Evaluation of Interleukin-6 and Tumor Necrosis Factor-alpha In Tears and Serum and Its Associated Factors in Age Related Macular Degeneration Patients

DR ABDUL HADI BIN ROSLI

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DISCLAIMER

I hereby clarify that the work in this dissertation is of my own except for quotations, some figures, and summaries which have been duly acknowledged. I declare that I have no financial of interest in the instruments and the computer software used in this study.

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(Dr Abdul Hadi Bin Rosli)

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ABSTRACT

Introduction:

Age-Related Macular Degeneration (AMD) is a progressive neurodegenerative disease that affect the macula lutea. AMD is the leading cause of irreversible central vision loss in elderly population in developed countries. AMD is a multifactorial disease. The development of AMD involve continuous interaction between genetic factors, oxidative stress and environmental factors. Recent studies has been showing that inflammation plays a critical role in pathogenesis of AMD. Increased in Interleukin-6 (IL-6) and Tumor necrosis factor alpha (TNF-α) in the serum and intraocular fluid has been associated with AMD and its progression. Measurement of IL-6 and TNF-α in tears will provide a potential non-invasive biomarker for the progression and monitoring of AMD.

Objectives:

Our objective was to evaluate IL-6 and TNF-α in tears and serum between AMD patients and Control group as well as between Early AMD and Late AMD. Our objective also was to determine the association between IL-6 and TNF-α in tears with duration of AMD, serum level of IL-6 and TNF-α, smoking status and AMD status.

Methods:

A comparative cross-sectional study was conducted at a tertiary hospital in Malaysia, Hospital Universiti Sains Malaysia (USM) from June 2018 till May 2021. This study involved patients with Early AMD, Late AMD and Control group who attended Ophthalmology Clinic. Tears and serum samples were collected. The samples were analysed using commercial Human IL-6 and TNF-α ELISA kit to measure IL-6 and TNF-α levels in tears and serum. Statistical analysis was done using SPSS Inc Version 24.

Results:

A total of 142 patients (56 early AMD, 56 late AMD and 30 Control group) were recruited and analysed in this study. In the late AMD group, there was no late dry AMD and only include late neovascular AMD (nAMD). The adjusted mean for IL-6 in tears was significantly higher in AMD compared to Control group (21.91 (95%CI: 19.89, 23.93) vs 16.27 (95%CI: 12.32, 20.22), respectively, p= 0.014) after adjusted with covariates. The adjusted mean for IL-6 in serum also was significantly higher in AMD compared to Control group (12.01 (95%CI: 10.93, 13.08) vs 8.51 (95%CI: 6.41, 10.62), respectively, p= 0.004) after adjusted with covariates. The adjusted mean IL-6 in serum was significantly higher in Late nAMD compared to Early AMD (13.97 (95%CI: 12.43, 15.52) vs 10.03 (95%CI: 8.49, 11.58), respectively, p= 0.001) after adjusted with covariates. The adjusted mean TNF-α in serum was significantly higher in AMD compared to Control group (18.49 (95%CI: 17.11, 19.86) versus 13.96 (95%CI: 11.27, 16.65), respectively, p= 0.004) after adjusted with covariates. There was no significant association found between IL-6 and TNF-α level in tears with duration of AMD, serum level of IL-6 and TNF-α, smoking status and AMD status.

Conclusion:

Systemic IL-6 level was significantly higher in Late nAMD. There was no different in the level of IL-6 and TNF-α in tears between Early AMD and Late nAMD. There was also no significant factors associated with IL-6 and TNF-α in tears among AMD patients. A large cohort study is needed for further evaluation of tears inflammatory biomarkers level in AMD patients.

ABSTRAK

Pengenalan:

Degenerasi Makula yang berkaitan umur (AMD) adalah penyakit neurodegeneratif progresif yang mempengaruhi makula lutea. AMD adalah penyebab utama kehilangan penglihatan pusat di kalangan populasi warga tua di negara maju. AMD adalah penyakit multifaktorial. Perkembangan AMD melibatkan interaksi berterusan antara faktor genetik, tekanan oksidatif dan faktor persekitaran. Kajian terkini menunjukkan bahawa keradangan memainkan peranan penting dalam patogenesis AMD. Peningkatan Interleukin-6 (IL-6) dan Tumor necrosis factor alpha (TNF-α) dalam cairan serum dan cairan intraokular telah dikaitkan dengan AMD dan perkembangannya. Penganalisaan IL-6 dan TNF-α dalam cairan air mata akan memberikan potensi dari aspek biomarker bukan invasif bagi tujuan melihat perkembangan dan pemantauan AMD.

Objektif:

Objektif penyelidikan ini adalah untuk menilai tahap IL-6 dan TNF-α dalam air mata dan darah pesakit AMD dan kumpulan kawalan, dan juga antara kumpulan AMD Awal dan Lewat. Kajian ini juga untuk mengkaji hubungan antara tahap IL-6 dan TNF-α dalam air mata pesakit AMD dengan faktor-faktor seperti tempoh AMD, tahap IL-6 dan TNF-α dalam cairan serum, status merokok dan status AMD.

Kaedah:

Sebuah kajian keratan rentas telah dijalankan di sebuah hospital utama di Malaysia, Hospital Universiti Sains Malaysia (USM) antara Jun 2018 dan Mei 2021. Kajian ini melibatkan pesakit AMD (Awal dan Lewat) dan kumpulan kawalan yang hadir ke Klinik Oftalmologi. Sampel air mata dan cairan serum pesakit telah dikumpulkan. Analisis dijalankan untuk menguji paras IL-

6 dan TNF-α dalam air mata dan cairan serum menggunakan kit komersial Human IL-6 dan TNF-α ELISA kit. Analisis statistik dilakukan dengan menggunakan Pakej Statistik untuk Sains Sosial (SPSS Inc Versi 24).

Keputusan:

Sejumlah 142 pesakit (56 AMD Awal, 56 AMD lewat dan 30 kumpulan kawalan) telah terpilih untuk analisis dalam kajian ini. Dalam kumpulan AMD lewat, hanya AMD neovaskular lewat sahaja dipilih. Purata nilai diubahsuai IL-6 dalam air mata adalah lebih tinggi dengan signifikan dalam kumpulan AMD berbanding kumpulan kawalan (21.91 (95%CI: 19.89, 23.93) vs 16.27 (95%CI: 12.32, 20.22), secara berturutan, p= 0.014) setelah diubahsuai berdasarkan kovariat. Purata nilai diubahsuai IL-6 dalam darah juga adalah lebih tinggi dengan signifikan dalam kumpulan AMD berbanding kumpulan kawalan (12.01 (95%CI: 10.93, 13.08) vs 8.51 (95%CI: 6.41, 10.62), secara berturutan, p= 0.004), setelah diubahsuai berdasarkan kovariat. Purata nilai diubahsuai IL-6 dalam kumpulan nAMD Lewat adalah lebih tinggi dengan signifikan berbanding kumpulan AMD Awal (13.97 (95%CI: 12.43, 15.52) vs 10.03 (95%CI: 8.49, 11.58), secara berturutan, p= 0.001), setelah diubahsuai berdasarkan kovariat. Purata nilai diubahsuai TNF-α dalam darah adalah lebih tinggi dengan signifikan dalam kumpulan AMD berbanding kumpulan kawalan (18.49 (95%CI: 17.11, 19.86) vs 13.96 (95%CI: 11.27, 16.65), secara berturutan, p= 0.004), setelah diubahsuai berdasarkan kovariat. Tiada perbezaan yang signifikan diperolehi untuk hubungan antara tahap IL-6 and TNF-α dalam air mata pesakit AMD dengan tempoh AMD, tahap IL-6 and TNF-α dalam cairan serum, status merokok dan status AMD.

Kesimpulan:

Tahap IL-6 dalam darah adalah lebih tinggi dengan signifikan dalam kumpulan nAMD Lewat. Tiada perbezaan yang signifikan bagi tahap IL-6 dan TNF-α dalam air mata antara AMD Awal dan nAMD lewat. Tiada faktor yang mempunyai hubungan yang signifikan dengan tahap IL-6 dan TNF-α dalam air mata di kalangan pesakit AMD. Kajian kohort yang lebih besar disarankan bagi penilaian yang lebih lanjut dalam mengenalpasti tahap biomarker keradangan dalam pesakit AMD

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Study Introduction

Age-Related Macular Degeneration (AMD) is a progressive neurodegenerative disease that affect the macula lutea. Macula lutea is the most important structure for central vision, visualization of fine details and image resolution. Damage to the macula subsequently lead to central vision loss, metamorphopsia, central scotoma and blindness. As a consequence, AMD will definitely affect the patient's lifestyle due to disturbance in carry out daily life activity such as reading, driving and walking.

AMD is the leading cause of irreversible central vision loss in elderly population in developed countries. Worldwide, AMD affect approximately 8.7% of the elderly population and this number is expected to be increase to 196 million in 2020 and to 288 million in 2040 (Cascella R et al, 2014). Increasing number of AMD patients certainly will lead to global economic burden due to increasing treatment cost.

AMD can be classified into early AMD and late AMD. Early AMD is defined as presence of soft indistinct drusen or hard/ soft distinct drusen with pigmentary abnormality. Late AMD is defined as the presence of either geographical atrophy (GA) or neovascular AMD (nAMD). Central vision remain good until the disease progress to its advanced stage, characterized by GA or once it progress into nAMD leading to blindness. The main concern about nAMD is that the vision loss progress rapidly despite its painless course, and patient might seek treatment once haemorrhagic complication of the choroidal neovascularization (CNV) occurs or disciform macular scar already formed.

AMD is a multifactorial disease. Age is known to be the strongest risk factor for AMD due to increase number of retinal senescent cells lead to inflammatory reaction in retinal layers (Coleman HR et al, 2008; Chen M et al, 2015). Smoking also is the significant risk factor for AMD. Toxic substance and oxidative compound found in cigarette smoke lead to increased reactive oxygen species (ROS) formation and cause damage to the retinal pigment epithelium (RPE) cells (Cascella R et al, 2014). Other significant risk factors include female sex and white individuals (Coleman HR et al, 2008). Immunogenetic study of AMD showed association between AMD and complement factor H (CFH). CFH function to down regulate the alternative complement pathway. Dysregulation of complement cascade is believed to be contribute in the development of AMD (Knickelbein JE et al, 2015).

Pathogenesis of AMD involve complex process with interaction between inflammatory reaction and angiogenic process and restricted to the retina-choroid interface of the macula. The presence of drusen, which deposited between RPE and Bruch membrane is hallmark of AMD. It is known to contain many potentially damaging constituents including lipids, lipoprotein, RPE-derived cellular debris, oxidation by-product and inflammation-related factors.

The resulting macula cell hypoxia and death due to lack of oxygen and nutrient supply from the choriocapillaries subsequently will form areas of GA and bring the risk of visual loss. Cell hypoxia and ischaemia also will activate the inflammatory reaction. Pro-inflammatory cytokines such as Interleukin-6 (IL-6) and Tumor Necrosis Factor- α (TNF-α) are thought to play active role in the formation of angiogenesis complex (Naldini A et al, 2005: Yildirim Z et al, 2012). The IL-6 and TNF-α will cause activation of endothelial cells which in advance state,

lead to increase in vascular permeability and leakage of fluid and protein. The IL-6 and TNF-α also are found to cause up-regulation of Vascular Endothelial Growth Factor (VEGF) secretion. VEGF has potent angiogenic properties. VEGF will lead to formation of CNV, which is can lead to submacular hemorrhage.

Numerous data available from previous studies showed elevation of IL-6 and TNF-α in various ophthalmological disease (Da Cunha AP et al, 2017; Wan L et al, 2010; Forouhe ZJ et al, 2017). In AMD, there have been reports of elevated level of IL-6 and TNF-α in serum, aqueous fluid, vitreous fluid and neovascular membrane (Yildirim Z et al, 2012; Chen M et al, 2015; Sato K et al, 2018; Abcouwe SF et al, 2013; Wan L et al, 2010). Previous study also found that IL-6 and TNF-α cause up-regulation of VEGF (Naldini A et al, 2005: Yildirim Z et al, 2012). However, to the best of our knowledge, no research has been done on detecting tears level of IL-6 and TNF-α in AMD patients. We strongly believe this non-invasive study will help us to determine high risk early AMD patients that may eventually progress to late AMD if left untreated, thus facilitate us in providing early prophylactic treatment.

1.2 Study Rationale

The focus of this study is to evaluate the IL-6 and TNF-α levels in the tears and serum of patients with AMD. Previous study reported significant higher level of IL-6 and TNF-α in serum, aqueous fluid and vitreous of AMD patient. Previous study also showed significant correlation between IL-6 and TNF-α and VEGF. However, there is no study done to check the level of IL-6 and TNF-α in tears of AMD patient. Hence, measurement of IL-6 and TNF-α in tears will provide a potential non-invasive biomarker for the progression of AMD.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Age-Related Macular Degeneration

Age-Related Macular Degeneration (AMD) is a progressive neurodegenerative disease that affect the macula lutea (Cascella R et al, 2014). AMD is the leading cause of irreversible central vision loss in elderly population in developed countries (Cascella R et al, 2014; Parmeggiani F et al, 2013; Coleman HR et al, 2008). Worldwide, AMD affect approximately 8.7% of the elderly population and this number is expected to be increase to 196 million in 2020 and to 288 million in 2040 (Cascella R et al, 2014).

Macula lutea is the most important structure for central vision, visualization of fine details and image resolution. AMD will affect all retinal layers of macula involving photoreceptor, RPE and Bruch membrane overlying it. Damage to the macula subsequently lead to central vision loss, metamorphopsia, central scotoma and blindness. As a consequence, AMD will definitely affect the patient's lifestyle due to disturbance in carry out daily life activity such as reading, driving and walking.

2.1.1 Risk Factors for Age-related Macular Degeneration

AMD is a multifactorial disease. The development of AMD involve continuous interaction between genetic factors, oxidative stress and environmental factors (Cascella R et al, 2014; Coleman HR et al, 2008; Knickelbein JE et al, 2015). Age is the strongest risk factor for AMD due to increase number of retinal senescent cells lead to inflammatory reaction in retinal layers (Coleman HR et al, 2008; Chen M et al, 2015). Smoking also is the significant risk factor for AMD. Toxic substance and oxidative compound found in cigarette smoke lead to increased

reactive oxygen species (ROS) formation and cause damage to the RPE cells (Cascella R et al, 2014). Other significant risk factors include female sex and white individuals (Coleman HR et al, 2008).

Immunogenetic study of AMD showed association between AMD and complement factor H (CFH), an integral component of the alternative pathway of complement activation. CFH function to down regulate the alternative complement pathway. Dysregulation of complement cascade is believed to be contribute in the development of AMD. The gene for this protein located on chromosome 1q32 (Knickelbein JE et al, 2015).

2.1.2 Classification of Age-related Macular Degeneration

Based on Wisconsin Age-related Maculopathy Grading System (WARMGS) from clinical guideline of AMD (The Royal College of Opthalmology, 2013), AMD can be classified into early AMD and late AMD (Figure 2.1). **Early AMD** is defined as (1) soft indistinct or reticular drusen or (2) hard distinct or soft distinct drusen with pigmentary abnormality (RPE depigmentation or increased retinal pigment). **Late AMD** is defined as the presence of either (1) geographical atrophy (GA) or (2) neovascular AMD (nAMD).

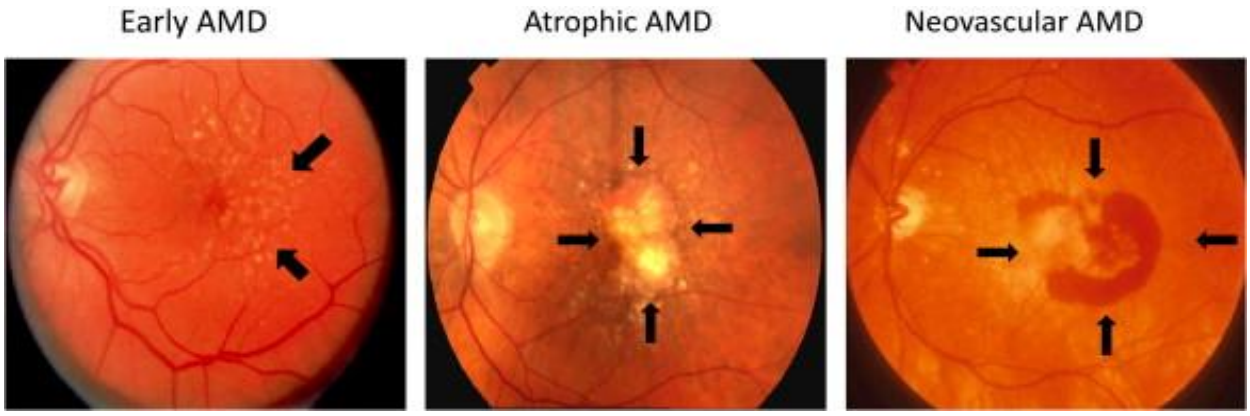

Figure 2.1: Classification of AMD by WARMGS

GA or atrophic AMD is a sharply demarcated retinal depigmentation at least 175 μm in diameter with visible choroidal vessel. It result from atrophy and complete loss of RPE cells. nAMD is characterised by serous hemorhagic detachment of either the RPE or sensory retina, the presence of subretinal fibrous tissue or scar. Neovascularisation developing under the retina which can leak or bleed. It can cause severe and vision loss (Coleman HR et al, 2008). About 12% of AMD patients may develop both GA and nAMD (Chen M et al, 2015).

Other classification used for AMD is the Age-Related Eye Disease Study Group (AREDS) (Klein R et al, 2014). AMD is classified into early, intermediate and advance form (Table 2.1). AREDS Classification is useful to determine treatment of vitamin supplementation for AMD patient. For this study, grading by WARMGS will be used.

Table 2.1: Classification of AMD by AREDS.

Classification	Description
No AMD (AREDS category 1)	No or few small drusen (<63 μm in diameter)
Early AMD (AREDS category 2)	Multiple small drusen, few intermediate drusen (63–124 μm in diameter), or mild RPE abnormalities
Intermediate AMD (AREDS category 3)	Numerous intermediate drusen or at least one large drusen (125 μm or larger) or GA not involving the center of the fovea
Advanced AMD (AREDS category 4)	GA of RPE involving the foveal center Neovascular maculopathy including choroid neovascularization, pigment epithelial detachment, retinal hard exudates, subretinal and sub-RPE fibrovascular proliferation, disciform scar.

Abbreviation: AMD: Age-Related Macular Degeneration; AREDS: Age-Related Eye Disease Study Group; RPE: Retinal Pigment Epithelium; GA: Geographical Atrophy

2.2 Pro-Inflammatory Cytokine

Cytokines are low molecular weight protein which are secreted by group of cell in response to cellular interaction and communication. Cytokines are involved in the immune response and it became the key modulator of inflammation by determine which group of cell or antibody will be involved in host defence mechanism.

Cytokine is general name. The specific name will be depending on its cell origin and function. For example, interleukin (cytokine produced by one leukocyte and acting on other leukocyte), monokines (cytokine produced by monocyte), lymphokine (cytokine produced by lymphocytes) and chemokine (cytokine with chemotactic activity). Regarding the action on cells, cytokines have autocrine action (act on cells that secrete them), paracrine action (act on nearby cells) and endocrine action (act on distant cells).

Pro-inflammatory cytokine can be produced by many cell group. However, it is predominantly produce by T-helper cell and macrophages (Jun-Ming Z et al, 2007). Pro-inflammatory cytokine directly causing up regulation of inflammatory process and participating in acute and chronic inflammatory reaction. Pro-inflammatory cytokine also act as chemotactive agent by which target cell release chemokine like Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-inducible Protein-10 (IP-10) and Keratinocyte Chemoattractant (KC) to recruit more inflammatory cell to the tissue/organ (Da Cunha AP et al, 2017). TNF-α, IL-6, IL-1β and IL-17a are the common pro-inflammatory cytokines, which found to be key player in inflammatory reaction (Da Cunha AP et al, 2017).

2.2.1 Pro-Inflammatory Cytokine in Ocular Inflammation and Angiogenesis

In Ophthalmology, these pro-inflammatory cytokines are known to be involved in various inflammatory and angiogenis ocular disease such as uveitis, dry eye syndrome, AMD, diabetic retinopathy and diabetic macular oedema (Da Cunha AP et al, 2017). Activation of pro-inflammatory cytokines can be due to multiple factors such as infection, hypersensitivity, tissue stress and injury, oxidative stress and aging tissues/cells (Kauppinen A et al, 2016; Wakefield D et al, 1992). Generally it contribute to the disease process by causing up-regulation of inflammatory reaction and regulate the recruitment of inflammatory cells to the ocular tissue.

Activation of inflammatory reaction by pro-inflammatory cytokines lead to activation of endothelial cells, which is the most critical phase at the early of inflammation process. Following endothelial cell activation, there is increased expression of leukocyte adhesion molecule, cytokine, growth factor and Human Leukocyte Antigen (HLA) molecules. These all process lead to increase of blood vessel permeability. The circulating leukocyte begin to make contact with the adhesion molecules expressed by endothelial cell. Subsequently, leukocyte able to leave the circulation and moving towards the damaged ocular tissue. Besides leukocyte, fluids and various plasma protein also gain access to the site of tissue damage (Kauppinen A et al, 2016).

In ocular disease such as wet AMD, proliferative diabetic retinopathy (PDR) and neovascular glaucoma, apart of inflammation, angiogenesis process plays an important role in the progression of the disease. Angiogenesis is a complex process. A specific microenvironment need to be establish to allow neovascularization to take place. Inflammatory cells such macrophages, monocyte and T-lymphocyte highly contribute to the angiogenic process.

Upon stimulation in inflammatory reaction, these cells will further increase the secretion of the pro-inflammatory and anti-inflammatory cytokines which subsequently control the endothelial cell proliferation, survival, migration and activation (Naldini A et al, 2005). For example, the macrophage will elicit further secretion of TNF-α which subsequently stimulate other production of pro-inflammatory cell, monocyte colonization protein and RPE-secreted VEGF. Increase production of VEGF will lead to neovascularization in ocular disease (Campa C et al, 2010). Besides that Sato K et al, (2018) and Chalam KV et al, (2014) reported that pro-inflammatory cytokines like IL-6 able to induce production of VEGF that lead to neovascularization.

2.2.2 Interleukin-6 (IL-6)

IL-6 is classified as T2-type of interleukin which are involved in humoral immune response. IL-6 is produced by variety of cells including inflammatory cells (lymphocyte, macrophage, monocytes, and neutrophils) and non-inflammatory cells (fibroblast, myocytes, osteoblast and endothelial cells) (Forouhe ZJ et al, 2017). Within the eye, few structures are able to secret IL-6 including RPE, iris, ciliary body and muller cells (Ahmed HM et al, 2014).

Human IL-6 consists of 212 amino acids, including 28-amino-acid signal peptides. Its corresponding gene is located on locus chromosome 7p21. The core protein is ∼20kDa, however, the glycosylation accounts for the size of 21–26 kDa of natural IL-6. The receptor-signalling system of IL-6 is formed by two receptor chains and downstream signalling molecules. The two receptor chains are, (i) IL-6 binding chain made up in 2 forms, 80 kDa transmembrane and 50–55 kDa– soluble IL-6 receptor (sIL-6R); (ii) signal transducing chain formed by 130 kDa gp130. Both of these proteins induce the pleotropic effect of IL-6 on

various cells. The pleotropic role of IL-6 explained by the number of genes regulated by this interleukin. The cellular signalling pathway shows both pro- and anti-inflammatory effects have been associated with IL-6 synthesis, which highlighting its importance in activation and regulation of the immune response (Tanaka et al, 2014). Figure 2.2 showed crystal structure of IL-6.

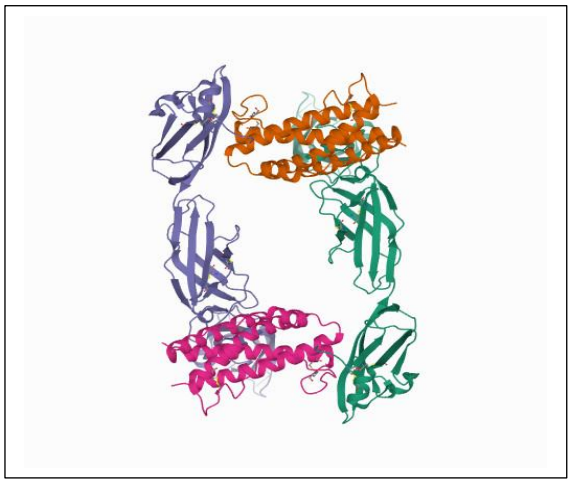

Figure 2.2: Crystal structure of IL-6

IL-6 is a multifunctional cytokines that has multiple role in human body. It plays an important role as pro-inflammatory cytokines in immune response regulation, acute phase reaction, haematopoiesis and host defence mechanisms (Shimizu E et al, 2002). Elevated levels of IL-6 have been detected in many ocular diseases such as glaucoma, central retinal vein occlusion, macular oedema, ocular neovascularisation, posterior capsule opacity formation, keratitis, dry eye disease, ocular autoimmune disease, cornea chemical burn and ocular inflammation (Forouhe ZJ et al, 2017; Shimizu E et al, 2002). IL-6 can be found present in tears fluid of healthy person eventhough it is a pro-inflammatory cytokine (Carreño E et al, 2010). However it is found to elevate in tears fluid in such case like dry eye disease (Yoon et al, 2007).

2.2.3 Tumor Necrosis Factor-α (TNF-α)

TNF-α is a member of the chemokines, plays an important inflammatory cytokines in metabolic and immunological respons. TNF-α is one of the earliest and most critical mediators in inflammation (Lacomba MS et al, 2001; Wan L et al, 2010). TNF-α secreted mainly by macrophages, monocytes, neutrophils, mast cells, T-lymphocytes and other cells such as stromal and malignant cells (Naldini A et al, 2005; Lacomba MS et al, 2001).

Human TNF-α is made up by 27-kDa (233 amino acid) protein, in which then cleaved proteolytically by a metalloprotease TNF-α-converting enzyme (TACE) to a 17-kDa (157 amino acid) molecules. The 27-kDa protein consists of highly conserved 76-amino-acid, and acts as a membrane integrated protein of TNF-α (mTNF-α).The 17-kDa is a soluble TNF-α (sTNF-α), consists of two antiparallel β-pleated sheets and antiparallel β-strands, form a 24 jelly-roll β-structure. Both these mTNF-α and sTNF-α involve in biological responses at autocrine/paracrine and endocrine levels, respectively (Parameswaran et al, 2010). Figure 2.3 showed crystal structure of TNF-α.

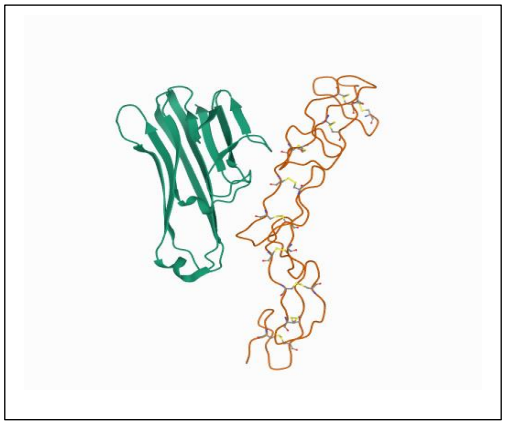

Figure 2.3: Crystal structure of TNF-α

TNF-α is well known as pro-inflammatory cytokines. Previous study showed significant increase in level of TNF-α in various inflammatory ocular disease such as AMD; in neovascular membrane (Wan L et al, 2010), PDR; in tears (Costagliola C et al, 2013), uveitis; in serum and aqueous humor (Wakefield D et al, 1992; Lacomba MS et al, 2001), dry eye syndrome, Sjogren disease; in tears (Yoon et al, 2007) and ocular cicatricial pemphigoid disease; in tears (Lee SJ et al, 1993).

2.3 Pro-Inflammatory Cytokines and Pathogenesis of Age-related Macular Degeneration

Pathogenesis of AMD involve complex process with interaction between inflammatory reaction and angiogenic process (Campa C et al, 2010) and restricted to the retina-choroid interface of the macula (Chen M et al, 2015). The presence of drusen is hallmark of AMD (Knickelbein JE et al, 2015). Drusen are focal areas of eosinophilic extracellular deposits located between RPE and Bruch membrane. It is known to contain many potentially damaging constituents including lipids, lipoprotein, RPE-derived cellular debris, oxidation by-product and inflammation-related factors (Kauppinen A et al, 2016). Drusen are formed by combination of basal deposit which has two distinct types; basal laminar and basal linear. Basal laminar composed of basement membrane protein and collagen while basal linear composed mainly of membranous material. Drusen can be classified into hard (hyaline) drusen which are discrete yellowish Periodic Acid Schift (PAS)-positive nodule composed of hyaline material, <63 μm with low risk for visual loss. It is common occurring in 80% of population. There are also soft drusen with amorphous, poorly demarcated boundaries, size >63 μm. Soft drusen are precursors to AMD.

During aging, macula damage occur via 2 main ways; (1) Bruch membrane thickened and decrease permeability (2) RPE function declines and density of choriocapillaries reduced. These will cause impaired oxygen and nutrient supply by the choroidal circulation to the macula as well as impaired disposal of the metabolic waste material of the retina through RPE/ Bruch membrane to the choroid. Later formation of drusen between RPE and Bruch membrane will further block the oxygen and nutrient from the choriocapillaries to access the RPE and photoreceptor cells. As a result, cells suffer from noxious insult, and will secrete number of pro-inflammatory cytokine and chemokines in attempt to promote tissue repair, maintain hemostasis and to restore functionality (Chen M et al, 2015). Further deterioration in oxygen and nutrient supply lead to atrophy and death of RPE and photoreceptor. In late stages, central atrophy of RPE and photoreceptors form areas of GA. GA will bring risks for visual loss due to foveal involvement.

In nAMD, cell hypoxia and ischaemia will further activate inflammatory reaction. These pro-inflammatory mediators will further stimulate recruitment of macrophage and monocyte to the site of inflammation as well as activate the tissue complement system. These inflammatory reaction able to degrade the integrity of the Bruch membrane, RPE and also outer blood retinal barrier (BRB). Retina represent an immune-privileged tissue provided by intact physical barriers which is BRB and Bruch membrane. This protective mechanism prevent inappropriate immune reaction to the retinal cells, hence reduce the risk of inflammation-mediated retinal damage. In nAMD, degradation of Bruch membrane increases likelihood of break allowing for buds of neovascularization tissue from choriocapillaries to perforate the outer aspect of retina. Degradation of BRB also allow inflammatory mediators to make contact with retinal cells, hence further damage the cells. Figure 2.4 showing the pathogenesis of AMD.

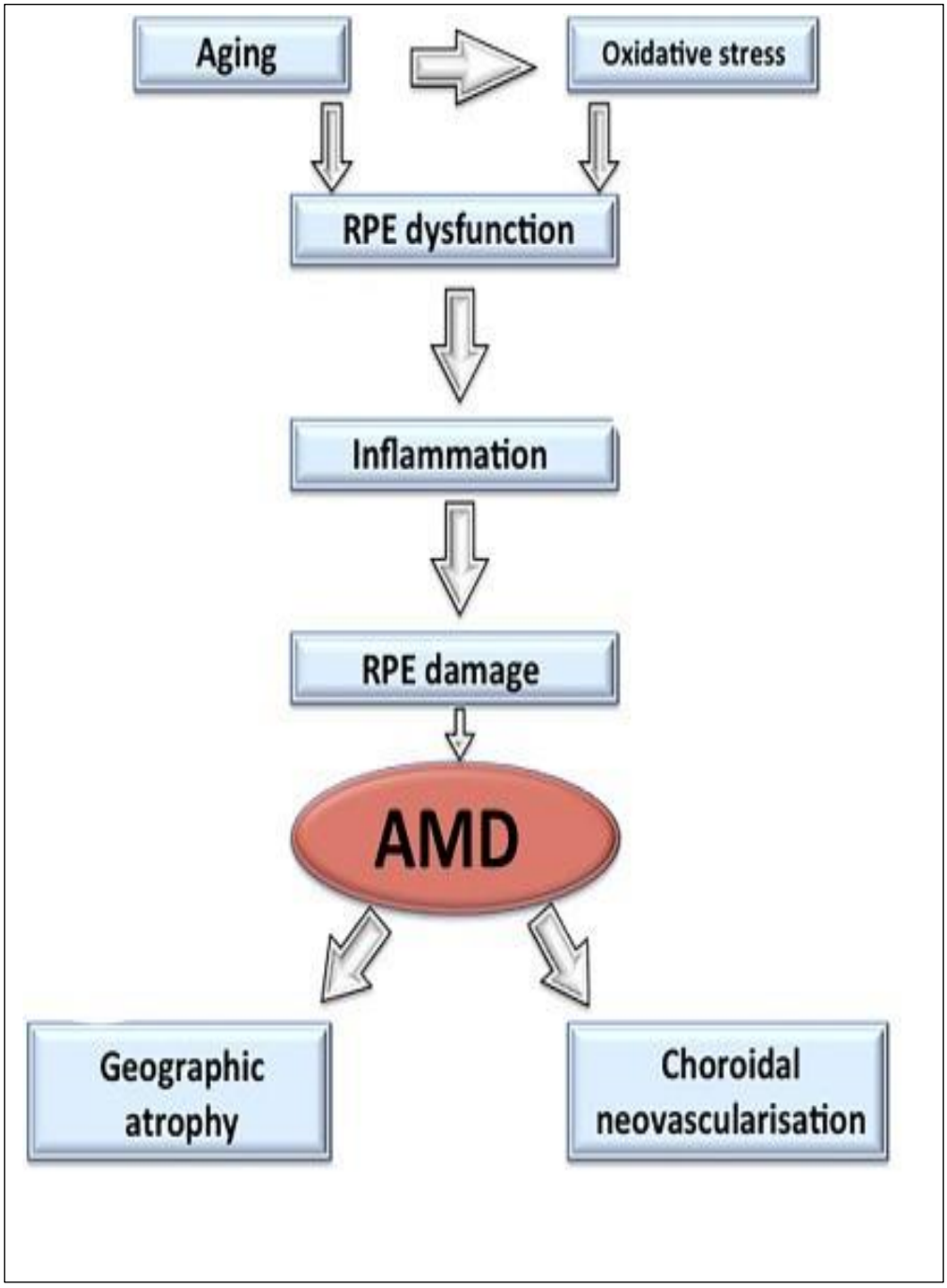

Figure 2.4: Pathogenesis of AMD

The formation of angiogenesis complex occur through action of pro-inflammatory cytokine that causes over-expression of VEGF. Pro-inflammatory cytokines such as IL-6 and TNF-α (Figure 2.5) are found to cause up-regulation of VEGF secretion (Naldini A et al, 2005; Yildirim Z et al, 2012). VEGF will lead to formation of CNV, which is can leak and bleed, and cause formation of subretinal fibrosis, scarring, serous retinal detachment, subretinal and intraretinal hemorrhage. It also enhances fibrovascular membrane formation around the CNV and inner aspects of Bruch membrane. These fibrovascular membrane complex may destroy normal architecture of Bruch membrane, RPE and photoreceptor to forms a discifom scar.

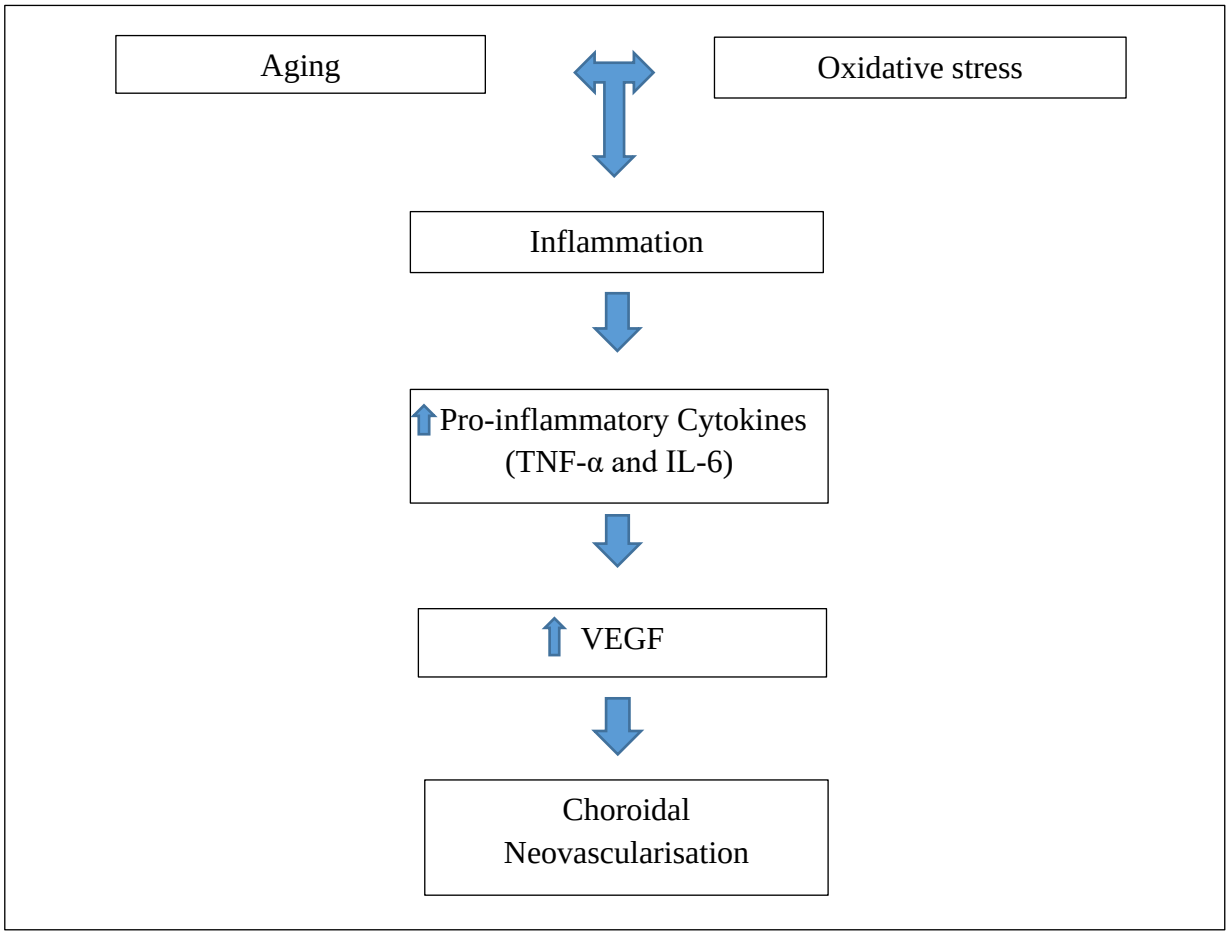

Figure 2.5: Pro-Inflammatory cytokine (IL-6 and TNF-α) in the pathogenesis of neovascular AMD

In AMD, following insult towards retinal cells, there is increased secretion of IL-6. The IL-6 will cause activation of endothelial cell which in advance state, lead to increase in vascular permeability and leakage of fluid and protein (Kauppinen A et al, 2016). Previous study done by Yildirim Z et al, (2012) showed increased serum level of IL-6 in AMD. IL-6 also has angiogenesis property and is thought to act as an inducer of VEGF. There is significant relationship between IL-6 and VEGF in aqueous and vitreous fluid (Sato K et al, 2018; Abcouwe SF et al, 2013; Ahmed HM et al, 2014). Systemic level of IL-6 has been shown to correlate with incidence of progression of AMD (Klein R et al, 2014; Seddon JM et al, 2005). Study by Chalam KV et al, (2014) found that aqueous IL-6 can be important marker of treatment response in AMD patient receiving intravitreal Bevacizumab injection, as IL-6 associated with the progression of choroid neovascularisation.

In progression of AMD, TNF-α plays a role as an inflammatory mediators and angiogenic agent. In inflammatory reaction, TNF-α found to cause activation of endothelial cells, which the most prominent process at the early phase of inflammation (Naldini A et al, 2005; Tracey KJ et al, 1994). TNF-α has angiogenesis property to stimulate development of new vessels (Wan L et al, 2010). It is believed, TNF-α causing neovascularisation by up-regulation of VEGF and by mediating macrophage-induced angiogenesis (Naldini A et al, 2005). Previous study by Knickelbein JE et al, (2015) showed systemic TNF-α are correlated with CFH activation in AMD patient. TNF-α also has been found in neovascular membrane of wet AMD (Wan L et al, 2010; Theodossiadis P G et al, 2009). The serum level of TNF-α also increased in AMD patient (Chen M et al, 2015).

CHAPTER 3

OBJECTIVES, RESEARCH QUESTIONS, RESEARCH HYPOTHESIS

3. OBJECTIVES, RESEARCH QUESTIONS, RESEARCH HYPOTHESIS

3.1 Research Objectives

3.1.1 General Objectives

i. To evaluate the level of IL-6 and TNF-α in tears and serum and its associated factors in AMD patients

3.1.1 Specific Objectives

i. To compare the level of IL-6 and TNF-α in tears between AMD and Control
ii. To compare the level of IL-6 and TNF-α in tears between Early and Late AMD
iii. To compare the level of IL-6 and TNF-α in serum between AMD and Control
iv. To compare the level of IL-6 and TNF-α in serum between Early and Late AMD
v. To identify the associated factors (AMD status, duration of AMD, serum level of IL-6 and smoking status) of tears IL-6 in AMD patients
vi. To identify the associated factors (AMD status, duration of AMD, serum level of TNF-α and smoking status) of tears TNF-α in AMD patients

3.2 Research Question

3.2.1 Is there any difference in level of IL-6 and TNF-α in tears between AMD and Control

3.2.2 Is there any difference in level of IL-6 and TNF-α in tears between Early and Late AMD

3.2.3 Is there any difference in level of IL-6 and TNF-α in serum between AMD and Control

3.2.4 Is there any difference in level of IL-6 and TNF-α in serum between Early and Late AMD

3.2.5 Is there any associated factors of tears IL-6 in AMD patients

3.2.6 Is there any associated factors of tears TNF-α in AMD patients

3.3 Research Hypothesis

3.3.1 There is significance difference in level of IL-6 and TNF-α in tears between AMD and Control

3.3.2 There is significance difference in level of IL-6 and TNF-α in tears between Early and Late AMD

3.3.3 There is significance difference in level of IL-6 and TNF-α in serum between AMD and Control

3.3.4 There is significance difference in level of IL-6 and TNF-α in serum between Early and Late AMD

3.3.5 There is associated factors of tears IL-6 in AMD patients

3.3.6 There is associated factors of tears TNF-α in AMD patients

CHAPTER 4

RESEARCH METHODOLOGY

4. RESEARCH METHODOLOGY

4.1 Study Design

A comparative cross-sectional study was conducted in the Ophthalmology Clinic Hospital Universiti Sains Malaysia (USM) from June 2018 till May 2021. The study population involved all AMD patients attending Ophthalmology Clinic Hospital USM during the study duration and fulfilled the selection criterias. A control group of non-AMD patient, who attended Opthalmology Clinic Hospital USM were recruited in the study for comparison.

4.2 Ethical Approval

This study was conducted after approval by the local Human Research Ethics Committee (Universiti Sains Malaysia [USM]/ Jawatankuasa Etika Penyelidikan Manusia [JEPEM]; Registration Number: 18100488] (Appendix A), with the highest respect for subjects according to the study protocol, the ethical principles that have their origin in the Declaration of Helsinki, and the International Conference on Harmonisation (ICH) – Harmonised Tripartite Guideline for Good Clinical Practice (GCP).

4.3 Selection Criteria

The study group include newly diagnose patients with Early and Late AMD, aged 45 to 70 years old. Any patients with vitreoretinal pathology such as diabetic retinopathy and retinal vein occlusion, refractive error more than -5.00DS, myopic maculopathy, macular dystrophies and any macular pathology were excluded. Other ocular exclusion criteria were: (a) History of ocular surgery last 6 months eg vitrectomy, cataract extraction; (b) History of ocular surface disease/corneal pathology including dry eye disease; (c) history of ocular inflammation/ocular autoimmune disease (eg uveitis, chorioretinitis) and (d) patients with systemic disease such as