THE ROLE OF MICROVASCULAR ENDOTHELIAL DYSFUNCTION AND GENETICS ON SEVERITY AND PROGRESSION OF PRIMARY OPEN ANGLE GLAUCOMA IN MALAYS

by

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ACG	Angle Closure Glaucoma
ACh	Acetylcholine
AGIS	Advanced Glaucoma Intervention Study
AU	Arbitrary Unit
AU	Arbitrary Perfusion Units
BES	Barbados Eye Study
BMES	Blue Mountain Eye Study
BMI	Body Mass Index
CCT	Central Corneal Thickness
CIGTS	Collaborative Initial Glaucoma Treatment Study
CV	Coefficient Of Variance
DBP	Diastolic Blood Pressure
EDCF	Endothelial Derived Constricting Factors
EDRF	Endothelial Derived Relaxing Factors
EDVFs	Endothelial-Derived Vasoactive Factors
EMGT	Early Manifest Glaucoma Treatment
EGPS	European Glaucoma Prevention Study
eNOS	Endothelial NOS
ET-1	Endothelin-1
FBF	Forearm Blood Flow
FBS	Fasting Blood Sugar

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ABSTRAK

Sehingga kini, pengetahuan terhadap persembahan klinikal dan faktor risiko untuk glaukoma sudut terbuka primer (POAG) dalam kalangan kaum Melayu masih kurang. Mengenalpasti faktor risiko yang berkaitan dengan tahap keterukan dan kadar keterukan penyakit. kadar keterukan POAG dalam kalangan kaum Melayu amat penting untuk merancang strategi pencegahan kebutaan dan rawatan yang lebih berkesan. Kajian ini dijalankan untuk menentukan tahap dan kadar keterukan POAG di kalangan pesakit POAG berbangsa Melayu. Selain dari itu, kajian ini juga bertujuan untuk menentukan peranan fungsi endotelial mikrovaskular dan genetik dalam menentukan tahap dan kadar keterukan POAG.

Satu kajian keratan rentas telah dijalankan yang melibatkan 215 subjek berbangsa Melayu (114 pesakit POAG dan 101 kawalan). Keterukan POAG adalah berdasarkan analisa medan penglihatan Humphrey (HVF). Ketentuan kadar keterukan adalah berdasarkan kombinasi skor Kajian Intervensi Glaukoma Lanjutan (AGIS) dan klasifikasi Hodapp, Parish dan Anderson. Manakala tahap keterukan POAG adalah berdasarkan skor AGIS HVF yang telah diubah suai; tahap keterukan ringan, sederhana dan teruk.

Fungsi endotelial mikrovaskular juga turut dinilai menggunakan ‘Laser Doppler Fluximetry’ (LDF) dan proses iontoforesis mengunakan asetilkolina (ACh) dan natrium nitroprusida. Darah vena juga turut diambil dari pesakit glaukona bagi kajian genetik. Ketulenan genom DNA yang tinggi juga telah diekstrakkan. Platform ‘Microarray Human Omni Express-12’ telah digunakan untuk mengenalpasti penanda genetik bagi kadar keterukkan penyakit POAG. rs1392912 dan rs1660029 dari gen KALRN, dan rs1210977 dari gen COL9A1 telah dikenalpasti sebagai penanda genetik yang berpotensi memainkan peranan dalam menentukan kadar keterukan penyakit dan fungsi endotelial mikrovaskular. Polimorfisma nukleotida tunggal (SNPs) ini kemudiannya telah didedahkan kepada penjujukan DNA.

Selepas purata 4.1 (3.0) tahun menjalani temujanji susulan didapati 35 pesakit menunjukkan perubahan dalam keterukan medan penglihatan. Berdasarkan kepada medan penglihatan semasa kajian ini dijalankan, 55 pesakit menunjukkan tahap POAG yang ringan, 29 sederhana dan 30 teruk. Terdapat pengurangan fungsi endotelial mikrovaskular yang signifikan dalam kalangan pesakit POAG berbanding kumpulan kawalan ($p<0.001$). Respon terhadap ACh dan s natrium nitroprusida berkurangan secara signifikan dalam kalangan pesakit yang mengalami POAG teruk ($p<0.001$). Terdapat juga pengurangan fungsi endotelial mikrovaskular yang signifikan di kalangan pesakit yang menunjukkan perubahan keterukan medan penglihatan ($p<0.001$). Didapati juga risiko perubahan keterukan penyakit POAG adalah 4.8 kali ganda (95% CI 1.52, 14.86) dalam kalangan pesakit dengan rs1392912GA dan 5.8 kali ganda (95% CI 1.85, 18.61) dalam kalangan pesakit dengan rs1660029AG. Walaubagaimana pun, tiada hubungkait variasi penanda

genetik rs1210977 dari COL9A1 dalam fungsi endotelial mikrovaskular pesakit Melayu yang menghidap POAG.

Kadar keterukan penyakit POAG adalah 8.5 pesakit/tahun di kalangan pesakit Melayu. Fungsi endotelial mikrovaskular memainkan peranan dalam tahap dan kadar keterukan POAG dalam kalangan bangsa Melayu. rs1392912GA dari gen KALRN adalah penanda genetik yang berpotensi dalam menentukan perubahan keterukkan penyakit POAG. Terdapat kemungkinan bahawa penambahbaikan fungsi endotelial mikrovaskular dan pengesanan awal pesakit dengan variasi genetik gen KALRN dapat membantutkan kadar keterukan penyakit POAG dikalangan bangsa Melayu.

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ABSTRACT

There is lack of knowledge of clinical presentation and risk factor for primary open angle glaucoma (POAG) in Malays. Identification of the risk factors for severity and progression of POAG in Malays is important to strategies prevention of blindness and effective management. This study was conducted to determine the severity and progression of POAG in Malay patients and to determine the role of microvascular endothelial function and genetics in progression and severity of POAG.

A cross-sectional study was conducted involving 215 Malay (114 POAG patients and 101 controls) subjects. Progression was determined based on Humphrey visual field analysis (HVF) of 114 POAG patients using combination criteria of Advanced Glaucoma Intervention Study (AGIS) scoring and Hodapp, Parish and Anderson classification. Severity of POAG was based on modified AGIS scoring of HVF. Microvascular endothelial function was assessed using Laser Doppler Fluximetry (LDF) with the process of acetylcholine (ACh) and sodium nitroprusside iontophoresis. Venesection was also conducted. High purity genomic DNA was extracted. Microarray Human Omni Express-12 platform was used to identify genetic markers. rs1392912 and rs1660029 from KALRN gene, and rs1210977of COL9A1 gene were identified as potential markers for progression and microvascular

endothelial function. These single nucleotide polymorphisms (SNPs) were then subjected to DNA sequencing.

After the mean 4.1(3.0) years of follow up, 35 patients showed evidence of visual field progression. Based on HVF at recruitment, 55 patients were mild, 29 moderate and 30 severe POAG. There was significant reduction of microvascular endothelial function in POAG patients compared to control ($p<0.001$)). ACh and sodium nitroprusside response was significantly reduced in severe POAG ($p<0.001$). There was significant reduction of microvascular endothelial function in patients who showed visual field progression ($p<0.001$). The risk of progression is 4.8 folds (95% CI 1.52, 14.86) in patients with rs1392912GA and 5.8folds (95% CI 1.85, 18.61) in patients with rs1660029AG. However, there was no association betweenrs1210977 of COL9A1 and microvascular endothelial function of Malay patients with POAG

The progression rate of POAG in Malay patients was 8.5 patients/year. Microvascular endothelial function play a role in progression and severity of POAG in Malays. rs1392912GA and rs1660029AG of KALRN gene are potential genetic markers for progression of glaucoma. Perhaps, improvement in microvascular endothelial function and early detection of patients with genetic variations of KALRN gene may retard the progression of POAG in Malays.

FLP	Fasting Lipid Profile
FMD	Flow Mediated Dilation
GCP	Glaucoma Change Probability
GHT	Glaucoma Hemifield Test
GON	Glaucomatous Optic Neuropathy
GTN	Glyceryltrinitrate
GWAS	Genome-Wide Association Studies
GWA	Genome-Wide Association
HFA	Humphrey Field Analyser
HR	Hazard Ratio
HUSM	Hospital Universiti Sains Malaysia
HVF	Humphrey Visual Field
IOP	Intra Ocular Pressure
ISGEO	International Society Of Geographical And Epidemiological Ophthalmology
JOAG	Juvenile- Onset Open Angle Glaucoma
LBF	Leg Blood Flow
LDF	Laser Doppler Fluximetry
LDI	Laser Doppler Imager
LOCS	Lens Opacities Classification System
LTH	Local Thermal Hyperaemia
MCh	Methacholine Chloride
MD	Mean Deviation

MESA	Multiethnic Study Of Atherosclerosis
MYOC	Myocilin
NO	Nitric Oxide
NOS	NO Synthase
NOS	Nitric Oxide Synthase
NSAID	Non-Steroidal Anti-Inflammatory
NTG	Normal-Tension Glaucoma
OAG	Open Angle Glaucoma
OBF	Ocular Blood Flow
OCT	Optical Coherence Tomography
OHT	Ocular Hypertension
OHTS	Ocular Hypertension Treatment Study
ONH	Optic Nerve Head
OPP	Ocular Perfusion Pressure
OPTN	Optineurin
PO2	Partial Oxygen Pressure
POAG	Primary Open Angle Glaucoma
PORH	Post Occlusive Reactive Hyperaemia
PSD	Pattern Standard Deviation
RBC	Red Blood Cells
RGC	Retinal Ganglion Cell
RGC	Retinal Ganglion Cells
RNFL	Retinal Nerve Fibre Layer

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAP	Standard Automated Perimetry
SES	Socioeconomic Status
SITA	Swedish Interactive Threshold Algorithm
SNP	Single Nucleotide Polymorphisms
SOPP	Systolic Ocular Perfusion Pressure
SPSS	Statistic Package For The Social Sciences
TM	Trabecular Meshwork
VCDR	Vertical Cup Disc Ratio
VCDR	Vertical Cup-To-Disc Ratio
VF	Visual fields
WHO	World Health Organization

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photoreceptor it has to pass through many layers. Once light reaches the photoreceptors, visual signal propagates back up to the ganglion nerves. These ganglion nerves, in turn course along the surface of the retina toward the optic disc and form the optic nerve running to the brain. Macula is pigmented area of the retina that responsible for central vision. Within the central macula lies the fovea, which is a small pit involves with extreme central vision. The fovea is very thin and derives its nutrition entirely from the underlying choroid. Choroid composed of blood vessels that lies right beneath the retina. Choroid supplies nutrition to the outer one-third of the retina which includes the rod and cone photoreceptors.

Optic disc is the entry and exit point of the eye. The central retinal artery and vein pass through here, along with the ganglion nerves that form the optic nerve. A physiologic divot or "cup" that is important in diagnosis and management of glaucoma. The optic nerve is made up of many nerve fibres, like an electrical cable containing numerous wires. Once these fibres are damaged, blind spots develop. These blind spots usually go undetected until the optic nerve is significantly damaged.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Glaucoma

Cornea is the clear anterior surface of the eye. The cornea-air interface provides the majority of the eye's refractive power. The cornea is avascular and gets its nutrition from tears on the outside, aqueous fluid on the inside, and from blood vessels located at the peripheral limbus. Iris, ciliary body, and choroid plexus are continuous with each other and are collectively known as uvea. Iris is coloured part of the eye and its primary function is to control the amount of light hitting the retina. The inner iris flows back and becomes the ciliary body.

The ciliary body has two functions: it secretes aqueous fluid and controls the shape of the lens. The lens is placed posterior to the iris. Ithas no innervation or vascularization. The lens receives its nourishment entirely from nutrients floating in the aqueous fluid. It also has the highest protein concentration of any tissue in the body (65% water, 35% protein). The lens is held in place by suspensory ligaments called zonular ligaments that insert around the periphery and connect to the muscular ciliary body. Contraction of the ciliary muscle causes the zonular ligaments to relax, allowing the lens to become rounder and increase its refracting power for close-up reading.

Retina is the sensory portion of the eye that act as a film of camera. Retina contains layers of photoreceptors, nerves and supporting cells. For light to reach the

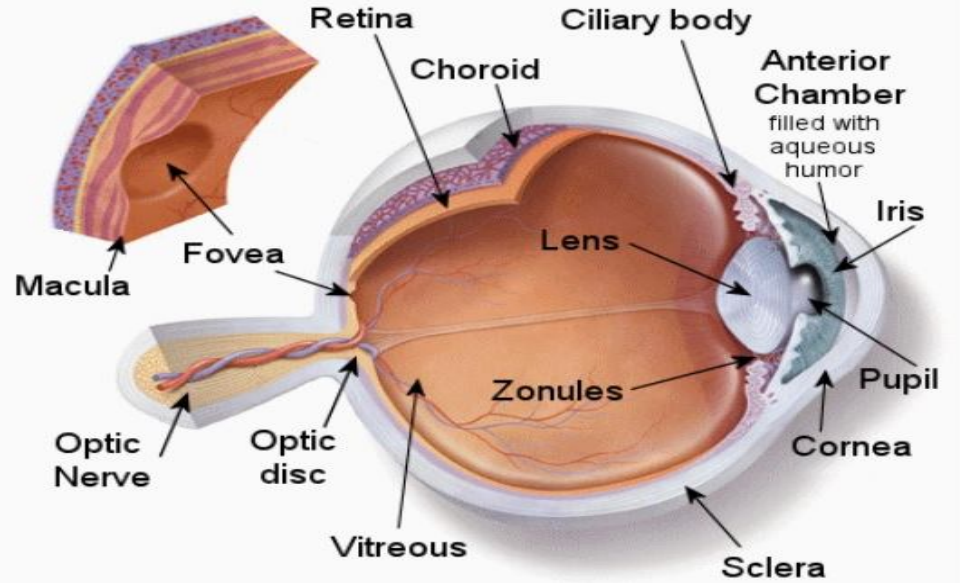

Figure 1-1: Cut section of the Eye, illustrating important anatomical structures (http://www.shutterstock.com/human+anatomy/search-vectors.html)

The word "Glaucosis" was first introduced in Hippocratic work in early Greece in 400 BC, as a disease resulting in blindness and occurring most commonly in the elderly. The term “Glaucosis” was used to describe various sight-threatening conditions in addition to glaucoma. In 1348, a Muslim scientist Sams-al-Din observed the elevation of intra-ocular pressure (IOP). However, his observation remained neglected for nearly three hundred years(Evans, 1939).Richard Bannister (1622), a renowned surgeon who wrote the first book on ophthalmology in English. He described glaucoma as an eye disease with a tetrad features: eye tension, long duration, no perception of light and a fixed pupil.’ (Keeler *et al.*, 2013).

In 1862, Donder described glaucoma as a disease of long duration with elevated pressure and fixed pupil (Haffmans and Schmidt, 1862). Later in 19th century, Antoine-Pierre Demours was the first to associate glaucoma with raised intra-ocular

tension (Arnold, 1963). For the past 700 years, the understanding of glaucoma has been further distinguished. Drance (Drance, 1960)introduced definition of glaucoma as a disease related to the optic nerve caused by several risk factors.

Currently, glaucoma is considered to be a group of eye conditions that cause damage to the optic nerve (Casson *et al.*, 2012; Resch *et al.*, 2009) . Glaucoma is a complex disease with possibilities of various underlying mechanisms. Initially increased IOP was identified as a causative factor of glaucoma (Drance *et al.*, 1973). However, with further understanding of the pathogenesis of glaucoma, IOP is considered as a risk factor; the only modifiable risk factor.

In 1990, glaucoma was considered to be the third leading source of blind people in the world by World Health Organization (WHO) (JMJ, 2002; Thylefors and Negrel, 1994). Glaucoma is responsible for 13% of world blindness, affecting approximately 5 million people in the world (Thylefors and Negrel, 1994)The recent reports by WHO ranked glaucoma as the second most prevalent cause of blindness (Resnikoff *et al.*, 2004). The number of people affected by glaucoma is expected to rise every year and may lead to a substantial public health challenge worldwide (Quigley, 1996). It was estimated that 67 million people were affected by this condition in year 2000 and over 6.7 million people were estimated to be bilaterally blind(Quigley, 1996). This estimation was based on 111 studies in 7 stratified populations with clear definition of glaucoma, random selection of samples, and large sample size (Quigley, 1996).

Prevalence of primary open angle glaucoma (POAG) is higher in African Americans and African Caribbean's compared to Caucasians. The difference was attributed to racial difference (Leske *et al.*, 1994). However, race is a complex and controversial

concept, which involves genetics, religion, culture, language, anthropology, geographic locations, inhabitant environment and population history(Wilson, 2003). Thus it is inappropriate to conclude that the observed difference in the prevalence of POAG is due to potential differences in environmental exposure, genetic predisposition or other factors (Kosoko-Lasaki *et al.,* 2006a). Such differences that is environmental exposure, genetic predisposition or other factors may occur between different ethnic groups of the same "race" as well. The prevalence of glaucoma has been studied extensively and has been shown to vary extensively between ethnic and racial groups (Quigley and Broman, 2006; Ramakrishan *et al.*, 2013; Rudnicka *et al.*, 2006).

Population based studies have shown estimated prevalence rates varying greatly between different populations ; ranging from 1-3% in Europe, 2-3% in Australia, 1-4% in Asia (Quigley and Broman, 2006; Rudnicka *et al.*, 2006). Higher prevalence of 7-9% was reported among African Caribbean's originating from West Africa (Leske *et al.*, 1994; Mason *et al.*, 1989).

The risk of developing glaucoma is increases exponentially with increasing age (Friedman *et al.*, 2004). This is most probably due to the improvement in health system and facilities. It was projected that over 8.4 million of the population will be bilaterally blind due to glaucoma in 2010 and further increases to 11.1 million by 2020 worldwide (Quigley and Broman, 2006). Based on 8 population based studies that include the Beaver Dam Eye Study, Barbados Eye Study (BES), Baltimore Eye study, Blue Mountain Eye Study (BMES), Proyecto Vision Evaluation Research, Kongwa Eye Project, Rotterdam Study, and the Melbourne Visual Impairment Project, the prevalence of glaucoma has been extrapolated to affect 3.36 million

Caucasians by 2020 (Friedman *et al*., 2004). Glaucoma is often asymptomatic and result in substantial visual damage before diagnosis is made (Fraser *et al.*, 2001). Late presentation is common and identified as a significant risk factor for a consequent blindness in glaucoma (Fraser *et al.*, 2001).

Among different subtypes of glaucoma, POAG is the commonest and responsible for more than 50% of all cases of glaucoma (Pang *et al.*, 2006). It is estimated that around 50% of patients with POAG remain undiagnosed in most Western communities (Kroese *et al.*, 2002). Visual acuity may not deteriorate significantly until advance stage of the disease, hence responsible for its delayed diagnosis or non-diagnosis (Topouzis, 2007).

Quigley and colleagues estimated that in 2010 half of the world's glaucoma patients were in Asia (Quigley and Broman, 2006). Even though more emphasis has been given to angle closure glaucoma (ACG), OAG is still the most common glaucoma in Asia (Foster and Johnson, 2001; Quigley and Broman, 2006). In population-based surveys in East Asia, Foster et al (2001) found higher rate of severe monoocular visual field loss from PACG patients than POAG patients (Foster and Johnson, 2001). PACG caused approximately 45% of monocular blindness (He *et al*., 2006), whereas POAG rates of severe vision loss were considerably lower (17%) (He *et al.*, 2006). The highest number of people affected by both ACG and OAG are in Asia followed by Europe(Quigley and Broman, 2006).

1.1.1 Definition of glaucoma

Diagnosing glaucoma remains a challenge due to inconsistent definition of glaucoma. This has generated a problem in not only its detection but also to determine its type and to institute appropriate treatment and to accurately assess its prevalence. Proper definition of disease is the foundation of epidemiological research whether measuring prevalence, studying risk factors, and or conducting clinical trials. In the past, definition included specific levels of IOP as the criteria, the definition has evolved for the past 30 years (Leske, 1983).Most of the population based studies defined glaucoma with special interest in structural changes of optic nerve head (ONH) and functional defect of visual fields, without specific reference to intra ocular pressure (IOP) (Mitchell *et al.*, 1996; Tielsch *et al.*, 1995; Tielsch *et al.*, 1991b).

IOP was then identified as a modifiable risk factor and not consider as a criteria for diagnosis of glaucoma (Sommer, 1989). Approximately 20% of subjects with open-angle glaucoma have their IOP within normal range (Bonomi *et al.*, 1998). Over the age of 40, around 2 in every 100 people developed open angle glaucoma but around 4 in 100 over 40 years developed ocular hypertension in the Blue Mountains Eye Study and the Melbourne Visual Impairment Project (Rait, 2014). Ocular hypertension was defined as an IOP above 21 mm Hg by applanation tonometry on repeated testing in the absence of typical glaucomatous field defects(Perkins and Phelps, 1982).Therefore, diagnosing glaucoma solely on IOP level may lead to over or under estimation. Most studies defined glaucoma based on vertical cup disc ratio

(VCDR) and visual field defects (Klein *et al.*, 1992; Leske *et al.*, 1994; Tielsch *et al.*, 1991b).

In some studies patients were further categorized as definite POAG and suspected cases on the basis of VCDR and previously obtained visual field (Dandona *et al.*, 2000; Foster *et al.*, 2000). International Society of Geographical and Epidemiological Ophthalmology (ISGEO) formed a special panel to develop a consensus on definition of glaucoma (Paul J Foster, 2002) . It is based on prevalence studies but has been adopted in many clinical studies. The definition was formulated from structural and functional evidence of glaucomatous damage (category 1 and 2). IOP and visual acuity along with visual acuity is considered as part of definition (category 3 and 4) when it is practically impossible to diagnose patients on structural and functional basis (Foster *et al.*, 2002).

Primary open angle glaucoma (POAG) is chronic, progressive optic neuropathy defined by changes in the optic nerve head (ONH) and visual field in the absence of primary causes (SEAGIG Glaucoma Guideline 2nd edition, 2008) (Brandt *et al.*, 2012). The relation between vertical cup-disc ratio (VCDR) and corresponding visual field abnormalities is complex (Foster *et al.*, 2002; Hoffmann *et al.*, 2007). Some patients have reproducible field defects but have a CDR that lies within normal range (Foster *et al.*, 2002). VCDR is well known to vary widely within the normal population (Jonas *et al.*, 1988a). There wasalso significant overlap in VCDR between normal and glaucomatous eyes. In a study by Jonas and colleagues, normal VCDR ranges up to 0.87(Jonas *et al.*, 1988a). It is well established that the vertical cup diameter increase in association with optic disc size (Bengtsson, 1976). Using 0.7 as

the cut off could potentially lead to high numbers of false negative in eyes with small optic discs and high numbers of false positive in larger optic discs.

The characteristic visual field defects are defined as asymmetrical points across horizontal midline located in the mid-periphery, clustered in neighbour test points, reproducible on at least two occasions and the visual field defects are not explained by any other disease (Foster *et al.,* 2002). However, these principles failed to address the characteristic visual field defect in severe cases. Thus, in severe cases, the glaucoma hemifield test graded as outside normal limits and a cluster of three contiguous points at the 5% level on the pattern deviation plot, using the threshold test strategy with 24-2 test pattern of Zeiss-HVF analyser 2 (Foster *et al.,* 2002).

The central idea of the suggested definition by International Society for Geographical and Epidemiological Ophthalmology is that the term glaucoma can be used only for people with recognizable, visually significant end organ damage and glaucoma can be characterized as an optic neuropathy associated with characteristic structural damage to the optic nerve with visual field loss that may be caused by various pathological processes. This diagnostic scheme limits the features required to make the diagnosis to direct measurements of the structure and function of the optic nerve.

1.1.2 Pathogenesis of Glaucoma

The concept of elevation of IOP in glaucoma was identified by the Arabs in the 10th century (AI Tabari), and in 1626 by Bannister (Keeler *et al.*, 2013). It was further emphasized by Mackenzie in 1830 (Duke-Elder and Jay, 1969). However, it was only after the publication of the work by Antoine-Pierre Demours (Arnold, 1963), that the

concept of increased IOP in glaucoma was fully established (Duke-Elder and Jay, 1969). For nearly 150 years, importance was given to IOP in the diagnosis of glaucoma. Only recently emphasis was given to the appearance of the optic disc and visual field loss.

The new concepts or theories of glaucoma evolved when patients with normal IOP developed typical visual field patterns with characteristics cup disc changes (Drance, 1972; Leighton and Phillips, 1972b; Sjögren, 1946). These theories were against the pressure-dependent theory. In addition, lowering intraocular pressure (IOP) alone would not retard progression of glaucoma (Mozaffarieh and Flammer, 2007; Tezel *et al.*, 2001). Pressure independent mechanism include inadequate perfusion of optic nerve head due to damage to microvascular endothelial cells may be more relevant (Adams, 2006; Grieshaber *et al.*, 2007b; Kaushik *et al.*, 2003; Nicolela *et al.*, 2003). Other possible pressure-independent mechanisms responsible for initiation and progression of glaucoma include oxidative stress (Izzotti *et al.*, 2003a; Sacca *et al.*, 2005), excitotoxicity (Kaushik *et al.*, 2003), abnormal glial-neuronal interactions (Tezel and Wax, 2000), neurotrophin starvation (Schuettauf *et al.*, 2002), and autoimmunity (Maruyama *et al.*, 2000) is believed to contribute to the initiation and progression of glaucoma. Genetics has also been identified as predisposing factor (Caprioli and Garway-Heath, 2007).

The pressure-dependent theory suggests the direct effect of mechanical pressure on axonal fibres. Pressure-independent theory focuses on decreased perfusion and development of intraneural ischaemia due to vascular resistance. Glaucoma is a multi- factorial disease with a final common pathway leading to optic nerve damage and visual field loss. Until now, there is no specific pathogenesis of glaucoma.

Glaucoma is generally accepted as neurodegenerative disease that is caused by various mechanisms. Based on the status of the angle structure of the eye, glaucoma is categorized as open angle glaucoma (OAG) and angle closure glaucoma (ACG). These classifications are further divided on the basis of aetiology: primary are those without any other known cause and secondary are those associated with other systemic pathologies.

1.1.2.1 Mechanical Theory

Currently the main aim of therapeutic treatment of glaucoma is to lower the IOP in order to retard the progression of ONH damage and visual field loss (NICE-CG85, 2009). Without doubt IOP is a relevant pathogenic feature in the development of GON (Nathan, 2000).

IOP is a mechanical entity, referring to the normal force per unit area applied by the intraocular fluids on the structure of the eye (Sigal and Ethier, 2009). It is indistinct whether alterations to the ONH are the primary event that precipitates retinal ganglion cell (RGC) damage or glaucomatous RGC death prompts the events that lead to ONH changes. There are evidences that indicate elevation of IOP trigger distinct compositional and structural changes in the ONH. These include increased synthesis of several extracellular matrix molecules such as tenascin (Pena *et al.*, 1999; Ricard *et al.*, 1999), matrix metalloproteinases (Ricard *et al.*, 1999), NCAM-180 (Ricard *et al.*, 2000), collagen IV, and elastin (Hernandez and Pena, 1997). In vitro studies found that reactive astrocytes may be responsible for remodelling of the

extracellular matrix (Hernandez and Pena, 1997; Ricard *et al.*, 2000). Reactive astrocytes drift to the nerve bundles is induced by increased IOP and may form big cavernous spaces through the expression of matrix metalloproteinases (proteolytic enzymes) (Guo *et al.*, 2005; Ricard *et al.*, 1999). The changes in the architecture of extracellular matrix may weaken the ONH and facilitate the collapse of the lamina cribrosa beams, ultimately leading to injury of the RGC axons that pass through these structures.

It is a widely accepted that lamina cribrosa is the site of early damage to the ganglion-cell axons (Gaasterland *et al.*, 1978; Quigley and Addicks, 1981). The lamina cribrosa consists of ten lamellar connective tissue sheets with apertures to allow passages for the axons of ganglion-cells (Quigley and Addicks, 1981; Radius and Gonzales, 1981). Raised IOP is proposed to push lamina cribrosa posteriorly (Levy and Crapps, 1984) with movement of the connective tissue layers in relation to each other and this change leads to narrowing of laminar apertures (Quigley *et al.*, 1981). The changes in lamina cribrosa are responsible for mechanical pressure to the nerve fibres that run through the apertures. This leads to the obstruction of anterograde and retrograde axoplasmic flow in neurons and subsequent neuronal death after duration of mechanical pressure (Gaasterland *et al.*, 1978; Quigley and Addicks, 1980). The overall susceptibility of the ocular structures to the effects of IOP however, appears to vary between individuals as a function of the individual eye's anatomy and composition (Bandyopadhyay *et al.*, 2011; Sigal and Ethier, 2009).

Both experimental as well as clinical studies have established the role of IOP and the benefits of IOP lowering treatment (AGIS, 2000). Evidence support that reduction in

IOP may improve the prognosis of patients but do not halt the progression in all patients. The postulation that IOP is the only causative factor is being increasingly challenged (Flammer, 1996). Normal-tension glaucoma (NTG) and ocular hypertension (increased IOP without recognizable damage) indicate that other factors are also involved in the pathogenesis of glaucomatous optic neuropathy (GON).

NTG incidence in women is twofold higher than men (Orgul *et al.*, 1995). African descent glaucoma patients have average IOP almost similar of Whites, yet the prevalence of glaucoma is four times higher (Sommer *et al.*, 1991b). NTG is the commonest type of glaucoma in Japan (Araie *et al.*, 1994) . The incidence rate of GON among Japanese is similar to Caucasian (Shiose, 1990) . Paradoxically, IOP diminishes with advancing age in Japan (Shiose, 1990). However it should be noted that despite this, Japanese patients with elevated IOP, like their Western counterparts, are more likely to have progressive GON than those with normal IOP (Shiose *et al.*, 1990). All these observations cannot be satisfactorily explained by the pressure theory alone, indicating that increased IOP is not necessary for the development of GON (Flammer and Orgül, 1998). Some eyes with high IOP may not develop disease; approximately 20% of subjects with open-angle glaucoma have IOP within normal range (Bonomi *et al.*, 1998; Lundberg *et al.*, 1987; Sommer *et al.*, 1991b). This raises a question: is it possible that at a given pressure level, the majority of people will never suffer GON, while others do? Furthermore, why glaucoma progression is only weakly related to IOP? (Martínez-Belló *et al.*, 2000). Finally pressure reduction, although significantly improves prognosis but it does not avoid damage in all patients (Stewart *et al.*, 2000).

1.1.2.2 Vascular mechanism

Duke-Elder described that glaucomatous visual field defects and optic disc cupping sometimes occur without raised intra-ocular tension, stressing the vasogenic factor in glaucoma (Duke-Elder, 1953) and considered instability of vascular control as an aetiology of glaucoma.Hayreh reported that the nerve fibre bundle defects and cupping of optic disc are due to damage of vessels in the prelaminar part of the optic disc and peripapillary choroid. Peripheral visual field constriction is due to the involvement of the peripheral centripetal vascular system to this part of the optic nerve (Hayreh, 1969)

A pressure independent mechanism mainly focused in the imbalance of optic nerve head (ONH) perfusion causing ischemia of ONH (Osborne *et al.*, 1999, Flammer, 1994; Flammer and Orgül, 1998). Based on this theory, reduction in perfusion of optic nerve head (Flammer, 1994; Flammer and Orgül, 1998) and ischaemia induced ONH damage and retinal ganglion cell death contributes to GON (Osborne *et al.*, 1999).

Ocular perfusion pressure (OPP) is dynamic but can be mathematically calculated; the difference between systemic blood pressure and intraocular pressure (IOP). OPP is affected by the resistance to flow and regulated by the size (calibre) of vessel lumen (Flammer, 1994). Vascular resistance plays a role in determining the amount of blood that flows through a vascular bed. Vascular resistance itself is affected by blood viscosity, vessel length and vessel diameter (Delaey and Van de Voorde, 2000). Due to constant capillary perfusion and lack of pre capillary sphincters in retinal vascular bed, alterations in the length of vessel are believed to not play a role

in the regulation of ocular blood flow (OBF) (Delaey and Van de Voorde, 2000; Henkind and De Oliveira, 1968). In spite of the alterations in blood viscosity have been found to substantially influence retinal blood flow but such alterations do not occur without the presence of underlying pathology, such as sickle cell anaemia and other blood disorders (Perry and Hoagland, 1976). Blood viscosity may not play significant role in regulation of OBF in healthy subjects.

Vessel diameter plays a major role in determining retinal vascular resistance and OBF (Delaey and Van de Voorde, 2000). According to Poiseuille's Law vascular resistance is known to be inversely proportional to the 4th power of a blood vessel radius (Hayreh, 2008). A small change in vessel diameter can significantly influence vascular resistance. For example, a 2-time increase in radius results in decreases of resistance by 16-time. Therefore, vessel resistance is precisely sensitive to changes in radius. Reduction or unstable OBF could therefore occur, not only because of reduced OPP, but also because of an increase in vascular resistance due to alterations in vessel diameter (Flammer *et al.*, 2002). Changes in vessel diameter may occur due to structural or mechanical changes in the vessel wall and in the presence of a functional dysregulation.

Autoregulation is believed to be responsible for maintaining optic nerve head perfusion (Flammer *et al.*, 2001; Moore *et al.*, 2008). Autoregulation refers to the ability of the vascular system to modify vascular resistance in order to maintain a constant blood supply despite variations in perfusion pressure (Johnson, 1986). Autoregulation is present in retinal, ONH and choroidal circulation (Dumskyj *et al.*, 1996; Movaffaghy *et al.*, 1998; Polska *et al.*, 2007). The basic mechanisms underlying autoregulation are still unclear. The proposed mechanisms include

metabolic, myogenic, neurogenic and humoral factors in the ocular circulation (Johnson, 1986; Pournaras *et al.*, 2008). Endothelial derived vasoactive agents are also believed to play a role in autoregulation (Johnson, 1986; Pournaras *et al.*, 2008).

A tight coupling mechanism is thought to exist between tissue metabolism and ocular perfusion. Alterations in the local concentrations of metabolites such as O2, CO2, potassium and hydrogen may influence ocular vascular tone (Johnson, 1986). Partial oxygen pressure (pO2) has been identified as one of the main driving force behind metabolic autoregulation (Kontos *et al.*, 1978). Systemic hyperoxia causes reduction in blood flow and pO2 to retina and ONH by vasoconstriction (Gilmore *et al.*, 2005; Harris *et al.*, 1996). However, hypoxic conditions increased retinal blood flow and allowing normalisation of pO2 by vasodilatation (Eperon *et al.*, 1975). The haemodynamic response of the retinal vasculature to hyperoxia is thought to be mediated by endothelin (Takagi *et al.*, 1996). Endothelial derived vasodilator factors are believed to be responsible for hypoxia-induced vasodilatation of the retinal vasculature (Busse *et al.*, 1983).

In general, myogenic autoregulation is considered to be mechanically independent of the endothelium and is considered as intrinsic function of vascular smooth muscle cells. Stretching of the vessel wall causes depolarisation of the smooth muscle cells membrane and that leads to vasoconstriction (Schubert and Mulvany, 1999). In the cerebral circulation, however, myogenic induced vasoconstriction has also been suggested to be at least partly mediated by endothelial factors (Harder, 1987). Myogenic regulation has been found in the ONH and retina (Weinstein *et al.*, 1983). The eye has a rich autonomic innervation but not in the retina and prelaminar portion of the ONH (Laties, 1967; Ye *et al.*, 1990). Stimulation of sympathetic innervation

triggers constriction of the choroidal blood vessels and increases choroidal vascular resistance during the periods of increased heart rate or blood pressure (Bill and Linder, 1976). It has been suggested that vasoconstriction of the uveal vasculature may protect the eye against over perfusion (Bill, 1962). Parasympathetic innervation stimulates vasodilatation response of choroidal vasculature and increase blood flow to ONH (Ehinger, 1966). Humoral control refers to the potential regulatory influence of numerous vasoactive agents present in the circulating blood. The effect is through either direct interaction with the vascular smooth muscle cells and pericytes or through mediation of endothelial cells causing changes in OBF (Rossitti *et al.*, 1995). Endothelium plays an important role as an active regulator of vascular tone (Bassenge and Busse, 1988; Furchgott and Zawadzki, 1980). Endothelium also play a role as inhibitor of vascular smooth muscle cell proliferation, regulator of inflammation, thrombosis and platelet aggregation, angiogenesis and controls the vascular permeability (Deanfield *et al.*, 2007; Landmesser *et al.*, 2004). The role of endothelium as an active regulator of vascular tone is accomplished through endothelial derived vasoactive factors. Nitric oxide (NO) and endothelin-1 (ET-1) has been identified as the most potent vasodilator and vasoconstrictor respectively(Haefliger *et al.*, 1992).

Ischaemia or vascular dysregulation occurs as a consequence of an imbalance between NO and ET-1 (Su *et al.*, 2006). According to the vascular theory, chronic reduction of blood supply to the ONH results in ganglion cell damage (Flammer, 1994). So it is advocated that reduced ONH blood flow, as experienced in glaucoma patients (Findl *et al.*, 2000) is related to an altered endothelial function in the supplying vessels (Haefliger *et al.*, 1999; Haefliger *et al.*, 1994b). Constant

formation of NO by the endothelial and neuronal isoforms of the enzyme NO synthase (NOS) helps in the regulation of a basal vasodilator tone in the optic nerve head (Haefliger *et al.*, 1993; Luksch *et al.*, 2000). Changes in NO formation may shift the balance between vasodilator and vasoconstrictor endothelial agents causing alteration of blood supply leading to ischaemia of ONH (Hayreh, 2008).

1.1.2.3 Oxidative stress

Oxidative stress is an imbalance between the production of oxidants and antioxidants, in favour of the former, with the potential for damage or having too many reactive oxygen species (ROS) in relation to the available antioxidants is said to be a state of oxidative stress (Halliwell, 2006; Sies, 1997). Oxidation is a harmful process biochemically due to the loss of electrons and/or gain of oxygen by a molecule and is brought about by the action of oxidizing agent or oxidants (Halliwell, 2006). Oxidizing agent may take the form of free radicals when there is one or more of unpaired electrons, which are usually highly reactive. It can also be present as non-radical species, derived from either oxygen (reactive oxygen species, ROS) or nitrogen (reactive nitrogen species, RNS).

The retinal ganglion cells (RGCs) and axons of the ONH are considered particularly susceptible to oxidative stress due to their direct exposure to light, their high proportion of polyunsaturated fatty acids and high levels of oxygen (O2) consumption. In addition, the lack of myelin sheaths in RGCs and high concentration of mitochondria in axons of ganglion cells further increase the susceptibility to

oxidative stress (Chan, 2014). Oxidative stress has been implicated to play a causative role in the elevation of IOP and subsequent development of GON (Mozaffarieh *et al.*, 2008). Several studies have demonstrated depletion of total antioxidant potential and enhanced antioxidant activity in the aqueous humour and the presence of oxidative damage in the trabecular meshwork (TM) cells of glaucoma patients (Bunin *et al.*, 1991; Ferreira *et al.*, 2004; Ghanem *et al.*, 2010; Izzotti *et al.*, 2003b; Kahn *et al.*, 1983; Sorkhabi *et al.*, 2011; Wang *et al.*, 2001). This suggests the potential evidence of oxidative stress as a pathogenesis of glaucoma.

Astrocytes are found in the ONH and provide biochemical support to the RGC axon (Wilson and Di Polo, 2011). They have a high susceptibility to reactive oxygen species (ROS). ROS are chemically reactive molecules that contain oxygen, for example per oxide, super oxide and hydroxyl redical. ROS on activation, start producing a variety of abnormal molecules that include NO and ET-1 hence altering the microenvironment of the cell (Prasanna *et al.*, 2011). Diffusion of NO from the astrocytes into the neighbouring RGC axons, where levels of ROS such as O2 are high can lead to the formation of very damaging peroxynitrate (ONOO-) (Neufeld *et al.*, 1997). It is proposed that subsequent diffusion of both O2 and ONOO- within the RGC axons, towards the retina and LGN trigger the apoptotic loss of RGCs (Feilchenfeld *et al.*, 2008; Flammer and Mozaffarieh, 2007; Luthra *et al.*, 2005). Astrocytes become activated due to elevated IOP through direct mechanical effect, or indirectly by the apoptosis of RGC axons via disruption of axoplasmic transport and deprivation of essential growth factor (Liu *et al.*, 2006; Zhang and Neufeld, 2005). In addition, elevated ET-1 levels as part of the changes of the raised IOP may also

trigger the activation of astrocytes and initiate the apoptosis of RGC and their axons (Prasanna *et al.*, 2011).

Unstable blood flow and or alteration of autoregulation in the ocular and systemic circulation may lead to ischaemia of ONH (Hayreh, 2008). The absence of oxygen during ischaemia leads to impairment in electron transport in the mitochondria that result in inefficient energy production and production of extra electrons. Improvement in the perfusion leads to the reaction of these spare electrons with the available now plentiful supply of O2 molecules, leading to the formation of damaging ROS (Grace, 1994). Mitochondria are abundant in the RGC axons of the ONH putting it at higher risk of damage by ROS. The development of mild recurrent reperfusion injury that continues over a sustained length of time may initiate chronic oxidative stress, endothelial dysfunction, ONH damage and the development of GON (Flammer and Mozaffarieh, 2007; Mozaffarieh *et al.*, 2008).

1.2 Severity of POAG

Most people with open-angle glaucoma do not notice any change in vision at the early stage of the disease due to the central vision and sharpness is maintained. In addition, any initial loss in visual field is barely perceptible in the peripheral vision. By the time the patient perceives any symptoms of vision loss, the disease is quite advanced, and the damage is permanent. Even if detection is made at the early stage, effective medications such as eye drops or surgical intervention can at best retard

further progression. Untreated, glaucoma can severely restrict the visual field and irreversible blindness (Hattenhauer *et al*., 1998).

Staging the severity of GON damage is an essential component in treatment decisions making and providing visual prognosis to the patients (Medeiros *et al*., 2012a). Stages of GON can be divided into mild, moderate, and severe (Hodapp *et al.*, 1993). Staging of severity enables an ophthalmologist to monitor progression (Susanna Jr and Vessani, 2008). Patients with severe damage are at increased risk of developing functional impairment and require more aggressive interventions (Medeiros *et al*., 2012a). Severe glaucoma needs aggressive treatment to reach target pressure. Target intraocular pressure can be defined as the level of intraocular pressure associated with minimal probability of pressure induced visual field or optic nerve damage, or of the existing damage progression (Popovic-Suic *et al.*, 2005), The 5-year probability of glaucoma development was 4.40% in the group of patients with 20% reduction from the initial intraocular pressure, and 9.50% in the control group without intraocular pressure reduction in OHTS (Palmberg, 2002). Similarly early manifest glaucoma treatment (EMGT)study showed that 25% reduction from the initial intraocular pressure decreased the risk of progression by 25% (Heijl *et al.*, 2002). The AGIS investigators report on a 20% decrease from initial intraocular pressure or intraocular pressure decrease to < 18 mmHg in advanced glaucoma to be a useful strategy to achieve a desirable level of intraocular pressure (AGIS, 2000).

Currently standard automated perimetry (SAP) is considered as the gold standard for the quantification and progression of glaucoma functional loss (Brusini and Johnson, 2007). In addition to its role in clinical care, visual field assessment has traditionally been chosen as the primary endpoint in most clinical trials evaluating disease

progression and severity (Medeiros *et al.*, 2012b). Several methods have been suggested to stage glaucoma severity over time using different approaches. These approaches can be broadly divided into two categories: event- versus trend-based methods (Hitchings, 2007). For event-based analysis, the criterion for progression (the event) is defined at the start of the study, and progression is confirmed when VF changes fall below a pre-set threshold, compared with baseline (Hitchings, 2007). Trend analysis evaluates all of the visual field results that have been collected over a series of sequential examinations via techniques for linear regression (Spry and Johnson, 2002).

Detection of advanced glaucoma at the initial presentation increases the annual health budget due to multiple medications and also surgery may be required to halt the progression and retaining useful vision (Lee *et al.*, 2007). Numerous SAP staging systems of glaucoma severity have been developed in the past three decades (Brusini, 1995; Gandolfo, 1987; Hodapp *et al.*, 1993; Investigators, 1994; Mills *et al.*, 2006; Musch *et al.*, 1999).

1.3 Progression of glaucoma

Determination and monitoring of glaucoma progression remains a challenge to the ophthalmologists and researchers. This is due to the absence of a standard independent reference that provide a precise assessment and monitoring of

progression. An ideal method must be able to give useful information on visual field defects objectively and it should be reproducible and user friendly. In general, progression is based on structural and functional changes of the ONH (Hood and Kardon, 2007; Kourkoutas *et al.*, 2014). The classification system for progression should be consistent with the structural damage and able to detect even a small functional loss (Susanna Jr and Vessani, 2008). There are many examples of standard automated perimetry methods to detect progression (Brusini, 1995; Gandolfo, 1987; Hodapp *et al.*, 1993; Investigators, 1994; Mills *et al.*, 2006; Musch *et al.*, 1999; Quigley *et al.*, 1996).To assess progression, clinicians have long relied on tests of function such as visual fields (VF) and optical coherence tomography (OCT) allow them to quantify structural component of eye. CIGTS and OHTS support the view that structural damage may be a more sensitive indicator of glaucomatous progression than functional damage in patients with glaucoma or ocular hypertension (OHT) (Kass *et al.*, 2002; Parrish *et al.*, 2009). However, in many cases functional damage was the first sign of glaucomatous development in OHT eyes (Kass *et al.*, 2002). Several investigators have reported that changes in the optic nerve head precede visual field loss (Drance, 1978; Hart *et al.*, 1978)but recently investigations have reported the opposite: functional glaucomatous deficits can occur before structural changes (Harwerth *et al.*, 2007; Hood and Kardon, 2007). Hood and colleagues presented a model of GON damage in which they showed that either structural or functional damage can occur prior to each other (Hood and Kardon, 2007). Structural tests like OCT and functional tests such as VF perform better and provide more useful information at different severity stages of the GON (Medeiros *et al.*, 2012c). VF usually performs poorly at detecting early damage in glaucoma and

underestimates the rate of progression that may be occurring. But like VF, OCT has its limitations. As disease advances, OCT becomes ineffective and can't detect any further damage or progression (Medeiros *et al.*, 2012c).

Since glaucoma is a chronic progressive disease, progression occurs over a period of time. Determination of progression requires long observation and monitoring. Some patients will progress very slowly and need only minimal therapy and others progress at faster rates that will quickly lead to visual disability (Ahrlich *et al.*, 2010). Nearly half of the patients with open angle glaucoma showed progression despite treatment (Erdem *et al.*, 2015). EMGT showed that 45% of patients continue to show glaucoma progression even with IOP lowering treatments (Leske *et al*., 2003). EMGT showed that progression rate of OAG treatment group was 45% as compared with 62% in the control group (Heijl *et al.*, 2002). In another study on angle closure glaucoma in Malays found that 26% of patients with IOPs of 13–21 mmHg progressed in five years of follow-up period (Liza-Sharmini *et al*., 2009). The overall progression rate for POAG was 1.31(SD+/-1.93) (Heijl *et al*., 2009). In the Collaborative Initial Glaucoma Treatment Study (CIGTS), Lichter and associates found that visual field loss occurred in 10%–13.5% of participants during 5 years of follow up (Lichter *et al.*, 2001). Collaborative Normal Tension Glaucoma Study (CNTGS) study reported a mean progression of -0.39dB per year and 33% of all patients progressed (Anderson *et al.*, 2003).

The major obstacle in monitoring progression is the lack of uniformly accepted, sensitive and specific criteria to detect glaucoma progression over time. Evaluation of VF defects and monitoring visuals fields and comparing them with previous fields remains the clinical method most frequently used to assess the severity and

progression of glaucoma and effectiveness of treatment. Structurally, new or progressive thinning or haemorrhage of neural rim, saucerization of cup and functionally, glaucomatous visual field worsening (Seidel's, paracentral, arcuate scotoma or nasal step, as well as appearance of altitudinal defect, temporal island, or deep diffuse depression) are generally considered as criteria's for progression of glaucoma(Liza-Sharmini *et al.*, 2009). Serial visual field assessment is used to determine the functionally changes.

Perimetry is the technique used to measure the sensitivity (or extent) of the visual field (Besharse and Bok, 2011). Perimetry is divided into static and kinetic depending on whether or not the stimulus moves. Goldmann perimetry is a common example of kinetic perimetry. The Humphrey Field Analyser (HFA) is a common example of static perimetry. In fact, both perimeters have the capability of doing both tests, but in practice Goldmann is used for kinetic perimetry and HFA for static. Perimetry can also be categorized as automated or manual, depending on whether the stimulus location is changed by a computer as in the Humphrey visual field (HVF), or if stimulus is moved by hand, as in the Goldmann (Dersu *et al.*, 2006). Automated perimetry is a static perimetry, measuring the central 25-30º of the uniocular visual field (figure 1.2). However Esterman visual field perimetry is a binocular ocular testing method which examines more than 130° of the field is also available on HFA (Ayala, 2012) (figure 3). The principle of automated perimetry is based on identification of light stimuli of varying different intensities at various retinal locations while the patient fixates on a central target (Wroblewski *et al.*, 2014). The location and intensity of stimuli observed by the patients are recorded based on their responses (Heijl and Patella, 2002). Loss of sensitivity in the visual field is a direct

indicator to the loss of signal carrying retinal ganglion cell (RGC) axons and dendrites that determines the effective functional loss experienced by the patient (Heijl, 2000).