EVALUATION OF TNF-α AND IL-6 IN SALIVA AMONG DIABETIC RETINOPATHY PATIENTS

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DISCLAIMER

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

(DR KHAIRUL ANWAR BIN IBRAHIM)

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And I would like to dedicate this dissertation to my late father, Ibrahim Malek. May you rest in peace, Al-Fatihah.

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ABSTRACT

Introduction:

Diabetes retinopathy (DR) is one of the major microvascular complication of diabetes mellitus (DM). It is known as the second most common cause of blindness in Malaysia and the 5th worldwide. The burden cause by DM and blindness due to DR is high. Multifactorial etiology involves in DR pathogenesis include genetic backgrounds and environmental risk factors. Recent evidence had showed that inflammatory process plays a crucial role in the development of DR. Increase in Tumor necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6) were associated with DR. Saliva protein biomarkers are a novel technique for clinical diagnosis. Thus, identification of TNF-α and IL-6 in saliva perhaps provide insight in the role of saliva as a diagnostic alternative in future.

Objective:

The objective of this study was to compare TNF and IL-6 level in saliva between DM patients and No DM, and also in between the different DR groups. Besides that, it also to determine the relationship between TNF-α and IL-6 level in saliva with level of HbA1c and duration of DM.

Methods:

A comparative cross-sectional study was conducted exclusively at a tertiary hospital in Malaysia, Hospital Universiti Sains Malaysia (USM) between January 2018 and November 2020. This study involved DM patients with DR, no DR and No DM. Unstimulated saliva samples were collected. Laboratory analysis using commercial Human TNF-α and IL-6 ELISA kit (LEGEND MAX™,

BioLegend, Inc.) was performed to measure TNF-α and IL-6 levels in saliva. Statistical analysis was done using SPSS Inc. Version 26.

Results:

A total of 120 patients were included into the study (DM no DR: 33 patients, DM with NPDR: 30 patients, DM with PDR: 32 patients, No DM: 25 patients). There was significantly higher mean saliva IL-6 in DM group (0.033 SD 0.005 pg/ml) compared to No DM group (0.027 SD 0.001 pg/ml) after adjusted with covariates ($p < 0.001$). There was no significant difference of mean TNF-α in saliva between DM and No DM after adjusted with covariates. Mean IL-6 in saliva were significantly higher in NPDR (0.036 SD 0.003 pg/ml) and PDR (0.093 SD 0.023 pg/ml) compare to No DR (0.027 SD 0.001 pg/ml) ($p < 0.001$ and $p < 0.001$ respectively). Mean TNF-α in saliva were significantly higher in NPDR (0.086 SD 0.022 pg/ml) and PDR (0.093 SD 0.023 pg/ml) compare to No DR (0.049 SD 0.011 pg/ml) ($p = 0.015$ and $p = 0.003$) respectively. However, there was no significant difference of mean IL-6 and TNF-α in saliva between NPDR and PDR. The HbA1c level and duration of DM were not associated with TNF-α and IL-6 in saliva. Comorbidities and DR stages were the factors associated with TNF-α in saliva. While only DR stages was the significant factor associated with IL-6 in saliva.

Conclusion:

This study showed that there is significant association between TNF-α and IL-6 level in saliva among DM patients. DR pathogenesis involved multiple pathway which is mediated by inflammatory mediators. They are found to be in high concentration in the eye and systematically

compare to non-DR patients. Further study with larger cohort and longer duration of follow-up needed to confirmed this finding.

ABSTRAK

Pengenalan:

Retinopati diabetes (DR) adalah salah satu komplikasi mikrovaskular di dalam penyakit diabetes melitus (DM). Ia adalah penyebab kebutaan yang kedua tertinggi di Malaysia and ke-lima di seluruh dunia. Beban yang disebabkan oleh penyakit DM dan kebutaan yang disebabkan oleh DR adalah tinggi. Pelbagai faktor memainkan peranan di dalam patogenesis DR termasuk faktor genetik dan faktor persekitaran. Kajian terkini telah membuktikan bahawa proses keradangan memainkan peranan penting. Peningkatan tahap *Tumor Necrosis Factor alpha* (TNF-α) dan *Interleukin 6* (IL-6) berkait rapat dengan DR. Protein penanda-bio di dalam air liur adalah merupakan teknik terkini bagi diagnosis klinikal. Oleh itu, pengesanan TNF-α dan IL-6 di dalam air liur diharap agar dapat menambah pemahaman terhadap penggunaan air liur sebagai kaedah diagnosis yang baru di masa hadapan.

Objektif:

Objektif kajian ini adalah untuk membandingkan paras TNF-α dan IL-6 di dalam air liur antara kumpulan pesakit DM dan bukan pesakit DM dan juga di antara kumpulan pesakit DR yang berbeza tahap keterukan. Selain itu, kajian ini juga ingin menilai kaitan antara TNF-α dan IL-6 di dalam air liur dengan tahap HbA1c dan jangka masa DM.

Kaedah:

Sebuah kajian keratan rentas telah dijalankan di hospital tertiary di Malaysia, Hospital Universiti Sains Malaysia (USM) diantara bulan Januari 2018 sehingga November 2020. Kajian ini melibatkan pesakit DM dengan DR, DM tanpa DR dan kumpulan tiada DM. Air liur tidak

dirangsang telah dikumpul. Analisis makmal menggunakan kit komersil TNF-α dan IL-6 manusia (LEGEND MAX™, BioLegend, Inc.) telah dijalankan bagi mengukur kandungan TNF-α dan IL-6 di dalam air liur. Analisis statistik telah dijalankan menggunakan SPSS Inc. Versi 26.

Keputusan:

Seramai 120 pesakit telah dipilih menyertai kajian ini (DM tiada DR: 33 pesakit, DM dengan DR tidak proliferatif (NPDR): 30 pesakit, DM dengan DR proliferatif (PDR): 32 pesakit, tiada DM: 25 pesakit). Purata paras IL-6 di dalam air liur lebih bererti di dalam kumpulan DM (0.033 SD 0.005 pg/ml) berbanding kumpulan tiada DM (0.027 SD 0.001 pg/ml) selepas dilaraskan dengan kovariat-kovariat. Purata paras IL-6 di dalam air liur lebih ketara dalam kumpulan NPDR (0.036 SD 0.003 pg/ml) dan PDR (0.093 SD 0.023 pg/ml) berbanding kumpulan tanpa DR (0.027 SD 0.001 pg/ml) ($p < 0.001$ dan $p < 0.001$ secara berturutan). Manakala purata paras TNF-α di dalam air liur paling ketara di dalam kumpulan NPDR (0.086 SD 0.022 pg/ml) dan PDR (0.093 SD 0.023 pg/ml) berbanding kumpulan tanpa DR (0.049 SD 0.011 pg/ml) ($p = 0.015$ dan $p = 0.003$ secara berturutan). Bagaimanapun tiada perbezaan yang ketara bagi purata paras IL-6 dan TNF-α di dalam air liur di antara kumpulan NPDR dan PDR. Paras HbA1c dan tempoh DM tidak mempengaruhi paras TNF-α dan IL-6 dalam air liur. Komorbiditi dan tahap DR adalah faktor signifikan yang mempengaruhi paras TNF-α dalam air liur. Manakala hanya tahap DR sahaja faktor yang signifikan mempengaruhi paras IL-6 dalam air liur.

Kesimpulan:

Kajian ini telah menunjukkan kaitan yang bererti diantara paras TNF-α dan IL-6 di dalam air liur di kalangan pesakit DM. Patogenesis DR mempunyai pelbagai laluan yang berperantara dengan

perantara keradangan. Paras TNF-α dan Il-6 didapati mempunyai kandungan yang tinggi di dalam mata dan di dalam sistem badan berbanding pesakit DM yang tidak mempunyai DR. Lebih banyak kajian perlu dilakukan dengan peserta yang lebih ramai dan dalam jangka masa pemantauan yang lebih lama diperlukan untuk mengesahkan hasil kajian ini.

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CHAPTER 1
INTRODUCTION

1. INTRODUCTION

1.1 STUDY INTRODUCTION

The increasing prevalence of type 2 diabetes mellitus (T2DM) demands for developing collateral screening strategies for early identification of high–risk individuals for the disease and/or its complications (Wild *et al.*, 2004). One of the major microvascular complication of this chronic disease is diabetic retinopathy (DR), in which is the leading cause of preventable blindness among working aged adults around the world (Yau *et al.*, 2012). The prevalence of DR among patients with diabetes for the period 2015 to 2019 is 27% worldwide (Thomas *et al.*, 2019) while DR was the cause of blindness in 10.4% of Malaysian geriatric population, as seen in the National Eye Survey in Malaysia (NESII) (Chew *et al.*, 2018). Nevertheless, blindness from DR is preventable if anchored with early detection of this devastating complicated (Kauffmann *et al.*, 1994), which indeed requires timely screening and treatment (Skaggs *et al.*, 2017).

DR is a complex process that involved multiple pathways that subsequently lead to the damages that are seen in the disease. Hyperglycemia, oxidative stress, advanced glycation end products (AGE) formation and hypertension all contributed to inflammation. Concurrently, inflammation propagates this pathway further through cytokines, adhesion molecules, VEGF signaling, enhanced receptor of advanced glycation end-products (RAGE) expression, changes in nitric oxide regulation, and nuclear factor κβ (NF-κβ) signaling. Cytokines cause inflammation in the retina causing the increase intraocular pressure through endothelial nitric synthase (eNOS) formation or new, weak vessels and their increased permeability due to VEGF which in turn lead to retinal

hemorrhages and leukostasis. This symptomatic hyperleukocytosisis is an important event in the pathogenesis, leading to capillary occlusions and reactive oxygen species (ROS)-mediated cell death as well as amplifying the inflammatory responses (Tarr *et al.*, 2013). Among inflammatory cytokines found to be affecting the response in formation of DR changes were IL-6, TNF-α, IL-12, IL-8, intercellular adhesion molecule (ICAM)-1 (Vujosevic and Simó, 2017).

IL-6 has been found elevated in the vitreous fluid of diabetic patients with DR and has been implicated in the pathogenesis of DR. It has been found that serum IL- 6 concentration correlates significantly with the severity of macular edema and could be a predictor of PDR. However, these results have not been confirmed in larger studies (Simó-Servat, Simó and Hernández, 2016).

In addition, TNF-α is a cytokine that promotes the irreversible adhesion of leukocytes to the endothelium (leukostasis), increases the production of ROS, and is implicated in blood-retinal barrier (BRB) breakdown. A strong correlation between plasma levels of TNF-α and PDR has been reported. It also has been reported that baseline circulating TNF-α is a predictor of DR incidence as well as of the progression of diabetic complications. Interestingly, it has been observed that the TNF-α level in tears is highly correlated with DR severity (Simó-Servat, Simó and Hernández, 2016).

In congruence 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 effortless, safe, cost-effective and non-invasive (Javaid *et al.*, 2016). In comparison to blood sampling, saliva might be an ideal diagnostic alternative for screening for inflammatory,

metabolic, and cardiovascular risk factors particularly among geriatric populations and chronic disease patients where blood sampling may be burdensome (Desai, 2014).

There have been few studies done previously to determine the possible association of salivary biomarkers and among T2DM (Malamud, 2011; Srinivasan *et al.*, 2015; Chee *et al.*, 2016). However, there is no previous study done on evaluating these biomarkers in saliva among different severity group of diabetic retinopathies in T2DM patients.

1.2 STUDY RATIONALE

This study is to assess the level of TNF-α and IL-6 in saliva among the severity of DR. The level of TNF-α and IL-6 in saliva may give a prediction of DR progression and paved way for non-invasive DR assessment.

The aforementioned benefit of this non-invasive investigation open doors for vast improvement in management of DR, either for patients' benefit or aiding ophthalmologist in preventing or delaying permanent vision loss.

CHAPTER 2
LITERATURE REVIEW

2.1 LITERATURE REVIEW

2.1.1 DIABETES MELLITUS

DM is a major public health concern in Malaysia. A recent National Diabetes Survey in 2019 reported the overall prevalence of diabetes in Malaysia among adults of 18 years and above was 20% (National Institute of Health Malaysia, 2019). More than half of this figure was undiagnosed diabetes. About 5.5% of respondents who were found to have diabetes came from 18-19 years age group and the percentage increases with the age. Compared to the previous National Health and Morbidity Survey (NHMS) 2015, there were 15% increment in the prevalence (Mustapha and Azmi, 2013). It is expected by year 2020, the prevalence of diabetes will be 21.6%, with an estimated 4.5 million Malaysians age 18 years and above (Mustapha and Azmi, 2013). Obesity, physical inactivity, poor proper diet intake, and low health literacy as reported in the recent NHMS study may contribute to this exponential increment. The increase in diabetes prevalence will put more strain to the country's health expenditure.

2.1.1.1 CLASSIFICATION OF DIABETES MELLITUS

Diabetes is classified in two distinct groups namely type 1 DM (T1DM) and T2DM. T1DM is characterized by destruction of the pancreatic β-cells of the islets of Langerhans which secrete insulin; a peptide hormone that promotes absorption of glucose from blood to human cells for energy metabolism. The destruction of β-cells is caused by an autoimmune process, which leads to absolute insulin deficiency. T2DM in the other hand is characterised by insulin resistance in

peripheral tissues and later progresses to insulin secretory defect of the β-cell. T2DM is related to unhealthy lifestyle including obesity, physical inactivity, and poor diet intake with underlying genetic predisposition. More than 90% of diabetic patients are T2DM.

2.1.1.2 COMPLICATIONS OF DIABETES MELLITUS

Long term irreversible vascular complications are the main cause of morbidity and mortality of diabetic patients. Vascular complications of diabetes can be divided into macro vascular and micro vascular complications. The macro vascular complications can manifest as coronary artery disease (heart attacks, angina), peripheral artery disease (leg claudication, gangrene), and carotid artery disease (strokes, dementia) (National Collaborating Centre for Chronic Conditions, 2008). The micro vascular complications include DR (sometimes cause blindness), diabetic neuropathy (resulting in amputation, painful symptoms, erectile dysfunction, and other problems), and diabetic nephropathy (may require dialysis or renal transplantation). The Malaysian prevalence of these complications based on a population-based study in 2012 was estimated from 11% to 15% (Mustapha and Azmi, 2013).

There are multiple risk factors associated with the complications of diabetes which require multiple treatment strategies. This includes lifestyle changes (diet and activity levels), management of obesity, plasma glucose control, blood pressure control, blood lipid control, reduction of thrombogenicity, laser therapy for eye damage, drug therapy to delay kidney damage, local foot care, and symptomatic treatments for various types of neuropathy (National Collaborating Centre

for Chronic Conditions, 2008). The complex nature of diabetic complications treatment further increases the overall disease burden.

2.1.2 DIABETIC RETINOPATHY

DR is one of the leading causes of legal blindness. Its prevalence varies between nations and ethnics. According to the National Diabetes Registry Report 2011, the overall prevalence of DR among diabetics was 15.2%. It is also found that 45% of population with known diabetes never had their fundus examined and from those who had their fundus examined, only 33% were examined within last one year. In term of ethnicity, Diabetic Eye Registry 2007 reported that more than 50% among Malay diabetic patients have some form of retinopathy followed by Chinese (20%) and Indian (16%). Among diabetic patients referred to eye clinic of government hospitals across Malaysia, 9.2% were found to have vision more than 3/60 (legal blindness) (Ngah, 2007).

Worldwide, DR is currently the 5th cause of legal blindness after cataract, glaucoma, uncorrected refractive error, and age-related macular degeneration (AMD). But it is showing global increase is age standardized prevalence from 1990 to 2020. This finding concerns the younger and economical active age group which can cause increase the financial burden to the family and country as a whole. The prevalence is found to be higher in Asia and Sub-Saharan Africa region (Adelson *et al.*, 2021).

2.1.2.1 PATHOPHYSIOLOGY OF DIABETIC RETINOPATHY

DR is a progressive micro vascular disease caused by hyperglycemia that altered certain biochemical pathways that led to endothelial vessels damage. A number of interconnecting biochemical pathways have been proposed as potential links between hyperglycemia and DR (Tarr *et al.*, 2013). These include increased polyol pathway flux, activation of diacylglycerol- (DAG-) protein kinase C (PKC) pathway, increased expression of growth factors such as VEGF and insulin-like growth factor 1 (IGF-1), haemodynamic changes, accelerated formation of AGE, oxidative stress, activation of the renin-angiotensin-aldosterone system (RAAS), and subclinical inflammation and leukostasis (Tarr *et al.*, 2013).

Diabetic macular oedema is a vision threatening subset of DR. It is defined as retinal thickening within 2 discs diameters of the center of the macular. It results from retinal micro vascular changes. The pathophysiology started with thickening of the basement membrane and reduction in the number of pericytes (reduced pericytes endothelial cells ratio). This leads to increased permeability and incompetence of the retinal vasculature which compromised the blood-retinal barrier and eventually leakage of plasma constituents into the surrounding retina. Hypoxia can develop as a result of compromised vasculature and it stimulates the up regulation of VEGF production (Mavrikakis, 2016).

2.1.2.2 CLASSIFICATION OF DIABETIC RETINOPATHY

The most widely used DR classification is the International Clinical Disease Severity Scale for DR. This classification is derived from the findings of Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) (Klein *et al.*, 1991) and the Early Treatment of Diabetic Retinopathy Study (ETDRS) (Group, 1987). The same classification is adapted by the Malaysian Diabetic Retinopathy Screening guideline (Ministry of Health Malaysia, 2011). There are five stages of DR which are easily recognizable based on the features of the fundus:

i. No DR or no apparent retinopathy
ii. Mild non-proliferative retinopathy (NPDR)
    a. Characterized by the presence of a few microaneurysms
iii. Moderate NPDR
    a. Characterised by the presence of microaneurysms, intraretinal hemorrhages or venous beading
iv. Severe NPDR
    a. 4:2:1 rule of the ETDRS
    b. 4 - More than 20 intraretinal haemorrhages in each of 4 quadrants
    c. 2 - Definite venous beading in 2 or more quadrants
    d. 1 - 1 or more quadrants have intraretinal microvascular abnormalities (IRMA)
v. Proliferative diabetic retinopathy (PDR)
    a. Characteried by neovascularization of the disc, neovascularization of the retina, neovascularization of the iris, neovascularization of the angle, vitreous hemorrhage or tractional retinal detachment.

Maculopathy is a sub classification of DR. It can present at any stages of retinopathy. Diabetic macular oedema can be presented in any severity as classified below:

i. Mild – some retinal thickening or hard exudates in posterior pole but distant from the macular
ii. Moderate – retinal thickening or hard exudates approaching the center of the macular but not involving the center
iii. Severe – retinal thickening or hard exudates involving the center of the macular

(Wilkinson *et al.*, 2003)

2.1.3 RISK FACTORS FOR DIABETIC RETINOPATHY

DR was found to be associated with many risk factors. One particular study, the Singapore Malay Eye Study which studied the prevalence of diabetic in Malay ethnics aged 40-80 years in Singapore reported that the presence of DR was associated with longer diabetes duration, poorer glycemic and blood pressure control, and lower levels of total and LDL cholesterol. Vision threatening retinopathy which occur in 35% of diabetic of this population was also found to be associated with stroke and chronic kidney disease (Wong *et al.*, 2016)

2.1.4 TREATMENT OF DIABETIC RETINOPATHY

General approach of treating DR is by controlling diabetes and maintaining the glycosylated hemoglobin (HbA1c) level in the 6%-7% range. Intensive treatment of glucose in T2DM delays onset and slows progression of DR (Cheung, Mitchell and Wong, 2010).

Specific treatment for DR is depending on the severity and the presence of diabetic macular oedema. There are a few modalities with several treatment strategies available for retinopathy treatment. In the ETDRS, it is found that laser surgery for macular oedema reduces the incidence of moderate visual loss from 30% to 15% over a 3-year period (Group ETDRS, 1987). However, scatter laser has no similar benefit.

The Diabetic Retinopathy Study on the other hand, has found that adequate scatter laser pan retinal photocoagulation (PRP) reduces the risk of severe visual loss by more than 50% in patient with severe NPDR. PRP was concluded not indicated for mild to moderate NPDR but should be considered as retinopathy approaches the high risk stage and usually should not be delayed when high risk stage is present (Group ETDRS, 1987).

Randomized trial evaluating ranibizumab plus prompt or deferred laser or triamcinolone plus prompt laser for diabetic macular oedema, has demonstrated that ranibizumab with prompt or deferred focal/grid laser achieved superior visual acuity and optical coherence tomography (OCT) outcomes compared with focal/grid laser treatment alone (Elman *et al.*, 2010).

2.2 PRO-CYTOKINES AND DIABETIC RETINOPATHY

DR is a complex process that involved multiple pathways that subsequently lead to the damages that are seen in the disease. Hyperglycemia, oxidative stress, AGE formation and hypertension all contributed to inflammation. At the same time inflammation propagates this pathway further through cytokines, adhesion molecules, VEGF signaling, enhanced RAGE expression, changes in

nitric oxide regulation, and NF-κβ signaling. Inflammation in the retina causing the increase intraocular pressure through eNOS, formation or new, weak vessels and their increased permeability due to VEGF which in turn lead to retinal hemorrhages and leukostasis. Leukostasis is an important event in the pathogenesis, leading to capillary occlusions and ROS-mediated cell death as well as amplifying the inflammatory responses (Tarr *et al.*, 2013). Figure 2.1 shows the DR pathways (Chous, 2013).

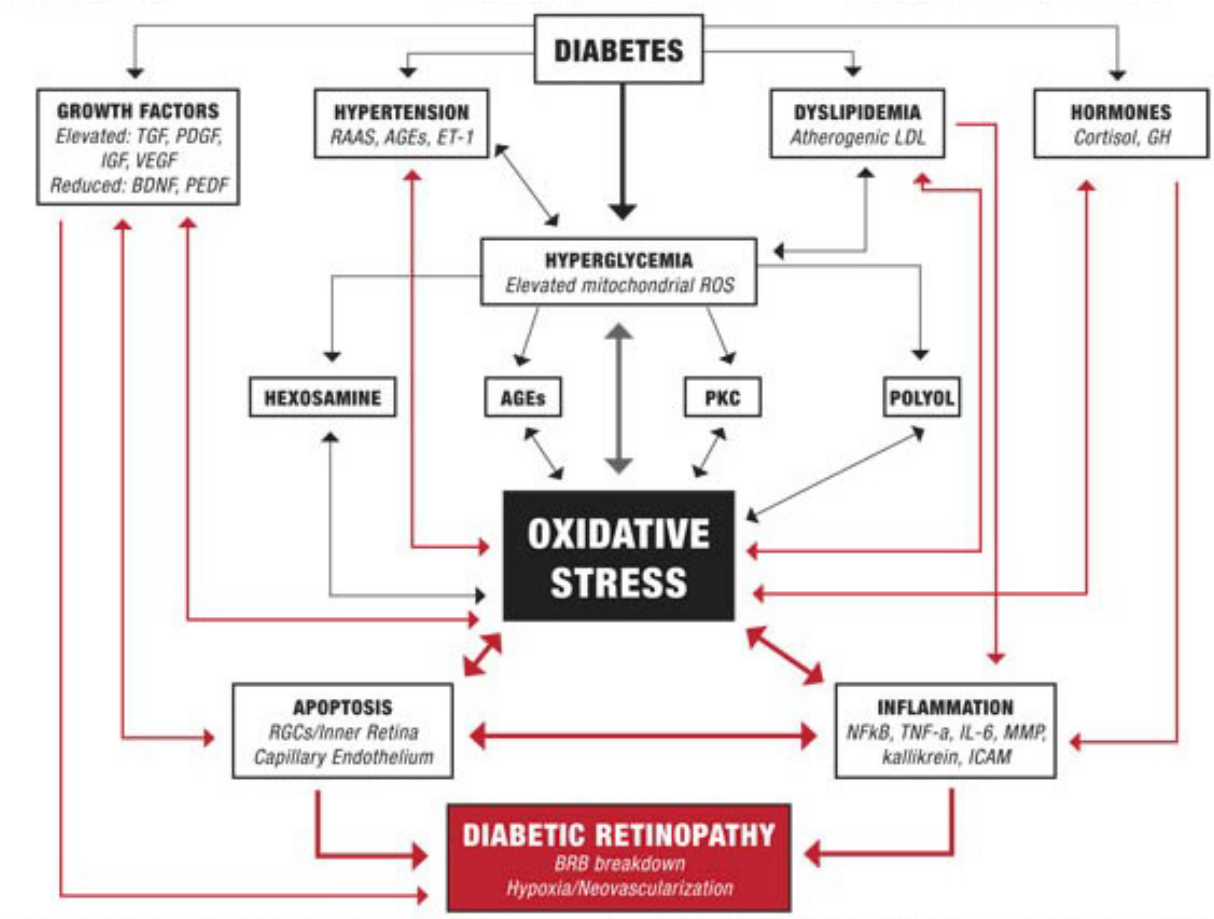

Figure 2.1: Diabetic Retinopathy Pathways (Chous, 2013).

2.2.1 TNF-α

TNF-α is a proinflammatory cytokines. It is produced by monocytes, macrophages, activated endothelial cells, fibroblasts and joint cartilages. It contributed to upregulation of the adhesive molecules of endothelial cells and leucocytes. In the event of DR, it involved in leucocytes activation, and endothelial apoptosis.

TNF-α regulates critical cell functions including cell proliferation, survival, differentiation, and apoptosis. Aberrant production and TNF receptor signaling have been associated with pathogenesis of several diseases, including rheumatoid arthritis, Crohn's disease, atherosclerosis, psoriasis, sepsis and obesity (Parameswaran and Patial, 2010). Figure 2.2 shows the structure of TNF-α (Eck and Sprang, 1989).

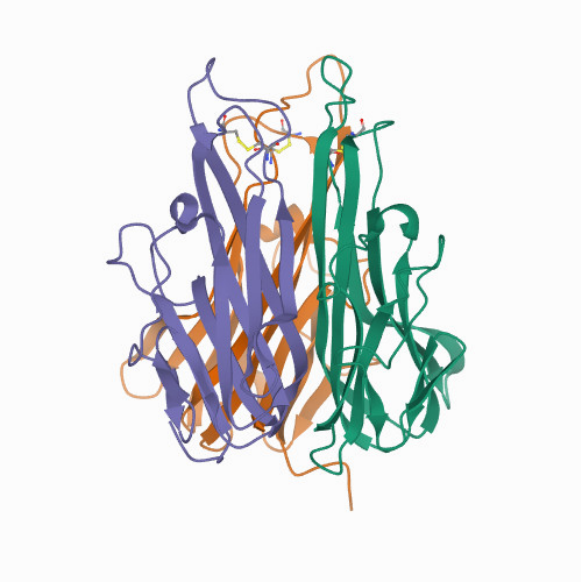

Figure 2.2: Structure of TNF-α (Eck and Sprang, 1989)

2.2.2 IL-6

IL-6 is non-specific inflammatory cytokines. In the eye, it is produced in the Müller cells (Liu *et al.*, 2015). During inflammatory response, it will be upregulated and contribute to the pathogenesis of the vascular leakage and neo-vascularization. It induces expression of VEGF which will further worsen the condition in the DR eye.

IL-6 is produced in response to infection and tissue injury, contribute to host defense through stimulation of acute phase responses, hematopoiesis and immune reactions. It is strictly controlled by transcriptional and posttranscriptional mechanism. Dysregulation of production can lead to pathological effect on chronic inflammation and autoimmunity. Other diseases that can produced high level of circulating IL-6 and being targeted for treatment such Graves' ophthalmopathy, rheumatoid arthritis (RA), polymyalgia rheumatica, giant-cell arteritis, systemic sclerosis, non-infectious uveitis, Bechet's syndrome, and juvenile idiopathic arthritis associate uveitis (Tanaka, Narazaki and Kishimoto, 2014). IL-6 also can be found in other bodily fluid such as serum, cerebrospinal fluid, urine, and synovial fluid (Frieling *et al.*, 1994). Figure 2.3 shows the structure of IL-6 (Xu *et al.*, 1997).

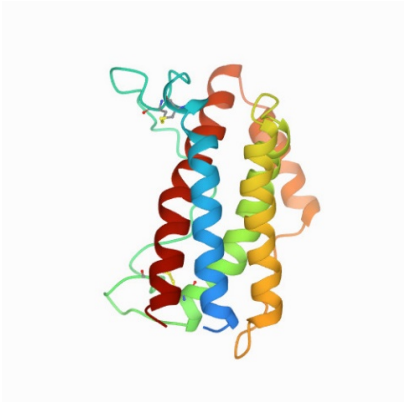

Figure 2.3: Structure of IL-6 (Xu *et al.*, 1997)

2.3 SALIVA BIOMARKERS

Saliva is a clear, slightly acidic, heterogenous fluid consists of water (99%), proteins (0.3%), and inorganic materials (0.2%). It is multifunctional and not only limited for digestion. It also functioning as food lubrication, swallowing, and also protection against pathogen. Saliva is produced by the acinar cells and collected in the salivary glands (>90% from parotid, submandibular and sublingual). While the rest are secreted by the minor gland of labial, buccal and palatal. Each salivary gland is highly permeable and enveloped by capillary. Researchers postulated that blood-derived molecules entering salivary gland via transcellular (passive or active) or paracellular. This suggest that circulating biomarkers can affect the saliva content and may contains circulating biomarkers as well (Yoshizawa *et al.*, 2013).

Saliva provides non-invasiveness and stress-free sample collection, easy and multiple sampling opportunities, reduced need for sample pre-processing, and minimal risk of contracting infectious organisms, and it is also an ideal biofluid for collecting specimens from patients in developing countries. Thus, making saliva a good source for diagnosis and monitoring disease progression.

Figure 2.4 shows the inflammatory biomarkers in saliva with various diseases. It showed that TNF-α in saliva detected in various diseases such as polycystic ovarian syndrome, Parkinson's disease, cancer, CKD and oral diseases compare to IL-6 (Prasad, Tyagi and Aggarwal, 2016).

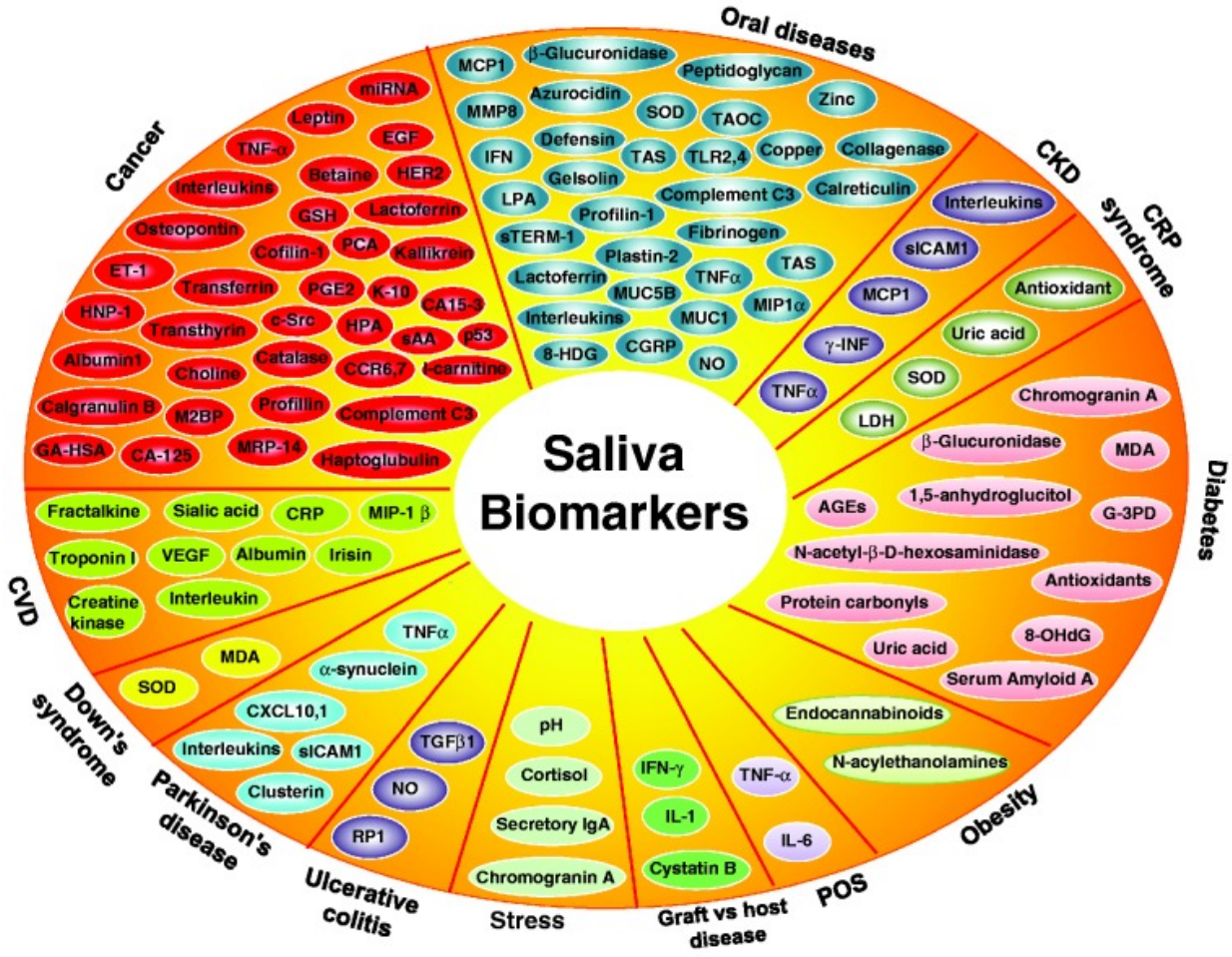

Figure 2.4: Saliva Biomarkers Related to Systemic Diseases (Prasad, Tyagi and Aggarwal, 2016).

CHAPTER 3
RESEARCH QUESTIONS, RESEARCH HYPOTHESIS AND OBJECTIVES

3. RESEARCH QUESTIONS, RESEARCH HYPOTHESIS, AND OBJECTIVES

3.1 RESEARCH QUESTIONS

i. Is there any difference in the level of TNF-α and IL-6 of saliva between T2DM and No DM?

ii. Is there any difference in the level of TNF-α and IL-6 of saliva in different stages of diabetic retinopathy among T2DM patients?

iii. Is there any relation between level of HbA1c and duration of DM with TNF-α and IL-6 level in saliva of T2DM patients?

3.2 RESEARCH HYPOTHESIS

i. There is difference in mean TNF-α and IL-6 level in saliva between T2DM and No DM.

ii. There is difference in mean TNF-α and IL-6 level in saliva in different stages of diabetic retinopathy among T2DM patients.

iii. There is relation between level of HbA1c and duration of DM with TNF-α and IL-6 level in saliva of T2DM patients.

3.3 OBJECTIVES

3.3.1 GENERAL OBJECTIVE

To evaluate TNF-α and IL-6 level in saliva among diabetic retinopathy in T2DM.

3.3.2 SPECIFIC OBJECTIVES

i. To compare the mean TNF-α and IL-6 level in saliva between T2DM and No DM.

ii. To compare the mean TNF-α and IL-6 level in saliva in different stages of diabetic retinopathy in T2DM patients.

iii. To determine the relationship of HbA1c level and duration of DM with TNF-α and IL-6 level in saliva among T2DM patients.

CHAPTER 4
RESEARCH METHODOLOGY

4. RESEARCH METHODOLOGY

4.1 STUDY DESIGN

A comparative cross-sectional study was conducted in the Ophthalmology Clinic of a tertiary centre, Hospital Universiti Sains Malaysia (USM), Kelantan, Malaysia from January 2018 to Nov 2020. The study populations included all DR patients who came to the Ophthalmology Clinic, Hospital USM who fulfilled the selection criteria. A Control group of No DM patients, who attended Ophthalmology Clinic, Hospital USM within the allocated time frame and fulfil the criteria after fundus examination 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:18070321) (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 GCP.

4.3 SELECTION CRITERIA

The study group include patients with mild NPDR, moderate NPDR, severe NPDR, and PDR, with duration of disease less than 15 years, aged 40 years to 60 years were recruited using non-probability sampling method. Sample collection was not depending on any eye since only saliva was collected for analysis. For the diagnosis of DR (Mild, Moderate, Severe, PDR):

- If the patient has similar findings in both eyes, then the diagnosis was either mild NPDR, moderate NPDR, severe NPDR, PDR based on the eye findings.
- If the patient has one eye with milder NPDR and the fellow eye with more severe NPDR, then the more severe form of DR was considered as the diagnosis for this patient.
- If the patient has only one eye with DR, then diagnosis was considered based on the pathological eye.

DR patients with any vitreoretinal pathology such as age-related macular degeneration (AMD), central retinal vein occlusion (CRVO) and pathological myopia with myopic maculopathy were excluded. Other ocular exclusion criteria were: (a) history of ocular surgery mainly vitrectomy and cataract extraction (within 6 months) prior to recruitment period; (b) history of ocular inflammation/ ocular autoimmune disease (e.g.: uveitis, chorioretinitis, keratitis); (c) history of photodynamic therapy (PDT) and intravitreal anti-VEGF.

For oral pathology, patients with pre-malignant lesion, oral carcinoma, chronic and severe periodontitis or gum bleeding were excluded. DM is an established risk factor for periodontitis.

People with DM have three times the risk of developing periodontitis compared to the general population (Wu *et al.*, 2021). We only choose the one with mild or moderate to control the possible outcome due to the disease. Systemically, patients who had uncontrolled hypertension, and those with systemic conditions that up-regulates inflammatory markers such as autoimmune disease, malignant tumour, rheumatoid arthritis or inflammatory bowel disease were excluded from this study.

The control group was patient who came to eye clinic, with aged 40 years to 60 years, without DM. The exclusion criteria for control group were same as the study group.