EVALUATION OF IL-6 AND TNF-α LEVEL IN SALIVA AMONG AMD PATIENTS

DR SAROJINI KRISHNAN

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF MEDICINE (OPHTHALMOLOGY)

UNIVERSITI SAINS MALAYSIA

2020

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.

Date: 15th November 2020

Dr Sarojini Krishnan

ACKNOWLEDMENTS

First and foremost, I am grateful to God for the good health and wellbeing that were necessary to complete this book.

I wish to express my greatest appreciation and gratitude to my supervisor, Prof. Datin. Dr. Zunaina Embong, Consultant Ophthalmologist, Department of Ophthalmology, School of Medical Sciences, Universiti Sains Malaysia for her earnestness in teaching, undivided guidance and providing continuous encouragement and support during the preparation of this dissertation.

I am thankful to my co-supervisors, Dr Wan Nazatul Shima Shahidan and Dr Siti Lailatul Akmar Zainuddin, School of Dental Sciences, Universiti Sains Malaysia for the great support in conducting this study. I also take this opportunity to express my appreciation to all lecturers in the Department of Ophthalmology, technicians, supporting staffs, and all my ophthalmology peers and colleagues for their constant support and ever willingness to help with the completion of this dissertation. In addition, I would like to extend my gratefulness to all the patients for their involvement.

Last but not least, I would also like to place on record my sense of indebtedness towards my family members for their undeterred support and encouragement. A special thank you to my beloved mother for all the support and sacrifices she had made for me in completing this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Introduction:

Age related macular degeneration (AMD) is known as a leading cause of blindness in developed countries and the third leading cause of blindness worldwide, in elderly population more than 50 years of age. Multifactorial etiology involves in AMD pathogenesis includes both genetic backgrounds and environmental risk factors. Recent evidence has been showing that inflammation has a crucial role in AMD development and progression. Increased in Interleukin 6 (IL-6) and Tumor necrosis factor alpha (TNF-α) were associated with AMD. Saliva protein biomarkers are fast development novel technique for clinical diagnosis. Thus, identification of IL-6 and TNF-α in saliva perhaps provide insight the role of saliva sample as a diagnostic alternative in future.

Objective:

Our objective was to evaluate IL-6 and TNF-α level in saliva between AMD patients and Control group, and also in between Early and Late AMD. Besides that, to determine the relationship between IL-6 and TNF-α level in saliva with duration of AMD.

Methods:

A comparative cross-sectional study was conducted exclusively at a tertiary hospital in Malaysia, Hospital Universiti Sains Malaysia (USM) between January 2018 and May 2020. This study involved patients with Early AMD, Late AMD and Control group. Unstimulated saliva samples were collected. Laboratory analysis using commercial Human IL-6 and TNF-α

ELISA kit (LEGEND MAX^{TM}, Biolegend, Inc.) was performed to measure IL-6 and TNF-α levels in saliva. Statistical analysis was done using SPSS Inc Version 24.

Results:

A total of 92 patients were included into the study and 88 were analysed after data cleaning (Early AMD: 29 patients, Late AMD: 30 patients, Control: 29 patients). There was no significant difference of mean IL-6 and TNF-α level in saliva between AMD and Control group after adjusted with covariates. Mean IL-6 in saliva was significantly higher in Late AMD (8.64 SD 6.58 pg/ml) compared to Early AMD (5.07 SD 4.29 pg/ml) (p=0.017), however, it was not significant after adjusted with the covariates (p=0.173). There was no significant difference of mean TNF-α in saliva between Early and Late AMD (-26.60 SD 1.29 pg/ml vs -26.13 SD 1.67 pg/ml, p=0.234). There was no significant association was found between duration of AMD with IL-6 and TNF-α level in saliva. Although few factors were significantly associated with IL-6 and TNF-α level in saliva on simple linear regression, however, none of the factors had significant association on multiple linear regression.

Conclusion:

This study showed that there was no significant associations between IL-6 and TNF-α level in saliva among AMD patients. AMD pathogenesis more confined to a local inflammatory process and ocular concentrations of inflammatory biomarkers in the pathological eye are not necessarily associated with systemic concentration. Furthermore, a large cohort study is needed for further evaluation to show the significant correlation between saliva inflammatory biomarkers level with ocular pathology.

ABSTRAK

Pengenalan:

Degenerasi makula yang berkaitan dengan usia (AMD) adalah penyebab kebutaan utama di negara maju dan penyebab ketiga kebutaan di seluruh dunia, pada populasi warga tua yang berumur lebih dari 50 tahun. Pelbagai faktor penyebab terlibat dalam proses penyakit AMD, termasuk genetik dan factor risiko persekitaraan. Penyelidikan terkini menunjukkan bahawa keradangan memainkan peranan penting yang cenderung untuk membuat AMD menjadi lebih teruk. Peningkatan Interleukin-6 (IL-6) dan *tumor necrosis factor alpha* (TNF-α) dikaitkan dengan AMD. Penganalisan biomarker protein dalam air liur adalah teknik terkini yang berkembang dengan pantas dalam membantu diagnosis klinikal. Oleh itu, pengenalpastian Il-6 dan TNF-α dalam air liur mungkin memberikan gambaran mengenai peranan sampel air liur sebagai alternatif diagnostik pada masa akan datang.

Objektif

Objektif penyelidikan ini adalah untuk menilai tahap pengenalpastian Il-6 dan TNF-α dalam air liur pesakit AMD dan kumpulan kawalan (kontrol), dan juga antara AMD Awal dan Lewat. Selain itu, untuk mengetahui hubungan antara tahap IL-6 dan TNF-α dalam air liur pesakit AMD dengan tempoh AMD.

Kaedah

Sebuah kajian keratan rentas telah dijalankan secara eksklusif di sebuah hospital utama di Malaysia, Hospital Universiti Sains Malaysia (USM) di antara Januari 2018 sehingga Mei 2020. Kajian ini telah melibatkan pesakit AMD (Awal dan Lewat) dan kumpulan kawalan. Sampel air liur yang tanpa rangsangan telah dikumpulkan. Analisis makmal dijalankan untuk menguji paras IL-6 dan TNF-α dalam air liur dengan menggunakan kit komersial Human

VEGF ELISA (LEGEND MAX^{TM}, Biolegend, Inc.). Analisis statistik dilakukan dengan menggunakan Pakej Statistik untuk Sains Sosial (SPSS Inc Versi 24).

Keputusan:

Seramai 92 pesakit yang terdiri daripada pesakit AMD dan kumpulan kawalan telah terlibat dalam kajian ini dan 88 pesakit telah terpilih untuk analisis (AMD Awal: 29 orang, AMD Lewat: 30 orang dan kawalan: 29 orang). Hasil kajian menunjukkan tiada perbezaan yang ketara dalam purata nilai IL-6 dan TNF-α dalam air liur antara pesakit AMD dan kumpulan kawalan setelah diubahsuai berdasarkan kovariat. Purata nilai IL-6 dalam air liur pesakit AMD Lewat (8.64 SD 6.58 pg/ml) adalah lebih tinggi dengan ketara daripada AMD Awal (5.07 SD 4.29 pg/ml) (p=0.017). Namun begitu, tiada perbezaan ketara diperolehi selepas diubahsuai berdasarkan kovariat (p=0.173). Tiada perbezaan yang ketara dalam purata nilai TNF-α dalam air liur antara pesakit Awal dan Lewat AMD setelah diubahsuai berdasarkan kovariat (-26.60 SD 1.29 pg/ml vs -26.13 SD 1.67 pg/ml, p=0.234). Tiada perbezaan ketara di perolehi untuk hubungan antara tahap IL-6 dan TNF-α dalam air liur pesakit AMD dengan tempoh AMD. Walaupun, beberapa faktor menunjukkan hubungan ketara dengan tahap IL-6 dan TNF-α dalam air liur pesakit AMD pada *simple linear regression*, namun, ia tidak mempunyai nilai signifikan pada *multiple linear regression*.

Kesimpulan.

Penyelidikan ini menujukkan bahawa tiada hubungan yang signifikan di antara tahap IL-6 dan TNF-α dalam air liur dengan pesakit AMD. Patogenesis AMD lebih terhad kepada proses keradangan tempatan dan tahap biomarker yang menilai keradangan penyakit mata tidak semestinya dihubungkaitkan dengan nilai sistemik. Tambahan pula, kajian kohort yang besar

diperlukan untuk penilaian lebih lanjut untuk menunjukkan hubungan yang ketara antara nilai biomarker keradangan dengan penyakit mata.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 STUDY INTRODUCTION

Inflammation has been postulated to have a major role in the pathogenesis of Age-related macular degeneration (AMD), where AMD may represent a chronic, age-related inflammatory disease manifested in the eye. Several epidemiologic studies have investigated the relationship between inflammation or presence of inflammatory biomarkers include Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α) among AMD patients. These biomarkers have been investigated systemically in serum/plasma and ocular fluids include vitreous, aqueous and tears, but not in saliva (Chalam KV et al, 2014; Yildirim et al, 2012; Tawwab et al, 2019). There is no study done before evaluating these biomarkers in saliva among AMD patients.

Pathogenesis of AMD is still poorly understood. Although the main determinant of AMD is aging, it must be considered a multifactorial aetiology that involves genetic backgrounds and environmental risk factors (smoking, family history, dietary habits, oxidative stress and hypertension). Mounting evidence shows the crucial role of inflammation and immune-mediated process also have been associated with AMD pathogenesis and progression. Inflammation is a complex response of the immune system to noxious stimuli and/or tissue damage to maintain homeostasis and restore functionality. However, in AMD, it has been linked to a low-level inflammation, which sustains for decades and increases in level with advancing age. Moreover, in wet AMD also, in addition to vascular endothelial growth factor (VEGF), choroidal neovascularization (CNV) involves a number of angiogenic molecules and inflammatory cytokines: Interleukin-6 (IL-6), Interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), intercellular adhesion molecule-1 (ICAM-1) and monocyte chemo attractant protein-1 (MCP-1) (Chalam KV et al, 2014).

Recent evidence has suggested that IL-6 may play important roles in the pathogenesis of ocular diseases such as proliferative diabetic retinopathy (PDR), ocular wound healing process and vision-threatening disease characterised by retinal neovascularization, where in neovascular AMD, levels of IL-6 are proven to be related with the activity of CNV (Cui W et al, 2014). Data also had shown significant reduction of aqueous IL-6 levels after treatment, more significantly than any other cytokine, suggest that aqueous IL-6 may be an important marker of treatment response or resistance (Chalam KV et al, 2014).

TNF-α has been implicated in the pathogenesis of inflammatory, edematous, neovascular, and neurodegenerative diseases of the eye. Retinal pigment epithelium (RPE) cells which exposed to proinflammatory cytokines TNF-α showed decreased expression of key genes in the visual cycle, epithelial morphology, and phagocytosis function. This may contribute to the RPE dysfunction implicated in AMD pathology (Kutty RK et al, 2016). Markomichelakis N et al (2005) had reported three cases of regression of CNV secondary to AMD in patients receiving systemic treatment with infliximab, an anti-TNF-α drug for inflammatory arthritis.

Understanding the molecular events leading to the pathogenesis of AMD help the identification of novel biomarkers as well as therapeutic targets in order to improve quality of life of AMD patients. Besides that, early detection is crucial as AMD can lead to blindness without any serious symptoms. The application of proteomics to the study of ocular disease had been reported and most of these studies have been devoted toward establishing retinal proteomes and proteome expression changes with disease. Yet, much more innovative research is needed to help ensure that this disease are diagnosed and treated early enough to save the sight of hundreds of thousands of people around the world.

With the fast development of mass spectrometry and proteomic technologies, saliva protein biomarkers are being explored for clinical diagnosis and prognosis of human disease as it is simple, safe and non-invasive. Recent studies also have shown that salivary concentrations of several inflammatory cytokines and insulin resistance indices (which may be lower than serum concentrations) may mirror alterations in systemic concentrations of such biomarkers. Saliva offers a promising diagnostic alternative, compared to blood sampling, for screening for inflammatory, metabolic, and cardiovascular risk factors particularly among geriatric populations where blood sampling may be difficult (Gauri SD et al, 2014).

In ophthalmological diseases, limited number of studies done involving saliva sample. A private Canadian company, ArcticDx Inc, launched the Macula Risk® test, which assess the risk of AMD progression from early or intermediate to advanced AMD by analyzing a saliva sample for genetic variations strongly associated with AMD (Josee D et al, 2009). However, to the best of our knowledge, no research has been done on detecting saliva inflammatory biomarkers level in AMD patients. We strongly believe this non-invasive study will help us to identify the individuals at risk. This will be beneficial to ophthalmologist for close monitoring and earlier diagnosis of AMD. This may help to prevent or delay permanent vision loss. Besides that, we may encourage people to modify their lifestyle to lower their risk, for example by giving up smoking and maintaining a healthier diet.

1.2 BACKGROUND

1.2.1 AGE-RELATED MACULAR DEGENERATION

AMD is one of the major causes of visual loss among elderly people. It is caused by the degeneration of cells in the macula which is responsible for central vision. Two major clinical subtypes of AMD have been established: the dry form (non-neovascular/ non-exudative) affecting 85–90% of patients and the wet form (neovascular/ exudative) affecting 10–15% of patients. The dry AMD consists of drusen accumulation and progressive GA of the RPE and retina, whereas, wet AMD is described by the presence of malformed leaky choroidal vessels into the retina. The dry form is usually an incidental finding during eye examinations for other cases and approximately10-20% of them may progress into wet AMD (Kanski JJ et al, 2011).

AMD causes severe vision loss and disability in all countries. The central vision remains good until the disease progress to its advanced stage. Therefore, the main concern about wet AMD is that the vision loss progress rapidly despite its painless course, and patient might seek treatment once haemorrhagic complication of the CNV occurs or macular scar already formed (The Royal College of Ophthalmologist, 2013). Patient will report on difficulties doing near task, unable to drive, seeing distorted wavy cloudy objects, and affect their performance at work. These results in high psychological impact and socio-economic burden globally. Many patients develop depression due to their limitation in doing daily activities as well as the need for huge sum of money for the treatment of the complications.

1.2.1.1 EPIDEMIOLOGY OF AMD

AMD is the leading cause of irreversible blindness in adults more than 50 years old and its accounts for 8.7% of all blindness worldwide (WHO, 2019). Whereas, the projected number of people with AMD in 2020 is 196 million increasing to 288 million in 2040. These show the substantial global burden of AMD in future in view of expensive treatment for this disease particularly for late AMD and not available to all patients in many countries.

Multiple population-based studies of AMD showed the pooled prevalence of early and late AMD to be 8.01% and 0.37% respectively. Between geographical distributions, for early AMD and geographic atrophy (GA) subtype had higher prevalence in European than Asians and Africans, whereby, there was no significant difference between Asians and Africans. For late AMD, the prevalence was similar between Europeans and Asians in which 0.59% and 0.56% respectively. No significant projected number noted in gender prevalence (Wong WL et al, 2014).

In Asian countries, AMD was less appreciated and was thought to be an uncommon disease among Asians because Asia had a relatively young population for a long time. However, a meta-analysis done in 2010 by Kawasaki R and colleagues found a relatively comparable pooled prevalence of early and late AMD among five Asian countries with the Western societies in which 6.8% and 8.8% respectively (Kawasaki R et al, 2010). Research also showed that Asians more likely to develop the variant of wet AMD, polypoidal choroidal vasculopathy (PCV) than white populations. Furthermore, an estimated growth of 42% in the number of wet AMD cases is expected by 2030. Two main scenarios were presented: (1) Projected number of wet AMD cases if patients were not taking preventive antioxidant vitamins; (2) projected number of wet AMD cases if patients were taking preventive antioxidant vitamins. The

estimated economic burden of wet AMD for scenarios 1 and 2 in 2030 for Singapore is SG $203.1 million and SG$162.9 million, respectively (Saxena N et al, 2016).

Irreversible central vision loss is highly incapacitating in multiple physical, social, and emotional areas of patients as well as is leading to increased health resource utilization and high societal cost burden (Alfredo GL et al, 2017). Thus, understanding the prevalence, burden, and impact on the lives of patients are important for adequate health care planning and provision, which require both precise and contemporary estimates of disease prevalence.

1.2.1.2 RISK FACTORS OF AMD

There are several risk factors have been postulated in AMD. The major risk factor for developing AMD is age, particularly after 50. Prevalence of early AMD would increase from 8% in persons 43 to 54 of age to 30% in those aged 75 and over. Prevalence of the advanced form of AMD is reported to increase from 0.1% to 7.1% in these two age groups. The increased risk with age related to the loss of photoreceptors approximately 30% during ageing process.

AMD is also known that men are affected more than women in various part of the world. AMD previously was higher in Caucasians, however recent studies has noted a comparable number of Asian and Western populations with AMD, in which 6.8% and 8.8 % respectively (Kawasaki R et al, 2010). In United States (US), white Americans (2.5%) have the greatest risk to develop AMD compare with blacks (0.94%), Hispanics (0.9%) and others (0.92%) (National Eye Institute, 2019). This distribution was postulated with high levels of melanin in blacks increases it free-radical scavenging potential of the RPE and Bruch's membrane. This was been protective mechanism in the development of AMD (Colak et al., 2012).

There is a substantial genetic component to AMD with estimates ranging from 25-75% (National Eye Institute, 2015). There were association between advanced AMD and complement factor H (CFH), which is an integral component of the alternative pathway of complement activation. Other factors such as factor B, ARMS2 gene and complement components (C2 and C3) are also associated with development and progression of AMD (Colak E et al, 2012).

A number of modifiable risk factors have been also associated with AMD. Smoking doubles the AMD risk (Vingerling JR et al, 1995). A few clinical studies had shown strong relationship

between wet AMD and history of cigarette smoking, 2.8 times for females and 3.2 times for men in active smokers (Colak E et al, 2012). Cigarette is associated with high number of toxic substances such as reactive oxygen species (ROS) and causes oxidative stress at RPE cell level. This contribute to AMD development by induce atherosclerosis, endothelial regulation and angiogenesis (Colak et al., 2012). Alcohol consumption increase the oxidative stress and causing the tissue damage. It also affects the protective mechanism against oxidative damage, in which low level of antioxidant nutrients such as serum carotene, Vitamin E and zinc found in heavy drinkers (Klein et al., 2002).

Fatty diet and low consumption of antioxidants and zinc also contribute to the development of AMD. Conversely, high concentration of high-density lipoprotein (HDL)-cholesterol, oral supplementation with high levels of antioxidants and mineral have been shown to lower the risk and slowing the progression of advanced stages of AMD. Diet with high intake of omega-3 fatty acids and fish had shown decrease the risk of late AMD (Colak E et al, 2012). Estrogen supplements in post-menopausal women reduced the risk of AMD in a study performed by the Eye Case Control Study Group ('Risk Factors for Neovascular Age-related Macular Degeneration', 1993).

Comorbidities such as diabetes mellitus (DM), cardiovascular disease, hypertension, and kidney disease also has influence in the development of AMD (The Royal College of Ophthalmologists, 2013 and Colak E et al, 2012). VEGF plays an important role in both late AMD and diabetic retinopathy (DR) by stimulating vasculogenesis and angiogenesis, even though the pathophysiology is different in both and inner retina was affected in DR and outer retina in AMD. However, good control in DM showed lower the risk of AMD development. A cohort study was also had shown DM had a 1.4-fold increase risk of AMD occurrence.

Furthermore, patient with DR changes had a 4-fold increase in early AMD and 3.9-fold increase in late AMD compare to patient without DR (He et al., 2018).

It was postulated that cardiovascular risk associated with AMD. The pathological mechanism of atherosclerosis and aging process in which causing lipid deposition in systemic arteries wall, similar to deposition of esterified lipid-rich and apolipoprotein-B lipid in the sclera and the Bruch membrane of the choroid with AMD. This leads to increase choroidal vascular resistance and choriocapillary pressure, further induce subretinal deposits (drusen) and development of AMD (Thomas et al., 2015).

Besides that, light also has been postulated to play a role in development of AMD with photosensitization reactions via synthesis of ROS such as superoxide, hydrogen peroxide and singlet oxygen. These ROS may damage the RPE and Bruch's membrane (Colak E et al, 2012).

1.2.1.3 PATHOGENESIS OF AMD

Numerous and heterogeneous pathological processes are likely to predispose an individual to AMD, which is considered an extremely complex, multifactorial disease. Aging represents its primary determinant, while environmental factors such as cigarette smoking, dietary habits and phototoxic exposure, together with several gene polymorphisms contribute to significantly increase the risk of AMD occurrence. Besides that, genome-wide association studies, also showed remarkable correlations between common or rare immunological/ inflammatory gene polymorphisms and AMD, unequivocally indicating the involvement of inflammation and immune-mediated processes in the pathogenesis of this disease. Thus, although AMD is not considered a classic inflammatory disease, immunocompetent cells, such as macrophages and lymphocytes, are present in the chorioretinal tissues affected by AMD (Parmeggiani F et al, 2012).

Inflammation is the first biological response to infection or irritant components in maintain tissue homeostasis and restore its functionality. Involvement of inflammatory process in AMD was first reported in 1916. Since then, few studies demonstrated the involvement of inflammatory cells (including macrophages, lymphocytes and mast cells) in RPE atrophy and Bruch's membrane damage. Microglia, the immunocompetent resident macrophages in retina, is activated in this degenerative process. It releases different inflammatory mediators such as cytokines, chemokines, ROS and nitric oxide (NO), which initially help in protecting retina tissue from chronic neuroinflammatory process during early steps of its degeneration. However, prolonged and persistence inflammatory stimuli results in locally cellular damage, together with drusen attract more reactive systemic immune cells to retina (Litwinska Z et al, 2019).

This excessive stimulation of the inflammatory and immune responses may increase the risk of degenerative disease, especially in tissues exposed to high oxidative stress and metabolic activity, such as in retina. In fact, macular retina represents an excellent example sample of such tissues because it's involves in high rates of oxygen consumption and mitochondrial oxidative pathways (phototransduction, neurotransmitter utilization, and protein/organelle transport). Thus, AMD had been related with inflammatory process which is a result of imbalance between stress oxidative damage and its repair process (Cascella R et al, 2014).

During degenerative process, the prolonged chronic inflammation stimulates overreactive neurotoxic microglia to release large amounts of pro-inflammatory and cytotoxic factors such as TNF-α, interleukin-1β (IL-1β), IL-6 and C-reactive protein (CRP). Subsequently, it creates an inflammatory condition favourable for further recruitment of retinal microglia and exogenous infiltrating monocytes. Recent findings also had shown the inflammatory responses were from both local and systemic in view of systemic activation of the complement cascade had been documented in AMD patients. Therefore, although the AMD mainly limited to retina, this significant finding points towards the possible systemic inflammatory response occurring with AMD patients (Litwinska Z et al, 2019).

The pathological hallmark of AMD is drusen. Drusen are focal areas of eosinophilic extracellular deposits located between the RPE and Bruch membrane. The material of which they are composed has a broad range of constituents, and is thought to be derived from immune mediated and metabolic process in the RPE (Kanski JJ et al, 2011).

Progressive engorgement of RPE with these functionless residues is associated with the extrusion of aberrant materials which accumulate in Bruch's membrane and aggregate in the

form of drusen and basal laminar deposits. Drusen may be classified into hard and soft depending on the size and appearance on fundus examinations (Algvere PV et al, 2016). Hard drusen appear as discrete yellowish periodic acid-Schiff (PAS) stain positive nodules composed of hyaline material, <63 μm, while soft drusen with amorphous, poorly demarcated, boundaries, usually >63 μm. Hard scant drusen is not pathognomonic for AMD as it may present in healthy ageing people, while soft and large drusen is characteristic for AMD (Kanski JJ et al, 2011). These excretions contribute to retinal pigment epithelial detachment (PED) and further deterioration of the RPE. In late stages, central atrophy of RPE and photoreceptors form areas of GA. GA brings risk for visual loss due to foveal involvement.

Another crucial age-related retinal change is the Bruch membrane alteration, characterized by an increased thickness, accumulations of basal laminar deposits and/or drusen formation, and frequently accompanied by pigmentary irregularities due to RPE cell hypertrophy, hyperplasia, or atrophy. Both basal laminar and basal linear deposits of the drusen causes a reduction in the permeability of Bruch membrane, thus hinder the nutrients transport from choriocapillaris to the RPE and photoreceptors as well as impair the waste transport removal from the RPE. It results in ischemic insults to the RPE and various active pro-inflammatory mediators will be released including IL-6 and TNF-α as well.

1.2.1.4 CLASSIFICATIONS OF AMD

Several classification systems are used to define AMD both clinically and for research purposes. Based on Clinical Guidelines of AMD (The Royal College of Ophthalmologists, 2013), the Wisconsin Age-Related Maculopathy Grading System (WARMGS) and the International Age-Related Maculopathy (ARM) Epidemiologic Study Group remain the standardized grading system until today. Funduscopic photo will be taken and used to detect the presence and the grade of macular degeneration (Ferris FL 3rd et al, 2013).

The WARMGS defined **Early AMD** as the absence of signs of advanced AMD and the presence of (1) soft indistinct or reticular drusen or (2) hard distinct or soft distinct drusen with pigmentary abnormalities (RPE depigmentation or increased retinal pigment). **Late AMD** is defined as the presence of either (1) GA or (2) exudative AMD. GA is a sharply demarcated area of partial or complete depigmentation reflecting atrophy of the RPE with scalloped margins. Exudative AMD is defined as the presence of any of the following exudative lesions: PED or age-related retinal detachment, subretinal hemorrhage, subretinal scar (subretinal fibrous scar), or laser treatment for exudative AMD (Vingerling JR et al, 1995).

In 1995, the International ARM Epidemiologic Study Group redefined AMD from the traditional wet and dry designations (Vingerling JR et al, 1995). Patients with minimal or moderate non-exudative age-related changes in the macula were reclassified as having ARM. An advanced RPE atrophy, GA or CNV was required to establish a diagnosis of non-exudative AMD and exudative AMD, respectively.

As a result of the International ARM Epidemiologic Study Group efforts, patients with ARM account for 85-90% of individuals with age-related macular changes and have only mild

drusen, RPE atrophy, and/or RPE hypertrophy. They usually asymptomatic or only minimally symptomatic with mild blurred central vision, color and contrast disturbances, and metamorphopsia (waviness). Conversely, the 10-15% of patients with macular changes defined as AMD tend to report painless, progressive, moderate-to-severe blurring of central vision and moderate-to-severe metamorphopsia, which can be acute or insidious in onset (Vingerling JR et al, 1995).

Another classification is based on The Age-Related Eye Disease Study Group (AREDS) (Klein R et al, 2014; The Royal College of Ophthalmologists, 2013). This classification of macular degeneration into early, intermediate and advanced forms is of value when discussing vitamin supplementation.

The classification of AMD from the AREDS is as follows:

- No AMD (AREDS category 1) represent the control group and is characterized by no or few small drusen (< 63 μm in diameter).
- Early AMD (AREDS category 2) is characterized by a combination of multiple small drusen, few intermediate drusen (63–124 μm in diameter), or mild RPE abnormalities.
- Intermediate AMD (AREDS category 3) is characterized by 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) is characterized by one or more of the following
    i. GA of RPE involving the foveal center or
    ii. Neovascular maculopathy that includes CNV, PED, retinal hard exudates, subretinal and sub-RPE fibrovascular proliferation as well as disciform scar.

1.2.1.5 TREATMENTS OF AMD

Vision loss in AMD is more associated with the late forms of AMD. Early detection is challenging and difficult as these processes are gradual and generally painless. There are currently no definite effective treatments for dry AMD while treatments for exudative AMD focus on preservation of the retina by targeting new blood vessel formation.

For prophylactic treatment of dry AMD, AREDS revealed a beneficial effect of very high doses of antioxidants (daily dose vitamin C 500 mg, vitamin E 400 IU, Beta-carotene 15 mg and zinc 80 mg (along with 2 mg copper to prevent anemia) in reducing patient's relative risk of progression to advanced AMD by 25%. These supplements also indicate in patients with advanced AMD in the fellow eye. The risk of developing advanced AMD was reduced by 27% at 10 years follow-up (Algvere PV et al, 2016).

Therapies for exudative AMD are range from laser treatments, intraocular injections and surgery. For some patients, thermal laser therapy may be used. The argon laser photocoagulation produces a small burn when it hits the area of the retina to be treated. This destroys the abnormal blood vessels, preventing further leakage, bleeding and growth. It leads to disappearance of drusen as well, however does not seem to reduce the risk of AMD progression (Kanski JJ et al, 2011).

During the past decade, the most meaningful therapy for wet AMD is anti-VEGF. Two most commonly used drugs are Ranibizumab and Aflibercept. This new paradigm shift in treatment of AMD can help keep patients from going blind, and in some cases, even restore vision. In addition, it is also recommended to combine anti-VEGF therapy with intravitreal steroid to

achieve better outcomes while minimizing the potential severe adverse effect of frequent intravitreal anti-VEGF therapy (Kanski JJ et al, 2011).

Surgical treatment also available in managing wet AMD in which retinal translocation surgery involving 360° retinotomy with retinal rotation is done in conjunction with extraocular muscle surgery to correct torsion (Kanski JJ et al, 2011). However, it carries a high risk of subsequent retinal detachment as well as an accelerated degeneration of "new" macula may occur, therefore not commonly practiced.

1.2.2 CYTOKINES AND AMD

Cytokines involve in many biological processes, including the inflammatory and immune responses. Whereby, chemokines are another class of proinflammatory molecules, which drive the migration of white blood cells to damaged tissue in response to external stimuli. The cytokines consist of soluble proteins, peptides, and glycoproteins, which produced on the pro- or anti-inflammatory response. In physiological conditions, the synthesis of both types of cytokines is finely regulated and balanced. However, the dysregulation or abnormal production of pro- and anti-inflammatory cytokines causes several inflammatory diseases, autoimmune diseases, or immune deficiency syndromes (Cascella R et al, 2014).

During the aging process, it had been postulated the dysregulation of reparative inflammatory mechanisms, particularly the down-regulation of pro-inflammatory cytokines and the up-regulation of anti-inflammatory cytokines by the RPE, in response to the stimulation by the deposition of advanced glycation end products (AGEs). This may lead to the low-grade chronic inflammation that contributes to the progression of AMD.

In AMD, the cytokines may play a role in the initiation, perpetuation or subsequent down-regulation of the immune response, eventually leading to wound healing by the formation of a fibrotic scar. Histologically, chronic inflammation at the RPE/choroidal interface found in early AMD patients, whereby, immunocompetent cells, such as lymphocytes and macrophages, had been documented in chorioretinal tissue in wet AMD patients. Thus, the molecular mechanisms in development and progression of CNV, the hallmark of wet AMD, are better understood than dry form of this disease (Spindler J et al, 2018).

1.2.2.1 INTERLEUKIN-6 (IL-6)

1.2.2.1.1 Structure and Cell Signalling Pathway

IL-6 is a pleiotropic cytokine that is involved in the acute phase of the inflammatory reaction, wound healing, angiogenesis and fibrogenesis. It contributes to immune system, through the stimulation of acute phase responses, hematopoiesis, and immune reactions (Tanaka et al, 2014). In addition to its pro-inflammatory role, IL6 has anti-inflammatory properties (Chalam KV et al, 2014).

It acts as a warning sign in an emergency occurrence with the strict regulation of IL-6 synthesis both gene transcriptionally and post transcriptionally. Various transcription factors had been related in regulation of IL-6 gene transcription. IL-6 is released by a variety of tissues, including immune-mediated cells, mesenchymal cells, endothelial cells, fibroblasts, and many other cells in response to various stimuli (Velazquez-Salinas et al., 2019; Tanaka 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 (Tanaka et al, 2014).Figure 1.1 showed crystal structure of 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 (Tanaka et al, 2014). Both of these proteins induce the pleotropic effect of IL-6 on various cells. Subsequently, the IL-6/IL-6R complex in turn

induces homodimerization of gp130 and initiates a downstream signal cascade (Velazquez-Salinas et al., 2019).

The signalling cascades of IL-6 is mainly associated with the Janus kinase (JAK) - signal transducer and activator of transcription (STAT) activation pathway. It triggers the transcription of various downstream genes which associated with cellular signalling processes such as cytokines, receptors, adaptor proteins, and protein kinases. In addition, it also controls the synthesis of proteins involve in regulation of gene expression (Tanaka et al, 2014; Sato et al., 2018).

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).

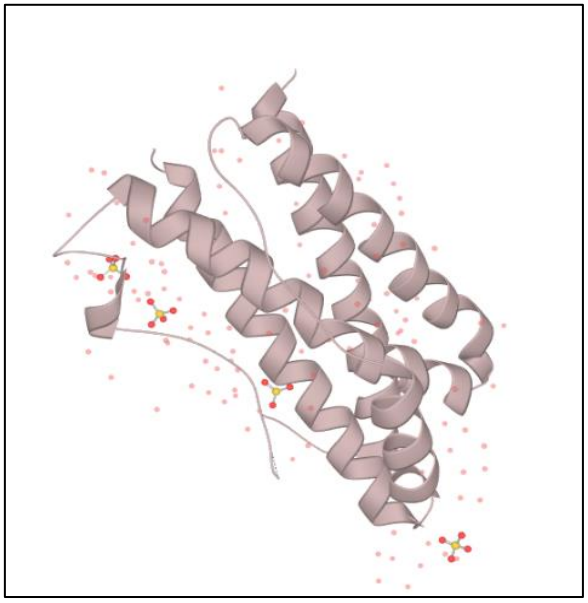

Figure 1.1 : Crystal structure of IL-6 ('IL6 - Interleukin-6 precursor - Homo sapiens (Human) - IL6 gene & protein').

1.2.2.1.2 Function and Role in Disease

The function of IL-6 in inflammation response are : (i) regulate the differentiation of monocytes into macrophages through the expression of macrophage colony-stimulating factor; (ii) increasing B-cell Immunoglobulin G (IgG) synthesis by regulating the expression of IL-21; (iii) activation of the STAT3 signalling pathway to down -regulate dendritic cell maturation; (iv) increase T helper 2 (Th2) response by inhibiting Th1 polarization, for these effects IL-6 stimulates secretion of Interleukin-4 (IL-4) and Interferon- γ (IFN-γ) respectively (Velazquez-Salinas et al., 2019).

In addition, IL-6 is a potent pyrogenic cytokine and play important role in lymphocyte production in lymphoid organ during febrile events. It is also modulating the host immune response for infection, together with Interleukin1 (IL-1) and TNF-α. Besides that, it's activity with transforming growth factor (TGF)-β in differentiation of naïve CD4 (cluster of differentiation 4) T cells to Th17, had shown its function in innate to acquired immune response. In converse, IL-6 also inhibits TGF-β-induced regulatory T cells (Treg) differentiation (Velazquez-Salinas et al., 2019). However, up-regulation of the Th17/Treg balance may lead to disruption of immunological tolerance. Therefore, IL-6 involved pathologically in autoimmune and chronic inflammatory diseases. It also regulates the differentiation of CD8 (cluster of differentiation 8) T cells into cytotoxic T cells. At certain circumstances, continuous differentiation of activated B cells into antibody-producing plasma cells, results in hypergammaglobulinemia and autoantibody production (Tanaka et al, 2014).

IL-6 production is strictly controlled by transcriptional and posttranscriptional mechanisms. However, imbalance in IL-6 production leads to a pathological effect on chronic inflammation and autoimmune diseases. Dysregulation of IL-6 related pathology was shown in cardiac

myxoma, synovial cells of rheumatoid arthritis (RA), swollen lymph nodes of Castleman's disease, systemic lupus erythematosus, myeloma cells, autoimmune related uveitis and inflammatory myopathies. It was also shown in peripheral blood cells or affected tissues in various other autoimmune and chronic inflammatory diseases and also malignant cells in cancers (Tanaka et al, 2014).

Tocilizumab, a humanized anti-IL-6 receptor antibody, approved for the treatment of RA and juvenile idiopathic arthritis (JIA). Furthermore, it is expected to be effective in other intractable immune-mediated diseases (Mihara et al, 2011). In this context, the mechanism for the continual synthesis of IL-6 needs to be elucidated to facilitate the development of more specific therapeutic approaches and analysis of the pathogenesis of specific diseases.

1.2.2.2 TUMOR NECROSIS FACTOR ALPHA (TNF-α)

1.2.2.2.1 Structure and Cell Signalling Pathway

TNF-α was found over 2 decades ago as a part of a super family of tumor necrosis factors (TNF) and their receptors (Parameswaran et al., 2010). It is an important pro-inflammatory cytokine with pleiotropic functions synthesised mainly by macrophages and T lymphocytes. TNF-α is upregulated in most of the inflammatory conditions leads to oxidative stress, disruption in macrovascular and microvascular circulation, changes in coagulation and induce apoptosis (Page et al, 2018). It also regulates the cell functions including cell proliferation, survival, differentiation, and apoptosis (Parameswaran et al., 2010).

TNF-α is a pleiotropic cytokine produced by various types of cells in the body, mainly monocytic lineage cells such as macrophages, astroglia, microglia, Langerhans cells, Kupffer cells, and alveolar macrophages. The TNF-α gene is encoded on human chromosome 6 (murine chromosome 17) as a single copy gene. Messenger RNA (mRNA) for TNF-α is present in a various cell, mainly monocytes and macrophages. Many factors regulate TNF-α gene expression at the transcriptional level [nuclear factor kappa b (NFκB) and nuclear factor activated T cells (NF-AT)] and translational level (UA-rich sequence in the 3′ untranslated region of human TNF-α mRNA) (Parameswaran et al., 2010; Lombardo et al., 2007).

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 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 1.2 showed crystal structure of TNF-α.

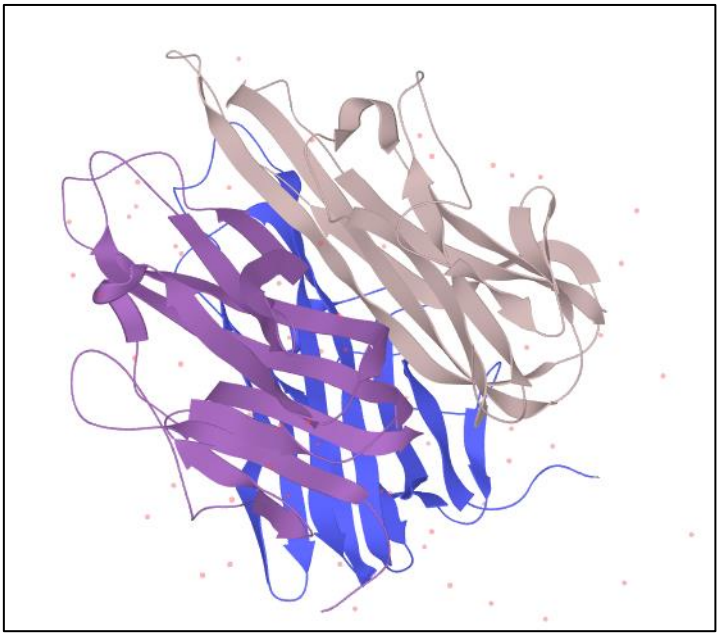

Figure 1.2 : Crystal structure of TNF-α (UniProt, 2016)

The transmembrane receptors of TNF-α are TNF receptor 1 (TNFR1), also known as p55 or p60, and TNF receptor 2 (TNFR2), also known as p75 or p80. TNFR1 is expressed in most of the mammalian tissues and binding of TNF-α-TNFR1 is irreversible. The TNFR2 is highly regulated and is mostly expressed in the immune cells and binding of TNF-α-TNFR2 is rapid on and off mechanism. Thus, TNFR2 plays role as a "ligand passer" to TNFR1 in some cells and increase the level of TNF-α at cell surface through rapid ligand binding and dissociation (Parameswaran et al., 2010; Lombardo et al., 2007).

Macrophages are innate immune cells that form the major defence system against invading microorganisms. Studies had shown the role of TNF-α signalling pathway and their biological activity related to macrophage, results in various physiological and pathophysiological