vs NDR	Yes	(Yang, Deng et al. 2014)

SNP of interest in KDR

- rs2071559

4.1.5.4 Sarcolemma-associated protein (SLMAP)

SLMAP is a tail-anchored membrane protein-coding gene associated with diabetes. Upregulation of SLMAP has been associated with vascular endothelial dysfunction in diabetes. Such changes are characterised by alterations in endothelium related contraction and relaxation of small mesenteric arteries in db/db mice (obese, diabetic mice). This suggests that changes in SLMAP expression may be a marker of microvascular dysfunction in diabetes and therefore was included in the panel of SNPs, of particular interest being the effect on vessel wall contraction.

SLMAP expression can be regulated by treatment with PPARy (Peroxisome proliferator- activated receptor-gamma) leading to a reversal of endothelial dysfunction however, the mode of action of SLAMP is not completely understood (Ding, Howarth et al. 2005). This study found that small mesenteric arteries upregulated SLAMP which is part of the excitation-contraction of the arteries regulating membrane function in muscle cells (Ding, Howarth et al. 2005).

Previous Studies in SLMAP

There has only been one study to date examining SLMAP SNPs, (Upadhyay, Robay et al. 2015). The authors found an association with rs17058639 in a T2DM

Qatari population, studying DR vs NDR/Healthy. (No association were found with rs1043045 or rs1057719).

Table 4.6 Previous studies investigating SLMAP polymorphisms

Gene	SNP	Population	Type	Disease	Association	Reference
SLAMP	rs17058639	Qatari	T2	DR vs NDR/health	Yes	(Upadhyay, Robay et al. 2015)
SLAMP	rs1043045	Qatari	T2	DR vs NDR/health	No	(Upadhyay, Robay et al. 2015)
SLAMP	rs1057719	Qatari	T2	DR vs NDR/health	No	(Upadhyay, Robay et al. 2015)

SNPs of interest in SLAMP

- rs17058639

4.1.5.5 Vitamin D Receptor (VDR)

The vitamin D receptor is a nuclear hormone receptor, which regulates numerous immune response pathways. Vitamin D effects the proliferation and differentiation of cells and is known to have anti-angiogenic actions (Lin and White 2004). VDR is expressed in the retinal pigment epithelium (RPE) and in most retinal cells including vascular endothelial cells. Its function is unclear but it is likely that this protein has a role in maintaining ocular function (Johnson, Grande et al. 1995). Interestingly, glucocorticoids are known to regulate expression of the gene encoding VDR (Pike and Meyer 2010).

Previous studies in VDR

Hong Y-J (Hong, Kang et al. 2015) found an association with rs1544410 in a Korean T2 population, DR vs No DR, also found by Zhong (Zhong, Du et al. 2015) Chinese T2DM DR vs NDR. This group also found an association with rs7975232. No association was found in a Caucasian population for rs2228570 Caucasian T2, DR vs NDR, Cyganek (Cyganek, Mirkiewicz-Sieradzka et al. 2006), but an association was found in a Chinese population of T2 DR vs NDR , Zhong (Zhong, Du et al. 2015) and Zhang (Zhang, Xia et al. 2016) Meta-analysis. The same meta-analysis T1/T2 found no association with rs731236.

Table 4.7 Previous studies investigating VDR polymorphisms

Gene	**SNP**	**Population**	**Type**	**Disease**	**Association**	**Reference**
VDR	rs1544410	Korean	T2	DR vs NDR	Yes	(Hong, Kang et al. 2015)
VDR	rs1544410	Chinese	T2	DR vs NDR	Yes	(Zhong, Du et al. 2015)
VDR	rs2228570	Caucasian	T2	DR vs NDR	No	(Cyganek, Mirkiewicz-Sieradzka et al. 2006)
VDR	rs2228570	Chinese	T2	DR vs NDR	Yes	(Zhong, Du et al. 2015) (Zhang, Xia et al. 2016)
VDR	rs731236	Meta-analysis	T1/T2	DR vs NDR	No	(Zhang, Xia et al. 2016)

VDR	rs7975232	Chinese	T2	DR vs NDR	Yes	(Zhong, Du et al. 2015)

SNPs of interest in VDR

- rs1544410
- rs2228570
- rs7975232

4.1.5.6 Transforming Growth Factor–beta (TGF-b)

Transforming growth factor-beta (TGF-b) is a cytokine which is stimulated by the growth factor angiopoietin 2 (Sharma 2019). TGF-b is involved in many functions such as angiogenesis, the formation of the extracellular matrix and adhesion (Battegay, Raines et al. 1995) (Nunes, Munger et al. 1998). In addition, over-expression of TGF-b is known to lead to tissue fibrosis hence possibly contributing to the pathology of DR (Wong, Poon et al. 2003). TGF-b may also inhibit endothelial cell functions and affect cell proliferation, differentiation and migration

Blocking TGFb signalling in the retina results in vascular endothelial cell apoptosis, basement membrane thickening, and breakdown of the BRB (Walshe, Saint-Geniez et al. 2009). Lastly, animal studies have demonstrated pathologic changes in mice that are similar to those found in NPDR and PDR in humans when TGF-b signalling is interrupted, a result of pericyte and endothelial cell loss (Braunger, Leimbeck et al. 2015). In view of the reported vascular changes that may be effected by TGFb, these SNPs were included in the panel.

Previous Studies in TGFb

Buraczynska et al. (Buraczynska, Baranowicz-Gaszczyk et al. 2007), in a Caucasian population of T2 DR vs NDR found a positive association, also confirmed by Rodrigues et al (Rodrigues, Pietrani et al. 2015) (2015) in a Brazilian population, with rs1800470. This positive link was also found by Lui et al (Liu, Jiao

et al. 2014) with T2 DR Vs NDR. A study of a Caucasian population of T2DM did not find an association between another TGF-b polymorphism, rs104894719, and DR (Simões, Lobo et al. 2014).

Table 4.8 Previous studies investigating TGF-b polymorphisms

Gene	SNP	Population	Type	Disease	Association	Reference
TGF-b	rs1800470	Caucasian	T2	DR vs NDR	Yes	(Buraczynska, Baranowicz-Gaszczyk et al. 2007)
TGF-b	rs1800470	Brazilian	T2	DR vs NDR/healthy	Yes	(Rodrigues, Pietrani et al. 2015)
TGF-b	rs1800470	Meta-analysis	T2	DR vs NDR	Yes	(Liu, Jiao et al. 2014)
TFG-b	rs104894719	Portuguese		DR vs NDR	No	(Simões, Lobo et al. 2014)

SNPs of Interest in TGFb

- rs1800470
- rs104894719

4.1.5.7 ARHGAP22

The ARHGAP22 gene encodes the Rho GTPase-activating protein 22 which is known to be involved in endothelial cell migration and capillary tube formation (Huang, Lin et al. 2011) (McAuley, Wang et al. 2014). The ARHGAP22 gene may be involved in the pathogenesis of PDR as well as increased capillary permeability and has been implicated in a pathway involving insulin (Huang, Lin et al. 2011).

ARHGAP22's role in endothelial cell angiogenesis and capillary permeability were reasons to be included in the selection of SNPs for analysis.

Previous Studies in ARHGAP22

Huang 2011, (Huang, Lin et al. 2011) found a positive association in a T1/T2 Taiwanese population with PDR for rs4838605, rs11101355 and rs11101357. The positive association with rs4838605 was confirmed by McAuley et al, (McAuley, Wang et al. 2014) in T1/T2 Caucasian population Severe/PDR vs Mild NPDR.

Table 4.9 Previous studies investigating ARHGAP22 polymorphisms

Gene	SNP	Population	Type	Disease	Association	Reference
ARHGAP22	rs4838065	Taiwanese	T1/T2	DR vs NDR	Yes	(Huang, Lin et al. 2011)
ARHGAP22	rs111101355	Taiwanese	T1/T2	DR vs NDR	Yes	(Huang, Lin et al. 2011)
ARHGAP22	rs11101357	Taiwanese	T1/T2	DR vs NDR	Yes	(Huang, Lin et al. 2011)
ARHGAP22	rs4838605	Caucasian	T1/T2	Severe NPDR/PDR vs mild NPDR	Yes	(McAuley, Wang et al. 2014)

SNPs of interest in ARHGAP22

- rs4838605
- rs11101355
- rs11101357

4.1.6 The Complement System

Introduction

The Complement system is an innate immune system, comprised of more than 30 different proteins, that plays a crucial role in inflammation and defence against infection and disease. It is permanently active to a certain degree in healthy

individuals and attacks pathogens by phagocytosis, opsonisation and cell lysis. Dead and dying cells, foreign cells and immune complexes are cleared from the blood by the complement system which plays an important role in regulating and enhancing immune and inflammatory responses (Mukai, Okunuki et al. 2018) (Merle, Church et al. 2015). Overstimulation or disruption of the complement system is associated with chronic inflammation, increased risk of infections and autoimmune disease (Merle, Church et al. 2015) and is thought to have a role in retinal pathologies such as AMD and DR. Despite limited understanding of the role of complement in retinal disease, targeting the complement system may lead to new treatments options for DR (Xu and Chen 2016).

4.1.6.1 Complement Pathways

The complement system comprises three pathways, the classic, lectin and alternative pathways (Merle, Church et al. 2015). All three pathways ultimately activate C3 (Charbel Issa, Chong 2011). Antibody-antigen complexes leading to the generation of C5 convertase trigger the classic pathway. C5 convertase leads to the formation of membrane attack complex (MAC), which destroys pathogens. The lectin pathway is similar to the classic pathway forming C3 convertase. The alternative pathway is not initiated by antibody-antigen complexes but by foreign molecules. In the alternative pathway, C3 molecules are activated by making contact with antigens. Factors B, D and H are also involved in the alternative pathway. All three pathways ultimately lead to MAC formation, lysis, inflammation and opsonisation (Dunkelberger, Song 2010).

4.1.6.2 Complement in PDR patients

Increased levels of CFH, C3, C5 and CFB were found in vitreous samples of those with PDR (Shahulhameed, Vishwakarma et al. 2020) (Chrzanowska, Modrzejewska et al. 2018) (Muramatsu, Wakabayashi et al. 2013). Studies have also found higher levels of complement components in the retinal tissue, retinal

vessels and choriocapillaris of donor eyes from those with DM compared to those without (Geri 2002) (Zhang 2002).

4.1.6.3 Complement in AMD

An association between CFH rs800292 and inflammatory and neovascular diseases has been widely reported (Wang, Yang et al. 2013). The polymorphism rs800292 has been shown to be associated with AMD and other inflammatory and neovascular diseases, due to an increased activation of the alternative complement pathway (Pechtl et al. 2011). As with DR, donor eyes with AMD show higher levels of complement factors than those without, mainly located in drusen and the Bruch's membrane/choroid complex (Charbel Issa, Chong 2011). Further evidence comes from studies showing increased complement proteins detected in the peripheral blood of those with AMD (Scholl, Charbel Issa 2008).

Retinal integrity during normal aging is preserved by low grade activation of the complement system and is regulated by complement regulators (Shahulhameed 2020). Disruption to the regulation and activation of the complement system can have a detrimental effect on retinal function and trigger inflammatory diseases like AMD, Alzheimer's disease and rheumatoid arthritis (Aitaz, Lupton 2012) (Di Muzio et al. 2011) (Wagner, Frank 2010) (Charbel Issa, Chong 2011).

The HF1/CFH polymorphism has been shown to increase the risk of AMD by 50% (Hageman, 2005).

Previous genetic studies of CFH, C5 and CFB

A study of a Chinese population with T2DM found a positive association between the polymorphism rs800292 in the gene for CFH and rs1048709 (CFB) and DR. Three other polymorphisms for CFB found no associations (Wang, Yang et al. 2013). Yang et al. (Yang, Wang et al. 2016) found two SNPs in the gene encoding C5 were associated with DR (rs17611 and rs1548782) in a Chinese cohort. Four other SNPs investigated in the study had no association with DR (rs12237774,

rs2269066, rs10985126 and rs1017119). A study of C5 SNPS in another Chinese study found no associations in two SNPs but a positive association between rs2269067 and PDR (Xu, Yi et al. 2016). A study of an Indian population with T2DM compared those with DR and those with no DR and found no association with CFH genes (rs1061170 and rs3753394) (Balasubbu, Sundaresan et al. 2010). A study of a Caucasian population found no association of the Y402 variant of CFH in T2DM with DR, but found a positive association with AMD in the same population (Doney, Palmer 2009).

Table 4.10 Previous studies investigating Complement polymorphisms

Gene	SNP	Population	Type	Disease	Association	Reference
CFH	rs800292	Chinese	2	DR vs NDR	Yes	(Wang, Yang et al. 2013)
CFH	rs1061170	Indian	2	DR vs NDR	No	(Balasubbu, Sundaresan et al. 2010)
CFH	rs3753394	Indian	2	DR vs NDR	No	(Balasubbu, Sundaresan et al. 2010)
CFH	(rs1061170)	Caucasian	2	DR vs NDR	No	(Doney, Lee et al. 2005)
CFB	rs1048709	Chinese	2	DR vs NDR	Yes	(Wang, Yang et al. 2013)
CFB	rs4151657	Chinese	2	DR vs NDR	No	(Wang, Yang et al. 2013)

CFB	rs2072633	Chinese	2	DR vs NDR	No	(Wang, Yang et al. 2013)
C5	rs17611	Han Chinese	2	DR vs NDR	Yes	(Yang, Wang et al. 2016)
C5	rs1548782	Han Chinese	2	DR vs NDR	Yes	(Yang, Wang et al. 2016)
C5	rs12237774	Han Chinese	2	DR vs NDR	No	(Yang, Wang et al. 2016)
C5	rs2269066	Han Chinese	2	DR vs NDR	No	(Yang, Wang et al. 2016)
C5	rs10985126	Han Chinese	2	DR vs NDR	No	(Yang, Wang et al. 2016)
C5	rs1017119	Chinese	2	DR vs NDR	No	(Yang, Wang et al. 2016)
C5	rs2269067	Chinese	2	DR vs PDR	Yes	(Xu, Yi et al. 2016)
C5	rs7040033	Chinese	2	DR vs PDR	No	(Xu, Yi et al. 2016)
C5	rs7027797	Chinese	2	DR vs PDR	No	(Xu, Yi et al. 2016)

As discussed, Complement may play a role in retinal vascular disease, therefore a selection of SNPs that have previously shown a positive association with DR development were selected for the panel.

SNPs of interest in Complement Factors

- CFB rs1048709
- CFH rs800292
- C5 rs17611
- C5 rs1548782
- C5 rs2269067

4.1.7 Discussion of SNPs' selection

Focus: VEGF, Ischaemia, Complement

Numerous candidate gene studies have investigated the role of VEGF, endothelial nitric oxide synthase (eNOS), receptor for advanced glycation end products (RAGE) and aldose reductase (AR) in DR. These proteins are believed to play an important role in the pathogenesis of DM or in the early stages of DR, with the exception of VEGF (Hampton, Schwartz et al. 2015).

The primary focus of this study is to better understand the three features of DR which appear once DR is established. The Candidate Genes were selected based on their implication in the development and progression of DR and their possible influence on the pathology of retinal vessels. Three key pathways were investigated: Complement, VEGF and Ischaemia-mediated pathways.

4.2 Methods

The study was granted approval by the South West-Cornwall and Plymouth Research Ethics Committee, (Research Ethics Approval Number: 10/H0203/14) the Health Research Authority and Trust management of Oxford Radcliffe Hospitals NHS Trust and King's College Hospital NHS Trust, and adhered to the

tenets of the Declaration of Helsinki. Informed consent was obtained from all participants.

A comprehensive literature search of Genetics and DR was previously completed by members of this group from 2014-2016. Their focus was T2DM and DMO. This collation of references was enhanced by a search from 2017-2019 by the author, with the primary interest being severe NPDR. The author performed a literature review to examine whether previously reported genes are relevant to our sample and selected a panel of single nucleotide polymorphisms (SNPs) relevant to severe NPDR. All English language journals were searched for studies examining cohorts of T1DM or T2DM between March 2017 and Jan 2019. Key words included in the search were diabetic retinopathy, single nucleotide polymorphism, genetic association studies. The literature review undertaken involved searching the US National Library of Medicine National Institutes of Health's search engine PubMed.gov for articles on the subject of the genetics of diabetic retinopathy. 520 articles were identified between 2002 and 2019. These were examined for relevance and determined the SNPs to be analysed (see above).

The search yielded a total of 108 SNPs of interest. Closer examination of the identified SNPs' relevance to this severe NPDR Caucasian population and to pathways of interest produced a selection of 28 SNPs to be examined. When selecting SNPs for examination in this study, I endeavoured to select SNPs which were relevant to the population and research question. As previously described, my interest was SNPs that may be involved in cell wall pathology, angiogenesis and ischaemia which may play a role in the development of DH, VB and IRMA. I selected studies of such SNPs that showed a positive association in Caucasian populations, with large sample sizes, where the studies showed significance and the groups studied were either DR vs no DR or PDR vs no DR. I found no studies examining the moderate/severe NPDR group alone, previous studies generally included three groups, no DR, DR, PDR. The focus was narrowed down to VEGF, ischaemia and Complement as previously described.

From the 108 SNPs identified, many were likely to be involved in the earlier disease process, hyperglycaemia-induced pathways such as the polyol pathway, aldose reductase, advanced glycation end products and reactive oxygen species. I also excluded SNPs which may contribute to microthrombin formation and leukostasis. A total of 59 such SNPs were therefore eliminated. The remaining 49 were in the 3 subgroups of interest. Twenty-one were eliminated as they either showed no association in previous studies or the sample sizes in the trials were small. I aimed to include studies which showed a positive association in a Caucasian population similar to the cohort in my study, however, the majority of studies were non-Caucasian populations, and in some SNPs of interest no studies had been reported in a Caucasian population,

SNPs of Interest VEGFA

- rs2010963
- rs699947
- rs833061
- rs13207351
- rs1570360
- rs2146323
- rs3025030
- rs3025039
- rs10434
- rs10738760

SNPs of Interest FGF2

- rs308398
- rs373341357

SNP of interest HFE

- rs1800562

SNP of interest KDR

- rs2071559

SNPs of interest SLAMP

- rs17058639

SNPs of interest VDR

- rs1544410
- rs2228570
- rs7975232

SNPs of Interest TGFb

- rs1800470
- rs104894719

SNPs of interest ARHGAP22

- rs4838605
- rs11101355
- rs11101357

SNPs of interest Complement Factors

- CFB rs1048709
- CFH rs800292
- C5 rs17611
- C5 rs1548782
- C5 rs2269067

4.2.1 Study Design and participants

This was a pilot study. The literature review identified a number of studies which compared DR vs no DR or NPDR vs PDR. The average sample size was a few hundred/ group, therefore, this was my starting point. However, as there were no studies examining the individual features of DR, the sample size choice was a

pragmatic one and I opted for 200/ group. The results of the study can inform future studies.

This was a case-control prospective study, involving Caucasians with Type 1 or Type 2 DM. Subjects were recruited to the no DR (control) group of the study from the Diabetic Retinopathy Screening Service at King's College Hospital, London. Subjects were recruited to the NPDR (case) group from the retinal clinics at King's College Hospital, London and Oxford Eye hospital. Patient eligibility was determined from medical record review. Approximately 20,000 Medisoft or Optimise notes were reviewed to identify those who were eligible.

Inclusion criteria for no DR group

- Type 1 or Type 2 DM for at least 5 years
- Caucasian
- Aged 18 years or older
- No diabetic retinopathy
- Able and willing to give informed consent

Inclusion criteria for NPDR group

- Type 1 or Type 2 DM
- Caucasian
- Aged 18 years or older
- Clinically confirmed moderate/severe NPDR
- Able and willing to give informed consent

4.2.2 Procedures

The study required one visit and all data was collected during the subject's scheduled regular appointment.

Imaging

Those recruited to the no DR group of the study had fundus photography as part of their routine care. After mydriasis, two images, one centred on the optic disc and one centred on the macular were taken with a digital fundus camera (Topcon Non-Mydriatic Retinal Camera TRC-NW8, Japan). These images were examined for signs of DR. If they met all the inclusion criteria, they were recruited to the control arm of the study.

Those recruited to the NPDR group of the study had ultrawide field scanning laser ophthalmoscope (UWFSLO) (Optomap 200Tx; Optos plc, Dunfermline, UK) Optomap images taken, after mydriasis, before the clinic appointment by a trained technician as part of the patient's routine care. This allows approximately 80% of the retinal surface to be captured by taking a 200° image of the retina. All patients who met the inclusion criteria were recruited as cases to the study. Also excluded were those who had previous PRP, intravitreal anti-VEGF or intravitreal steroid injections.

Questionnaire

A short questionnaire was administered, see appendix 7. This recorded the number of years' duration of DM, whether Type 1 or Type 2 DM, whether there was a history of angina, stroke, renal problems or laser treatment for diabetic eye disease. Current medication and smoking status were recorded and the participant's HbA1c recorded from their clinical notes.

Buccal Cell sample

A buccal cell sample was taken from the inside of the mouth of each participant with a buccal collection brush and stored in a tube of Cell Lysis Solution (Qiagen, UK) as per the standard operating procedure detailed in the Appendix 2. The samples were stored without any patient identifiable information. The samples were purified using the Gentra Puregene Buccal Cell Kit as described in the

Appendix 2. Once processed, the concentration of a DNA sample was measured using the Nanodrop 1000 (Thermo-Fisher). Nucleic acids absorb light with an absorbance maximum at 260 nanometres. 1µl DNA was pipetted onto the pedestal of the nanodrop and the arm lowered. The pedestal was then moved to automatically adjust to an optimal path length (0.05-1mm) forming a sample column. Light was then directed through the column and the nanodrop measured the amount of light that passed through the column to quantify the concentration of DNA in the sample. A second measurement was then taken at a wavelength of 230nm to quantify the quality of the sample. The optimal ratios at A260/A280 and A230/260 are 1.8 and 2 respectively indicating 'pure' DNA. In this cohort a ratio of 1.6-2.1 at A260/280 was deemed acceptable to be used for further analysis.

Sample plating – 14ul of DNA was aliquoted into individual wells of 96 hard-shell plates. The optimum sample are 30-150ng/ml of DNA with a 260/280nm value of 1.8-2.0 and a 260/230nm value of greater than 0.5. The plates were then sealed and sent for processing at the Wellcome Trust Centre for Human Genetics. An Illumina Infinium Genotyping v2 array is used to analyse the DNA samples.

4.2.3 Image Grading

The images from those in the control arm were examined to confirm the absence of DR. All Optomap images of those enrolled to the cases arm were anonymised and stored on the hospital server. The images from the cases were examined.

Images were included in the study if all central fields were gradable. Some peripheral areas were ungradable but as this did not affect the analysis, these images were included in the study.

The Early Treatment Diabetic Retinopathy Study (ETDRS) used seven standard field 35mm film stereoscopic colour photographs, each image being 30° to 35° wide to assess DR severity levels. Field 1M is centred on the temporal edge of the optic nerve, field 2 on the macular and field 3M on the temporal macular. The four outer fields image the superior temporal, inferior temporal, superior nasal and

inferior temporal fundus (see fig 5.2). A seven-field overlay, equivalent to ETDRS 30-degree seven standard field 35mm stereoscopic colour photographs, was superimposed on the Optomap image using Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA, USA). The two central ETDRS fields covered the macula and optic disc. This enabled the ETDRS seven-field area of the posterior pole to be accurately assessed (see fig 5.1) as well as the peripheral fundus. The area within the seven fields were graded for each quadrant. This method has previously been described by this group and that based in Joslin Diabetes centre, Boston (Price, Au et al. 2015) (Silva, El-Rami et al. 2017).

The images were graded using the ETDRS DRSS grading system. The seven standard fields were graded by quadrants; superior temporal, superior nasal, inferior nasal and inferior temporal. The grading quadrants were then extended to the periphery, using the same quadrants as well as an additional middle temporal area. Each quadrant was evaluated for the presence and severity of the three features of NPDR: deep haemorrhages (DH), venous beading (VB) and intraretinal microvascular abnormalities (IRMA).

The peripheral fundus was graded by the same quadrants as well as including a mid-peripheral area, previously used by other groups. Silva and colleagues (Silva, El-Rami et al. 2017) created 5 peripheral areas to grade.

The three features, as well as venous loops were graded with reference to the ETDRS standard photographs.

DH are recorded as:

- 0 not present
- 1 Present but less than ETDRS standard photo 1 (the mildest standard for haemorrhages/microaneurysms)

- 2 Greater or equal to standard photograph 1 but less than standard photograph 2A (the intermediate standard for haemorrhages/microaneurysms)
- 3 Greater or equal to photograph 2A (the severe standard for haemorrhages/microaneurysms)

IRMA are recorded as:

- 0 not present
- 1 definite IRMA but less than standard photograph 8A
- 2 Greater or equal to standard photograph 8A

VB are recorded as:

- 0 not present
- 1 definite beading, less than standard photo 6A
- 2 beading equal to or greater than standard photo 6A

Venous Loops were also graded as: 0 (Absent) or 1 (Present)

(See Table 10.1 Appendix)

The ETDRS grading protocol is based on seven field images. This study adapted the grading, taking out some categories, see below. This was changed as questionable did not seem relevant and the more severe grades were combined in line with the 4-2-1 rule.

For example, ETDRS graded Haemorrhage as follows:

Grade 0 = no haemorrhages and/or microaneurysms;

Grade 1 = questionable haemorrhages and/or microaneurysms;

Grade 2 = definite haemorrhages and/or microaneurysms less than standard photograph 1;

Grade 3 = haemorrhages and/or microaneurysms greater than or equal to standard photograph 1 but less than standard photograph 2A;

Grade 4 = haemorrhages and/or microaneurysms greater than or equal to standard photograph 2A but less than standard photograph 2B;

Grade 5 = haemorrhages and/or microaneurysms greater than or equal to standard photograph 2B;

Grade 8 = cannot grade.

IRMA ETDRS Classification:

Grade 0 = no IRMA;

Grade 1= questionable IRMA;

Grade 2 = definite IRMA, less than standard photograph 8A;

Grade 3 = IRMA greater than or equal to standard photograph 8A but less than standard photograph 8B;

Grade 4 = IRMA greater than or equal to standard photograph 8B; grade 8 = cannot grade

In this study grades 1 and 4 were removed

VB ETDRS classification:

Grade 0 = no beading;

Grade 1 = questionable beading;

Grade 2 = definite beading, less than standard photograph 6A;

Grade 3 = beading greater than or equal to standard photograph 6A but less than standard photograph 6B;

Grade 4 = beading greater than or equal to standard photograph 6B;

Grade 8 = cannot grade

In this study grades 1 and 4 were removed

(ETDRS Paper 10, 1991)

Fig 4.1 Optomap Image illustrating the areas graded, the central seven fields and five peripheral areas

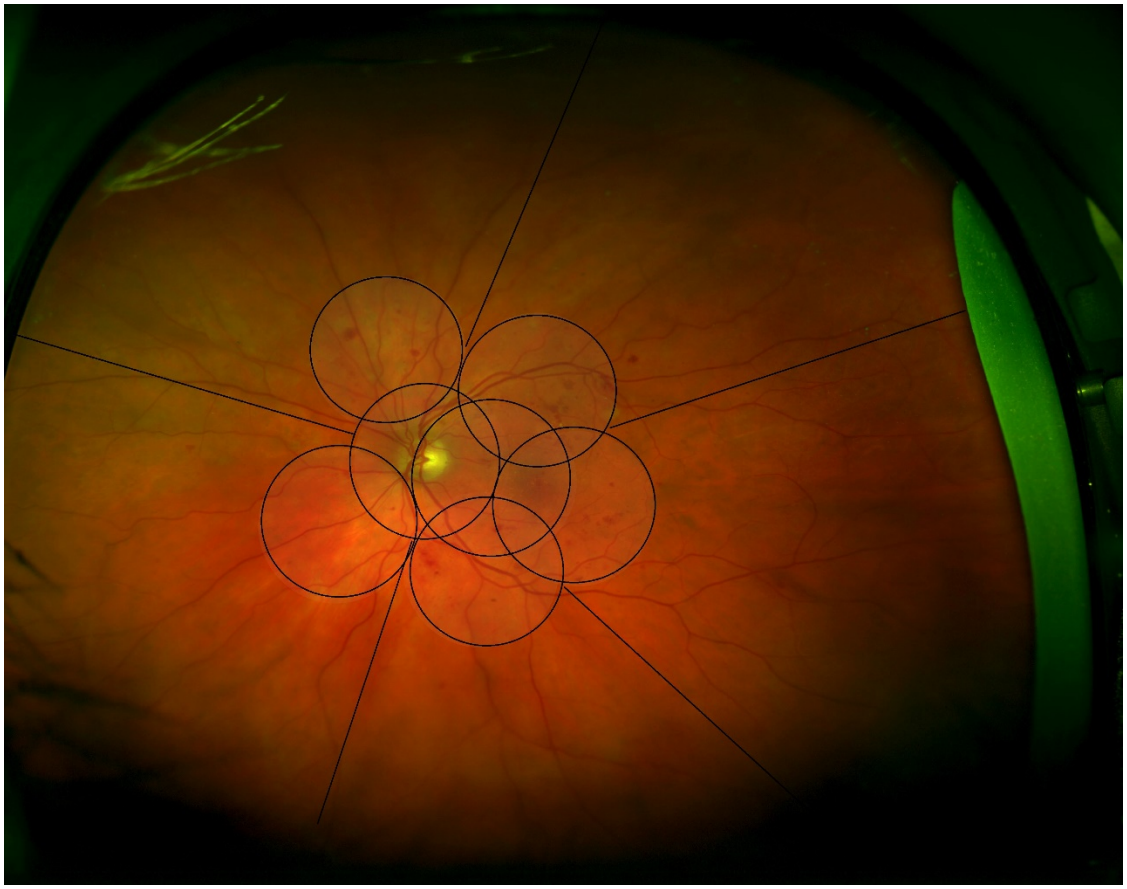
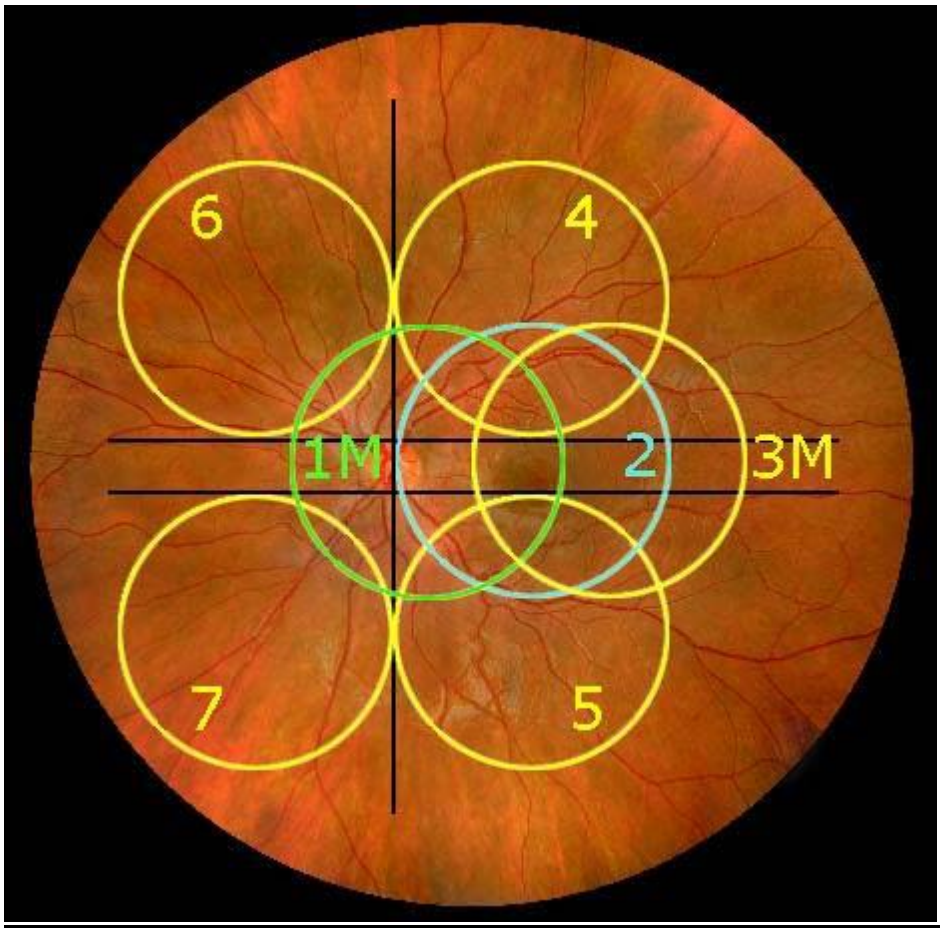


Fig 4.2 ETDRS 7 standard fields Photograph:
http://eyephoto.ophth.wisc.edu/photography/tutorial/slide22.html



A grading audit was performed. A sample of 10% of images were re-graded by the primary grader and a sample of 10% of images graded by another experienced grader and the results compared.

Grading Audit Results

Intra-grader agreement was found to be 87.5% within one DRSS step and 100% within two DRSS steps. Inter-grader agreement was found to be 85% within one DRSS and 100% within two DRSS steps. When differences were found cases

were discussed and consensus sought. This process involved comparing colour fundus photograph (CFP) images previously graded with VB and IRMA by the Diabetic Retinopathy Screening Service with Optomap images of the same patient at their first referral visit to the hospital, which is no longer than 20 weeks later. In some cases, the presence of IRMA and VB was confirmed clinically by comparing to the acquired image. The advice of Medical Retina Consultants was also sought in some cases.

Figure 4.3 Colour Fundus Photograph. VB Superior temporal arcade

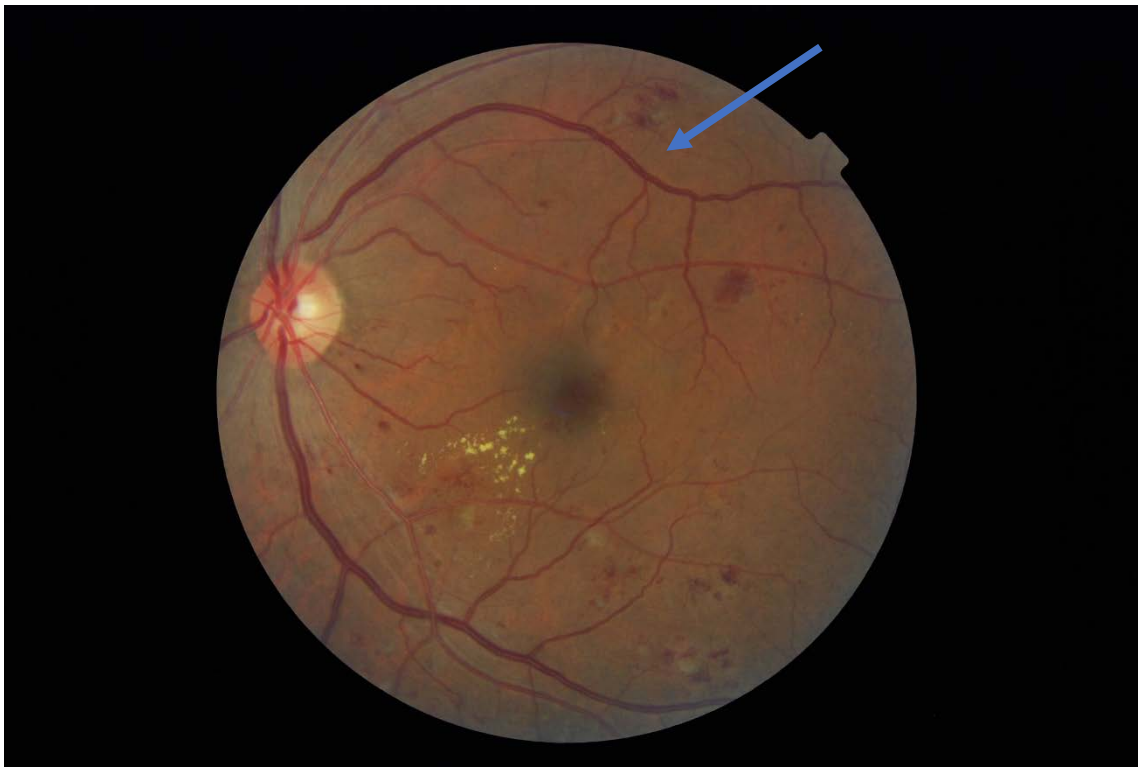
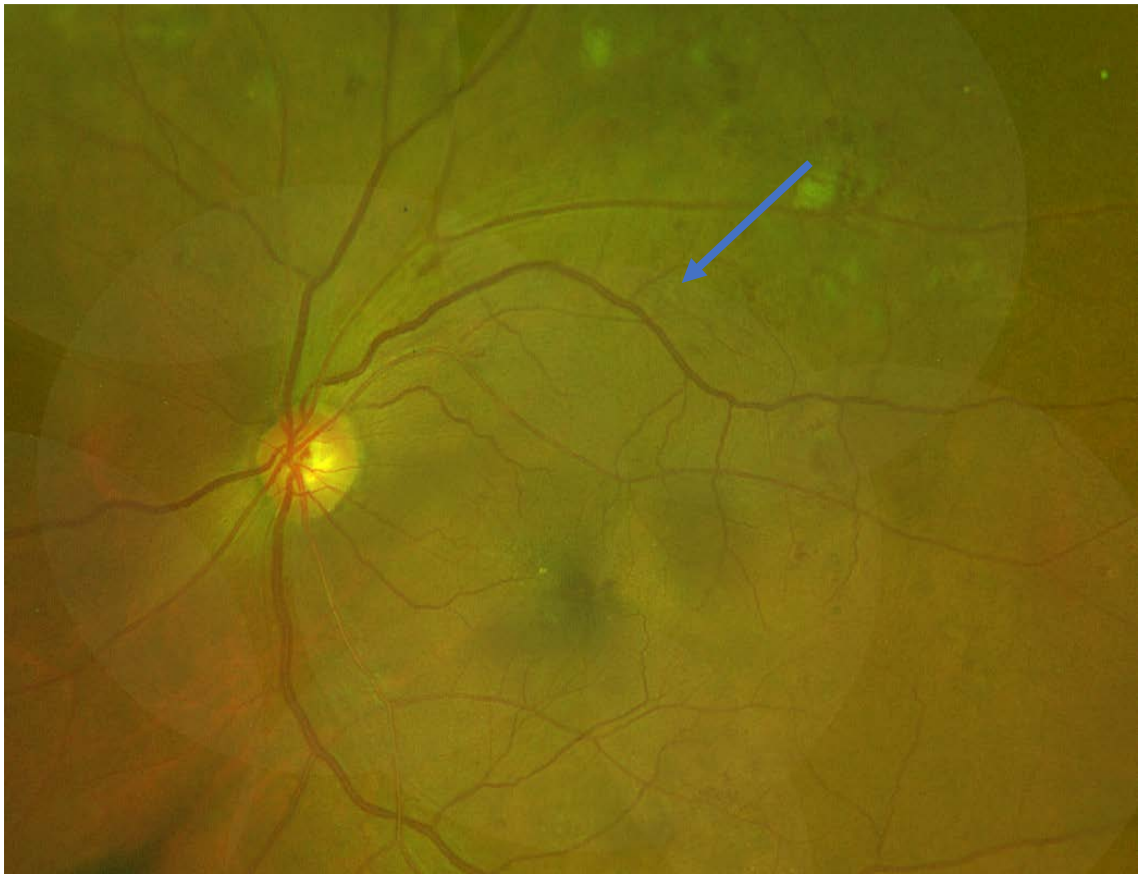


Fig 4.4 Optomap Image of the same eye as Fig 4.3



Subsequently, a further 5% of images were graded and inter-grader agreement was found to be 100% within one DRSS step.

4.2.4 SNP Analysis

A panel of SNPs was selected based on those identified by the literature review and the differences between two groups were compared:

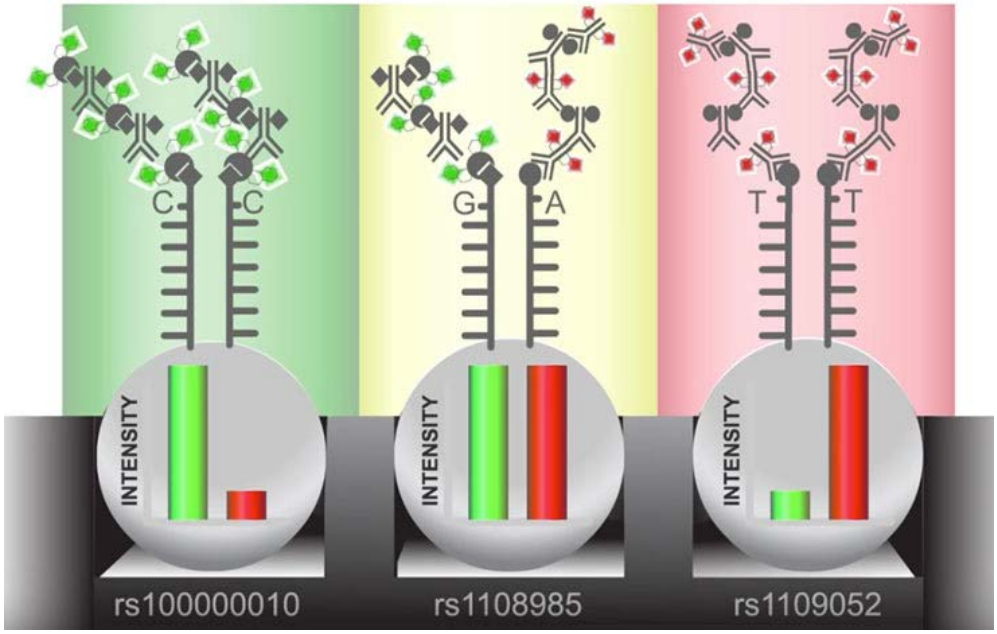
- those with moderate - severe NPDR and no DR
- those with VB and no DR
- those with severe IRMA and no DR

4.2.4.1 Infinium Genotyping

The samples were sent to the Wellcome Trust Centre for Human Genetics, Oxford. Their Infinium© HTS assay on Global Screening-24v2.0x bead-chips was used to analyse the DNA samples. The Array is an ‘off the shelf’ array with in excess of 200k SNPs covering many genes. This particular array was selected on the advice of the Wellcome Trust as being most appropriate for our study and most likely to examine the SNPs of interest identified from the literature review.

The Wellcome Trust firstly analysed the quality of the samples to remove suboptimal samples or to reprocess them. The purified DNA samples were then denatured and amplified up to one thousand-fold to increase the quantity of DNA. They were then fragmented into optimal lengths for hybridization to the array, the process cleaves the DNA into 300-600 base pairs. The samples were then purified by precipitation and re-suspended. They were then applied to an array comprised of small spherical beads. During this hybridization process each bead was specific to a locus of interest. Probes on the beads were stained with red or green fluorescent molecules in order to discriminate between the different alleles for each bead. The illumina scanner uses lasers to excite the fluorophores and measures the red and green intensity signal from each bead on the array. For example, the homozygote TT produces primarily red signal and the homozygote CC primarily green. The heterozygote GA will produce approximately red and green signals; therefore, heterozygote bead types appear yellow in intensity images as they omit both red and green fluorescence at the same bead location. The red and green intensities are written to an intensity data file which Genome Studio coverts into genotype calls.

Fig 4.5. Once laser excited the nucleotide label emits a signal which is detected by an Illumina scanner. Intensity values for each colour convey information about the allelic ratio of a given locus (Illumina.com)



4.2.5 Statistical Analysis

Chi-square test was used for testing relationships between categorical variables and student T-test was used for all continuous variables (Microsoft Excel).

For analysis of our genetic data PLINK v1.9 software was used. (PLINK)

The original data received from the Oxford Wellcome centre consisted of a ped file. This ped file was changed into a transposed text fileset using the - - tfile command.

Phenotype data comparing cases and controls were manually added to the phenotype file for each analysis. For instance, comparing DR vs no DR, DR was entered as case, whilst no DR as control.

Using the - - assoc command, it wrote the results of a 1df chi-square allelic test to the plink.assoc report file. Using this command, PLINK reports allele counts, rather than frequencies, in cases and controls. The asymptotic p-value of each tested SNP was included in the report.

Consider a genetic marker consisting of a single biallelic locus with alleles *a* and *A* (i.e., a SNP). Unordered possible genotypes are then *a/a*, *a/A* and *A/A*. The risk factor for case versus control status (disease outcome) is the genotype or allele at a specific marker. The disease penetrance associated with a given genotype is the risk of disease in individuals carrying that genotype. Standard models for disease penetrance that imply a specific relationship between genotype and phenotype include multiplicative, additive, common recessive and common dominant models.

We did not know whether a specific genetic marker would be a dominant model or recessive model, and also unknown was if the effect were additive or multiplied. We decided to use the default option and report the p-value based on allele counts.

4.3 Results

In total, 596 participants were recruited to the study including, 199 with the moderate/severe NPDR (NPDR group) and 397 diabetes for at least 5 years but no DR (No DR group). Twenty samples failed to yield enough DNA to be analysed, leaving 576 samples that were sent to the Wellcome Trust, Oxford Genomics Centre to be analysed. Of these, 44 were removed from analysis as they failed to yield meaningful data.

So in total, 532 were analysed, in which 181 and 351 were in the NPDR and No DR respectively.

The demographic and features analysis included the 532 whose samples were able to be analysed. In a few cases, age, sex, HBA1c and duration of DM were not recorded.

4.3.1 Illumina Report

Overview of genotyping results: 576 Human genomic DNA samples were processed using the Infinium© HTS assay on Global Screening-24v2.0x bead-chips. The manifest file (which describes the SNP/probe content on a beadchip) for this project was GSA-24v2-0_A2.bpm. As the project consisted of more than 96 samples, the samples were clustered against each other.

Sample QC review: Quantification was carried out by the customer.

Technical errors: None.

Controls review: In general, all the samples performed well across all controls. Samples removed: None.

Sample Summary: Data QC was performed using Genome Studio v2.0.4. The call rate cut-off for the Global Screening Array-24 v2.0 bead-chips is 98% as it an off-the-shelf array.

Please note that out of 95 samples that failed 51 had a call rate between 95% and 97.9%. Although this group of samples falls below the threshold they should still give meaningful data. This is highlighted in the samples table. Therefore, the Wellcome Trust included the 51 samples that didn't meet the optimal call rate of 98% as these samples had call rates of between 95 and 97.9% which the Wellcome Trust considered high enough to still yield meaningful data. This means that only 44 sample sent to the Trust were excluded.

Table 4.11 Quality of samples report from Wellcome Trust

		Samples		Call Rate	
P190323	Plates	Total in Batch	No. failed Call Rate (<98%)	Average (%) with fails	Average (%) without fails
	6	576	95	96.7	98.3

The call rate is the percentage of genotypes successfully assigned. A call rate of 98% means a genotype can be assigned in 98% of SNPPs, so less than 2% of data missing.

Failure rate: 16.49%

Provided Results Files:

P190323_RawData

P190323_Final Report.txt: These files contain the complete list of all the genotypes (all the markers and all the samples) in several formats as outlined below:

Top: The ATGC allele from the top strand.

Forward: The ATGC allele from the forward strand.

Design: The ATGC allele defined in the design file. AB: The allele coded as either A or B.

P190323_PLINK folder containing final report in PLINK format.

P190323_SamplesTable.txt: These tables show the call rates for all the samples.

4.3.2 Demographic Data of those with genetic analysis

Table 4.12 Demographics Data, No DR vs NPDR (n=532)

	No DR (n=351)	**NPDR (n=181)**
Mean Age (years)	66.18	59.19
Sex (Male)	57.81%	60.48%
HbA1c	7.65	8.68
Mean duration of DM (years)	12.15	18.49

A value of $p \leq 0.05$ was considered statistically significant.

The No DR group was significantly older, $p<0.0001$, had a lower HbA1c, $p=0.0003$ and a shorter duration of diagnosed DM, $p=0.0049$.

As the control group can become cases in later life but were also older at the time of analysis, the findings are likely to become more significant, hence I did not adjust for age. There was no difference between sex for cases and controls (Table 4.14) therefore no adjustment was made.

Table 4.13 No DR vs NPDR by Diabetic Type (n=528)* and by sex (n=532)

	Type 1	Type 2	F	M
NPDR	25	153	77	104
No DR	10	340	146	205

$p<0.00001$, Chi Square=23.86 for diabetes Type

$p=0.834$, Chi-Square=0.044 for sex

* Information about Diabetic Type was not available for all patients

In both groups, Type 2 was much more prevalent. When looking at the prevalence of DR amongst Type 1 and Type 2, despite much larger numbers of those with Type 2, those with Type 1 were more likely to have DR than those with Type 2 (Table 4.13).

There was no significant difference between sexes in the proportion with DR compared to no DR (Table 4.13).

Table 4.14 Mean Age, duration of DM, HBA1c of patients with different IRMA severity in NPDR group, and with or without VB

	Age	Duration of DM	HBA1c
No or mild IRMA (n=102)	60.94	16.26	8.81
Severe IRMA (n=79)	56.90	21.30	8.53
p value	0.059	0.309	0.378
No VB	60.13	19.48	8.59
VB	56.70	16.02	8.94
p value	0.178	0.303	0.38

Table 4.15: Demographic data (sex) of patients with different IRMA severity, and with or without

	F	M	
No or mild IRMA	44	58	
Severe IRMA	26	53	p=0.161, Chi Square=1.963 n=181
No VB	50	81	
VB	20	30	p=0.821, Chi-Square=0.051 n=181

p=0.161, Chi Square=1.963 n=181

Analysis of the NPDR group revealed the mean age of those with severe IRMA showed a trend (p=0.059) to be lower than those without. Those with severe IRMA had a slightly longer duration of DM and slightly lower HBA1c (Table 4.15) but not reaching statistical significance. Severe IRMA appeared to be more common in male, but it was not statistically significant (Table 4.15).

The findings for VB were similar to those with severe IRMA in terms of age, patients were slightly younger, but the duration of DM was shorter and HBA1c were slightly higher (the opposite of IRMA), although these results were not statistically significant (table 4.15).

Fig 4.6 Optomap Image Participant with Temporal IRMA

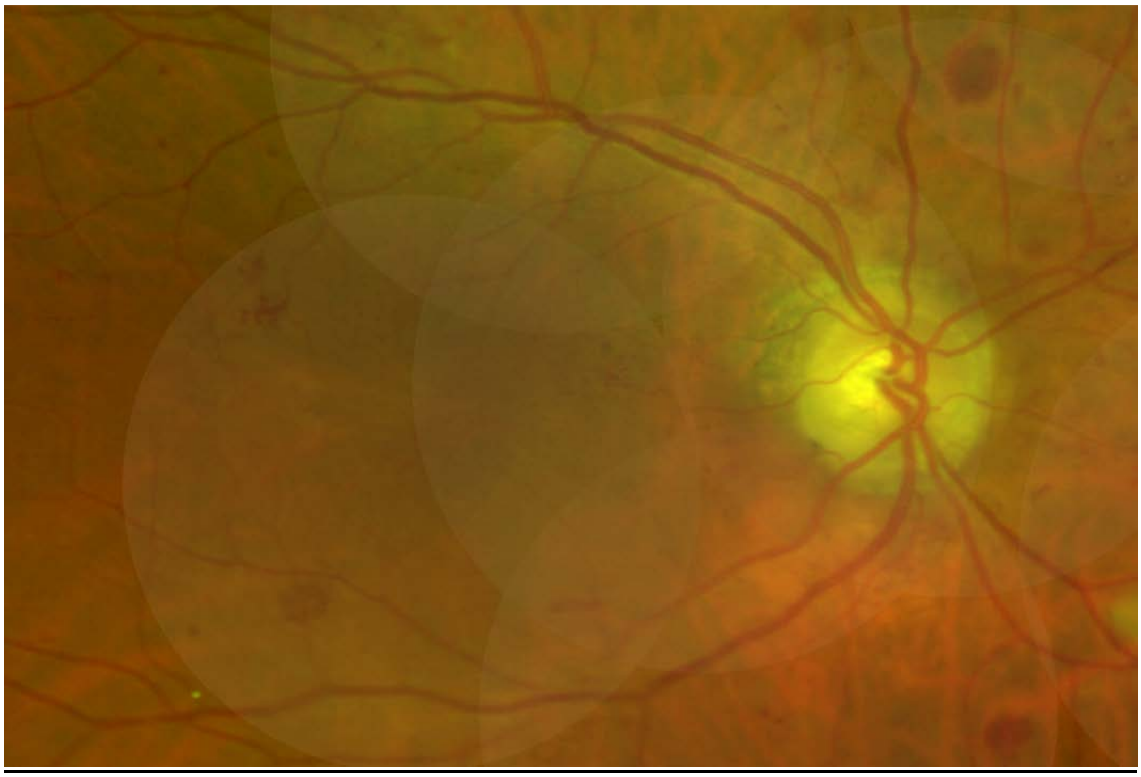
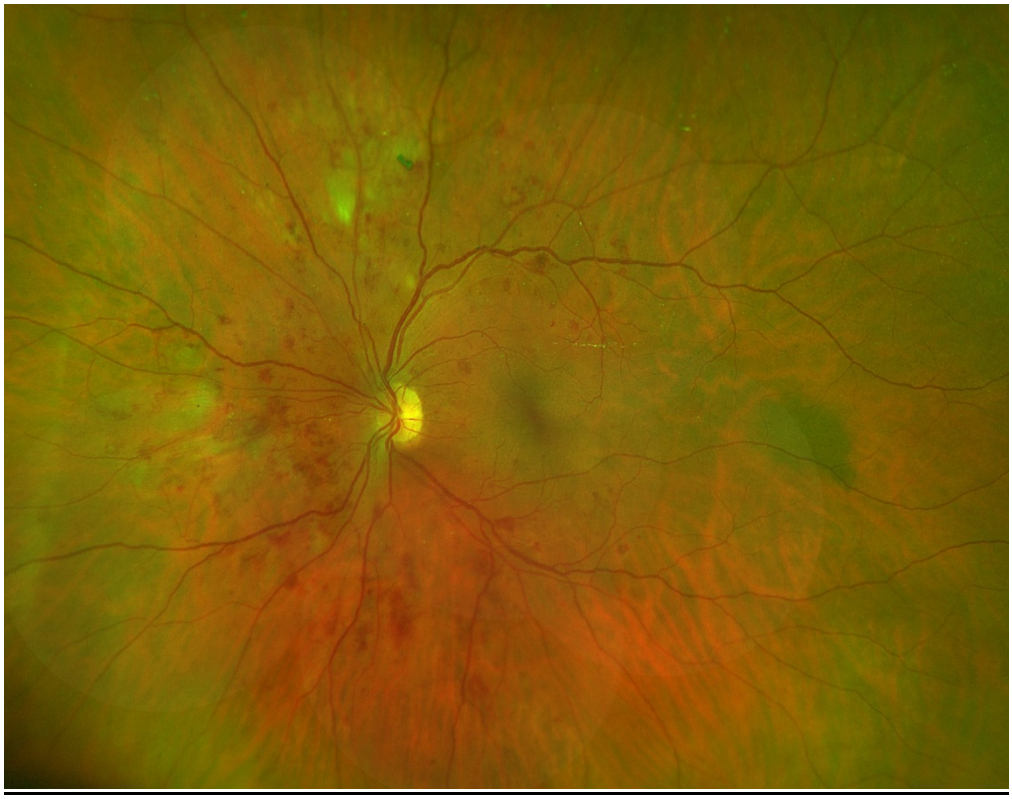


Fig 4.7 Optomap Image of Participant with DH,VB and IRMA



4.3.3 Results of Illumina Analysis

Table 4.16 Results of Illumina Analysis with uncorrected p-value

Gene	**SNP**	**NPDR vs No DR** n=181 NPDR n=351 No DR	**VB vs No DR** n=50 VB n=351 No DR	**Severe IRMA vs No DR** n= 79 severe IRMA n=351 No DR
CFB	rs1048709	0.042	0.199	0.173

CFH	rs800292	0.36	0.228	0.669
C5	rs17611	0.501	0.557	0.36
C5	rs2269067	Not on array	Not on array	Not on array
C5	rs1548782	Not on array	Not on array	Not on array
VEGFA	rs2010963	0.083	0.487	0.09
VEGFA	rs699947	0.34	0.916	0.046
VEGFA	rs833061	N/A	N/A	N/A
VEGFA	rs13207351	0.357	0.952	0.035
VEGFA	rs1570360	0.459	0.69	0.05
VEGFA	rs2146323	N/A	N/A	N/A
VEGFA	rs3025030	0.731	0.451	0.884
VEGFA	rs3025039	0.378	0.184	0.592
VEGFA	rs10434	0.304	0.109	0.27
VEGFA	rs10738760	0.368	0.589	0.945
FGF2	rs308398	Not on array	Not on array	Not on array
FGF2	rs373341357	Not on array	Not on array	Not on array
HFE	rs1800562	0.955	0.918	0.527

KDR	rs2071559	N/A	N/A	N/A
SLMAP	rs17058639	0.035	0.106	0.22
VDR	rs1544410	0.431	0.438	0.288
VDR	rs7975232	0.66	0.378	0.93
VDR	rs2228570	N/A	N/A	N/A
TGF-b1	rs1800470	0.015	0.226	0.02
TGF-b1	rs104894719	Not on array	Not on array	Not on array
ARHGAP22	rs4838605	0.065	0.691	0.048
ARHGAP22	rs11101355	Not on array	Not on array	Not on array
ARHGAP22	rs11101357	Not on array	Not on array	Not on array

Of the 28 SNPs selected, 7 were not included in the Illumina Array and therefore could not be analysed. There were another 5 SNP excluded due to a significant number of samples with missing data.

The TGF-b1 SNP rs1800470 showed the highest correlation (p=0.015) for the group NPDR vs No DR, followed by the SLMAP SNP rs17058639 (p=0.035) and CFB rs1048709 (p=0.042). None of the SNPs selected showed any association with those with VB. Four of the VEGFA SNPs, the ARHGAP22 SNP rs4838605 and the TGF-b1 SNP also associated with NPDR showed statistically significant associations with the presence of IRMA.

Table 4.17 SNPs with an association, NPDR vs No DR

Gene	SNP	p
CFB	rs1048709	0.042
SLMAP	rs17058639	0.035
TGF-b1	rs1800470	0.015
VEGFA	rs2010963	0.083
ARHGAP22	rs4838605	0.065

Table 4.18 SNPs with a trend association VB vs No DR

Gene	SNP	p
SLAMP	rs17058639	0.106

Table 4.19 SNPs with an association, Severe IRMA vs No DR

Gene	SNP	p
VEGFA	rs2010963	0.09
VEGFA	rs699947	0.046
VEGFA	rs13207351	0.035
VEGFA	rs1570360	0.05
TGF-b1	rs1800470	0.02
ARHGAP22	rs4838605	0.048

4.4 Discussion

The results displayed are not corrected for multiple testing. A p value of 0.0031 would have to be met if this were the case, as 17 SNPs were analysed. No SNP reached this level of significance. The purpose of this study was to identify differences between VB and IRMA by multiple approaches: genetic profiling, retinal distribution, proximity to ischaemia and responses to anti-VEGF therefore no Bonferroni correction was made. (see 8.4 for more detail)

4.4.1 DR vs no DR Results

TGF-b1 rs1800470 p=0.015

The strongest association in the comparison between DR and no DR was found with SNP TGF-b1 rs1800470. The p value of this was 0.015, this confirms the findings in many other studies. (Buraczynska, Baranowicz-Gaszczyk et al. 2007) (Liu, Jiao et al. 2014) (Rodrigues, Pietrani et al. 2015).The result ties in with the findings that TGF-b1 has a role in angiogenesis, maintaining retinal capillaries, and is involved in cellular proliferation and differentiation, it's inhibition leading breakdown of the BRB and impaired retinal perfusion (Walshe, Saint-Geniez et al. 2009). Another study found that TGFb signalling was important for maintaining pericyte function. As discussed in Chapter 2, the loss of pericytes in the basement membrane of retinal vessels appears early on in DR and may contribute to vascular leakage and haemorrhage and microaneurysm development.

SLMAP rs17058639 p=0.035

SLMAP was also positively associated with DR vs no DR in our study. SLMAP has a role in vascular endothelial dysfunction and may be a marker of microvascular dysfunction in diabetes. A previous study found this SNP to be associated with a higher risk of DR (Upadhyay, Robay et al. 2015). As previously discussed, vascular endothelial cell death is widespread in DR and leads to hypoxia and capillary closure.

CFB rs1048709 p=0.042

The role of Complement in the development of DR is not completely understood. As previously discussed, the Complement System helps regulate the immune response and inflammation and its dysfunction is linked to autoimmune disease. DM affects the production of complement proteins and elevated levels of CFB has been detected in the vitreous of patients with PDR, despite also being implicated in the early stages of DR (Chrzanowska, Modrzejewska et al. 2018). Evidence from previous studies suggests the complement system is involved in the development of DR. The SNP rs1048709 was previously found to have an association with DR versus no DR in a Chinese population (Wang, Yang et al. 2013) and had a significant association with the presence of DR compared to no DR in our study. Our findings therefore strengthen the case of CFB‘s role in the development of DR.

VEGFA rs2010963 p=0.083, ARHGAP22 rs4838605 p=0.065

One VEGF SNP showed a positive association with DR, as well as an ARHGAP22 SNP, although these were not statistically significant (p= 0.083 and 0.065 respectively).

4.4.2 VB vs no DR Results

No association was found with any SNPs studied and the presence of VB. SLMAP rs17058639 was not significant but showed a trend association, p=0.106. SLAMP was found to show a positive association with the development of DR vs no DR (p=0.035) but not with IRMA. This finding may be explained by SLMAP being positively associated with DH but as DH were so common in the NPDR group, its genetic associations were not studied separately. SLMAP has been implicated in vascular endothelial dysfunction in diabetes potentially leading to DH. Furthermore, if confirmed in future studies of its importance in VB, the pathophysiology of VB may be related to vessel wall damage secondary to endothelial dysfunction.

Nonetheless, most interestingly, none of the ischaemic driven genes showed any significance. This may be due to the relatively small number of patients with VB, however, significant associations were shown with severe IRMA with only a slightly higher number of patients (see below).

4.4.3 Severe IRMA vs No DR Results

Four of the VEGF SNPS; rs2010963 p=0.09, rs699947 p=0.046, rs13207351 p=0.035 and rs1570360, p=0.05 showed a correlation with IRMA compared to those without. There was a weaker association with one VEGF SNP and DR vs no DR. As previously described, VEGF has an important role in retinal angiogenesis, and endothelial cell permeability and is activated by microvascular changes resulting from elevated blood glucose levels and hypoxia (Simó-Servat, Hernández et al. 2013). As Chapter 6 details, IRMA improve after anti-VEGF therapy so these associations are not unexpected. Numerous studies have found positive associations with DR and SNPs associated with VEGF as discussed previously, indeed VEGF has the greatest number of SNPs reportedly associated with DR (Han, Lando et al. 2019).

TGFb-1 rs1800470 also showed a positive association with IRMA compared to those without, p=0.02, suggesting it has a role in the development of DH and IRMA.

ARHGAP22 rs4838605 (p=0.048) was found to be significant in the group with IRMA compared to no IRMA. The ARHGAP22 may be involved in endothelial cell migration and angiogenesis, specifically capillary tube formation and thereby influence IRMA development.

Previous studies have shown an association between VEGF and TGFb1, our results confirming this are not surprising. Less prevalent was an association with DR and CFB, ARHGAP22 and SLAMP in previous studies.

4.4.4 Discussion and summary

Examination of the demographic data of both groups found that those with DR were likely to be younger, have T1DM and have a higher HbA1c, as well as a longer duration of diagnosed DM. The younger age of the NPDR may be explained by their being T1DM which develops at an earlier age than in T2DM, however, the sample size was too small to be sure. In both groups, T2DM was more prevalent. No difference was observed between the number of male or female in the No DR and the NPDR group, despite a previous study showing that males were more likely to have severe NPDR (Lee, Lee et al. 2017). As the NDPR group had a longer duration of DM, it is reasonable to suggest that the no DR participants with the significant alleles may develop NPDR in the future with increasing duration of DM. This theory may be confirmed and the genetic association may be more significant if the duration of DM inclusion criteria was more than 20 years as opposed to five years in this study.

In Chapter 2 I discussed risk factors for DR development and progression. As part of my questionnaire, I recorded the participant's age, DM Type, history of angina, heart attack, stroke or renal problems, 'other medical problems', year of diagnosis of DM, whether their DM was controlled by Insulin, tablets or diet, whether they smoked or previously smoked and if they had laser treatment for diabetic eye disease.

When examining the importance of the risk factors described in Chapter 2, the most important factors for DR were duration of DM, diabetic control, and age. All these factors were therefore included in my analysis. I decided not to examine hypertension and hyperlipidaemia as this would mean including a blood draw and blood pressure measurements into the procedures which is a significant deterrent for patients to join studies. Hypertension and hyperlipidaemia were considered modest risk factors for DR. In the case of hypertension, it was suggested that lowering may delay the onset of DR, and may have more of an effect in delaying

DMO. Other covariates like comorbidities have been recorded and will be available for future analysis as such data is easy to collect and is likely to be helpful for future analyses. Baseline levels of retinopathy were generally not available for analysis.

In this study no correction for these risk factors, glycaemic control, age and duration of DM was made. As expected, those with DR were found to have a longer duration of DM and had higher HbA1c levels. Those with no DR were significantly younger than those with DR, as explained in 4.3.2, which means that the result can only become more significant with time.

In the VB and IRMA groups, there was no difference in age, duration of diabetes and HbA1c levels. As this study was designed to identify differences between VB and IRMA and no meaningful differences were found, no correction was therefore made.

Analysis of the NPDR group showed no significant association with age, sex, duration of DM or HbA1c levels when looking at the presence or absence of VB or severe IRMA. However, it is interesting that patients with VB (16.02 years) have shorter durations of DM as compared with those with severe IRMA (21.30 years) despite being very similar in age at 56.70 years for VB and 56.90 years for IRMA. This may suggest that VB develops first and then progresses to PDR without developing IRMA. Indeed, this theory would be consistent with the observation that VB are rarely observed alone, whilst severe IRMA may occur with or without VB.

In a complex disease such as NPDR, there are a number of systemic factors that can modify the disease. Also, some patients may have already progressed to PDR and therefore are not represented in the NPDR group who may also have the genetic association, thereby explaining why the results were not highly significant. Nonetheless, the differences in association with VEGF and TGFb-1 polymorphisms, and to a lesser extent CFB and ARHGAP22, between NPDR and no DR may be driven by the presence of IRMA, for which a number of VEGF SNPs

and TGFb-1 and ARHGAP22 SNPs showed a positive correlation with IRMA's presence. Conversely, we found no gene to be associated with the presence of VB.

These findings of VEGF, TGFb-1 and ARHGAP22 SNPs' positive association with those with IRMA tie in with the hypothesis that IRMA may be precursors of new vessels as they all play a role in angiogenesis, cellular proliferation and differentiation. The pathophysiology of VB may be different, and may be more related to endothelial damage in the vessel wall.

Chapter 5: Venous Beading and IRMA location and prevalence in patients with severe NPDR

5.1 Background

Our understanding of the three features of severe NPDR, deep haemorrhages (DH), venous beading (VB) and intra-retinal microvascular abnormalities (IRMA) is limited. Very few studies have examined these features and their individual risk of progression to PDR since the landmark trials of the 1980's (DRS and ETDRS). A more detailed characterisation of the three features may improve prediction of visual prognosis and help us better understand the pathophysiology.

5.2 Purpose

- To analyse the relative prevalence of the three features of severe NPDR.
- To analyse the location and distribution of the three features of severe NPDR.
- To see if the three features always co-exist or are likely to appear in sequence.

This will help to determine whether they have the same pathophysiology, for example if both VB and IRMA always appear together for example adjacent to an area of ischaemia then they are likely to share the same aetiology. IRMA are commonly found adjacent to areas of ischaemia but it is unknown how frequently VB are found near ischaemia. A recent study by Chen et al found that VB were associated with capillary non-perfusion (Chen, Zhang et al. 2018). As ischaemia can be quite regional, in this study individual quadrants of the fundal images were examined for the three features. If VB and IRMA always co-localise, other local factors may play a role, e.g. localized cytokines or blood flow changes. Currently, clinicians and researchers only grade features by their presence or absence or their size and extent. Traditional grading combines the three features of severe NPDR together for simplicity. This study examined the individual features in more detail and looked for differences in the distribution of VB and IRMA, and also

whether VB and IRMA are more prominent in one quadrant. If they always appear in sequence i.e. DH followed by IRMA followed by VB, again they may share the same pathophysiology. As we do not have longitudinal data, this cross-sectional study examined the distribution of the three features.

5.3 Methods

Participants were recruited from the Diabetic Retinopathy Clinics of King's College Hospital, London and Oxford Eye Hospital. The study adhered to the tenets of the Declaration of Helsinki and was approved by the South West Cornwall and Plymouth Research ethics Committee, the Health Research Authority and Trust management of Oxford Radcliffe Hospitals NHS Trust and King's College Hospital NHS Trust.

Literature Review

A literature review was undertaken of PubMed.gov for articles on venous beading, IRMA, severe NPDR, Ultrawide field imaging in DR, genetics of DR. For section 4, a comprehensive literature search of Genetics and DR was previously completed by members of this group from 2014-2016. Their focus was Type 2 and DMO. This collation of references was enhanced by a search from 2017-2019 by the author.

Statistical Analysis

We were advised that as this is a pilot study and the research question not previously studied, an accurate sample size would not be possible to calculate. The Chi-square test was used for testing relationships between categorical variables and Student T-test was used for all continuous variables (Microsoft Excel).

Imaging

We elected to study ultrawide field images as opposed to seven-field fundus photography images. The reason for this being that seven-field fundus

photography is time consuming and uncomfortable for the subject and is not routinely done in clinical practice. The two centres routinely use Optomap imaging for patients with diabetic retinopathy. Historic ultrawide field scanning laser ophthalmoscope (UWFSLO) (Optomap 200Tx; Optos plc, Dunfermline, UK) images from the Hospital Eye Service were identified. Five hundred images with DRSS level 43 to 53 (moderate and severe NPDR) were included in the study. This DRSS level was chosen as our interest is in the three features of moderate/severe DR, namely deep haemorrhage (DH), venous beading (VB) and intraretinal microvascular abnormalities, (IRMA). The medical notes of those attending the medical retina clinics at King's College Hospital and Oxford University Hospital were scrutinised and Optomap images of all those recorded as having moderate-severe NPDR examined.

In a more detailed analysis of the co-location of IRMA and VB, in eyes with both VB and IRMA, the quadrant in which IRMA or VB in the individual eye were most severe was recorded. These were then analysed to assess whether these quadrants were the same.

5.4 Results

A total of 308 patients were examined. Initially, the study eye was defined as the more severe of the two if both eyes of a patient were eligible. A sensitivity analysis was undertaken to assess if there was a significant difference between study eyes and eligible fellow eyes. No statistical significance was found between the groups using Chi Square Tests for distribution of DH, VB or IRMA. Hence, all 504 eyes were included in the analysis and graded as described. (Sensitivity analysis see Appendix 3).

Imaging and grading

Initially identifying IRMA and VB with certainty proved difficult. As previously discussed, an experienced Diabetic Retinopathy Grader was consulted and a grading audit undertaken. Images were discussed together after the first results

found discrepancies. Experienced Medical Retina Consultants' advice was also sought, as well as comparing recent colour fundus photograph (CFP) images to those taken with Optomap and clinically examining the fundus of those with suspected VB and IRMA with a digital high-magnification lens (Volk). These difficulties have previously been reported; Nderitu et al found that identifying IRMA and VB in Optomap images is more difficult than identifying them on digital fundus photographs (Nghiem, Nderitu et al. 2019). It has previously been reported that IRMA and VB are difficult to recognise (Wilkinson, Ferris et al. 2003) and that ETDRS 35mm colour slides are better able to detect subtle diabetic lesions such as small haemorrhages and microaneurysms and IRMA than digital images (Hubbard, Sun et al. 2011). However the study supported the transition from film to digital imaging for assessing DR and the quality of digital imaging has improved since this report.

The quality of the images captured varied significantly, being dependent on the expertise of the technician and the ability of the participant to co-operate.

As the Optomap uses an ellipsoid mirror to produce images, the peripheral fundus appears more distorted, making detection of IRMA in the periphery very difficult. The peripheral retinal vessels also appear distorted, making VB hard to identify. The peripheral retinal areas were graded during the study but not included in the analysis as it became clear that the distortion of the peripheral images made it impossible to discern VB outside the seven field area. A previous study of 54 eyes with VB found that only 10% were observed in the fourth branch and 3% in the fifth branch, using ophthalmoscopy and fluorescein angiography (Sato, Kamata et al. 1993). In this study, IRMA in the peripheral area were rarely seen. This may be because they are harder to detect due to the peripheral distortion and poor image resolution in the periphery as well as the effect of eyelids in some participants.

5.4.1 Prevalence and distribution of lesions per quadrant

The three features were present as follows:

DH >ETDRS Standard Photo (SP) 1 in at least one quadrant=484 (96%)

DH ≥ETDRS SP 2A in at least two quadrants=255 (50.6%)

VB, all grades =109 (21.63%)

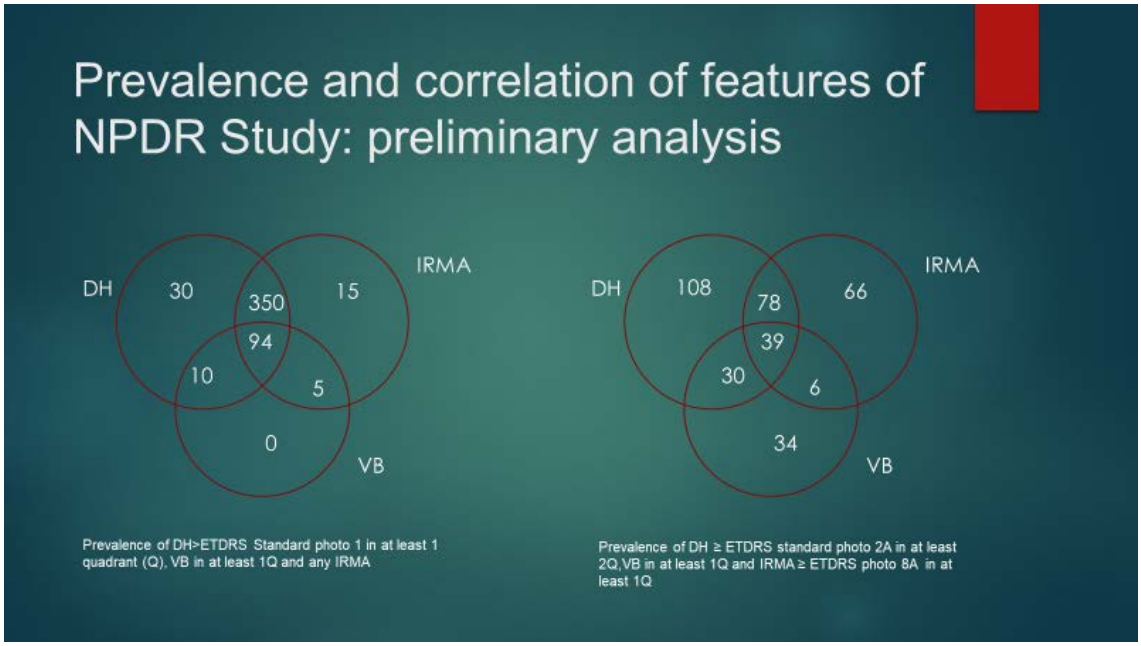
IRMA (any) definite but less than SP 8A = 464 (92.1%)

IRMA ≥ETDRS SP 8A in at least one quadrant= 189 (37.5%)

The presence and distribution of the three features of NPDR: DH, VB and IRMA were examined. The prevalence of the three features, at different severity levels, whether they are present of absent and if the eye has 1, 2 or 3 features was examined and is detailed below in figure 5.1.

The first graph in Fig 5.1 includes all levels of lesions and in this category, it looks as if many have DH and IRMA and only a few have all three lesions. In the second graph in Fig 5.1, when the severity level examined is raised to levels 47-53 then the features are more evenly distributed.

Figure 5.1 Prevalence of the 3 features of moderate-severe NPDR



5.4.2 Location of DH, VB and IRMA

Five hundred and four images were analysed and graded as described. The prevalence of features in the temporal and nasal retina were compared as well as in the superior and nasal retina.

A p value of ≤0.05 was selected as significant.

5.4.2.1 Comparison of Superior and Inferior Retina

Table 5.1 Superior vs Inferior with DH Grade 2

	Superior	Inferior
Yes	172	219
No	332	285

p=0.002, Chi-square=9.23

Table 5.2 Superior vs Inferior with DH Grade 3

	Superior	Inferior
Yes	291	212
No	213	292

p=<0.00001, Chi-square=24.766

Table 5.3 Superior vs Inferior with DH Grade 2 and 3

	Superior	Inferior
Yes	463	431
No	41	73

p=0.0015, Chi-square=10.128

Table 5.4 Superior vs Inferior with IRMA Grade 1

	Superior	Inferior
Yes	325	263
No	179	241

p=0.00008, Chi-square=15.69

Table 5.5 Superior vs Inferior with IRMA Grade 2

	Superior	Inferior
Yes	91	34
No	413	470

p<0.00001, Chi-square=29.67

Table 5.6 Superior vs Inferior with any IRMA

	Superior	Inferior
Yes	416	297
No	88	207

p<0.00001, Chi-square=67.865

Table 5.7 Superior vs Inferior with VB (all)

	Superior	Inferior
Yes	74	68
No	430	436

p=0.587, Chi-square=0.295

5.4.2.2 Comparison of Temporal and Nasal Retina

Table 5.8 Temporal vs Nasal with DH Grade 2

	Temporal	Nasal
Yes	187	198
No	317	306

p=0.476, Chi-square=0.509

Table 5.9 Temporal vs Nasal with DH Grade 3

	Temporal	Nasal
Yes	272	234
No	232	270

p=0.0167, Chi-square=5.73

Table 5.10 Temporal vs Nasal with DH Grade 2 and 3

	Temporal	Nasal
Yes	459	432
No	45	72

p=0.079, Chi-square=7.049

Table 5.11 Temporal vs Nasal with IRMA Grade 1

	Temporal	Nasal
Yes	289	296
No	215	208

p=0.655, Chi-square=0.1996

Table 5.12 Temporal vs Nasal with IRMA Grade 2

	Temporal	Nasal
Yes	100	21
No	404	483

p<0.0001, Chi-square=58.615

Table 5.13 Temporal vs Nasal with IRMA (all)

	Temporal	Nasal
Yes	389	316
No	115	188

p=<0.0001, Chi-square=25.146

Table 5.14 Temporal vs Nasal with VB (all)

	Temporal	Nasal
Yes	59	82
No	445	422

p=0.037, Chi-square=4.362

Milder haemorrhages were more likely to be found in the inferior retina than the superior, however, more severe grades of DH, and combined severities (all DH), were more likely to be located in the superior retina. All these results were statistically significant. More severe DH were more likely to be present in the temporal retina compared to the nasal, the result being statistically significant, p=0.0167.

All categories of IRMA (mild, severe and combined) were observed more extensively in the superior retina than inferior, all results being statistically significant. Combined severity grades and the more severe category of IRMA were more likely to be found in the temporal retina compared to nasal, p=<0.0001.

No difference in VB distribution between the superior and inferior retina were found, p=0.587, however, VB were more likely to be found in the nasal retina than temporal, p=0.037.

Summary of Distribution

- DH were more likely to be located in the superior rather than inferior retina and in the temporal rather than nasal retina.
- IRMA were also more likely to be located in the superior rather than inferior and temporal rather than nasal retina.

- VB were more likely to be present in the nasal rather than temporal retina but no difference was found in the prevalence of VB when comparing the inferior and nasal retina.

The colocalisation of VB and severe IRMA in the same eye was also examined. Ninety-nine eyes had both VB and IRMA. In these eyes the most severe quadrant of VB and IRMA were the same in 31 eyes and not so in 68. Hence, they are slightly more likely to be in the same quadrant but this was not statistically significant p=0.345.

5.5 Discussion

Cliniacally, vascular lesions are more frequently observed in the area temporal to the macula corresponding to the 'watershed zone' but the phenomenon is not fully understood. The term 'watershed zone' describes an area of tissue that is supplied by two or more end arteries (Hayreh, 1990). This means that the tissue located in the watershed zone has relatively reduced vascularity and is therefore more susceptible to ischemia. The watershed zone in the retina is much easier to visualise with FFA (Hayreh, 1990). Reports from investigations of the watershed zone found that DH and IRMA are more commonly found in the temporal retina (May, Rutkowski 2019). Our study found that IRMA are more prevalent in the temporal retina and adds weight to the hypothesis that IRMA are largely driven by ischaemia.

This study found that VB were more commonly located in the nasal retina. This was unexpected as their being ischaemia driven would suggest they would also be more prevalent in the watershed zone. The significance of VB being more prevalent in the nasal retina is not clear. The number with VB was smaller than that with DH or IRMA and a different result may possibly be observed with a larger sample size.The only previous study, Sato (Sato, Kamata et al. 1993) of venous abnormalities (mainly, 96%, VB but also including venous loops and reduplications) in a similar sized group of 56 eyes found no differences between superior and

inferior nor nasal and temporal. No other studies to date have described the distribution of the individual lesions. The study concluded that the presence of venous loops and reduplications were associated with advanced stages of retinopathy, PDR. In this study, the presence of loops and reduplications was so small that they were not analysed, a total of 9 were identified. A more recent study of DR lesions using UWFSLO, Optomap, found that VB, DH, IRMA and NVE were more frequently located in the temporal fields than the nasal. This study looked at distribution in the entire retina and found that VB were more likely to be present in the central seven field area than in the periphery (Silva, 2015). The numbers of those with VB were similar to this study,

When looking at all levels of lesions present, many had DH and IRMA and only a few had all three lesions. This suggests that the development of lesions could be sequential i.e. DH and IRMA first, then VB. When the severity level examined is raised to levels 47-53 the features were more evenly distributed, implying that some patients may be more disposed to the development of either VB or IRMA. (See Fig 5.1). Another interesting finding was that the most severe of VB and IRMA lesions were found in the same quadrant only slightly more often than if the observation was by chance which also suggest that they are do not share the same aetiology.

In summary, DH and IRMA were more commonly located in the temporal and superior retina, VB in the nasal, suggesting that VB and IRMA do not occur together and are independent of each other. DH were common in this moderate/severe NPDR group and were found in almost all participants. This is consistent with the genetic findings (see Chapter 4) that VB and IRMA may have different pathophysiologies.

Chapter 6: Analysis of NPDR features response to anti-VEGF in DR patients

The response of DH, VB and IRMA to anti-VEGF was examined in a retrospective analysis of two studies, the CLARITY Study (which recruited those with PDR) and the Randomised Trial of wide-field guided PRP for diabetic macular oedema treated with Ranibizumab (RDP) study of those with NPDR.

6.1 CLARITY STUDY

6.1.1 Introduction

As reported in Chapter 1, the number of people diagnosed with DM is increasing at an alarming rate. Consequently, the numbers of people with microvascular ocular complications grouped together as diabetic retinopathy (DR) is also rapidly increasing.

Non-proliferative diabetic retinopathy (NPDR) is the earliest stage of DR and progresses from mild to moderate and then to severe. Some may progress to proliferative DR (PDR) which often leads to severe visual loss. Rates of progression of NPDR to sight-threatening DR can vary. The rates of progression in 5-years from moderate NPDR and severe NPDR to PDR was estimated to be 27.2% and 45.5% respectively (Denniston, Chakravarthy et al 2017).

Proliferative diabetic retinopathy (PDR) is a common cause of severe vision loss in people with diabetes. Fragile new vessels which develop in PDR as a result of ischaemia bleed into the vitreous, leading to tractional retinal detachment and vision loss.

As discussed in Chapter 2, baseline fundus photographs were examined for the presence or absence of the different features of DR and the presence of deep haemorrhage (DH), venous beading (VB) and intraretinal microvascular abnormalities (IRMA) were found to be the most important factors in predicting progression to PDR in the Early treatment diabetic retinopathy study (ETDRS Report 12,1991). VB was the most powerful predictor of subsequent development

of PDR. (ETDRS Report 12, 1991). This ETDRS study also led to the development of a grading and classification system, the Diabetic Retinopathy Severity Scale (DRSS) which divides DR into 13 levels from no retinopathy to advanced PDR (ETDRS Report 12, 1991). It also refers to the distribution and quantity of individual features of DR (ETDRS Reports 7, 10, 12).

The DRSS score in most cases progresses with time and does not generally improve by optimising blood glucose levels or blood pressure. However, recent clinical trials comparing the effect of anti-VEGF on diabetic macular oedema (DMO) to sham (RISE and RIDE, NCT00473382 and NCT00473330) (Ip, Domalpally et al. 2012) and anti-VEGF compared to laser (VIVID, VISTA) (Staurenghi, Feltgen et al. 2018) also looked at the effect of anti-VEGF on the participant's DRSS score.

As discussed in Chapter 2, the RIDE and RISE trials showed that participants receiving anti-VEGF injections for DME showed a significantly lower rate of progression of DR (worsening of ≥2 or ≥ 3 steps DRSS). Participants in the anti-VEGF arm also had a greater likelihood of improving their DRSS score by 2 or more steps than those in the sham arm. (See 2.9.9)

The VIVD, VISTA trials showed that the proportion of patients with ≥2 step DRSS score improvement were greater in the intravitreal aflibercept group versus laser. (See 2.9.9)

The VIVID, VISTA, RISE and RIDE trials reported the overall DRSS score changes but not the individual features of NPDR, namely DH, VB and IRMA.

More recently, the PANORAMA study (NCT02718326), an ongoing trial, is looking at the efficacy and safety of intravitreal aflibercept injections in patients with moderately severe NPDR (DRSS 47) or severe NPDR (DRSS 53) compared to sham. So far, the study reported the overall DRSS score, not individual feature grading. Results show, on the primary endpoint at one year, 80% and 65% of patients receiving aflibercept (anti-VEGF) on an every 8- and every 16-week

interval (after an initial monthly dosing period), respectively, experienced a two-step or greater improvement from baseline on the DRSS, compared to 15% of patients receiving sham injection ($p<0.0001$). In year two, at week 100, the same level was achieved by 62% and 50% of 16-week and 8-week eyes respectively, versus 13% of sham eyes (Lim 2020).

There is no reported data as to whether there is a differential response of these NPDR features to anti-VEGF therapy. These features are the hallmark of NPDR but are also present in eyes with PDR. I reanalysed the CLARITY images to see whether there is a differential response.

The CLARITY (Clinical efficacy of intravitreal aflibercept versus panretinal photocoagulation for best corrected visual acuity in patients with proliferative diabetic retinopathy at 52 weeks) has been reported in detail (Sivaprasad, Prevost et al. 2015) (Sivaprasad, Prevost et al. 2017) (Nicholson, Crosby-Nwaobi et al. 2018). In brief, it compared panretinal photocoagulation (PRP) (standard care) to repeated intravitreal aflibercept, the primary outcome being the change in best-corrected visual acuity at 52 weeks (ISRCTN 32207582). It also measured the effect of intravitreal aflibercept compared to PRP on other visual function and quality of life outcomes. In this study, both PDR patients who were treatment naïve and those patients with previous laser treatment were included in the study. Patients were randomized to PRP or intravitreal aflibercept. In the anti-VEGF arm, the patient had 3 intravitreal aflibercept at baseline, week 4 and week 8. If there was active neovascularization, further aflibercept was given, if not, the injections would be stopped. Patients were followed up on a monthly basis and as soon as reactivation of neovascularization was seen, a further aflibercept injection would be given. Nonetheless, the median number of injections beyond the mandatory injections was only one until week 52.

6.1.2 Methods

In the current study, we reanalysed the retinal photographs focusing on the 3 features of NPDR and their response to anti-VEGF, as opposed to looking at the overall DRSS which was an outcome measure of other previously mentioned trials. The majority of patients with PDR also have some or all of these three features. In this reanalysis, I only included patients randomized to the aflibercept arm of the CLARITY study who were treatment naïve at baseline as we were not sure the effect of previous laser on these NPDR features. I also included the fellow eyes of these patients for comparison if they were treatment naïve and without PDR.

Retinal photographs were either Optomap (Optos, Dunfermline, Scotland) images or 7 field fundus photographs. For Optomap images, only the central 7 fields of each eye were included using a "7 field mask" which covered the equivalent area of 7 fields (see Fig 4.1).

DH, VB, IRMA and venous loops are graded by quadrant at baseline, week 12 and week 52 as follows. The severity of the separate features, in all quadrants of the retina, were recorded at these visits and compared with standard ETRDS photos.

The three features, as well as venous loops were graded with reference to the ETDRS standard photographs (ETDRS paper 10).

DH are recorded as:

- 0 not present
- 1 Present but less than or equal to ETDRS standard photo 1 (the mildest standard for haemorrhages/microaneurysms)
- 2 Greater or equal to photograph 1 but less than standard photograph 2A (the intermediate standard for haemorrhages/microaneurysms)
- 3 Greater or equal to photograph 2A (the severe standard for haemorrhages/microaneurysm)

IRMA are recorded as:

- 0 not present
- 1 definite IRMA but less than standard photograph 8A
- 2 Greater or equal to standard photograph 8A

VB are recorded as:

- 0 not present
- 1 definite beading, less than standard photo 6A
- 2 beading equal to or greater than standard photo 6A

Venous Loops were also graded as:

- 0 not present
- 1 definite present

Statistical Analysis

The Chi Square test was used to test relationships between categorical variables.

6.1.3 Results

Of the 232 participants recruited to the CLARITY study, 112 were randomized to the aflibercept arm and 56 of these were treatment naïve. One was lost to follow up after baseline, one was ungradable after baseline and one had PRP at week 12. Four were lost to follow-up after week 12 and one had PRP at week 20 leaving 49 participants included in the study.

The prevalence of the three features examined is shown in the table below.

Table 6.1 Prevalence of Deep Haemorrhage, Venous beading and IRMA at the three study visits

Feature n=49	Baseline	Week 12	Week 52
Deep hemorrhage (DH) in at least one quadrant	26	43	39
Deep hemorrhage ≥2A in at least one quadrant	23	6	6
Venous beading (VB) <6A in at least one quadrant	18	18	19
Venous beading (VB)≥6A in at least one quadrant	1	1	1
Intravitreal microvascular abnormalities (IRMA) <8A in at least one quadrant	9	20	17
IRMA ≥8A in at least one quadrant	29	8	10

DH, VB and IRMA responses to aflibercept at week 12 and at week 52

Severe DH (>2A) and severe IRMA (>8A) improved in approximately 75% of eyes at week 12 and these eyes mostly remained improved at week 52, VB remained unchanged in all eyes at week 12 but 1 more eye developed VB at week 52.

IRMA Reappearance

Of the 13 participants in whom IRMA reappeared between week 12 and week 52, seven returned in the same area, i.e. in the same quadrant but not the exact

location, one in a different quadrant and five reappeared in the exact location as at screening i.e. the IRMA may have refilled.

DH and IRMA changes in relation to number of anti-VEGF Injections

There was a significantly greater improvement in DH and IRMA appearance if at least one injection was received after week 8.

Table 6.2: DH responses. Changes in DH appearance from week 12 to week 52, comparing no injection post 8 weeks to one or more injection post 8 weeks, p=0.007

	No further anti-VEGF injection post week 8	One or more anti-VEGF injections post 8 weeks
DH improved or stable at week 52	7	32
DH deteriorated at week 52	6	4

Table 6.3: IRMA responses. Changes in IRMA appearance from week 12 to week 52, comparing no injection post 8 weeks to one or more injection post 8 weeks, p= 0.007

	No further anti-VEGF injection post week 8	One or more anti-VEGF injections post 8 weeks
IRMA improved or stable at week 52	4	15
IRMA deteriorated at week 52	5	4

Duration of improvement post anti-VEGF

The following tables summarize how the length of time improvements in DH and IRMA are maintained after an anti-VEGF injection. It shows that the improvements of DH and IRMA are more likely to be maintained if there was less than 16 weeks since the last anti-VEGF injection.

Table 6.4: Responses of DH in relation to the timing of last injection when reviewing week 52 images compared to week 12, p=0.022

	16 weeks or less since last injection	More than 16 weeks since last injection
Improvement in DH at week 52	25	14
Deterioration of DH at week 52	1	9

Table 6.5: Responses of IRMA in relation to the timing of last injection when reviewing week 52 images compared to week 12, p=0.0019

	16 weeks or less since last injection	More than 16 weeks since last injection
Improvement in IRMA at week 52	14	5
Deterioration of IRMA at week 52	1	8

Venous Beading (VB) and Venous Loops (VL)

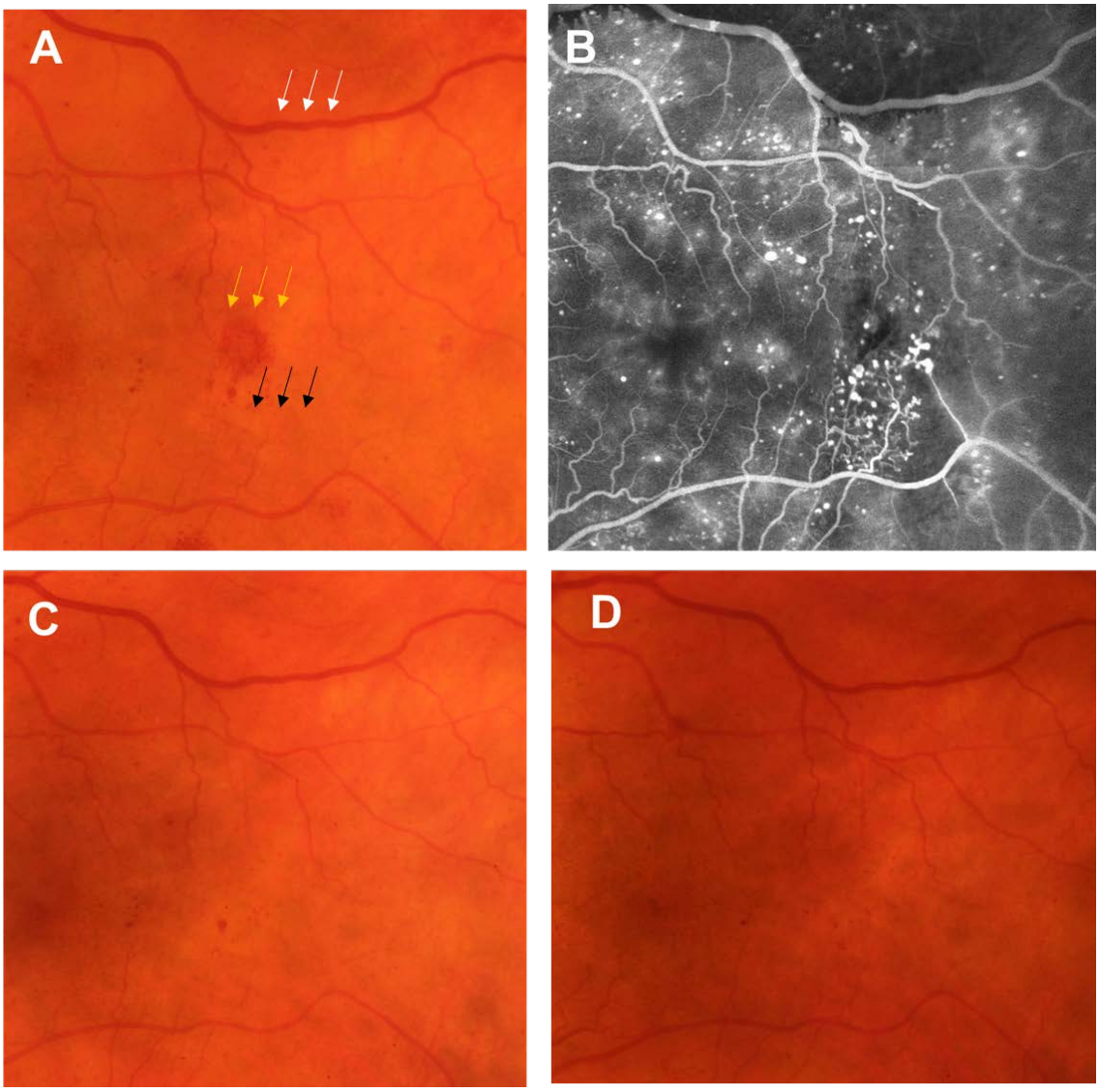
Nineteen participants had VB at baseline. Of these, none improved at week 12 and one developed VB by week 52. Fifteen participants had venous loops at baseline, all remained unchanged at week 12 and week 52 suggesting anti-VEGF has no impact on venous changes.

Fellow eyes

Eighteen fellow eyes of treatment naïve participants were studied. All these fellow eyes were also treatment naïve and without PDR. Nine of these fellow eyes showed an increase in the three features, DH, VB and IRMA by week 52. Seven appeared unchanged. Two showed an improvement in these features, however, one of them has documented anti-VEGF treatment for DMO. It was unclear whether the other participant showing improvement had received anti-VEGF or not. This result is consistent with the natural history of NPDR patients.

Figure 6.2 Responses to ant-VEGF.

A: Baseline B: Baseline FFA C: Week 12 D: Week 52. DH (yellow arrows) and IRMA (black arrows) disappeared with no changes on VB (white arrows) at week 12, IRMA starting to return at week 52



6.1.4 Discussion

All participants had the same number of injections in the first 12 weeks of the trial, at baseline, week 4 and week 8. The number of injections after week 8 varied from patient to patient depending on how much regression of the neovascularisation had occurred as mandated by the CLARITY study protocol.

As expected, DH, VB and IRMA are commonly found in PDR eyes. DH and IRMA appeared to respond similarly to anti-VEGF i.e. when one improved, the other did as well. The chance of DH and IRMA improving or not at week 52 was influenced by the number of injections after the first 3 mandatory injections, it seemed if at least one injection was received after that, these two features continue to improve or at least maintained the improvement. Furthermore, there was a much higher chance of reduction in DH and IRMA severity being maintained if the last injection was less than four months before week 52 review. This is in keeping with the expectation that anti-VEGF can only temporarily improve the retinopathy.

Venous Beading (VB) and venous loops appeared unchanged by anti-VEGF treatment. In one, an increase in VB was noted by week 52, which may be an indicator of disease progression despite anti-VEGF treatment. VB describes localized changes in retinal vessel calibre which sometimes resemble a string of beads (ETDRS Report 10, 1991). The aetiology of VB is unclear, as discussed in 2.7.5.5. Sympathetic vasomotor innervation is not present in retinal veins, therefore changes in vessel calibre may be caused by responses of the vessel wall to local stimuli, possibly increased ischaemia. Alternatively, VB could be a response to vasoactive agents within the circulation. Venous beading in DR is considered a predictor of progression to PDR (ETDRS Report 12, 1991) (Chen, Zhang et al. 2018) (Wong 2011). Anti-VEGF therapy, administered in the treatment regimen of the CLARITY study, appeared to have no effect on VB suggesting VEGF is not involved in the pathophysiology of venous changes, or that these changes might be anatomical and cannot be improved over a relatively short period of time of one

year. This is a similar observation to that of anti-VEGF having little effect on retinal ischaemia.

The patients included in the CLARITY study had PDR. Although venous changes may be present for a shorter time in moderate to severe NPDR compared to PDR patients, they are resistant to anti-VEGF treatment for up to 12 months post anti-VEGF therapy. We are unable to exclude the possibility that a more frequent anti-VEGF regimen may have improved the venous changes, a PRN regimen was followed based on the activity of new vessels which may be more sensitive to anti-VEGF than venous changes. Alternatively, VB may be more established in PDR and therefore less likely to regress, which may not be the case in NPDR. However, we reviewed the images of the 'Randomised trial of wide-field guided PRP for diabetic macular oedema (DMO) treated with Ranibizumab' (RDP) Study (Talks, Bhatia et al. 2019) in which DMO patients with NPDR were randomized to ranibizumab alone or ranibizumab with wide-field guided PRP. Twenty-five participants were enrolled into the ranibizumab alone arm, only 15 patients had baseline and week 52 images for analysis. The average number of injections in the ranibizumab only arm was 6.84. Out of these 15 patients, the DH (n=15) and IRMA (n=11) improved at week 52, but venous beading (n=2) did not (unpublished data). This is supportive of our general conclusion.

A recent study looked at a similar population and examined the effect on anti-VEGF therapy (aflibercept, ranibizumab or bevacizumab) on IRMA using OCTA. This study showed less of an effect on IRMA than our findings. They found 31% of IRMA regressed, 44% were unchanged, 13% progressed and 11% disappeared. There are a number of possible explanations for this. The number of participants was smaller, 15, the follow up period was shorter (mean 35 days). In addition, OCTA is able to detect smaller IRMA and smaller changes more successfully than colour fundus photographs (Schaal, Munk et al. 2019).

Limitations to our study include the small sample size. However, the changes between DH, IRMA and VB were very different so despite a small sample size it is highly significant. Some images were seven-fields CFP and some were Optomap UWF and it is possible that subtle IRMA were missed with Optomap, which has previously been reported in another study (Nghiem, Nderitu et al. 2019). However, all patients had the same imaging modality throughout the trial, so comparisons of the changes remained consistent. It is not a masked study as the grader had to directly compare the week 12 to the week 52 images to see whether there is an improvement or deterioration, as reference images did not show clear demarcation. Hence, there is some degree of bias, although a high level of caution was taken with only significant improvements or deterioration recorded. As previously mentioned, this study examined patients with PDR, it is possible that NPDR patients behave differently, we therefore encourage PANAROMA investigators to look at this aspect in their analysis.

If this data is confirmed in patients with NPDR, anti-VEGF may have very little effect in patients with mainly venous changes. Nonetheless, it is unclear whether treating patients with mainly venous changes with NPDR can prevent the development of PDR. Our data suggests that anti-VEGF, at least in this cohort with relatively low treatment, did not halt the development of venous beading.

6. 2 RDP Study (Randomised trial of wide-field guided PRP for diabetic macular oedema (DMO) treated with Ranibizumab

6.2.1 Introduction

In the RDP study, patients with OCT confirmed DMO and Wide field Fundus Fluorescein Angiography (WFFA) confirmed moderate/severe NPDR were randomised to PRP + ranibizumab or ranibizumab monotherapy. The primary outcome was the number of repeat ranibizumab injections required after the first six months until one-year post treatment. The purpose of the study was to evaluate whether treating peripheral ischaemia with PRP in patients with macular oedema would reduce VEGF levels and ultimately reduce the need for anti-VEGF injections for diabetic macular oedema.

In this sub-analysis we assessed the effect of IVT anti-VEGF injections on the features of moderate/severe NPDR in a treatment naïve population.

6.2.2 Methods

Images of eyes randomised to the ranibizumab arm of the trial were examined at baseline and at week 52, the final visit. The presence of the three features of NPDR (DH, VB and IRMA) were recorded at baseline and the final visit at week 52 as in the Clarity study (see 7.1.2 Methods).

Inclusion criteria:

- Treatment naïve
- Randomised to ranibizumab monotherapy

6.2.3 Results

Fifteen eyes were included in this study. Ten eyes were excluded, 8 had missing data and 2 had poor quality images.

Table 6.6 Presence of DR features at baseline and responses to anti-VEGF

Feature	Baseline	Week 52 Improved	Week 52 unchanged	Week 52 deteriorated
DH	15	14	1	0
VB	2	0	2	0
IRMA	11	11	0	0

Fifteen participants had DH at Baseline and 14 of these improved at week 52, one remained unchanged. Eleven of the participants had IRMA, all of which were improved at week 52. Two participants had VB; both were unchanged at week 52.

Two participants had both VB and IRMA. Both showed some co-location of VB and IRMA but also quadrants of IRMA alone.

6.2.4 Discussion

SEE CLARITY DISCUSSION (6.1.4)

This cohort differs from those in the Clarity study as the participants have moderate/severe NPDR (DRSS levels 43-53). The responses of the three features to anti-VEGF appear similar in eyes with different levels of disease. Despite only two participants having VB, this did not alter after treatment with anti-VEGF. DH and IRMA improved in most participants at week 52.

Chapter 7: Optical Coherence Tomography Angiogram (OCTA) in Diabetic Retinopathy

7.1 Background

As previously discussed in Chapter 2, OCTA is a relatively new technique, which provides high-resolution images of retinal and choroidal blood flow and structure (de Carlo, Bonini Filho et al. 2016) (See 2.10). OCTA is able to view both superficial and deep retinal layers and has advantages over FFA, being non-invasive and quick to perform as well as providing higher resolution images of the retinal vasculature.

As previously discussed, both VB and IRMA are observed near areas of ischaemia (Chen, Zhang et al. 2018) (Stitt, Curtis et al. 2016). OCTA allows acquisition of images of the retinal vasculature at high-resolution (Samara, Shahlaee et al. 2017). With OCTA imaging, IRMA appear as abnormal branching, dilated vessels, see Fig 2.7. The advantage of imaging with OCTA is that their exact location in the retina can be observed and easily distinguished from neovascularisation as the vessels have not penetrated the ILM. In addition, ischaemia is clearly imaged with OCTA, appearing as dark areas.

Enlargement of the foveal avascular zone (FAZ) shows correlation with progression of DR (Mansour, Schachat et al. 1993). OCTA has facilitated improved imaging of the (FAZ). As it can visualise the retinal microvasculature in multiple layers of the retina, it can assess retinal capillary density in both the superficial and deep retinal layers unlike FFA which assesses the superficial vasculature only, and images are not compromised by fluorescein leakage (Al-Sheikh, Akil et al. 2016). OCTA shows good correlation with FFA and it also provides a numerical measure of the vascularity around the fovea providing an opportunity to study macular ischaemia in detail. It is possible to extract angiographic data, including FAZ area, vascular density, areas of no flow from OCTA scans (Agemy, Scripsema et al.

2015) (Shahlaee, Samara et al. 2016). As OCTA is non-invasive, its role in monitoring retinal vascular changes is important.

7.2 Methods

The DMI Study (A prospective study of structure-function correlation in patients with diabetes with varying sizes of foveal avascular zone) aims to identify and estimate the frequency of patients with diabetic macular ischaemia (DMI) losing ≥ 6 letters at 52 weeks from baseline, describe their characteristics and compare them to DMI patients losing < 6 letters. (Protocol number: SIVS1048)

Dabetic Macular Ischaemia (DMI) causes visual loss in those with DR. No treatment is currently available and there is very limited data on the progression of DMI over time. This study aims to provide information on the natural history of DMI and help inform the best clinically meaningful change in clinical end-points for future potential treatments.

Subjects enrolled to the study undergo baseline examination including visual acuity in ETDRS letters and low luminance visual acuity, ultrawide field scanning laser ophthalmoscopy, (Optomap 200Tx, Optos plc, Dunfermline, UK), MAIA microperimetry (MAIA, Centervue, Padova, Italy), spectral domain optical coherence tomography (SD OCT) (Heidelberg Engineering) and OCT angiography, RTVue XR Avanti SD-OCT device (Optovue, Fremont, CA) using the prototype AngioVue OCTA software. The aim is to identify structural markers that are associated with functional loss at 52 weeks in all those meeting the inclusion criteria: Stable PRP treated PDR patients with no active neovascularisation for 6 months, or NPDR patients with no treatment in the preceding 6 month and with a large foveal avascular zone (FAZ), BCVA of 54 letters or better.

Enlarged FAZ was defined as those with ≥0.5mm² area in superficial capillary plexus (SCP) present on Optical coherence tomography angiography. If the FAZ was <0.5mm² then an enlarged peri-foveal inter-capillary space in at least 1

quadrant was sufficient for inclusion. The subjects were followed at three-monthly intervals for 12 months. Current analysis

In this analysis, baseline OCTA and Optomap images of 30 participants were reanalysed. The 6x6 mm scans of the superficial layer were examined for the 30 subjects enrolled in the trial. This scan captures the optic disc and macula. All images had a Signal Strength Index (SSI) of more than 5/10. For each OCTA image, the number of IRMA and VB were counted. This was cross-referenced with the Optomap image to verify the presence of the features.

Areas of ischaemia were located on the OCTA images and the proximity to IRMA and VB were assessed. Image J software (NIH, Image J; National Institute of Health, Bethesda, MD, USA) was used to view the Optovue images and to measure the distance of features from ischaemia and the sizes of adjacent ischaemic areas. To measure actual sizes on the images, the software's 'Analyse' option is selected which gives the option of setting the scale. The average size of an Optic disc is 1.5mm vertically (Crowston, Hopley et al. 2004). This was entered into the 'known distance' box and the 'Pixel aspect ratio' was set at 1.0. A line was then drawn with the measurement tool from the top to the bottom edges of an optic disc. Once the 'Global' box is ticked, the scale is set for all further measurements. Straight line distances can be measured as well as areas in mm and mm^2. As optic disc sizes vary, the measurements are not absolute.

Statistical Analysis

The Chi-square test was used for testing relationships between categorical variables and student T-test was used for all continuous variables (Microsoft Excel).

7.3 Results

Of the 30 images analysed, one set of images (OCTA and Optomap) were too poor to be assessed accurately. All other images analysed were of good quality with no focal areas of reduced image quality. Seven images had neither IRMA nor VB.

Twenty-one eyes had at least one patch of IRMA, 43 patches of IRMA were detected in total. Seven eyes had VB, one eye had two beaded veins. Thirty-four patches of IRMA were directly adjacent to areas of ischaemia and 2 beaded veins were directly adjacent to an area of ischaemia. Six patches of IRMA were not directly adjacent to areas of ischaemia, but in close proximity. Four areas of VB were not directly adjacent to areas of ischaemia but in close proximity.

Table 7.1 Number of IRMA and VB adjacent to areas of ischaemia

	IRMA	VB
Adjacent to Ischaemia	34	2
Not adjacent to ischaemia	9	6

Chi-Square test: $p<0.05$ $p=0.002$

Table 7.2 Average size of ischaemic areas directly adjacent to IRMA and VB

	IRMA (n=34)	VB (n=2)
Average area of adjacent Ischaemia, (mm^2)	0.715	0.23

Student's t-Test, p=0.17

Table 7.3 Average distance of IRMA and VB to the closest area of ischaemia

	IRMA (n=6)	VB (n=4)
Average distance from Ischaemia if not adjacent (mm)	1.806 mm	1.704

Student's t-Test, p= 0.905

Fig 7.1 Optomap image with the area of OCTA captured highlighted by the black box

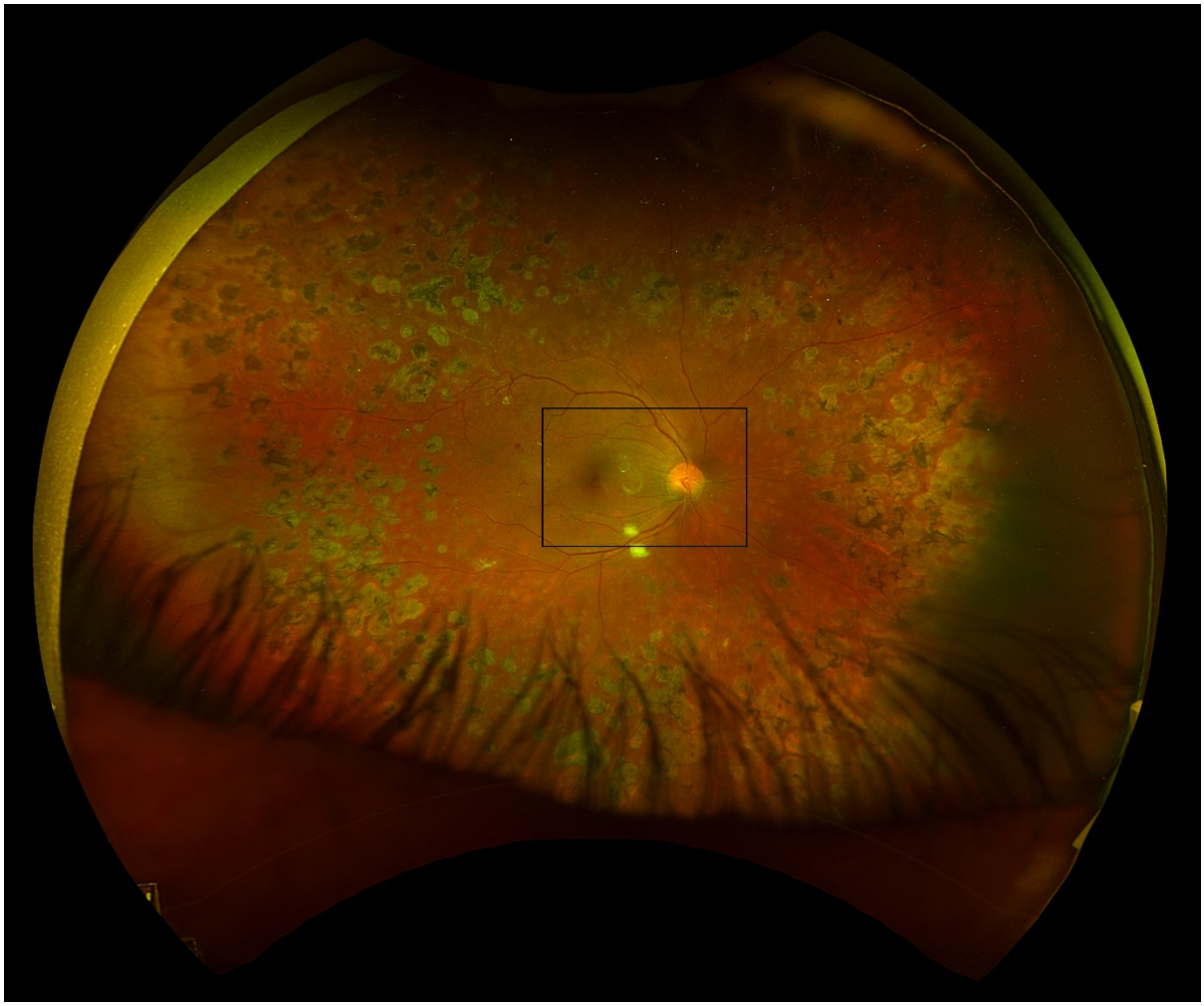


Figure 7.2 IRMA (outlined in blue) next to ischaemia (the ischaemia extends beyond the border of the OCTA Image)

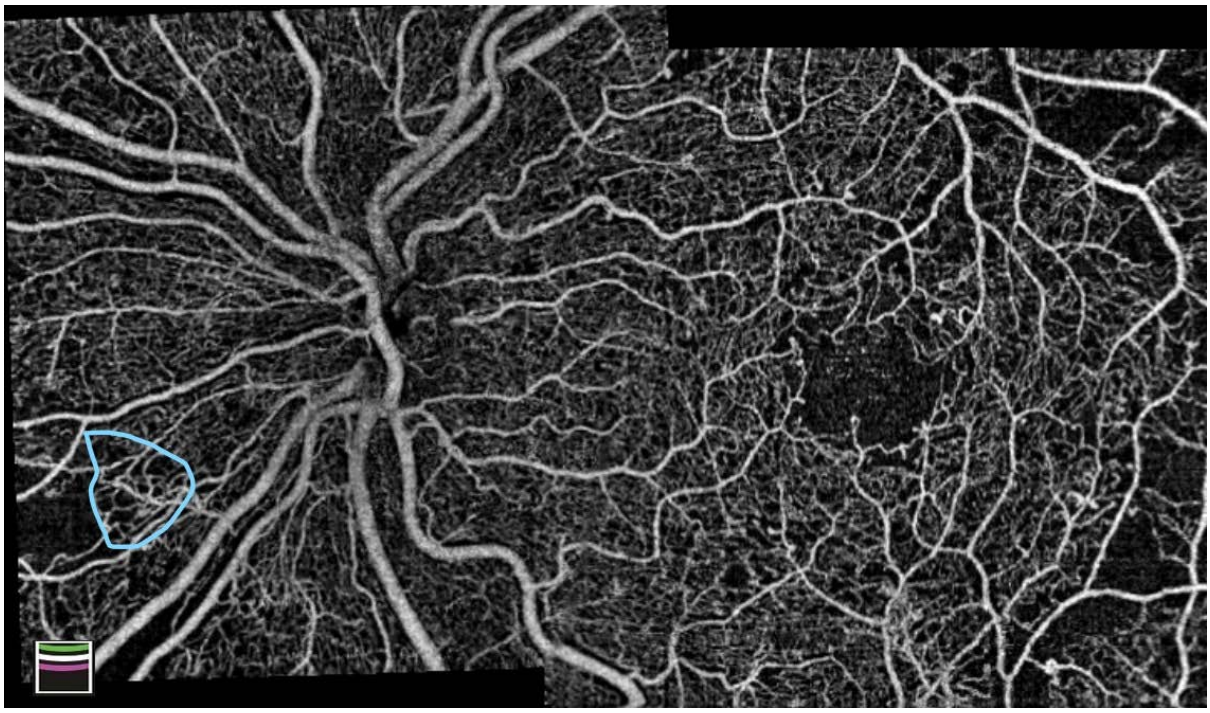


Fig 7.3 Patches of IRMA adjacent to ischaemia

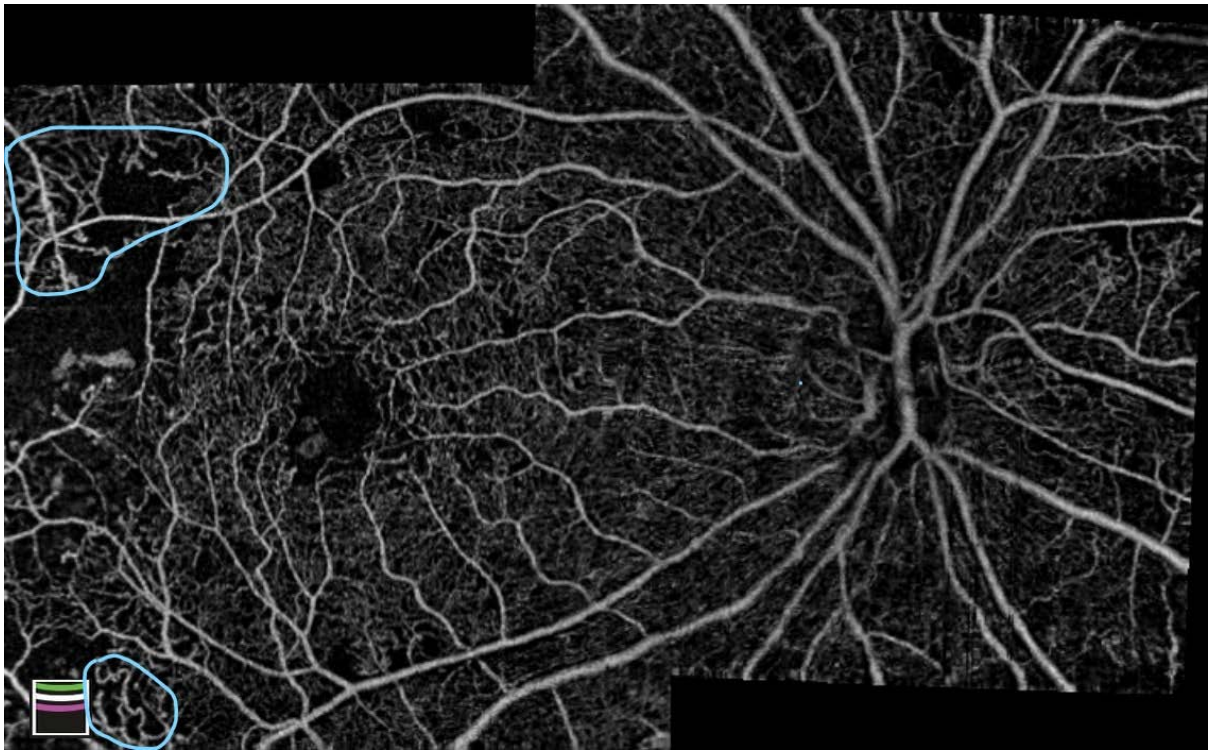


Fig 7.4 VB (highlighted in red) not near Ischaemia and a patch of ischaemia temporally which is likely to extend further temporally (highlighted in green). Adjacent to this ischaemic area is a patch of IRMA not captured by the OCTA Image but seen on the Optomap Image.

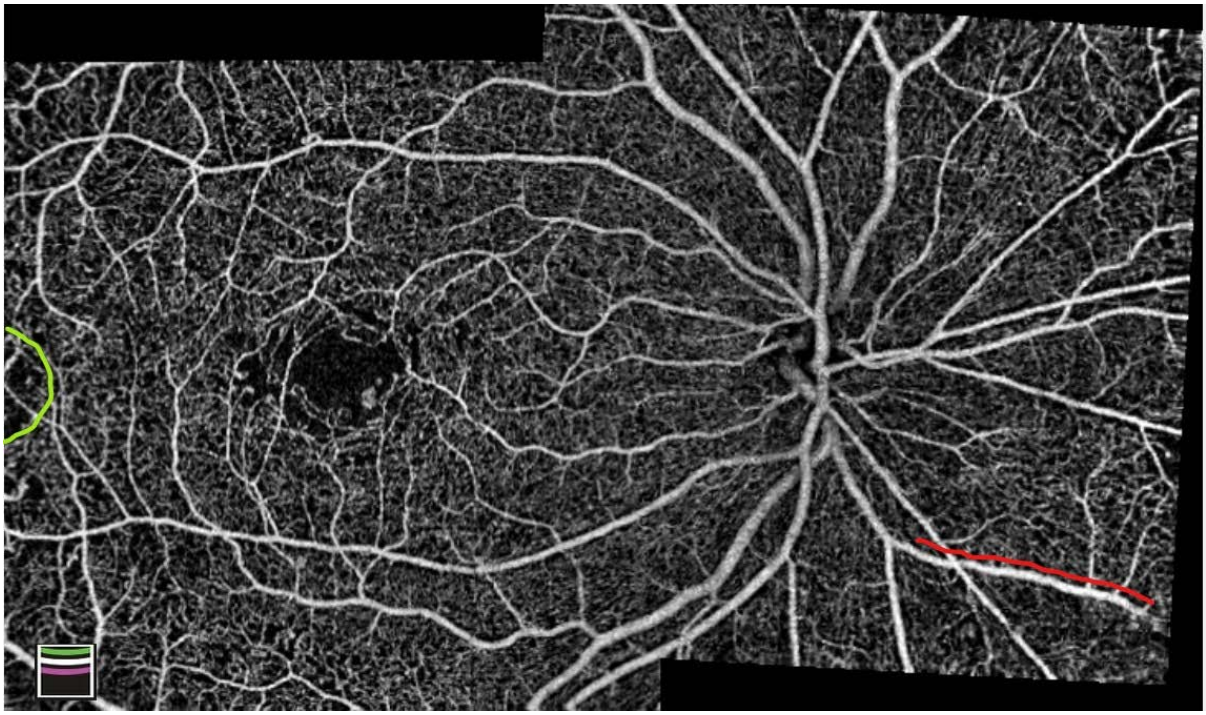
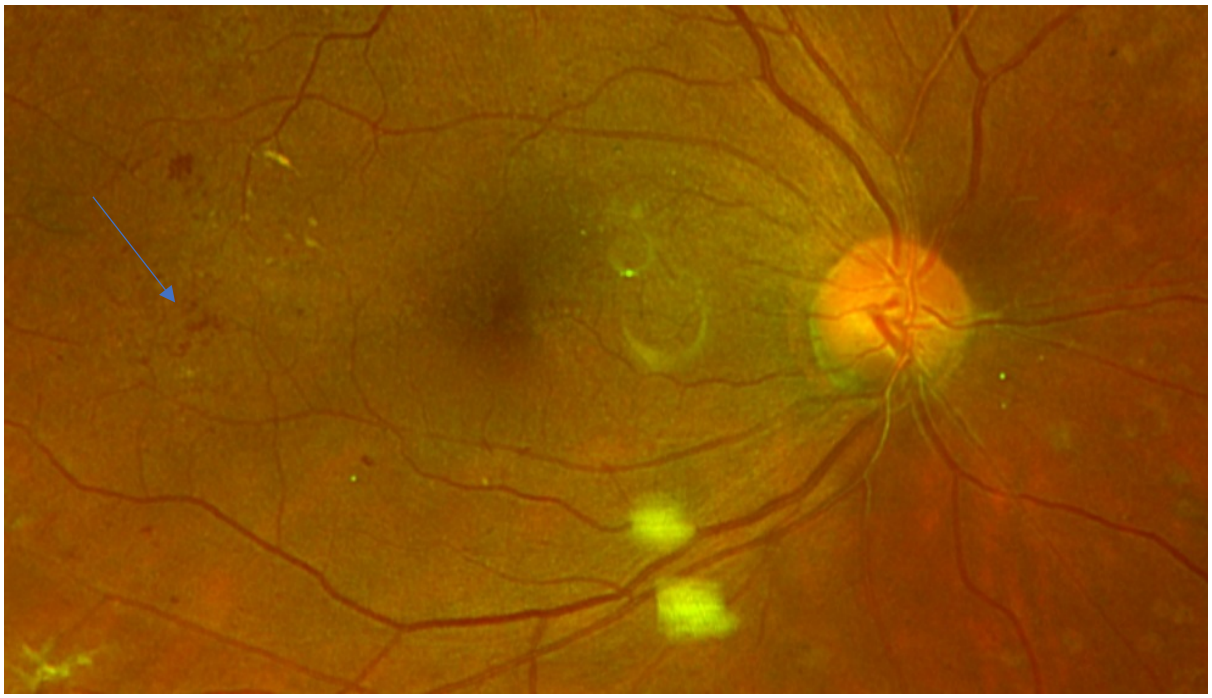


Fig 7.5 Corresponding Optomap image showing an area of IRMA more temporal to the ischaemic area which are not captured by the OCTA image (Blue arrow)



7.4 Discussion

Previous studies utilising FFA have shown VB to be located near areas of non-perfusion (Chen,Zhang et al. 2018) and IRMA are known to develop next to, and supply areas of non-perfusion (Wong.Cheung et al. 2016). These findings have been confirmed by OCTA studies (Sorour, Mehta et al. 2020), (Schaal,Munk et al. 2019) identifying IRMA near areas of capillary dropout. In addition, because of its ability to accurately locate lesions in the retinal layers, OCTA has proved useful in distinguishing IRMA from NVE as IRMA lie within the retinal layers whereas NV protrude though the ILM (Arya, Rashad et al. 2018).

OCTA has been shown to visualise IRMA in more detail and even identify different types of IRMA based on their variable morphological appearance (Shimouchi et al. 2020), (Sorour, Mehta et al. 2020). This detailed imaging of IRMA may help further

classify different subtypes and help our understanding of the natural history of IRMA. Indeed, a more recent study used OCTA to examine IRMA pre and post PRP and found differing responses, some remained unchanged whilst some reperfused. Despite the significance of the different responses remaining unclear, this imaging modality is likely to add to the understanding of IRMA structure and may help identify different subtypes (Shimouchi et al. 2020).

In this study, IRMA were easier to detect with OCTA than with Optomap. Optomap was used to confirm their presence but small IRMA were much easier to detect with OCTA and may have been missed if relying on Optomap alone.

This study found that IRMA were more prevalent in the 6x6 mm area captured by the OCTA image. IRMA were also more likely to be directly adjacent to an area of ischaemia ($p=0.002$). The areas of ischaemia directly adjacent to IRMA were numerically larger than those next to VB, (average 0.715 mm^2 in IRMA compared to 0.23 mm^2 in VB). However, this was not statistically significant ($p=0.17$), which may be partly due to the large imbalance in numbers (IRMA n=34, VB n=2).

Where IRMA and VB were not directly adjacent to areas of ischaemia but were close by, the average distance from the nearest ischaemic area for VB was 1.806 mm and 1.704 mm for IRMA, which was not statistically significant ($p=0.9$). IRMA and VB not directly adjacent to areas of ischaemia were located at similar distances from areas of ischaemia. As these areas are unlikely be important to the development of either feature, these distances are probably less relevant. In addition, the closest area of ischaemia may not have been captured by the OCTA image.

The findings suggest that IRMA may be more closely associated with ischaemia but this may be because more IRMA were detected in the macular area.

In all subjects, more IRMA and VB were observed in the Optomap images outside the area covered by OCTA. Limits to the study were the small number included, although this is a pilot study. The OCTA images covered a small area of the retina.

Because of this, not all areas of ischemia were captured by the OCTA and they may have been in closer proximity to IRMA and VB than is possible to record.

A future study would provide more information about the proximity of IRMA and VB to areas of ischaemia if a larger area were captured by OCTA. A 15x9 and a 12x12 mm scan are now available in some of the newer generation of OCTA devices.

Chapter 8: Discussion

8.1 Key findings of this Thesis

- Genetics may play a role in the development of IRMA, particularly by ischaemic driven genes.
- VB and IRMA do not always coexist.
- DH, VB and IRMA may not develop sequentially.
- DH and IRMA improve with anti-VEGF therapy.
- IRMA appear more likely to be driven by focal localised ischaemia.
- Some IRMA do not response to anti-VEGF and are not near areas of localised ischaemia suggesting there may be at least 2 types of IRMA.

The DRSS was developed based on the findings of the ETDRS. Using mathematical modelling, DH, VB and IRMA were found to be independent risk factors for progression from NPDR to PDR and eventually visual loss (ETDRS paper 12, 1991). There is a degree of inconsistency in the grading; on one hand it was claimed that VB is the most significant risk factor for progression to PDR. Yet, to be graded as severe NPDR (DRSS 53) an eye requires two quadrants of VB. However, if the eye has only one quadrant of severe IRMA, it is also severe NPDR (DRSS 53), that is, considered an equivalent risk factor for progression. This inconsistency is reflected further down the scale, at DRSS 47. At this level, VB in one quadrant has a score of 47, yet four quadrants of mild IRMA (less than 8A) are required to be DRSS 47. Despite being identified as independent risk factors, they often coexist, for instance VB alone without DH and IRMA are rare, as was confirmed by our study. In this project, we aimed to better understand these three features in patients with DRSS 43 to 53 (moderate/severe NPDR). As DH were common we turned our focus to VB and IRMA. It is claimed that both VB and IRMA are secondary to ischaemia, (Stitt, O'Neill et al. 2011),(Chen, Zhang et al. 2018) and related to vascular closure (Stitt, Curtis et al. 2016) and pericyte loss (Imesch,

Bindley et al. 1997). In radiation retinopathy, VB was observed in areas of ischaemia but with less pericyte loss (Archer, Amoaku et al. 1991). Nonetheless, the evidence came from a very small case series and is theoretical at best.

At the beginning of the project, I aimed to study the genetics of NPDR and eventually narrowed this down to VB and IRMA. This is a follow on from the group in Oxford's study of the genetics of DMO. There were limitations to the study which will be discussed in more detail later, however, with similar numbers of patients with severe IRMA and VB, we found distinctly different genetic profiles. Genes that are well known to be related to ischaemia showed positive associations with IRMA but not with VB. The association with IRMA may not be highly significant due to the number of patients, however, the differences between IRMA and VB were clear. The only weak genetic association with VB is with SLMAP. The result may be a coincidence, but SLMAP may play a role in vascular endothelial cell dysfunction, and its association with the dysfunction of the contraction regulation of the small mesenteric arteries in db/db mouse (a genetic diabetic mouse model) is interesting. It is possible that SLAMP may be involved with the contraction regulation of retinal venules leading to venous beading.

Using a large cohort of retinal images with moderate/severe NPDR, as previously mentioned, DH were found in most subjects. I examined whether VB and IRMA coexist and when they do, whether the most severe of these features in an individual eye were in the same quadrant. As reported in Chapter 5, in only 99 eyes, out of just over 500, VB and IRMA coexist in the same eye. When they were observed in the same eye, the most severe of each phenotype were found to be slightly more likely to be present in the same quadrant (31%), rather than the expected 25% if it were completely random. This finding was not statistically significant. Once again, this supports that IRMA and VB may have different local pathophysiologies, for example local ischaemia.

I then identified the location of DH, VB, and IRMA in the eye. VB were more commonly located in the nasal retina whereas DH and IRMA in the temporal. Other retinal pathologies have been observed predominantly in one area of the retina, such as sectoral Retinitis Pigmentosa (Coussa, Basali et al. 2019) which almost always affects the inferior retina. The hypothesis being that the inferior retina has greater exposure to light which may have a detrimental effect. In subjects with NPDR, DH and IRMA are reported more frequently in the temporal retina, the watershed zone (May, Rutkowski 2019), which was confirmed by this study, both being more frequently located in the superior and temporal retina. This may be because this area has fewer blood vessels and therefore oxygen supply, adding further weight to the role of ischemia in IRMA's development. VB were more commonly found in the nasal retina in our study. The only other study of VB location found no differences between nasal and temporal or superior and inferior distribution but it was also a small study. No other retinal pathologies report greater severity in the nasal retina. The significance of this finding remains unclear.

When all grades of lesions were examined, it appeared that DH and IRMA were widespread in those with moderate NPDR and few have all three features, suggesting their appearance could be sequential, with VB appearing later. However, when examining more severe NPDR, DRSS 53, the features are more evenly distributed contradicting the original observation. Furthermore, patients with VB were younger than those who had severe IRMA. Therefore, it does not follow that VB develop later than IRMA. This supports our conclusion that some patients are more predisposed to developing VB or IRMA. It is however possible that if a particular patient with DH and IRMA goes on to develop VB, they may then develop PDR very quickly and hence would have been excluded from our study, as the window to capture them may be small. Indeed, this was suggested by the

Chinese group (Chen, Zhang et al. 2018), that VB were much more common in PDR patients.

Analysis of responses of the three features to anti-VEGF showed that DH and IRMA respond similarly but there appeared to be no impact on VB. This was demonstrated in both a PDR cohort and a less severe cohort of moderate-severe NPDR. This could have implications for clinical trial outcomes, which are assessed in terms of DRSS in trials examining improvements in DR severity. A patient can have the same DRSS score with differing presentations of DR. For example, DRSS 53 can present as VB in two or more quadrants, or severe IRMA in one quadrant. As our analysis of the Clarity study showed, if the treatment was anti-VEGF, the first patient described, with VB and no IRMA may show no change in their DRSS score with treatment whereas the second may improve to DRSS level 47 or 43 (one or two steps) as their IRMA resolve.

My study showed anti-VEGF treatment improves DH and IRMA, however, the underlying ischaemia and retinal perfusion does not improve (Couturier, Rey et al. 2019). Anti-VEGF prevents angiogenesis and therefore IRMA formation but not ischaemia and is therefore only a temporary measure, or a cosmetic improvement. This is evidenced by the need for repeated anti-VEGF therapy and the return of DH and IRMA if treatment was not administered (based on the presence or absence of new vessels as per the CLARITY protocol). Anti-VEGF does not therefore prevent progression to neovascularisation or DMO as the underlying stimulus of ischemia is still present. To treat DR effectively, therapy is needed to increase reperfusion to achieve a more permanent halt in progression of the disease. This study's findings may also explain why a number of IRMA do not respond to anti-VEGF, these being shunts, rather than originating by angiogenesis.

The different genetic findings for those developing VB and IRMA further strengthens the theory that these two features of DR have different aetiologies. If these findings are confirmed in larger studies, this could pave the way for

personalised treatment options for those more at risk of developing different features of NPDR.

Investigations of OCTA images of the macula suggest that IRMA may occur more frequently near areas of ischaemia, more so than VB. Nonetheless, not all IRMA were found adjacent to areas of ischaemia. This may strengthen the idea that there may be at least two types of IRMA. IRMA can be considered as part of the repair mechanism, arising either from shunts from existing vessels or by angiogenesis to provide additional oxygen supply to ischaemic areas. These localised ischaemic areas are likely to be due to localised vessel occlusion and may be the stimulus for shunts and therefore IRMA formation. The number with VB observed in this study were small as was the area of the retina examined, but the findings tie in with our theory that the features don't share the same pathophysiology, as do our findings that different genes are implicated in the individual's predisposition to their development.

To date little is understood about the aetiology of VB and IRMA. Both are frequently found adjacent to areas of ischaemia, suggesting ischaemia's role in their aetiology. This study confirms they may be considered independent risk factors for the development of PDR and behave differently in terms of location, genetic origin and response to anti-VEGF.

8.2 New findings that have added to literature

Analysis of the Clarity data found that DH and IRMA improved with intravitreal anti-VEGF but that VB are unchanged (Pearce, Chong et al. 2020). This suggests that VEGF may not be involved in the pathophysiology of venous beading, or that these changes are anatomical and cannot be improved over a relatively short period of time of one year. There is a possibility that more frequent anti-VEGF dosing or longer treatment may improve venous beading. These findings were observed in both NPDR and PDR groups. For those patients with mainly venous changes, anti-VEGF might have very little effect. Nonetheless, it is unclear whether treating

patients with anti-VEGF with mainly venous changes with NPDR can prevent the development of PDR. We encourage the Panorama and the Diabetic Retinopathy Clinical research Network (DRCR.net) Protocol W to examine this.

The duration of improvement of the DH and IRMA was related to the number and timing of the injections. At the 52 week review, if at least one injection was received after the mandatory course of three, as well as if the last injection was received less than four months before the final week 52 review, these improvements were more likely to be maintained, or further improvements achieved. This is supported by similar findings in the Panorama study results of the second year when, in one arm, patients were given aflibercept as needed leading to a deterioration of DRSS in some patients.

This study highlighted the need to assess the individual features of NPDR when considering treatment options.

We also published a review of risk factors for progression to PDR which highlights the need for evaluating NPDR lesions in more detail and suggests incorporating them into a new DR classification system. According to the 4:2:1 rule, a patient with severe IRMA in one quadrant has the same risk of progression to PDR as one with severe IRMA in four quadrants as they have the same DRSS score. It is unclear why the presence of IRMA is classified so broadly and more detailed assessments of the number of these lesions may better predict the risk of progression to sight threatening vision loss. If IRMA have different aetiologies (some originating via angiogenesis and some from vascular remodelling), further stratification of these separate types of IRMA may help assess differential impacts on progression to PDR.

We also suggest using individuals' relevant concomitant medication, evaluation of whether lesions are predominantly peripheral or centrally located, as well as the turnover of lesions to assess an individual with NPDR's risk of progression to PDR (Sivaprasad and Pearce 2018).

8.3 Strengths of our studies

A large number of images were analysed for the examination of prevalence and distribution. The three features of NPDR were investigated in detail by different imaging modalities as well as genetic analysis and response to anti-VEGF treatment. The studies confirmed the ETDRS findings that VB and IRMA are independent risk factors for progression to PDR by approaching the question from four directions; whether VB and IRMA co-locate, whether they share similar genetic profiles, their proximity to ischaemia and their responses to anti-VEGF. No previous validation of the ETDRS findings has been published.

8.4 Limitations of our studies

Limitations of our study are the small numbers, particularly of those with VB, but trends were identified. The purpose of the study was to detect similarities or differences in IRMA and VB. A future study powered to detect this would need a much bigger sample size.

The far periphery of the fundus is more distorted and therefore harder to grade and identify lesions than the posterior pole and patient's eyelids often preclude adequate imaging. Detection of IRMA in the periphery is very difficult due to poor image resolution and the retinal vessels appear distorted, making VB very hard to identify outside the seven-field area. This is because the Optomap uses an ellipsoid mirror to produce images. In addition, the quality of the images captured varied significantly, being dependent on the expertise of the technician and the ability of the participant to co-operate. Similar difficulties have previously been reported by Nderitu et al, who found identifying IRMA and VB in Optomap images more difficult than identifying them on digital fundus photographs (Nghiem, Nderitu et al. 2019). For these reasons the peripheral retinal areas were graded during the study but not included in the analysis. A future project evaluating the peripheral retina in relation to the central 7 Fields, in particular the presence of DH in those with NPDR may add to our understanding of this population.

A further limitation to the study is that most subjects with no DR had two field CFP and not Optomap imaging. This may have identified DR lesions. However the diabetic screening service takes additional images of the peripheral retina with the Topcon camera in all those with more than 8 years duration of diabetes, which would have identified peripheral lesions. Optomap was not the imaging modality of choice for the controls as this is not routine care for those under the diabetic screening service.

As mild IRMA are harder to detect with Optomap compared to CFP, only severe IRMA were included in the genetic analysis. The results could therefore be different if all grades of IRMA were included.

Multiple testing correction for the genetics study was not undertaken. We used a p value of 0.05 as statistically significant. A Bonferroni adjustment for different comparisons will reduce the p value below 0.05. A decision was made not to use this correction as this is a pilot project, which aimed to identify polymorphisms of interest to potentially investigate in more detail. In addition, the main reason for the study was to identify differences between VB and IRMA, and to identify trends. A more detailed analysis of the genetic data can be undertaken but this is beyond the scope of this study. Previous studies have shown significance as they have only studied one or two SNPS, this study was not looking for new SNPs but to examine whether SNPs previously identified as significant were driven by VB and/or IRMA.

8.5 The need for a new classification of DR

Previous studies of the features of DR have classified VB and IRMA as individual risk factors for progression to PDR. These studies have confirmed this. The findings imply that IRMA may be more driven by ischemia. Previous classifications suggest both are independent risk factors for DR progression but the reasons for this were not elucidated or explained.

Currently, the Diabetic Retinopathy Severity Scale (DRSS), (see table 2.2), groups those with different amounts of DH, VB and IRMA at the same DRSS score.

For example, a patient with DRSS level 53, may present with severe retinal haemorrhages in all four quadrants, venous beading in two quadrants or IRMA in one quadrant. As these features respond differently to anti-VEGF, a patient with DH or IRMA only, meeting the severity scale 53, may show an improvement in DRSS if treated with anti-VEGF, whereas a patient with just VB at level 53 would be unlikely to show any improvement. Currently, all those with DR meeting DRSS level e.g. 53 are considered as one group regardless of which features they have. Therefore a more detailed classification of the features of DR is required.

As our study shows, DH and IRMA respond to anti-VEGF, an approved treatment for NPDR, this has highlighted the absence of effective therapy for VB.

Furthermore, it has been reported recently that patients with DRSS 47 induced by anti-VEGF (i.e. patients with higher DRSS treated by anti-VEGF and regress to DRSS 47) have a much higher risk of progression than treatment naïve DRSS 47 patient (Lim JI 2020).

OCTA may also add to the classification of DR by adding the areas of ischaemia and may also be able to distinguish different types of IRMA. However, the smaller 6x6 mm OCTA scan used in this study only captures a small area of the retina and therefore identifies only a small proportion of ischaemic retina. The development of wide-field OCTA may allow assessment of the majority of the retina. The FAZ size measured by OCTA has also been used as a risk prediction to progression of PDR and DMO in a pilot study, although further clarification is needed (Sun, Tang et al. 2019). It remains unclear whether this is an independent risk factor or whether the FAZ size correlates with the level of DRSS.

8.6 Conclusion

VB and IRMA do not always co-exist, neither do they co-localise. The genetic profiles appear to be different, with IRMA more likely to be ischaemia driven. IRMA may be more likely to present adjacent to areas of localised ischaemia than VB. DH and IRMA respond to anti-VEGF unlike VB. Some IRMA have different genetic and response profiles suggesting there are at least two classes of IRMA.

Chapter 9: A list of potential new studies that should be done to expand this thesis

Natural history studies of VB and IRMA may further inform the sequence of progression to sight-threatening DR and advance our understanding of the disease progression. Further OCTA studies of VB and IRMA, examining larger areas of the retina, with wide-field OCTA may better inform the extent of VB and IRMA's coexistence with ischaemia, help sub-divide the different classes of IRMA, as well as inform their risk of progression. A future study with a larger, sample size, appropriately calculated based on the data collected in these studies could closely monitor the progression of IRMA. Including UWF OCTA, would allow identification of IRMA adjacent to iscaemia. Such a longitudinal study could monitor responses of IRMA to anti-VEGF and identify if those that do not respond to anti-VEGF eventually become NVE, thereby helping to categorise different types of IRMA. If the SNPs identified as showing a trend for IRMA are confirmed, genetic testing could be incorporated into this study. This would add to our understanding of the different phenotypes of IRMA based on proximity to ischaemia, response to anti-VEGF, progression of those that do not respond and the genetic profiles of those with different types of IRMA. Regular follow up of participants in this study may also inform our understanding of possible turnover of DR lesions. At present it is not certain whether IRMA resolve and reappear as review of those with DR in clinics is generally undertaken at 4-6 month intervals or longer. Enrolling those with longer durations of DM, e.g. 20 years or more would provide more robust genetic data as it is unknown whether those with the SNPs identified as showing a trend for IRMA development and no DR will develop DR in the future. This approach will better help identify those in the moderate/severe group at most risk of progression to PDR.

Machine learning may assist quantification and location mapping of these lesions. This may in turn lead to individualising management of diabetic retinopathy particularly when more treatment becomes available.

Further investigation of the genes identified as playing a role in DR and IRMA (VEGF and TGFb-1) may increase our understanding of DR. Despite no genetic link being observed in those with VB, the trend of an association with SLAMP warrants further investigation as it was also associated with the development of DR. Further investigation of the SNPs investigated with an appropriate correction for multiple testing would be of interest although unlikely to find significance.

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Appendices

Appendix 1

Table 10.1. Spreadsheet for grading Optomap images

	ST	SN	IN	IT	PST	PSN	PIN	PIT	PMT	DRSS Grade
DH										
IRMA										
VB										
Venous Loops										

This table illustrates how the grading is recorded, ST superior temporal, SN superior nasal, IN inferior nasal, IT inferior temporal, PST peripheral superior temporal, PSN peripheral superior nasal, PIT peripheral inferior temporal, PMT peripheral middle temporal

Appendix 2

Standard Operational Procedure DNA Extraction-Buccal Cell Swab

1. Purpose

The purpose of this standard operating procedure is to describe the standard procedures when extracting DNA using a buccal cell swab technique.

2. Introduction

Each participant in the CGDME study requires DNA to be extracted for genetic testing of Diabetic Retinopathy. In this trial, buccal cell swab is the preferred method of DNA extraction.

3. Scope

This SOP is for buccal swab collection for DNA analysis.

4. Definitions

CGDME ……The Cytokine Profile and Genetics of Diabetic Macular Oedema Study

DNA……Deoxyribonucleic Acid

PPE……Personal Protective Equipment

5. Responsibilities

The collection of the buccal swab will be carried out by Research Nurses and research Assistants named on the delegation log (filed in the TMF). They are trained to carry out the buccal swab test with handling and storage of the sample. The samples will be processed in the research laboratory, conducted by a trained individual under the supervision of the PI.

6. SPECIFIC PROCEDURE

The initial part of the process is to gain informed consent from the patient, having explained the rationale behind the procedure and the technique to be employed when extracting cells from the cheek. Patients' details to be confirmed as correct.

Ascertain as to whether the patient has had anything to eat or drink within the last 4 hours, as food debris in the cheeks can compromise the samples collected.

Prepare the equipment to carry out the procedure. Swab, pot with preservative, scissors and pen.

Hand hygiene and application of PPE (Personal protective equipment) prior to beginning the procedure.

Ask patient to open their mouth before bringing swab tip to the inside of their cheek.

Gently rub and rotate swab along inside of cheek for 5-10 seconds, ensuring entire swab tip makes contact with cheek.

Remove swab, do not touch swab tip against teeth, tongue, gums or lips.

Place swab into pot provided, using scissors to cut the swab stick to the correct length so that pot lid can be closed and the sample contained within.

Use marker pen to label pot with patient's study identification number.

Dispose of PPEs and once again wash hands.

Protocol: DNA Purification from a Buccal Brush Using the Gentra Puregene Buccal Cell Kit

This protocol is for purification of genomic DNA from 1 buccal brush using the Gentra Puregene Buccal Cell Kit.

Things to do before starting:

Preheat water baths to 55°C for use in step 3b and 65°C for use in steps 3a and17 of the procedure.

Procedure

1. To collect buccal cells, scrape the inside of the mouth 10 times with a Buccal Collection Brush.

For best results, wait at least 1 hour after eating or drinking to collect buccal cells.

DNA may be purified immediately or samples may be stored on the collection brush for up to 1 month at room temperature (15–25°C).

2. Dispense 300 µl Cell Lysis Solution into a 1.5 ml microcentrifuge tube. Remove the collection brush from its handle using sterile scissors or a razor blade, and place the detached head in the tube.

Samples are stable in Cell Lysis Solution for at least 2 years at room temperature.

If 300 µl Cell Lysis Solution is not sufficient to cover the head, the protocol must be scaled up to use a larger volume.

3. Complete cell lysis by incubating at 65°C for at least 15 min (up to 60 min for maximum yield).

Mix by inverting 25 times

4. Remove the collection brush head from the Cell Lysis Solution, scraping it on the sides of the tube to recover as much liquid as possible.

5. Add 100 µl Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.

6. Incubate for 5 min on ice.

7. Centrifuge for 3 min at 13,000–16,000 x g.

The precipitated proteins should form a tight pellet. If the protein pellet is not tight, incubate on ice for 5 min and repeat the centrifugation.

8. Pipet 300 µl isopropanol and 0.5 µl Glycogen Solution (cat. no. 158930) into a clean 1.5 ml microcentrifuge tube, and add the supernatant from the previous step by pouring carefully.

Be sure the protein pellet is not dislodged during pouring.

9. Mix by inverting gently 50 times.

10. Centrifuge for 5 min at 13,000–16,000 x g.

11. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.

12. Add 300 µl of 70% ethanol and invert several times to wash the DNA pellet.

13. Centrifuge for 1 min at 13,000–16,000 x g.

14. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for 5 min.

The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet, as the DNA will be difficult to dissolve.

15. Add 100 µl DNA Hydration Solution and vortex for 5 s at medium speed to mix.

16. Incubate at 65°C for 1 h to dissolve the DNA.

17. Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.

The samples are then checked for quality and concentration of the DNA using a NanoDrop spectrophotometer

Appendix 3

Sensitivity Analysis

Study eyes n=308, all eyes n=504

Deep haemorrhage Grade 2= ≥ than 1A but less than 2A

	ST	SN	IN	IT
Study eyes n=	39 (12.67%)	47 (15.26%)	89 (28.90%)	61 (19.81%)
All eyes n=	167 (33.13%)	199 (39.48%)	178 (35.32%)	201 (39.88%)
p, Chi Sq	0.00001, 42.32	0.0001, 53.12	0.059,3.57	0.0001, 35.26

Deep haemorrhage Grade 3 ≥ 2A

	ST	SN	IN	IT
Study eyes n=	173 (56.17%)	143 (46.43%)	112 (36.36%)	128 (41.56%)
All eyes n=	260 (51.59%)	213 (42.26%)	166 (32.94%)	191 (37.90%)
p, Chi Sq	0.204, 1.61	0.246, 1.35	0.318, 0.99	0.3, 1.075

Deep haemorrhage Grade 2 and 3

	ST	SN	IN	IT
Study eyes n=	212 (68.83%)	190 (61.69%)	201 (65.26%)	189 (61.36%)
All eyes n=	427 (84.72%)	381 (75.6%)	335 (66.47%)	361 (71.63%)

p, Chi Sq	0.0001, 28.79	0.00003, 17.72	0.724, 0.124	0.002, 9.214

IRMA Grade 1

	ST	SN	IN	IT
Study eyes n=	123 (39.94%)	140 (45.45%)	83 (26.95%)	94 (40.52%)
All eyes n=	199 (39.48%)	226 (44.84%)	125 (24.80%)	162 (32.14%)
p, Ch Sq	0.899, 0.016	0.865, 0.029	0.497, 0.462	0.629, 0.233

IRMA Grade 2

	ST	SN	IN	IT
Study eyes n=	96 (31.17%)	39 (12.66%)	15 (4.87%)	58 (18.83%)
All eyes n=	125 (24.8%)	48 (9.52%)	18 (3.57%)	75 (14.88%)
p, Ch Sq	0.048, 3.913	0.161, 1.969	0.363, 0.827	0.140, 2.178

IRMA Grade 1 and 2

	ST	SN	IN	IT
Study eyes n=	219 (71.10%)	179 (58.12%)	98 (31.82%)	152 (49.35%)
All eyes n=	324 (64.29%)	274 (54.37%)	143 (28.37%)	237 (47.02%)
p, Ch Sq	0.048, 3.912	0.296, 1.09	0.297, 1.087	0.520, 0.415

VB Grade 1

	ST	SN	IN	IT
Study eyes n=	33 (10.71%)	33 (10.71%)	38 (12.34%)	18 (5.84%)
All eyes n=	44 (8.73%)	46 (9.13%)	51 (10.12%)	21 (4.17%)
p, Ch Sq	0.349, 0.877	0.459, 0.548	0.326, 0.954	0.278, 1.177

VB Grade 2

	ST	SN	IN	IT
Study eyes n=	0	1 (0.33%)	1 (0.33%)	0
All eyes n=	0	1 (0.2%)	1 (0.2%)	0
p, Ch Sq	0	0.725, 0.124	0.725, 0.124	0

VB Grade 1 and 2

	ST	SN	IN	IT
Study eyes n=	33 (10.71%)	34 (11.04%)	39 (12.66%)	18 (5.84%)
All eyes n=	44 (8.73%)	47 (9.33%)	52 (10.32%)	21 (4.17%)
p, Ch Sq	0.349, 0.877	0.429, 0.625	0.304, 1.056	0.278, 1.177

Appendix 4

Patient Information Sheet

PATIENT INFORMATION BOOKLET

THE CYTOKINE PROFILE AND GENETICS OF DIABETIC MACULAR OEDEMA (CGDME) STUDY

Ethics Number: 10/H0203/14

Date & Version: 03/07/2017 v0.9

What is the Cytokine Profile and Genetics of Diabetic Macular Oedema Study?

This is a study investigating how genes relate to an eye condition found in some patients with diabetes called **diabetic retinopathy**. Diabetic retinopathy is the most common diabetic eye disease and is caused by changes in the blood vessels of the retina.

Invitation to join a research study

We would like to invite you to take part in our research study on diabetic retinopathy.

Before you decide if you would like to participate, we would like you to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully and feel free to discuss it with your family and friends.

Part 1 tells you the purpose of the study and what will happen if you take part.

Part 2 gives you more detailed information about the conduct of the study.

One of our team will go through this information with you and answer any questions that you have. We expect that this should take about 20 minutes. It can be done whilst you are waiting to be seen at your appointment.

PART 1

The Purpose of the Study

Diabetic retinopathy is a common complication of diabetes and if it is not treated can lead to blindness.

Because diabetic retinopathy can be sight-threatening, it is a high priority for us to try to understand it as completely as possible. We need to understand why problems occur in some diabetic people but not others. We also want to know why it forms certain patterns in different people's eyes. For reasons that remain unclear, the currently available treatment is not always effective.

Our aim with this research is to examine the relationship between genes and retinopathy in patients with diabetes. A better understanding of retinopathy will help us to identify more effective ways of treating it than we have at the moment. This could help us to preserve vision in more patients in the future.

Why have I been invited to take part?

You have been invited to take part because you have been diagnosed with retinopathy in the eye clinic. We are inviting all patients with retinopathy to take part.

Do I have to take part if I meet the entry criteria for the study?

No. Participation in this research study is entirely voluntary. This means that we leave it up to you to decide if you would like to join the study. We will describe the study and go through this information booklet. You are free to withdraw at any time, without giving a reason. This will not affect the standard of care you receive in any way.

What will happen if I decide to take part?

If you decide to take part we will ask you to sign a consent form. This just states that you understand what the study is about, and that you are willing to go ahead with it. We will also ask you to complete a questionnaire about any medical problems you

may have had in the past, the medication you are taking and whether or not you smoke. This will help us to interpret the results of the tests done in the study more accurately. We will take one buccal cell swab sample from the inside of your cheek.

The buccal cells will be used to examine your genes, looking for any inherited patterns that might have increased your risk of developing retinopathy.

We need to determine the relevance of any patterns found in your genes to the health of your eyes. We would therefore like to examine images of your eyes. These will have been done routinely, at retinopathy screening or in the King's College Hospital as part of your normal diabetic eye care. No extra images will be taken for the study.

We will let your GP know if you choose to take part in this study.

Summary of what is involved for those who take part in the study

1. **Signing the consent form to state that you understand the purpose of the study and that you would like to take part**
2. **Collecting one buccal cell sample**
3. **Completing a short questionnaire about other medical conditions you may have, what medication you are taking and whether or not you smoke**
4. **Providing a contact telephone number so that we can contact you if you are unsure of your past medical history or what medication you are taking**
5. **We will inform your GP that you are taking part in the study**

What are the potential risks or burdens associated with taking part?

This is a low risk study. We are planning to take one buccal cell sample for testing. Buccal cells are cells from the inside of your cheek. We will collect them using a

buccal swab which is a small, short bristled brush attached to the end of a plastic stick. A member of the clinical care team will rub the swab against the inside of your cheek a few times to collect the cells. This should only take a few seconds and feels like rubbing a toothbrush against your cheek. We will also examine the imaging of your eyes that you would already be having as part of your routine eye care. There will be no change to your normal treatment.

We will store your information securely on NHS computers. Only the research team, and those checking that the study is progressing appropriately, will have access to any information from which you could be identified. If we need to collaborate with other scientists at any stage they will not have access to any information from which you could be identified. Published reports on this study will not include any information that could lead to anyone taking part in the study being identified.

What are the potential benefits of taking part?

If you take part in the study you will have helped to provide valuable information on the links between genes and diabetic retinopathy. The results of the study may help us to identify new ways to try to treat diabetic retinopathy. This is important because the current treatment does not always work well. We hope that the study will help us to understand why some diabetic patients are more likely to develop a certain type of diabetic retinopathy than others. If we can identify those most at risk, we may be able to reduce their risk of developing this condition.

What happens when the research study stops?

When we have completed the study, we will write up and publish the results for the benefit of other people who are interested in our findings. If you wish to be informed directly of the outcomes of the study, we will provide you with our results.

What if there is a problem?

Any complaint about the way you have been dealt with during the study, or any possible harm you might suffer, will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. We will, however, with your consent, also inform your GP that you are taking part in the study. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation in this study, please read the additional information in Part 2 before making any decision.

PART 2

What if relevant new information becomes available?

It is unlikely that new information that affects what we are collecting will become available during the study.

What will happen if I do not wish to carry on with the study?

If you withdraw from the study, we will destroy all your identifiable samples, but we would like to use the data collected up to the time of your withdrawal. This will not prejudice any future treatment.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. Contact details are provided at the end of this booklet.

In the event that something does go wrong and you are harmed during the research, and this is due to someone's negligence, then you may have grounds for legal action

for compensation, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in the study be kept confidential?

Yes. If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons actively involved in the research team. They may also be looked at by authorised people, to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

Involvement of the General Practitioner/ Family Doctor (GP)

We will inform your GP of your involvement in the study if you give us your consent to do so.

What will happen to the buccal cell sample that I give?

The buccal cell sample will be coded and then stored without any of your personal details. When we are ready to begin the tests, the sample will be processed in a laboratory. The results will be stored on a secure computer database, ready for us to analyse in more depth. The analysis will involve correlating the buccal cell results with what we can see on retinal photographs, and/or retinal scans and angiograms that have already been done.

How long will we keep the buccal cells for?

We plan to process the cells very soon after collection. The genetic material extracted from the cells (DNA) may be kept for longer than 3 years. Although no further studies are planned at this stage, we would like to store the DNA so that they could potentially be used in future studies related to the work done in this study.

What will we do with the results of the study?

Given that we expect this study to yield new and valuable information about genes in diabetic retinopathy, we intend to publish our results in research journals to make them as widely available as possible. We would also like to make our results available to patient groups that are interested in diabetic eye disease, and to any participants in the study who would like to be informed of the results once it has been completed. If you wish to participate and would like to receive a summary of the results, you can indicate this on the consent form when you join the study.

Study organisation

This research is being organised by doctors and researchers at the Oxford Eye Hospital and King's College Hospital. It is taking place in the Oxford University Hospitals NHS Trust and King's College Hospital NHS Trust. All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the Cornwall and Plymouth Research Ethics Committee.

Further information

If you require any more information about the study then please do not hesitate to contact us.

CONTACT DETAILS:

Prof Susan Downes

Consultant Ophthalmologist

Oxford Eye Hospital

John Radcliffe Hospital

Oxford, OX3 9DU

Email: [redacted]

Nicki Timson
Ophthalmology Department

1st Floor Normanby Building
Kings College Hospital

Denmark Hill
SE5 9RS

Telephone: [redacted]

Liz Pearce

Ophthalmology Department

1st Floor Normanby Building

Kings College Hospital

Denmark Hill

SE5 9RS

Telephone: [redacted]

Email: [redacted]

Appendix 5

CONSENT FORM

THE CYTOKINE PROFILE AND GENETICS OF DIABETIC MACULAR OEDEMA (CGDME) STUDY

Please initial box

1. I confirm that I have read and understand the information sheet dated..... (Version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I have freely chosen to participate in this study and I understand that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that my medical notes and information collected during the study may be looked at by the research team, regulatory authorities or individuals from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study. ☐

5. I agree to take part in the above study. ☐
6. I agree to have one buccal cell swab taken from the inside of my cheek and that the DNA extracted from these cells can be stored and may be used in future related studies. ☐
7. I agree to the DNA extracted from my buccal cells being stored securely and allow it to be used in future related studies. ☐
8. I wish to be contacted with the results of the study once complete. ☐

Signatures

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Name of person taking consent	Date	Signature

Appendix 6

GP details

DEAR DR

Re: Patient's details

THE CYTOKINE PROFILE AND GENETICS OF DIABETIC MACULAR OEDEMA (CGDME) STUDY

Research Ethics Approval Number: 10/H0203/14

Your patient has agreed to participate and has given consent to take part in a research project investigating the cytokine and genetic profile of diabetic macular oedema. This is a matched case-control study being organised by the Diabetic team at the Oxford Eye Hospital in collaboration with the Oxford University Nuffield Laboratory of Ophthalmology.

The main objective of the study is to describe the cytokine and genetic profiles of adult patients with and without diabetic macular oedema. The study team have collected details of your patient's medical history and medication followed by a buccal swab for DNA analysis.

A patient information sheet is enclosed for your records. If you require any further information please contact us at the above address.

With Best Wishes

Yours sincerely

Appendix 7

Patient Questionnaire

The Cytokine Profile and Genetics of Diabetic Macular Oedema Study

Research Ethics Approval Number: 10/H0203/14

Inclusion Criteria

•Participant is willing and able to give informed consent for participation in the study ☐

•Male or Female, aged 18 years or above ☐

•Diabetes mellitus (type 1 or type 2) ☐

•Clinically confirmed diabetic retinopathy ☐

Exclusion Criteria

•Age under 18 ☐

•Unable or unwilling to give consent for participation in the study ☐

The purpose of this questionnaire is to determine if you have any medical conditions which may affect the way in which we interpret the results of your blood tests.

Please answer "**YES**" or "**NO**" in response to the following questions and supply further information if required.

1. Do you have, or have you ever had, any of the following medical conditions?

ANGINA or a HEART ATTACK **YES / NO** Date if "**YES**" _____

STROKE **YES / NO** Date if "**YES**" _____

KIDNEY PROBLEMS or RENAL FAILURE **YES / NO** Date if "**YES**" _____

2. Do you have, or have you ever had, any other medical problems?

YES / NO

If “**YES**” then please list them below

---

3. Do you take any regular medication? **YES / NO**

If “**YES**” the please list all of your current medication below

---

---

4. Year of diagnosis of diabetes. _____

5. Are you using insulin now? **YES/NO**

6. Are you using any tablet for diabetes? **YES/NO**

7. Are you a current smoker? **YES/NO**

8. Are you an ex-smoker? **YES/NO** Date quit smoking if "**YES**" _____

9. Did you ever have laser treatment for diabetic eye disease **YES/NO**

(if yes, ask medical staff to complete question 10 and 11)

10. Is there history of having macular laser treatment for diabetic macular oedema? **YES/NO (need medical staff to complete)**

11. Is there history of having panretinal laser treatment for proliferative diabetic retinopathy? **YES/NO (need medical staff to complete)**

Appendix 8

Ethics Approval

NHS
Health Research Authority

South West - Cornwall & Plymouth Research Ethics Committee

Level 3
Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

09 May 2017

Professor Susan Downes
Oxford Eye Hospital
West Wing, LG1
John Radcliffe Hospital, Oxford
OX3 9DU

Dear Professor Downes

Study title:	**The cytokine profile and genetics of diabetic macular oedema**
REC reference:	**10/H0203/14**
Protocol number:	**n/a**
EudraCT number:	**n/a**
Amendment number:	**3**
Amendment date:	**03 August 2016**
IRAS project ID:	**30820**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

The Sub-Committee reviewed the following amendment:

1. Change of CI from Victor Chong to Susan Downes.
2. Protocol clarifications.

3. Addition of King's College Hospital London as an additional recruiting centre for controls

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Notice of Substantial Amendment (non-CTIMP) [AmendmentForm3_CGDME_VC_260417.pdf]	3	03 August 2016
Other [RecForm_CGDME_SD_260417.pdf]		26 April 2017
Participant information sheet (PIS) [Generic CGDME patient info booklet tracked change (cases)AF V0.8 300916.docx]	0.8	30 September 2016
Participant information sheet (PIS) [Generic CGDME patient info Booklet Clean copy (cases) AFv0 8_300916.docx]	0.8	30 September 2016
Participant information sheet (PIS) [Generic PIS CGDME_controls AF (3) tracked change.docx]	2.0	04 April 2016
Participant information sheet (PIS) [Generic PIS controls CGDME_AF (2) clean copy.docx]	2.0	04 April 2016
Research protocol or project proposal [CGDME amended protocol 280317 AF_SD clean copy.docx]	0.7	04 April 2016
Research protocol or project proposal [CGDME amended protocol 280317_ AF_SD tracked (1).docx]	0.7	04 April 2016
Summary CV for Chief Investigator (CI) [Signed CV_Susan Downes_29.06.2015.pdf]		29 June 2015

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

10/H0203/14:	**Please quote this number on all correspondence**

Yours sincerely

pp. [redacted]

Canon Ian Ainsworth-Smith
Chair

E-mail: nrescommittee.southwest-cornwall-plymouth@nhs.net

Copy to: *Heather House, Research and Development Department*
Ms Heather House, Oxford University Hospital NHS Foundation Trust

South West - Cornwall & Plymouth Research Ethics Committee

Attendance at Sub-Committee of the REC meeting via correspondence

Committee Members:

Name	*Profession*	*Present*	*Notes*
Canon Ian Ainsworth-Smith	Retired Hospital Chaplain	Yes	
Mr Robert Wosley	Deputy Service Line Cluster Manager	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Lucy Roberts	REC Manager

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

Health Research Authority

Professor Sobha Sivaprasad
Consultant Ophthalmologist
Moorfields Eye Hospital NHS Foundation Trust
162 City Road
London
EC1V 2PD

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

07 March 2019

Dear Professor Sivaprasad

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**A prospective study of structure-function correlation in patients with diabetes with varying sizes of foveal avascular zone.**
IRAS project ID:	**254164**
Protocol number:	**SIVS1048**
REC reference:	**19/NI/0030**
Sponsor	**Moorfields Eye Hospital NHS Foundation Trust**

I am pleased to confirm that **HRA and Health and Care Research Wales (HCRW) Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales?
You should now provide a copy of this letter to all participating NHS organisations in England and Wales, as well as any documentation that has been updated as a result of the assessment.

This is a single site study sponsored by the site. The sponsor R&D office will confirm to you when the study can start following issue of HRA and HCRW Approval.

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) has been sent to the coordinating centre of each participating nation. You should work with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?
The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

I am a participating NHS organisation in England or Wales. What should I do once I receive this letter?
You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

Name: Sobha Sivaprasad

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **254164**. Please quote this on all correspondence.

Yours sincerely,

Natalie Wilson
Assessor

Email: hra.approval@nhs.net

Copy to: *Ms Maria Hassard, Moorfields Eye Hospital NHS Foundation Trust, Sponsor contact*

Ms Tania West, Moorfields Eye Hospital NHS Foundation Trust, Lead NHS R&D contact

List of Documents

The final document set assessed and approved by HRA and HCRW Approval is listed below.

Document	*Version*	*Date*
Covering letter on headed paper [REC reply]		19 February 2019
GP/consultant information sheets or letters [GP letter]	1.0	27 September 2018
IRAS Application Form [IRAS_Form_06022019]		06 February 2019
IRAS Checklist XML [Checklist_19022019]		19 February 2019
Letter from funder [Funder Letter]		16 July 2018
Letter from sponsor [Sponsor Letter]		06 December 2018
Letters of invitation to participant [Invitation Letter]	1.0	27 September 2018
Non-validated questionnaire [Distress Protocol]	1.0	19 February 2019
Participant consent form [Consent form V2.0]	2.0	19 February 2019
Participant information sheet (PIS) [PIS Version 2.0]	2.0	19 February 2019
Research protocol or project proposal [Study Protocol]	1.0	27 September 2018
Summary CV for Chief Investigator (CI) [Chief Investigator CV]		01 December 2018

Summary of assessment

The following information provides assurance to you, the sponsor and the NHS in England and Wales that the study, as assessed for HRA and HCRW Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England and Wales to assist in assessing, arranging and confirming capacity and capability.

Assessment criteria

Section	Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	This is non-commercial, single site study taking place in the NHS where the single participating NHS organisation is also the study sponsor. Therefore, no study agreements are expected.
4.2	Insurance/indemnity arrangements assessed	Yes	No comments
4.3	Financial arrangements assessed	Yes	No comments
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	
5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics	Yes	No comments

Section	Assessment Criteria	Compliant with Standards	Comments
	Committee favourable opinion received for applicable studies		
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	
6.3	Devices – MHRA notice of no objection received	Not Applicable	
6.4	Other regulatory approvals and authorisations received	Not Applicable	

Participating NHS Organisations in England and Wales

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.

This is a non-commercial, single site study. There is one site-type involved in the research. Activities and procedures as detailed in the protocol will take place at participating NHS organisations.

If this study is subsequently extended to other NHS organisation(s) in England or Wales, an amendment should be submitted, with a Statement of Activities and Schedule of Events for the newly participating NHS organisation(s) in England or Wales.

The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England and Wales in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. Where applicable, the local LCRN contact should also be copied into this correspondence.

If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England and Wales which are not provided in IRAS, the HRA or HCRW websites, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net or HCRW at Research-permissions@wales.nhs.uk. We will work with these organisations to achieve a consistent approach to information provision.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and Wales, and the minimum expectations for education, training and experience that PIs should meet (where applicable).

A Principal Investigator (PI) is expected at participating NHS organisations. Sponsor will confirm any training requirements with research staff directly.

GCP training is not a generic training expectation, in line with the HRA/HCRW/MHRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken

No Honorary Research Contracts, Letters of Access or pre-engagement checks are expected for local staff employed by the participating NHS organisations. Where arrangements are not already in place, research staff not employed by the NHS host organisation undertaking any of the research activities listed in the research application would be expected to obtain an honorary research contract. This would be on the basis of a Research Passport (if university employed) or an NHS to NHS confirmation of pre-engagement checks letter (if NHS employed). These should confirm enhanced DBS checks, including appropriate barred list checks, and occupational health clearance.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England and Wales to aid study set-up.

The applicant has indicated that they intend to apply for inclusion on the NIHR CRN Portfolio.

Health Research Authority

Professor Susan Downes
Oxford Eye Hospital
West Wing, LG1
John Radcliffe Hospital, Oxford
OX3 9DU

Email: hra.approval@nhs.net

10 May 2017

Dear Professor Downes

Letter of HRA Approval for a study processed through pre-HRA Approval systems

Study title:	**The cytokine profile and genetics of diabetic macular oedema**
IRAS project ID:	**30820**
Sponsor	**Oxford University Hospital NHS Foundation Trust**

Thank you for your request for HRA Approval to be issued for the above referenced study.

I am pleased to confirm that the study has been given **HRA Approval.** This has been issued on the basis of an existing assessment of regulatory compliance, which has confirmed that the study is compliant with the UK wide standards for research in the NHS.

The extension of HRA Approval to this study on this basis allows the sponsor and participating NHS organisations in England to set-up the study in accordance with HRA Approval processes, with decisions on study set-up being taken on the basis of capacity and capability alone.

This letter incorporates HRA Approval for Substantial Amendment 3 dated 3 August 2016, which may be implemented in accordance with the amendment categorisation email of 3 May 2017. The submitted amendment includes the addition of a new NHS organisation in England and this is also approved and should be set up in accordance with HRA Approval processes (e.g. the organisation should be invited to assess and arrange its capacity and capability to deliver the study and confirm once it is ready to do so).

Participation of NHS Organisations in England

Please note that full information to enable set up of participating NHS organisations in England is not provided in this letter, on the basis that activities to set up these NHS organisations is likely to be underway already.

The sponsor should provide a copy of this letter, together with the local document package and a list of the documents provided, to participating NHS organisations in England that are being set up in accordance with HRA Approval Processes. It is for the sponsor to ensure that any documents provided to participating organisations are the current, approved documents.

For non-commercial studies the local document package should include an appropriate Statement of Activities and HRA Schedule of Events. The sponsor should also provide the template agreement to be used in the study, where the sponsor is using an agreement in addition to the Statement of Activities. Any changes that are appropriate to the content of the Statement of Activities and HRA Schedule of Events should be agreed in a pragmatic fashion as part of the process of assessing, arranging and confirming capacity and capability to deliver the study. If subsequent NHS organisations in England are added, an amendment should be submitted to the HRA..

It is critical that you involve both the research management function (e.g. R&D office and, if the study is on the NIHR portfolio, the LCRN) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

After HRA Approval

In addition to the document, *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC Favourable Opinion, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/.

If you have any queries about the issue of this letter please, in the first instance, see the further information provided in the question and answer document on the HRA website.

Your IRAS project ID is 30820. Please quote this on all correspondence.

Yours sincerely

Isobel Lyle | Senior Assessor
Health Research Authority
Room 002, TEDCO Business Centre, Rolling Mill Rd, Jarrow NE32 3DT
Hra.approval@nhs.net or [redacted]
T: [redacted]
www.hra.nhs.uk

Copy to: *Ms Heather House, R&D, Oxford University Hospital NHS Foundation Trust*