Tan Sri Jeffry Cheah School of Medicine and Health Sciences
Monash University Malaysia
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Professor Dato' Dr Khalid Abdul Kadir, Dr Badariah Ahmad, Assoc Prof Uma Palani, Dr Nevein Bottross, Dr Suzanne Tan, Dr Chuah Yilynn, Dr Ng En Yng, Dr Gerald Tan Chen Lie, Dr Ng Yeek Tat

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A. Study Title

Tocotrienol-rich Vitamin E (Tocovid) and its Effects in Diabetes and Diabetic Microvascular Complications: Nephropathy, Retinopathy and Neuropathy

B. Literature review

1.0 INTRODUCTION

1.1 Burden of Diabetes

Diabetes is one of the biggest and most challenging global health crises of the 21st century, affecting approximately 422 million people worldwide in 2014. This figure had almost quadrupled from 108 million people in 1980. The main risk factor is the rising prevalence of obesity all over the world. Furthermore, the incidence of diabetes in low- and middle-income countries are growing the most rapid, making it no longer a disease of the affluence. (1) According to the latest National Diabetes Registry (2011), 15.2% of Malaysians have diabetes, a one-third increase from 11.6% in 2006, over a span of just 5 years. (2) In fact, our previous study showed that the overall prevalence of diabetes among 4341 subjects recruited in Malaysia was 22.6% which was significantly higher than the national statistics. (3) Therefore, diabetes is a major public health issue in Malaysia. Type 2 diabetes mellitus (T2DM) is the most common type of diabetes worldwide and it is characterized by four major metabolic abnormalities: obesity, impaired insulin action, insulin secretory dysfunction and increased endogenous glucose output. Obesity increases insulin resistance in the body causing increased compensatory secretion of insulin in order to maintain normal glucose tolerance. However, the beta cell function in the pancreas declines over time due to oxidative damage, thus decreasing the amount of insulin secreted. Lack of insulin to suppress the endogenous glucose output causes hyperglycemia. As a result, patients with normal glucose tolerance will eventually progress to impaired glucose tolerance to Type 2 diabetes with mainly postprandial hyperglycemia and finally, to diabetes with fasting hyperglycemia. (4) Over time, chronic inappropriate hyperglycemia is associated with the onset of acute and chronic complications of diabetes. (4, 5) World Health Organization (WHO) reported 4 million deaths annually from diabetic complications such as cardiovascular disease, stroke and kidney failure, also known as end-stage renal disease (ESRD). (1) Patients with ESRD require costly renal replacement therapy in the form of dialysis or kidney transplantation in order to survive. (6) There is approximately 2 million people worldwide suffering from ESRD and this figure is increasing at a rapid rate of 5-7% per year. (7) Currently, 50% of all ESRD cases in developed countries are due to diabetic nephropathy (DN). DN is also associated with an increased risk of cardiovascular disease 6 events, hospitalizations (4), cognitive dysfunction (8) and poor quality of life. (9, 10) Furthermore, the impact of diabetes on the global economy is enormous. WHO estimated in their global report on diabetes that the losses in Gross

domestic products worldwide from 2011 to 2030, due to direct and indirect costs of diabetes, will sum up to a grand total of US$ 1.7 trillion. (1) Therefore, prevention of the onset of diabetes and its complications is crucial to overcome this global pandemic.

1.2 Big Trials on Diabetes and its Implications

The first big trial on diabetes was the Diabetes Control and Complications Trial (DCCT) study published in 1993. This study showed that 6.5 years of intensive glucose control effectively delays the onset and slows the progression of diabetic nephropathy, retinopathy and neuropathy in patients with Type 1 Diabetes (T1DM). For every reduction in HbA1c by 2%, the conferred relative risk reduction of microalbuminuria and albuminuria was 39% and 54% respectively, diabetic retinopathy was 76% and clinical neuropathy was 60%. (11) In the UK Prospective Diabetes Study (UKPDS) published in 1998, 10 years of tight glucose control in patients with Type 2 diabetes (T2DM) resulted in only 0.9% reduction in HbA1c which conferred a relative risk reduction of only 34% in albuminuria and 21% in retinopathy. The overall reduction in microvascular complications was 25%. (12) However, both studies showed no significant reduction in the risk of macrovascular complications such as cardiovascular events. Overall, strict glucose control was proven more effective in DCCT patients compared to UKPDS patients and the risk reductions in both studies seem to be directly proportional to the HbA1c levels. In other words, the higher the reduction in HbA1c levels, the higher the reduction in the risk of microvascular complications. Therefore, the results from the UKPDS study are consonant with the DCCT trial results. (11, 12) Researchers have speculated that strict glucose control in UKPDS patients resulted in lower reduction of HbA1c and risk of microvascular complications compared to DCCT patients because UKPDS patients had T2DM whilst DCCT patients had T1DM. The pathogenesis of diabetic complications in T2DM is due to a combination of factors including insulin resistance, dyslipidemia and poor blood pressure control. In the Steno2 study published in 2003, 7.8 years of intensified multi-target intervention aimed at concomitant risk factors such as hypertension, dyslipidemia and hyperglycemia resulted in 50% reduction in the risk of both cardiovascular and microvascular events among patients with T2DM and microalbuminuria. Therefore, multi-target intervention for T2DM in Steno-2 study is superior to the single target approach in the UKPDS study as it reduced the risk of cardiovascular events which were not seen in the UKPDS study. Furthermore, the risk of microvascular complications including diabetic nephropathy were significantly reduced at a higher rate compared to the UKPDS study (50% vs 34%). In fact, the risk reductions were almost equivalent to the DCCT trial results. (13) On the other hand, Type 1 diabetic complications are purely due to insulin deficiency causing hyperglycemia. Therefore, a single risk factor intervention approach like strict glucose control in patients with T1DM will definitely result in greater reduction of HbA1c compared to patients with

T2DM leading to lower risk of diabetic complications, as shown by the DCCT and UKPDS study, which is consistent with the results of one meta-analysis. (14) In fact, intensive glucose control in Type 1 diabetic patients should have logically prevented the risk of diabetic nephropathy on a whole instead of just 54% reduction in albuminuria since insulin deficiency is the primary cause of T1DM complications. This is probably because the results of the studies were correlated to the HbA1c levels. This is further supported by the results from the follow-up trials of DCCT, UKPDS and Steno-2 studies. (15-17)

1.3 Good Metabolic Memory

The DCCT-EDIC trial is an 18-year follow-up study of the DCCT trial. Prior to the initiation of the EDIC trial, all of the participants in the conventional group (CON) were offered training in intensive therapy (INT). By the fourth year of follow-up, 75% of the former CON group was still following the INT and their HbA1c started to decrease. For the INT group, although 95% of them were was still following the same insulin treatment, their HbA1c started to rise. At the end of the study, the HbA1c levels of the two groups converge and eventually reach the same level of 8.0%, as shown in Figure 1 below. However, the INT group had 35-76% lower risk of microvascular complications and 58% lower risk of cardiovascular disease events such as fatal and nonfatal myocardial infarct and stroke or death compared to the CON group, indicating the benefits of tight glucose control persisted and became even greater long after the DCCT trial has concluded. The researchers called this —metabolic memory. (15)

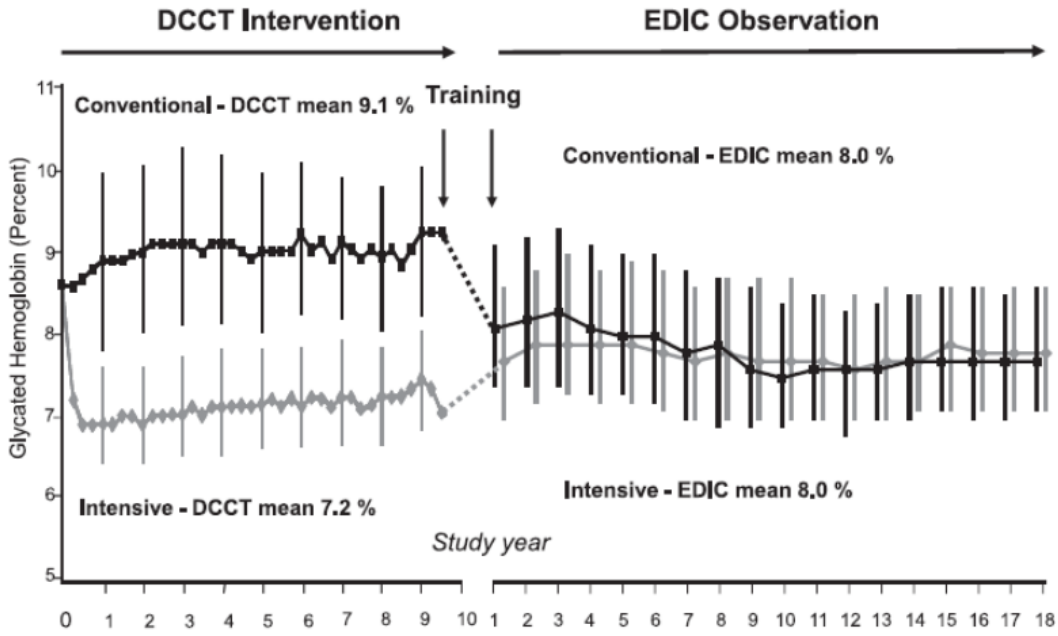

Figure 1: Median HbA1c concentrations during DCCT and EDIC follow-up. [15]

Figure 1 Median HbA1c concentrations during DCCT and EDIC follow-up. Adapted from Nathan 2014. (15)

The UKPDS follow-up study of 10 years also showed similar findings because the relative reductions in risks persisted at 10 years despite the loss of glycemic differences in HbA1c between the two groups after the first year of follow-up. The effects that persisted was the reduction in the risk of any diabetes-related end point by 9%, microvascular complications by 24%, myocardial infarction by 15% and death from any cause by 13%. (16) Besides that, the Steno-2 Study also reported similar findings during a follow-up of 5.5 years after a prior multifactorial risk reduction among patients with T2DM. In this study, enhanced risk reductions persisted up to almost 6 years despite the loss of within-trial differences in glucose levels (HbA1c), blood pressure and lipid levels, indicating the persistence of beneficial effects from earlier intensive metabolic management. The INT group was associated with 54% lower risk of death from any cause, 43% lower risk of death from cardiovascular causes and 41% reduction in cardiovascular events. (17) Therefore, — metabolic memory or the so-called — legacy effect that conferred a long term protection against microvascular and macrovascular complications of diabetes is unlikely to be related to the HbA1c levels.

Table 1 below summarizes the mean relative risk reduction (%) of different outcomes with intensive therapy (INT) compared to conventional group (CON) in patients with Type 1 and Type 2

diabetes during the DCCT, UKPDS and STENO-2 randomized controlled trials (RCT) and its follow-up studies. (18)

Outcomes	Clinical Trials					
	DCCT/EDIC		UKPDS		STENO-2	
	RCT	Follow-up	RCT	Follow-up	RCT	Follow-up
Duration (years)	6.5	18	10	10	7.8	5.5
HbA1c (%) (INT vs CON)	7.2 vs 9.1	Both 8.0	7.0 vs 7.9	Both 7.8	7.9 vs 9.0	7.7 vs 8.0
Retinopathy (%)	76	75	21		58	43
Nephropathy (%) (albuminuria)	54	53	34		61	56
Microvascular complications (%)			25	24		
Any DM-related (%)			12	9		
Any CV (%)		58			53	59
MI (%)			NS	15		
DM-related death (%)			NS	17		
Total mortality (%)			NS	13	NS	46

Table 1: Mean relative risk reduction (%) of different outcomes with INT compared to CON in large clinical trials and its follow-up studies. Adapted from Aschner et al. (2012) [18]

1.4 Bad Metabolic Memory

Metabolic memory has also been used to describe the contrary, which is the prolonged harm caused by chronic hyperglycemia due to poor glycemic control in patients with long-standing Type 2 Diabetes. As a matter of fact, they are two sides of the same coin. (18) This theory was proven in three landmark studies, namely the Veterans Affairs Diabetes Trial (VADT), Action to Control Cardiovascular Risk in Diabetes (ACCORD), and Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) study. The VADT, ACCORD and ADVANCE studies involved high risk patients who were 6, 8 and 12 years older respectively, than the patients in the UKPDS study (mean age is 60). Furthermore, the patients in the ADVANCE, ACCORD and VADT trials had diabetes for about 8, 10 and 12 years respectively, while the UKPDS patients had newly diagnosed diabetes. Moreover, at least 30% of the ADVANCE, ACCORD and VADT patients had a history of macrovascular disease compared to only 7.5% of the UKPDS patients. (19) Intensive glucose control (INT) in the ADVANCE study resulted in only 14% reduction in the risk of microvascular complications (albuminuria) but no significant reduction in the risk of macrovascular complications after a period of 5 years. (20) Similarly, the VADT and ACCORD study had no significant difference in the risk of cardiovascular events or the risk of death from any cause

between both groups after 5.6 and 3.7 years respectively. However, the INT group of both studies had higher rates of adverse events compared to the CON group. (21, 22) In fact, the ACCORD study had to terminate early due to an increased risk of mortality rate by 22% among patients in the INT group. (22) These studies showed a minimal to no reduction in the risk of diabetic complications in the short term despite a reduction in the HbA1c levels among patients with longstanding T2DM and high CVD risk. This indicates that prior poor glucose control has sustained detrimental effects that persisted even after normalization of glycemic control. Therefore, it was probably too late to reverse the effects of longstanding diabetes through strict glycemic control. (23) Table 2 below summarizes the reduction in HbA1c seen in these studies.

Outcomes	Clinical Trials								
	VADT			ACCORD			ADVANCE		
	Initial	CON	INT	Initial	CON	INT	Initial	CON	INT
HbA1c (%)	9.4	8.4	6.9	8.3	7.5	6.5	7.5	7.3	6.5

Table 2: HbA1c levels before and after the trials in both CON and INT groups. [20 - 22]

In summary, it is clear that HbA1c is not a long-lasting biomarker that can accumulate over time and correlate to diabetic complications. Experimental and even clinical studies have suggested that this “Bad” metabolic memory can start to develop very early in diabetes without any relation to the blood glucose levels or HbA1c levels. (18) Therefore, the irreversible damage was probably caused by years of accumulation of a more permanent biomarker called Advanced Glycation End Product (AGE), which is the current concept proposed by many researchers today. (23)

2.0 ROLE OF AGE IN METABOLIC MEMORY

2.1 Pathogenesis of AGE in Diabetic Complications

The state of chronic hyperglycemia in T2DM is one of the major contributors to the development of microvascular and macrovascular complications. Prolonged exposure to hyperglycemia increases the production of reactive oxygen species (ROS), particularly superoxide anion (O2-). Superoxide anion is the main —primary ROS because it is precursor to a bulk variety of other ROS found in mammalian cells, namely hydrogen peroxide and hydroxyl radical. Overproduction of these free radicals leads to oxidative stress. Increased oxidative stress causes β cell dysfunction, insulin resistance and formation of AGE. (24) The formation of AGE is normally part of a physiological aging process but under hyperglycemic conditions, AGE is produced at an accelerated rate. (25) The

accumulation of AGEs in the body can persist even after glycemic control is restored because AGEs-modified collagens are hard to degrade and can remain in diabetic vessels, kidneys and the heart for a prolonged period of time. As a result, excessive production and accumulation of AGEs will enhance chronic inflammation damaging the cells and tissues of various organs in the body. (24) Studies have correlated AGE levels in tissues and serum to the development of Alzheimer's disease, osteoporosis (26) and diabetes-related complications such as the diabetic retinopathy (27) (28, 29), nephropathy (30, 31) and cardiovascular diseases (30, 32, 33). There are several pathways involved in the pathogenesis of AGEs, namely non-enzymatic glycosylation, glucose autoxidation, lipid peroxidation, glycolysis and polyol pathways. (24) The non-enzymatic glycosylation pathway, also known as the Maillard or —browning reaction, is the most common pathway for AGE formation. In this reaction, sugar molecules such as glucose or fructose are glycated to mitochondrial proteins, lipids or nucleic acids without the help of an enzyme leading to the formation of AGEs. A more indepth explanation of this glycation process is the reaction between the carbonyl group of reducing sugars with the amino group of proteins, lipids or nucleic acids in the mitochondria leading to the production of Schiff bases that rearrange to form Amadori products. The Amadori products are, however, unstable causing it to convert to highly reactive carbonyl compounds (glyoxal, methylglyoxal or 3-deoxyglucosone) and eventually to irreversible AGEs. This highly reactive carbonyl compounds are also by-products or intermediates of glucose autoxidation, polyol pathway and lipid peroxidation. Therefore, all of these pathways eventually lead to the 11 formation of AGEs such as Nε-carboxymethyl-lysine (CML), glyoxal-derived lysyl dimer (GOLD), methylglyoxal-derived lysyl dimer (MOLD) and many more, as shown in Figure 2 below. (24) Besides that, AGEs can also be obtained exogenously through diet or tobacco smoking even before the onset of diabetes. This is because food processing involving heat and dehydration such as broiling, searing, and frying) can accelerate the production of new reactive dicarbonyl derivatives through glycoxidation and lipoxidation, as shown above. In a single AGE-rich meal, about 10% of AGEs are absorbed into the circulation and two-thirds of this will remain in the body even after 72 hours, an interval that is adequate enough to cause tissue injury. Thus, nutrient-derived AGE can increase the risk of diabetes as well as diabetic vascular diseases. (34) Furthermore, without altering the HbA1c or blood glucose levels among patients with T2DM and T1DM, 6 weeks of low-AGE diet led to a significant reduction in the markers of inflammation and endothelial dysfunction such as hsCRP, TNFα and VCAM-1, including AGE levels (25, 35) as well as improvement in insulin sensitivity (36), indicating that the progression of diabetes may depend on oxidant (AGE) overload, beyond hyperglycemia. (34) Figure 2 below illustrates the formation of AGE from various pathways.

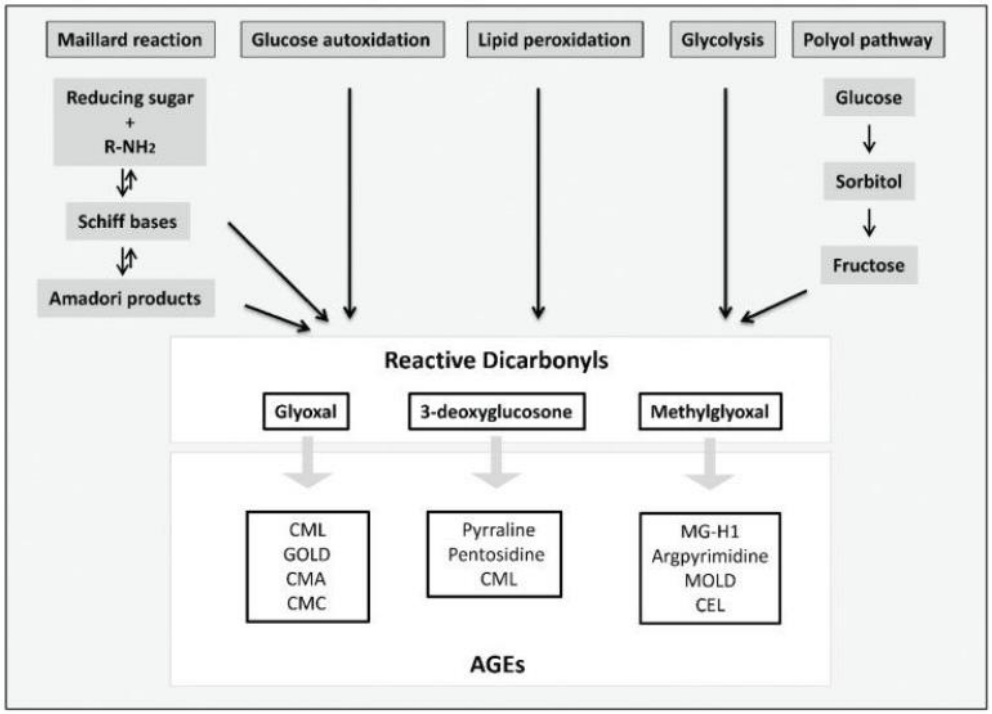

Figure 2 Formation of reactive dicarbonyls and AGEs through different pathways. (24)

2.2 Mechanism of AGE in Metabolic Memory

The exact molecular mechanism of —metabolic memory remains unclear. However, recent research have postulated that the —metabolic phenomenon is linked to chronic hyperglycemia exerting a long lasting detrimental effect on the cardiovascular system through the overproduction of ROS and accumulation of AGEs. The interaction between AGE and the receptor for AGE (RAGE) causes disruption in intracellular DNA synthesis, vascular hyperpermeability, pathological angiogenesis and thrombogenic reaction through the production of vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor-1 (PAI-1). These biochemical reactions are found to have long-lasting effects on the body, linking it to the metabolic memory and tissue damage seen in diabetic complications. (23) Besides that, the binding of AGEs to their receptors (RAGE) also increases the production of ROS leading to a vicious cycle of the —bad metabolic memory. [18] Furthermore, AGE and ROS production via hyperglycemia-induced epigenetic changes may cause an up-regulation of certain pro-inflammatory genes such as the monocyte chemo attractant protein-1 (MCP-1), the p65 subunit of NF-kB and vascular cell adhesion molecule-1 (VCAM-1) genes in target cells. As a result, these overly-expressed genes will cause low grade inflammation, direct tissue damage and prothrombotic state that persist despite returning to normoglycemia state. (23)

In addition, this epigenetic changes also down-regulate endogenous antioxidants such as superoxide dismustase which persist even after optimization of glycemic control. (18) Over time, this epigenetic modulation involving changes in DNA methylation will cause histone alterations in the chromatin structure and deterioration of the miRNA signature which may become irreversible over time. Thus, long-term accumulation of AGE and ROS might lead to a permanent altered gene expression. (23)

Figure 3 below illustrates the link between hyperglycemia, AGEs and genetic predisposition, with the target organs' damage through the —metabolic memory phenomenon. (23) The strength of this hypothesis is based on several evidences shown in animal, pre-clinical and clinical trials. (37) Figure 4 below illustrates the vicious cycle of the metabolic memory. (38)

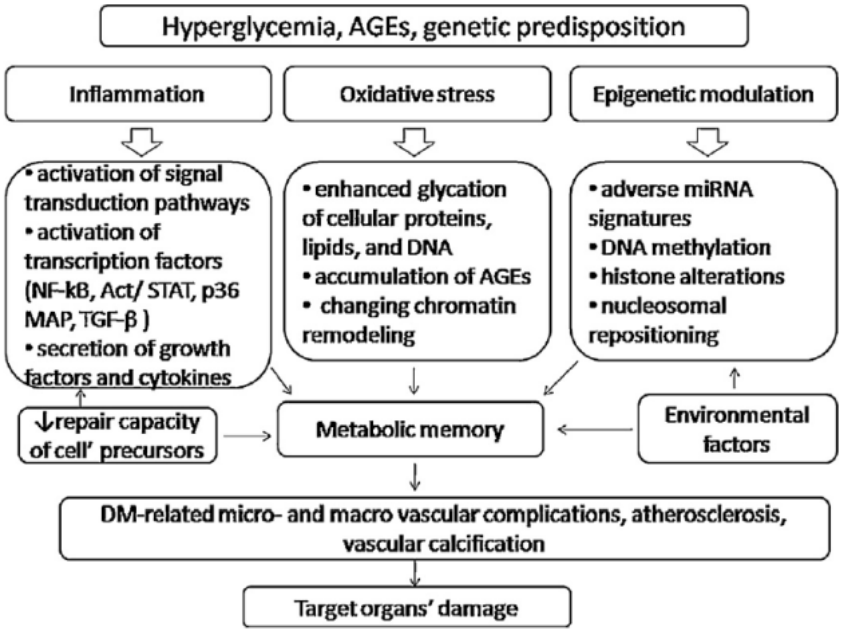

Figure 3 Links between hyperglycaemia, AGE and genetic predisposition (37)

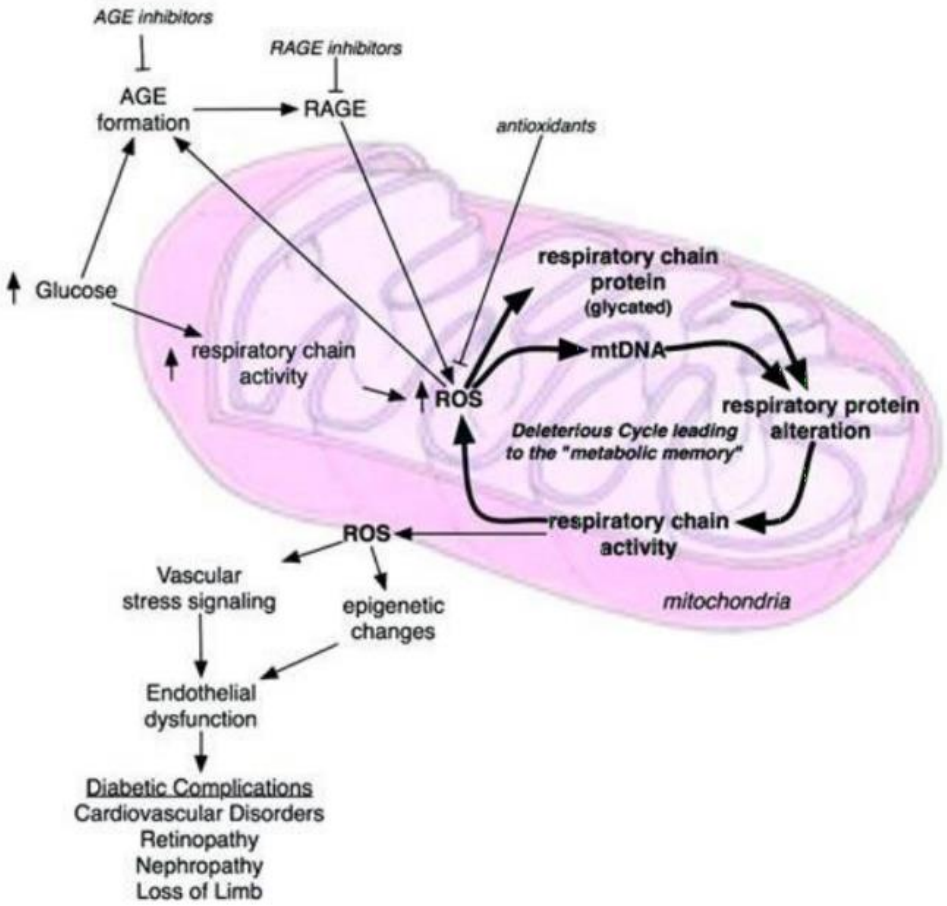

Figure 4 Vicious cycle of Metabolic Memory (38)

2.3 AGE as a Predictor for Risk of Diabetic Complications

Despite normalization of HbA1c levels in patients with long-standing diabetes, the accumulation of AGEs and risk of diabetic complications persisted, indicating a strong correlation between AGE and risk of diabetic complications. (26) This was proven in the DCCT-EDIC trial that showed a combination of AGEs in the skin collagen called furosine (glycated collagen) and carboxymethyllysine (CML) were able to predict the risk of future 10-year progression of diabetic retinopathy and nephropathy even after adjustment for mean HbA1c. In fact, the predictive effect of HbA1c disappeared after the adjustment, further strengthening the role of glycation of proteins and AGE formation in the pathogenesis of diabetic retinopathy and nephropathy. (39) Therefore, AGE could become a better marker than HbA1c in reflecting cumulative diabetic exposure leading to diabetic complications. (26) Furthermore, plasma or serum concentrations of AGEs have been shown to predict total and cardiovascular mortality in both T1DM and T2DM patients. (40, 41) This was also seen among community dwelling elderly women in Baltimore, in which high circulating

carboxymethyl-lysine (CML) and soluble form of RAGE (sRAGE) levels were found to predict CVD mortality. (42) In Tuscany, Italy, it was found that non diabetic adults aged 65 years old and above with plasma CML at the highest tertile had sustained higher all cause mortality and death rate due to CVD. (43) This is expected because AGE levels increase with aging; therefore older people are more likely to have higher levels of AGEs. However, diabetes causes AGE to increase at a faster rate, thus increasing their risk of diabetic-related CVD mortality. Besides that, the ratio of serum AGEs concentrations to the sRAGE was found to be an independent predictor of endothelial dysfunction (39) as well as associated with reduced levels of adiponectin which is an adipocytokine with anti-inflammatory and insulin-sensitizing properties. (44) In contrast to RAGE, the function of sRAGE is to neutralize the RAGE ligands effects, thus inhibiting the RAGE signals seen in diabetes. This sRAGE was found to be inversely related to atherosclerosis. (45) Therefore, circulating concentrations of AGE, RAGE, and the AGE:sRAGE ratio may replace HbA1c and become novel biomarkers capable of predicting risk of future diabetic complications. (26) In our study, AGE and RAGE will be used to predict progression of diabetic nephropathy among T2DM patient with stable glucose control, thus eliminating HbA1c as a confounding factor in our study.

3.0 DIABETIC MICROVASCULAR COMPLICATIONS

3.1 Diabetic Nephropathy (DN)

3.1.1 Role of AGE in DN

Current literatures have shed a new light on the pathogenesis of diabetic nephropathy on a genetic and molecular level. As a result, the traditional view of renal injury in diabetes due to metabolic and hemodynamic changes has transformed significantly. Metabolic factors involving the production of ROS through glucose dependent pathways, as well as hemodynamic factors such as blood pressure control regulated by the renin angiotensin system, are only a partial aspect of a much more complex picture. It is now found that immune mediated inflammatory processes are involved in the pathogenesis of diabetic nephropathy, thus becoming the main focus of today‘s research studies. The immunologic and inflammatory cells implicated are leukocytes, monocytes, and macrophages while the inflammatory molecules involved are chemokines, adhesion molecules, growth factors (VEGF, growth hormone, IGF, TGF-β1), nuclear factors (NFκB) and enzymes (cyclooxygenase-2, nitric oxidesynthase). Overproduction of AGE stimulates the formation of these inflammatory markers contributing to the immune-mediated inflammatory process in diabetic nephropathy. (46) Chronically high levels of AGE causes apoptosis and expression of VEGF in the mesangial cells (47) which are specialized pericytes found in the glomerulus of the kidney. These mesangial cells are vital structures in the kidneys because it helps to modulate glomerular filtration

as well as give structural support. (48) Therefore, loss of mesangial cells or further up regulation of VEGF will lead to enhanced vascular permeability causing hyper filtration and proteinuria in kidney diseases. This suggests that AGE plays a pivotal role in causing diabetic nephropathy. Furthermore, it has been shown that the expression of MCP-1 in mesangial cells is also up regulated by AGE. (49) MCP-1 is a chemokine that modulates the migration and infiltration of macrophage/monocyte, therefore the up regulation of MCP-1 by AGE may precipitate inflammation in renal tissues. Besides that, it is also demonstrated in vitro that AGE can induce TGFβ expression (49, 50) which is associated with elevated expression of extracellular matrix proteins leading to glomerular hypertrophy in vivo. (51) Studies have also reported the interaction between AGE and RAGE can induce TGFβ expression. (52, 53) Therefore, AGE and RAGE are the major perpetrators involved in the progression of diabetic nephropathy. In essence, the mainstay treatment for diabetic nephropathy which is tight glucose and blood pressure control are no longer sufficient to provide an ideal protection against renal progression. (46) Alternative or additive approaches are now crucial to address this new inflammatory pathway involving AGE and RAGE in order to maximize reno-protection and halt the progression of diabetic nephropathy.

3.1.2 Current Treatment for AGE Reduction in DN

Over the years, there were many pharmacological treatments proposed or invented to inhibit the production of AGE in order to delay the progression of diabetic nephropathy. They are benfotiamine, Angiotensin Converting Enzyme (ACE) inhibitors, Angiotensin Receptor Blockers (ARB), aminoguanidine, statin, ALT-711 (alagebrium) and thiazolidinediones. Currently, the results from studies on these drugs are conflicting and limited. (54) For instance, benfothiamine was shown to be effective in preventing oxidative stress and endothelial dysfunction among diabetic patients given meals rich in AGE. (55) However, another study showed the opposite; three months of benfothiamine did not reduce urine albumin excretion rate, plasma or urinary AGEs, inflammation and endothelial dysfunction in patients with type 2 diabetes. (56, 57) Therefore, the role of these drugs in reducing AGE among patients with diabetic nephropathy is still inconclusive. (54) Furthermore, many of these anti-AGE drugs are still under investigation in the preclinical phase (animal testing) such as ACE inhibitor and ALT-711 despite having beneficial anti-AGE effects in diabetic nephropathy. (57, 58) On the other hand, ARB, statin, thiazolidinediones and aminoguanidine have entered clinical trials and most of them showed favourable results. A study on ARB (candesartan) for 3 months had significantly reduced the AGE and albumin levels in 25 patients with diabetes and hypertension (57) while statins (cerivastatin) for 3 months in 69 patients with pre-diabetes or diabetes have improved the lipid profile and reduced the CML levels (58). Thiazolidinediones, an oral anti-diabetic drug have proven to reduce AGE due to its PPAR γ-agonist

activity that increases sRAGE expression. (59) Although these drugs are effective, they are not free from side effects. In fact, the ACTION II study comparing amino guanidine to placebo in 599 patients with T2DM and nephropathy had to be discontinued prematurely because the patients in the high dose group started having severe side effects from the study drug including flu-like symptoms, gastrointestinal problems, hepatic abnormalities and anaemia. (60) Therefore, a more established intervention proven to be safe and readily available in the market is crucial and our study proposes the use of antioxidants. As oxidative stress is a major stimulus for AGE production, antioxidants such as Vitamin A, C and E, glutathione, glutathione peroxidase, glutathione reductase, catalase and enzymes superoxide dismutase play a crucial role in preventing oxidative stress. These antioxidants delay or inhibit cellular damage primarily through their "free radical scavenging" property in the plasma. (34) In diabetes, this endogenous antioxidant defense mechanism is impaired by hyperglycemia causing it to lose its ability to counterbalance toxic ROS in the cells resulting in cell and tissue damage. (61) However, high doses of exogenous Vitamin E have been proven to reduce serum AGE levels in-vitro and *in vivo*. (62-64) In fact, our previous study has shown that high doses of Tocotrienol-rich vitamin E reduced the AGE and HbA1c levels in rats with metabolic syndrome as well as suppressed RAGE expression in liver. (65) Therefore, this clinical trial is the next step of our prior animal studies on vitamin E and we will be investigating the role of Tocotrienol-rich vitamin E in patients with T2DM and early diabetic nephropathy.

3.1.3 Other Biomarkers for DN

Besides AGE, other biomarkers capable of predicting the risk of diabetic nephropathy is Cystatin C, nephrin, liver-type fatty acid-binding protein (L-FABP), neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1) and many more. (66) In our study, Cystatin C was chosen in addition to AGE and RAGE. Although Cystatin C is not involved in the AGE-RAGE pathway, numerous studies have shown Cystatin C has the highest sensitivity and specificity for detecting early diabetic nephropathy. According to a study, the sensitivity and specificity of serum Cystatin C to detect stage 2 Chronic Kidney Disease (CKD) in patients with normolbuminuria was 71.1% and 77.3% respectively and micro- and macroalbuminuria was 78.7% and 83% respectively. In fact, many studies have suggested that cystatin C-based eGFR is a better diagnostic instrument for predicting early diabetic nephropathy in the microalbuminuria stage as compared to creatinine-based eGFR. (67) This is crucial because 29.1–61.6 % of people with T2DM may have kidney damage even before the appearance of microalbuminuria, the gold standard for early diagnosis. (68) Therefore, our study aims to investigate if Tocotrienol-rich Vitamin E can reduce not only AGE, RAGE and microalbuminuria but also cystatin C in patients with T2DM and early nephropathy.

3.2 Diabetic Retinopathy (DR)

Retinopathy is a major complication of diabetes mellitus and is the most common cause of blindness in middle-aged individuals despite the availability of treatment. (69) Fortunately, 90% of visual impairment caused by DR can be prevented with proper management. (70) However to date, diabetic retinopathy (DR) can only be diagnosed through formal clinical examination by trained healthcare personnel using sophisticated ophthalmological instruments. The sensitivity of detecting sight-threatening DR using direct ophthalmoscopy varies among practitioners. Ophthalmological examination by general practitioners produce a sensitivity of 41%-67%, ophthalmologists 65%, optometrists 48%-82%, while diabetologists and hospital physicians give a sensitivity of 27%-67%. (71) DR diagnosis through photographic methods with subsequent grading by trained personnel increases the sensitivity of detecting sight-threatening DR to 87%-100%, giving specificity of 83%-96%. (71) Photographic methods significantly improve the ability of practitioners to detect sight-threatening DR correctly. However, the method requires costly equipment and adequate operator training, which may be a challenge for developing countries.

3.2.1 Potential Biomarkers for DR

A large number of potential biological markers have been explored with the advancement of molecular medicine. Older biological markers include inflammatory markers, advanced glycosylated end products (AGEs), and vascular endothelial growth factor (VEGF). (72) Newer proteins have been explore including pigment epithelium derivedfactor (PEDF), brain derived neorotrophic factor (BDNF), brain natriuretic peptide (BNP), SA100A12, pentraxin 3, apelin 3, and chemerin. (72) In addition, researches on DR biological markers have expanded to investigate metabolites such as homocysteine, folate, and lipoprotein A, as well as microRNA. (72) However, the definitive biological marker for DR remains elusive due to the complex pathogenesis of DR as shown in the figure below. Figure 5 illustrates the multiple pathways which are involved in the development on DR. (72)

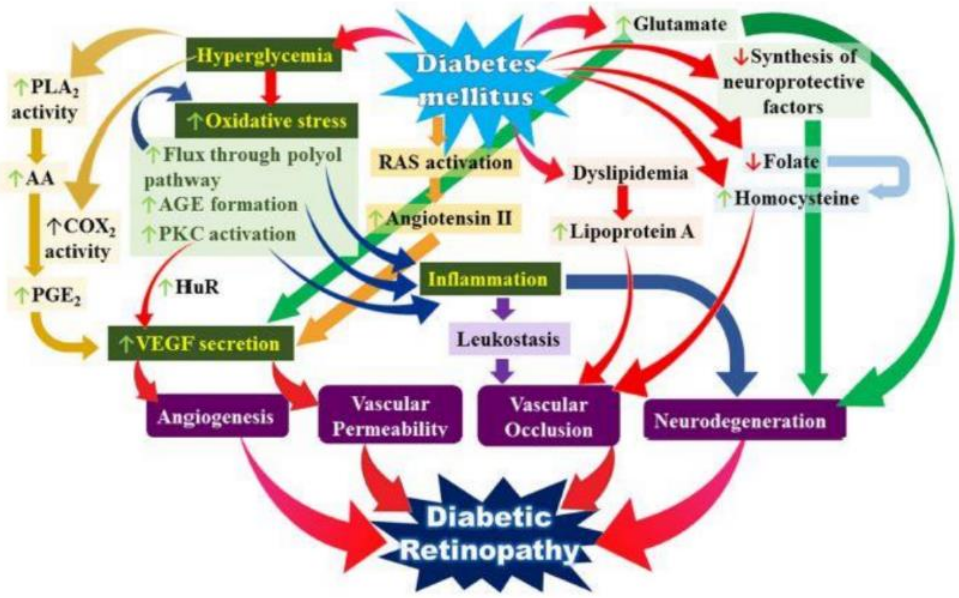

Figure 5 Overview of multiple interrelated pathways leading to the development of diabetic retinopathy. (72)

3.2.2 Evidence of Tocotrienol rich Vitamin E on DR

The use of tocotrienol in preventing or improving diabetic retinopathy is still at its infancy. More clinical studies should be conducted to explore this topic given its promising role as a powerful antioxidant. The following tables (Table 3 and 4) summarizes the results of Vitamin E in both animal and human (clinical) trial studies.

Citation	Study Type	Conclusion
Bursell et al (1999) (73)	Clinical (N=45)	Vitamin E improved retinal blood flow and creatinine clearance
Chida et al (1999) (74)	In vitro (Bovine retina)	Palm oil-derived vitamin E conferred significant protection in the retina, retinal pigment epithelium and rod outer segments
Nakagawa et al (2007) (75)	Animals (Rats)	Tocotrienol is a powerful anti-angiogenetic inhibitor
Montero et al (2013) (76)	Systematic reviews & Meta-analysis	Vitamin E and/or C supplementation improves endothelial function in non-obese cohort

Table 3 Summary of studies showing effects of tocotrienol on glycemic and metabolic control in diabetes. (73-76)

3.2.3 Justification for Vitamin E as potential treatment for DR

Tocotrienol rich Vitamin E had significantly reduce retinal microhaemorrhage in T2DM patients with DR in our previous pilot study. However, there was no difference in AGE, RAGE, and homocysteine levels between treatment and control groups. This suggests that Vitamin E may reduce retinal hemorrhage through another pathway other than reducing inflammation. The potential protective effect of Vitamin E against the development of DR has not been thoroughly evaluated in epidemiologic studies. We now wish to extend the study to include more subjects for longer duration of therapy. Plasma thromboxane B2 (TXB2) will be investigated to discover the potential antithrombotic effect of Vitamin E, particularly tocotrienol compound. We will conduct a prospective cohort study on patients with Type 2 diabetes mellitus (T2DM) with retinal microhemorrhages, comparing the effect tocotreinol-rich Vitamin E versus placebo on mild to moderate DR. The main aim of the study is to determine the serum Vitamin E level in subjects and assess the correlation of serum Vitamin E level and mild to moderate DR.

3.3 Diabetic Peripheral Neuropathy (DPN)

Diabetes mellitus leads to neuropathic syndromes which are often classified to acute or chronic subtype and focal or diffused neurological deficit. (77) Among all the subtype classifications, typical diabetic peripheral neuropathy (DPN), also known as distal symmetrical polyneuropathy (DSPN), accounts for approximately 75% of diabetic neuropathies, (78, 79) and is the commonest neuropathic syndromes in diabetic patients. (77-80)

3.3.1 Postulated Pathophysiology of DPN

Over the years, the pathogenesis of DPN is attributed to chronic hyperglycemia but the exact etiology is yet to be found. Based on previous experimental studies, the pathogenesis of DPN is multifactorial, in which hyperglycemia plays a principal role in the various interrelated pathways of DPN development. (81, 82) Current views regarding pathogenesis of DPN mainly focus on metabolic dysfunction among diabetics which subsequently leads to oxidative and inflammatory stress response.(79) The metabolic pathways include hexosamine pathway, polyol pathway, poly(ADP ribose) polymerase (PARP), protein kinase C (PKC) pathway, formation of advanced glycation end-products (AGEs), increased pro-inflammatory cytokines, reduced nerve growth factors (NGF), increased neurotrophin 3, hedgehog (Hh) signaling pathway and autophagic pathway.(81) Nonetheless, only AGEs formation polyol pathway, oxidative stress, increased pro-inflammatory cytokines and reduced NGF will be discussed in this write-up as other pathways are beyond the scope of this research project.

3.3.1.i Maillard Reaction and Amadori Product

Increased AGEs and their receptors have been proposed to be one of the important mechanisms which results in diabetic complications including DPN. (82, 83) In chronic hyperglycemia among diabetic patients, Maillard reaction takes place in which the carbonyl group of reducing sugars reacts non-enzymatically with free amino groups of proteins. (84) This reaction is divided into 3 stages, namely early, intermediate and late stages. In the early stage, the reaction forms an unstable Schiff base product that subsequently rearrange to form a relatively stable amadori product. The amadori products then degrades to various forms of dicarbonyl compounds in the intermediate stage. Finally, during the late stage of Maillard reaction, oxidation, dehydration and condensation reactions occur, forming irreversible AGEs (Figure 1). (83, 85, 86)

3.3.1.ii Chronic hyperglycaemia and Receptors for AGE (RAGE)

The AGEs that was produced will bind to receptors for AGE (RAGE), which in turn affects cellular tissues by altering the intracellular protein structures as well as the extracellular matrix constituents, thereby modifying their normal function. Besides, AGEs alter the cellular plasma proteins by their interactions with RAGE on macrophages, microglial, endothelial cells and mesangial cells. Consequently, reactive oxygen species (ROS) is then produced due to the AGE-RAGE interactions. This ligation also triggers the inflammatory pathway through activation of transcription factor nuclear factor-κβ (NF-κβ). Thus, a pro-inflammatory expression of gene is induced, producing cytokines such as TNF-α and IL-1β. Moreover, NF-κβ activation also stimulates programmed neuronal cell death.(82) With reference to DPN, AGE-RAGE interactions occur at the neuronal level as RAGE expression is enhanced in diabetes, particularly in the Schwann cells as well as the endothelial cells of the perineural and endoneural blood vessels. Consequently, the end product of this interaction is nerve fibers degeneration and atrophy.(83)

Formation of AGEs, polyol pathways as well as other pathways are linked to oxidative stressed in diabetes, which showed the importance of this pathway in DPN development (Figure 2).(87, 88) Chronic hyperglycemia causes excessive production of superoxide, a precursor of reactive oxygen species (ROS),(89) in the mitochondria.(90)

3.3.1.iii Reactive Oxidative Species (ROS) and other Inflammatory biomarkers

The superoxide produced will then be converted to hydrogen peroxide and other ROS's which will cause damage to the cellular tissues in diabetic patients, leading to neuropathy, nephropathy, retinopathy and cardiomyopathy.(90) In DPN, superoxide overproduction in the neuronal tissues activate the formation of AGEs, polyol pathways, hexosamine pathway and PKC pathway, leading to neuronal dysfunction.(88)

Furthermore, diabetes displays chronic inflammation features and triggers the proinflammatory pathways which are interconnected with the other metabolic pathways mentioned above.(91) This inflammatory mechanisms upregulates proinflammatory cytokines including tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6 and IL-8.(81, 92) Also, there are increased circulating intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) which indicate chronic inflammation in diabetic patients.(92, 93) All these molecules have been associated with development of diabetic complications.

Last but not least, NGF, a neurotrophic factor, also plays a crucial role in development of DPN as it is involved in neuronal survival and development.(87) In previous studies on animals, it was shown that NGF was reduced in diabetes.(81) In addition, a study conducted by Sun Q et al showed that NGF is reduced in diabetic population, and markedly reduced among those with DPN.(94) Figure 3 depicts other pathogenesis

3.3.2 Diagnosis of DPN

Nerve conduction study (NCS) should be used as a standard diagnostic tool for DPN given its objective quantification even in the early progression of the disease.(80) Moreover, NCS is able to detect subclinical changes of DPN among diabetic patients (Figure 4). (95) NCS is able to determine the functionality of the nerve, whether it is normal, sustained axonal injury or demyelinated. (95) For instance, in the response latency and nerve conduction velocity will be prolonged in demyelinated nerves; amplitude is reduced in axonal injury.(96) According to a research study done in 2008 by Kong X et al, NCS is suitable in aiding the diagnosis of DPN.(97) However, this test is recommended to be done only for controlled clinical trials or epidemiology surveys because it requires specific devices, trained examiners, is time consuming and does not have standardized criteria in diagnosing DPN. (98) Besides, NCS is sensitive only sensitive in picking up large fiber neuropathy but not small fiber neuropathy. (78, 99) Figure 6 below illustrates the subclinical and clinical symptoms of DPN and shows the diabetic peripheral neuropathy assessment with reference to progression of the condition. (95)

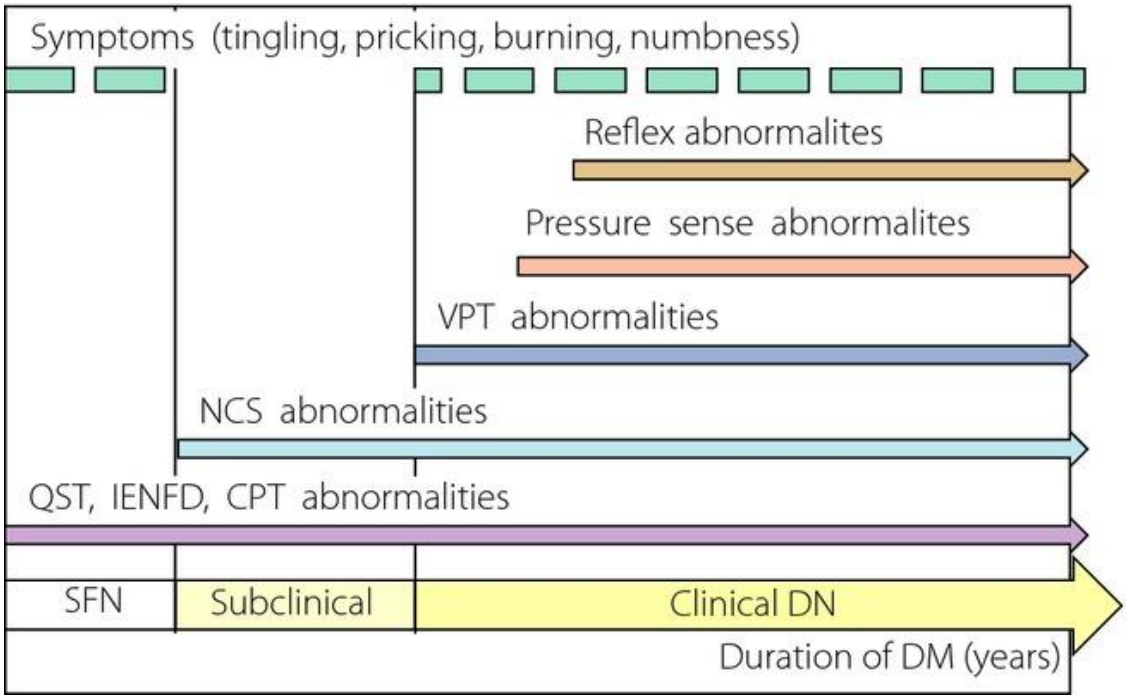

Figure 6 Subclinical and Clinical Symptoms of DPN in NCS tests (95)

4.0 VITAMIN E

4.1 Background of Vitamin E: Tocopherol vs Tocotrienol

Vitamin E was first discovered by Herbert Evans and Katherine Bishop in 1922 in lipid-rich plant products and vegetable oils. There are 8 naturally-occurring isoforms of Vitamin E, namely α-, β-, γ- and δtocopherol and α-, β-, γ- and δ-tocotrienol. Usually these Vitamin E sources contain a mixture of tocotrienol and tocopherol isoforms. However, the sources that are primarily rich in tocotrienol are palm oil, rice bran, coconut oil, cocoa butter, soybeans, barley, wheat germ, latex and annatto seeds. The most abundant among these natural sources is bran oil, palm oil and annatto seeds; the ratio of tocotrienol to tocopherol is 55:45, 70:30 and 100:0 respectively. Vitamin E derived from palm oil, also known as Elaeis guineensis, is readily available in Malaysia but annatto seeds have to be imported from overseas. On the other hand, sources that are mainly rich in tocopherol are sunflower, peanut, almond, avocado, sesame and olive oils. (66) The bioavailability of tocotrienol in the body is much more limited compared to tocopherol because of the poorer absorption rate of tocotrienol in the liver as compared to tocopherol. This is because the αtocopherol transport protein in the liver has 8.5 fold lower affinity to bind to tocotrienol than tocopherol. As a result, the unbounded tocotrienols are vulnerable to catabolization via cytochrome P450-initiated-ωhydroxylation and oxidation by ω-hydroxylase. Hence, tocotrienol has a much shorter plasma half-life compared to tocopherol. Furthermore, the bioavailability of tocotrienol is also affected by

the nutritional status, presence of tocopherol and food processing. This sparked a huge debate for many years on whether tocotrienol, if administered orally, can reach vital organs in the body. Consequently, the research on tocotrienols dampened in the 1990s. (66, 100) Besides that, Tocopherol was the first Vitamin E isoform to be identified and it was deemed to be the major isoform with the most superior antioxidant and biological activity. Thus, it became the main focus of vitamin E research since the 1920s. Research on tocotrienol has only garnered special attention over the recent years because numerous studies have shown otherwise; tocotrienol-rich vitamin E was a 50x more potent antioxidant compared to tocopherol-rich vitamin E. The reason behind this lies in the unique ability of tocotrienol in achieving a better uniform distribution in the phospholipid bilayer of the plasma membrane. The more even the distribution, the more efficient the collision of alpha-tocotrienol with free radicals, therefore maximizing its scavenging ability. (66) The potent antioxidant property of tocotrienol is also attributed to its distinctive structural form compared to tocopherol. Tocotrienols have three unsaturated bonds in the carbon side chain and only one chiral center, whereas tocopherols have no unsaturated bonds, only a long saturated carbon side chain with many chiral centers. This distinctive characteristric promotes the efficient metabolic 19 function of tocotrienols, allowing it to penetrate tissues with saturated fatty layers, such as the brain and liver, more readily as compared to tocopherols. (66, 100) Figure 7 below illustrates the different structural forms of tocopherol and tocotrienol.

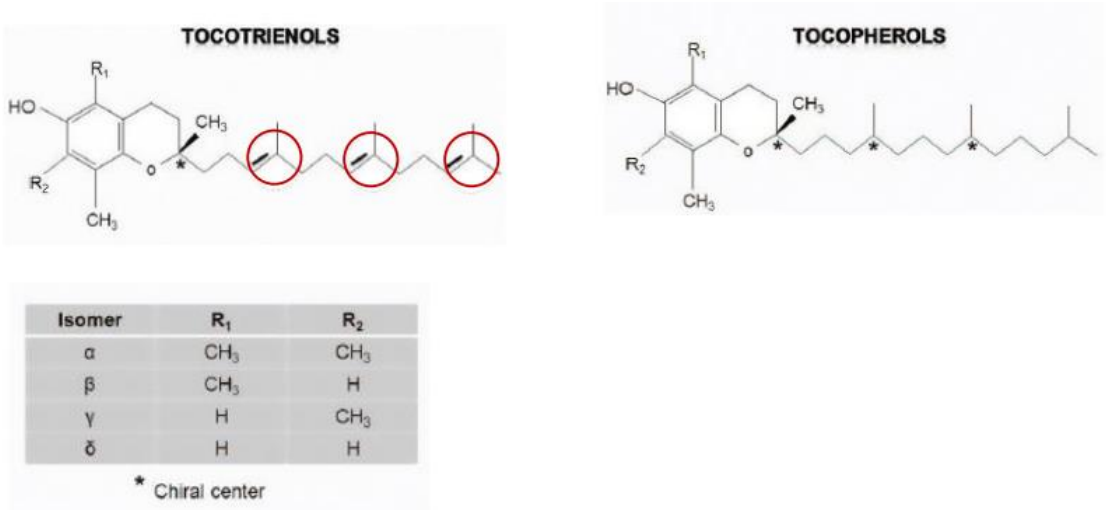

Figure 7 The structures of tocotrienols and tocopherols. (66) The double bonds are in the circles.

4.2 Role of Vitamin E in Diabetes

As a potent antioxidant, Vitamin E plays a crucial role in the treatment of diabetes, a disease caused by hyperglycemia and oxidative stress as mentioned previously. According to a prospective cohort

study, the intake of vitamin E for 23 years reduced the risk of developing T2DM due to its antioxidant effects. (101) This therefore indicates the beneficial role of vitamin E in diabetes. In our study, tocotrienol-rich vitamin E from palm oil was chosen due to its superior antioxidant properties. The following section will explore the benefits of tocotrienol-rich vitamin E in diabetes and diabetic nephropathy. Figure 4: The structures of tocotrienols and tocopherols. The double bonds are in the circles. Adapted from Peh 2016. (66)

4.3 Tocotrienol

4.3.1 Benefits of Tocotrienol-Rich Vitamin E

Besides being a superior antioxidant, tocotrienol-rich vitamin E has also proven to triumph over tocopherol with its superior anti-glycemic, anti-inflammatory, anti-cholesterolemic, anti-cancer, cardioprotective and neuroprotective properties. The anti-cholesterolemic properties are unique to tocotrienol only because it inhibits the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, thus lowering the cholesterol levels. Besides that, tocotrienol is capable of attenuating inflammation by downregulating the activation of transcription factor NF-κB which is not seen in tocopherol. Additionally, tocotrienol is a powerful radioprotectant against radiation damage giving it a unique anti-cancer property. (66) In fact, our previous studies have demonstrated most of these properties as shown in Table 4 below.

Ongoing and completed clinical trials of tocotrienols[a].

Drug	Disease conditions	Phase	Status (last updated)	Clinical trial
Naturally occurring tocotrienols				
δ-Tocotrienol and TRF	Metastatic breast cancer	I	Completed (2014)	NCT01571921
δ-Tocotrienol	Pancreatic cancer	I	Ongoing (2015)	NCT01450046 NCT01446952 NCT00985777
Tocotrienols	Pharmacokinetics	I	Completed (2010)	NCT01185769
Tocotrienols	Attention deficit disorder	III	Completed (2013)	NCT01855984
Tocotrienols	Cognitive impairment in types 1 and 2 diabetes mellitus	IV	Ongoing (2015)	NCT01973400
Tocotrienols	Ischemic stroke	III	Ongoing (2014)	NCT02263924
Tocotrienols	Ovarian cancer	II	Ongoing (2015)	NCT02399592
Tocotrienols	Osteoporosis	II	Ongoing (2015)	NCT02058420
Tocotrienols	Pharmacokinetics	I	Completed (2014)	NCT00678834
Tocotrienols	Stroke	–	Recruiting (2015)	NCT01858311
Topical and oral tocotrienols	Scar	I	Ongoing (2015)	NCT01579227, NCT00700791
γ-/δ-TRF	Metabolic syndrome	–	Recruiting (2012)	NCT01626430
TRF	Breast cancer	Pilot trial	Completed (2010)	NCT01157026
TRF	Cerebrovascular disorders	II	Completed (2014)	NCT00753532
TRF	Cholesterol lowering	III	Ongoing (2014)	NCT02142569
TRF	Chronic haemodialysis	–	Recruiting (2015)	NCT02358967
TRF	Platelet aggregation in metabolic syndrome	I	Completed (2013)	NCT01631838
Modified tocotrienols				
EPI-743	Friedreich's ataxia	II	Ongoing (2015)	NCT01962363
EPI-743	Leber's hereditary optic neuropathy	Single patient	Completed (2014)	NCT02300753
EPI-743	Leigh syndrome	II	Ongoing (2015)	NCT02352896
EPI-743	Mitochondrial disorders	II	Ongoing (2015)	NCT01642056
EPI-743	Pearson syndrome	II	Ongoing (2015)	NCT02104336

TRF: tocotrienol-rich fraction.
[a] Data obtained from http://clinicaltrials.gov/ in September 2015.

Table 4 The list of previous studies on Tocotrienol-rich Vitamin E. (102-106)

Thus, our previous studies have demonstrated that tocotrienol-rich Vitamin E does have an effect on the AGE-RAGE pathway and therefore, the role of tocotrienol-rich Vitamin E in diabetes is

paramount. Numerous other studies have further supported our findings that tocotrienol-rich vitamin E is a superior antiglycemic compound than tocopherol in Type 2 Diabetes. (46, 107-109)

4.3.2 Evidence of Tocotrienol in the treatment of Diabetic Nephropathy

Although the antioxidant, anti-glycemic and anti-cholesterolemic properties of tocotrienol-rich vitamin E in diabetes are well-established, there are currently very limited studies that reported the beneficial effects of tocotrienol in preventing or delaying diabetic nephropathy. (107) A study on streptozocin-induced diabetic rats reported that tocotrienol-rich vitamin E ameliorated diabetes and even reversed the damage to the kidneys through regulation of caspase-3 and TNF-α-induced NF-κβ signaling pathway. (46) In another study, tocotrienol-rich vitamin E attenuated lipid-induced nephropathy because it normalized the glycemic control, serum lipid profile as well as the kidney function in type-2 diabetic rats. Tocotrienol-rich vitamin E also reduced the progression of diabetic nephropathy through downregulation of TGF-β, fibronectin and collagen type IV expression in diabetic rats. (107) At the moment, there is only one clinical trial on vitamin E and diabetic nephropathy. This study demonstrated that high doses (1500 IU/d) of oral vitamin E supplementation for 12 weeks reduced the oxidative stress, inflammation and biomarkers of kidney injury in 60 diabetic patients with nephropathy. (110) This clinical trial is similar to our study because the biomarkers used to show improvement in diabetic nephropathy are proteinuria, urine protein-to-creatinine ratio (UPCR), blood urea nitrogen (BUN), serum creatinine (eGFR) and serum total protein. The only difference is that we are using microalbuminuria and urine albumin to creatinine ratio (UACR) instead of proteinuria and UPCR to detect early diabetic nephropathy. Furthermore, it was not stated whether that the vitamin E used was tocopherol-rich vitamin E or tocotrienol-rich vitamin E, whereas in our study, tocotrienol-rich vitamin E is used because of its superiority as mentioned previously. Table 6 below summarizes the studies available on vitamin E and diabetic nephropathy.

Citation	**Study Type**	**Conclusion**
Kuhad et al (2009) (46)	Animal (Rats)	Tocotrienol reversed Diabetic Nephropathy through modulation of TNF-αinduced NF-κβ signaling pathway and caspase-3
Siddiqui et al (2013) (107)	Animal (Rats)	Tocotrienol improved lipid-induced nephropathy by its hypoglycemic, hypolipidemic and antioxidant activities & by down-regulating TGF-β expression
Khatami et al (2016) (110)	Clinical Trial (N=60)	Vitamin E reduced proteinuria, urine protein-to-creatinine ratio, AGE, MDA, TNF-α, MMP-2 and MMP-9.

Table 5 Vitamin E and Diabetic Nephropathy Studies. (46, 107, 110)

4.3.3 Controversy Surrounding the Role of Vitamin E in Diabetes

The role of Vitamin E in DM has been heavily debated for the past few decades. Several systematic reviews have been conducted to gather evidences regarding the effect of Vitamin E on glycemic control and antioxidant status of patients with DM.

4.3.3.i Limited Evidence for Vitamin E in treatment of diabetes and its complications

Earlier systematic reviews have shown that Vitamin E is only beneficial to a subgroup of individuals with T2DM. Suksomboon et al conducted a systematic review on RCTs up until November 2008 to evaluate the effect of Vitamin E supplementation on glycemic control in individuals with T2DM.(111) The study involved nine RCTs with 418 patients identified from EMBASE, EBM reviews, MEDLINE, and Cochrane Library databases. (111) They concluded that there was no significant beneficial effects exhibited by Vitamin E in improving glycemic control. (111) However, Vitamin E supplementation does improve glycemic control in participants with baseline HbA1c of $\geq$8% and in subgroups of participants with low serum vitamin E levels. (111)

Later, Xu et al published another systematic review in year 2014 investigating the beneficial effects of Vitamin E on glycemic control in individuals with T2DM. (112) The study involved 14 RCTs with 714 participants identified from EMBASE, Cochrane Library, and PubMed databases up until April 2013. (112) Similarly, they concluded that Vitamin E supplementation did not significantly improve HbA1c, fasting insulin, and fasting glucose levels. (112) However, subgroup analysis revealed that participants with low baseline Vitamin E levels achieved significant improvement in HbA1c and fasting insulin as shown in figures below. (112) In summary, the findings from systematic reviews by Suksomboon et al and Xu et al suggest that Vitamin E may act as a complementary targeted therapy for T2DM patients with HbA1c >8% and low serum Vitamin E levels.

4.3.3.ii Encouraging Evidence for Vitamin E in treatment of diabetes and its complications

However, recent systematic review suggests otherwise. Balbi et al has published a systematic review in March 2018 which investigated the effect of vitamin supplementation on glycemic index and antioxidant status of individuals with T2DM. (113) 12 randomized controlled trials (RCT) were included from Web of Science, Scopus, and PubMed databases up until December 2017. (113) After comparing to Vitamins C, D, and E with placebo, they concluded that Vitamin E significantly improved HbA1c and blood glucose levels in T2DM compared to placebo as shown in figures below. (113) Concurrently, they revealed that Vitamin E is able to improve the oxidation status in T2DM through different mechanisms through markedly reducing malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) levels, in the

meantime significantly elevating glutathione peroxidase (GPx), total antioxidant capacity (TAC), and superoxide dismutase enzyme (SOD) levels compared to placebo.(113)

4.3.3.iii Justification or Rationale of Vitamin E as complementary treatment

The use of Vitamin E as a complementary therapy in DM is gaining acknowledgement. However, a huge majority of these articles focused on isoform α-tocopherol. As mentioned earlier, tocotrienols exhibit unique biological functions and are functionally distinct from tocopherol. Therefore, researches and investigations on tocopherols should not be extrapolated to tocotrienols. Although the evidence regarding tocotrienol is still scarce, there has been an increasing trend in tocotrienol publications over the past two decades as shown in Table 5. (66)

4.3.4 Summary of Evidence of Vitamin E on Diabetes and its Complications

Summary of studies showing effects of tocotrienol on glycemic and metabolic control in diabetes in animal studies and one clinical study. (46, 61, 103, 106, 108, 114-121)

Citation	**Study Type**	**Conclusion**
Kuhad et al (2009) (114)	Animal (Rats)	Reduced diabetes-associated cognitive impairment by NFκβ signalling pathway
Kuhad & Chopra (2009a) (115)	Animal (Rats)	Reduced neuropathic pain by modulating oxidative-nitrosative stress and inflammatory mechanisms
Kuhad & Chopra (2009b) (46)	Animal (Rats)	Improved kidney function by attenuation of TNFα induced NFκβ and capase 3 mobilisation
Muharis et al (2010) (116)	Animal (Rats)	Improved vascular endothelial function
Patel et al (2011) (117)	Animal (Rats)	Prevent and reverse metabolic and cardiovascular damage
Wan Nazaimoon & Khalid (2002) (103)	Animal (Rats)	Improved blood glucose and HbA1C in diabetic rats Reduced AGE in normal rats
Baliarsingh et al (2005) (118)	Clinical (N=19)	Improved lipid profile in T2DM patients
Budin et al (2009) (106)	Animal (Rats)	Lowered blood glucose and improved dyslipidaemia in diabetic rats Reduced oxidative stress and maintained vessel wall integrity
Fang et al (2010) (119)	Animal (Rats)	Improved insulin sensitivity in mice through activation of PPARS
Siddiqui et al	Animal	Palm oil derived TRF more effective hypoglycaemic and

(2010) (120)	(Rats)	nephroprotective agent in diabetic nephropathy than rice bran oil TRF
Kanaya et al (2004) (121)	Animal (Rats)	Protective effect against oxidative damage in DM
Mantough et al (2014) (108)	Animal (Rats)	Inhibit lipid peroxidation and increasing levels of antioxidant status

Table 6 Summary of studies showing effects of tocotrienol on glycemic and metabolic control in diabetes in animal studies and one clinical study. (46, 61, 103, 106, 108, 114-121)

5.0 FUNDING for project:

Dr Badariah and co-workers have been awarded RM177000 under the FRGS Grant 212094-267016 from 2018-2019. Previous Study Pilot Project was completed 2017-2018 with 48 patients under exactly the same protocol. Significant findings were found and was published in Nutrients 2018.

C. Research Protocol

1.0 General Information

1.1 Study Title

Tocotrienol-rich Vitamin E (Tocovid) and its Effects in Diabetes and Diabetic Microvascular Complications: Nephropathy, Retinopathy and Neuropathy

1.2 Protocol Details:

Protocol Number: Not available yet (NMRR ID: 45140)

Protocol Version: 1

Protocol Date: 7 February 2019

1.3 Sponsor

Name and Address of Sponsor	Sponsored Items
Jeffrey Cheah School of Medicine and Health Sciences **Monash University Malaysia** Jalan Lagoon Selatan, Bandar Sunway, 47500 Subang Jaya, Selangor Darul Ehsan Telephone No: +603-5514 6000	All items except tocovid and placebo. This includes: • Remuneration for test subjects • Additional tests (e.g. ECG strips, urine dipsticks) • Consumables (e.g. gloves, syringe and needle) Refer to Finances and Budget for a detailed

Fax No: +60355146001	list.
Hovid-Integrated Global Pharmaceutical Partner Berhad Headquarters 121, Jalan Tunku Abdul Rahman, 30010 Ipoh, Perak Telephone No: +605-506 0690	• Tocovid 200mg capsule • Placebo capsule

1.4 Name and Institution of Local Investigator/s

Dr Gerald Tan Chen Jie[1]

Dr Ng Yeek Tatt[1]

Dr Ng En Yng[1]

Professor Dato' Khalid Abdul Kadir[1]

Dr Badariah Ahmad[1]

[1]Monash University Malaysia, Bandar Sunway

For Correspondence

Professor Khalid Kadir

E-mail: khalid.kadir@monash.edu

E-mail: badariah.ahmad@monash.edu

1.5 Investigation Sites, Study Team & Appropriateness

Investigation Site & Address	**Clinical Research Centre,** **Jeffrey Cheah School of Medicine and Health Sciences** **Monash University Malaysia** No 20 & 22, Jalan PJS 11/5, Bandar Sunway, 46150 Petaling Jaya, Selangor Darul Ehsan.
Study Team	**Investigators:** Professor Dato' Khalid Abdul Kadir Dr Badariah Ahmad Dr Gerald Tan Chen Jie Dr Ng Yeek Tatt Dr Ng En Yng **Research Nurse**: Noras'kin binti Mohamad

	Clinical Site Coordinator: Zulaikha binti Ungku Omar
Appropriateness	**Facilities:** This clinical research centre is fully equipped to conduct clinical trials. **Expertise:** Under the co-supervision of Prof. Khalid Abdul Kadir and Dr Badariah Ahmad **Patient Populations**: Patients with T2DM who have volunteered to participate in the study and fulfilled the inclusion and exclusion criteria. (Refer to **Section 5.4** and **Section 5.5** for the inclusion and exclusion criteria)

1.5.1 Potential Recruitment Centres

Clinical Research Centre, Monash University Malaysia has enrolled more than 200 patients into clinical trials conducted in the past and at present. These patients have varying degrees of diabetic control and complications. Patients who fulfill the inclusion and exclusion criteria will be obtained from this existing pool.

Additionally, potentially eligible patients may be referred to our Clinical Research Center from the following places for further screening:

Potential Recruitment Centres	**Address/Location**
Klinik Kesihatan Tanglin, Kuala Lumpur	Jalan Cenderasari, Tasik Perdana, 50480 Kuala Lumpur, Wilayah Persekutuan Kuala Lumpur.
Tun Hussein Onn Eye Hospital	Lot 2, Lorong Utara (B), Pjs 52, 46200 Petaling Jaya, Selangor
Sunway Medical Centre	5, Jalan Lagoon Selatan, Bandar Sunway, 47500 Petaling Jaya, Selangor
Klinik Kesihatan Kelana Jaya	38294, Jln.SS6/3A, Ss 6, 47301 Petaling Jaya, Selangor
Hospital Sultanah Aminah, Johor Bahru	Jalan Abu Bakar, Masjid Sultan Abu Bakar, 80000 Johor Bahru, Johor

1.6 Declaration of Conflict of Interest

The authors of this study declare that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this study.

There are also no potential conflict(s) of interest or any competing interest(s) among the study team members.

2.0 Background/Literature Review

Please refer to the attached document for the complete literature review.

3.0 Objectives and Purpose

1. To demonstrate the anti-oxidant effect of Tocovid by measuring circulating levels of AGE, MDA and TNFR1 in patients with diabetic nephropathy.
2. To demonstrate the anti-inflammatory effect of Tocovid by measuring circulating levels of IL-6 and VCAM-1 in patients with diabetic nephropathy.
3. To establish renal protective effect of Tocovid by measuring microalbuminuria (UACR) and serum creatinine (eGFR) in patients with diabetic nephropathy.
4. To establish correlation between changes in circulating AGE, MDA, TNFR1, IL-6 and VCAM-1 to changes in diabetic nephropathy as assessed by microalbuminuria (UACR) and serum creatinine (eGFR) in patients with diabetic nephropathy.
5. To establish retinal protective effect of Tocovid by measuring intraretinal microhemorrhage(s) in patients with diabetic retinopathy.
6. To determine the anti-thrombotic effect of Tocovid by measuring the circulating levels of thromboxane B2 levels in patients with diabetic retinopathy.
7. To establish correlation between changes in circulating serum thromboxane B2 levels and intraretinal haemorrhage(s) as assessed with retinal photography in patients with diabetic retinopathy.
8. To establish baseline nerve conduction parameters (i.e. conduction velocity) in patients with diabetic peripheral neuropathy.
9. To demonstrate the effects of Tocovid by measuring circulating NGF and AGE and other inflammatory markers in patients with diabetic peripheral neuropathy.

10. To establish correlation between changes in circulating NGF, AGE, TNF-α and other inflammatory markers with nerve conduction parameters in patients with diabetic peripheral neuropathy.

11. To establish the levels of Vitamin E sub-types in extracellular fluids and erythrocytes in patients treated with high dose Tocovid (i.e. Tocovid 200 mg BD).

4.0 Study endpoints

4.1 Primary endpoint:

(i) Improvement in early diabetic nephropathy as assessed by microalbuminuria (UACR) and serum creatinine (eGFR) in patients given tocotrienol-rich vitamin E from palm oil (Tocovid).

(ii) Improvement in early diabetic retinopathy and reduction in intraretinal microhaemorrhage as assessed by fundal photography in patients given tocotrienol-rich vitamin E from palm oil (Tocovid).

(iii) Improvement in diabetes peripheral neuropathy as assessed by nerve conduction parameters in patients given tocotrienol-rich vitamin E from palm oil (Tocovid).

4.2 Secondary endpoint:

(i) Reduction of AGEs, MDA, TNFR1, IL-6 and VCAM-1 in patients with early diabetic nephropathy given tocotrienol-rich vitamin E from palm oil (Tocovid).

(ii) Reduction of AGEs, MDA, TNFR1, IL-6, VCAM-1 and Thromboxane 2 in patients with early diabetic retinopathy given tocotrienol-rich vitamin E from palm oil (Tocovid).

(ii) Reduction of AGEs, MDA, TNFR1, IL-6, VCAM-1 and NGF in patients with diabetes neuropathy given tocotrienol-rich vitamin E from palm oil (Tocovid).

4.3 Research description

The over aching aim of the research is to establish the potential mechanisms of action(s) of Vitamin E on diabetes and its diabetes microvascular complications, namely nephropathy, retinopathy and neuropathy.

In this study, we aim to demonstrate the various mechanisms of actions(s) of Vitamin E; namely its anti-oxidant, anti-inflammatory, anti-thrombotic effects as well as establishing Vitamin E potential role as renal, retinal- and/or neuro-protective by measuring its renal, retinal and nerve parameters respectively. We will also be measuring circulating levels of anti-oxidants and anti-inflammatory markers and determine any correlation with the severity of diabetes

microvascular complications (i.e. nephropathy, retinopathy and neuropathy). In addition, we will establish the levels of Vitamin E sub-types found both in extracellular fluid and erythrocytes.

This is a prospective, randomized, double-blinded, placebo-controlled study. The study will take 12 months to complete. Patients (n=112) will be randomized into intervention and control groups. The interventional (INT) group will receive active treatment (200 mg Tocovid BD) and the control group will receive placebo (200 mg placebo BD) for 6 months. These patients will be followed up for 12 months and attend a total 8 visits. The detail timeline of the study will be provided in the relevant section below. The improvement of the microvascular complications will be assessed by (i) UACR & eGFR for renal parameters (ii) Intraretinal hemorrhages for retinal parameters and (iii) nerve conduction test for peripheral neuropathy. In addition, we will be measuring circulating inflammatory markers such as AGEs, MDA, TNFR1, IL-6, VCAM-1, Thromboxane 2 and NGF in these patients.

5.0 Methodology

5.1 Study Design

This is a prospective, randomized, double-blinded, placebo-controlled study. The study duration and timeline are shown in the Figure 8 below.

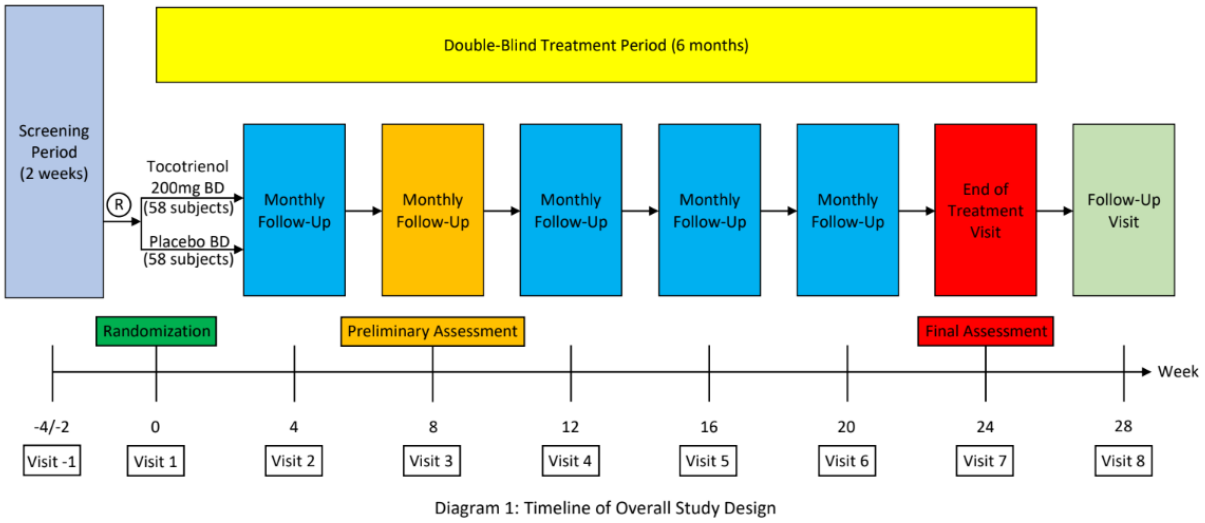

Diagram 1: Timeline of Overall Study Design

Figure 8 Overall study design

5.1.1 Screening (Visit -1)

Potentially eligible patients from the existing pool of patients at the Clinical Research Centre, Monash University Malaysia will be invited to participate in the clinical trial. Additional participants may be referred to the Clinical Research Centre from other sites as mentioned in **Section 1.5**.

Informed consent will be obtained from the participants before the commencement of screening (refer to Section 14.0). All local standard operating guidelines and procedures for health screening, management and referral (if necessary) pertaining to the comorbidities of interest be abided by investigators for all participating subjects.

Prior to the screening visit, potential participants will be advised to fast for at least 8 hours beforehand (no food/drinks, only plain water allowed), avoid strenuous activity the night before, skip their morning dose of anti-diabetic medications but take the rest of their other medications. Participants are also advised to bring their medications and past test results with them. If the participant is a premenopausal female participant, it must be ensured that she is not having her menses during the visit. If the participant has fallen sick, their appointment will be postponed. This is important because it may affect the accuracy of the test results if the instructions are not followed. Participants will be reminded via telephone call the day before all visits.

In the event the participants do not adhere to the advice given, they will be requested to return on the next earliest suitable day so that appropriate tests can be done. Female subjects who are menstruating will be advised to return after their menses have ended.

Informed consent will be taken from the participants before starting the screening. After that, a complete history-taking and physical examination will be done to ensure the participants meet the inclusion and exclusion criteria. (Refer to **Section 5.4 and Section 5.5** for the inclusion and exclusion criteria). Patients' blood pressure and anthropometric measurements such as weight, height and waist circumference will be routinely carried out in every visit.

Additional safety and screening tests will only be done on participants who are still eligible thus far. A total of 40mL of blood samples (5 tubes) and urine samples will be obtained and processed on the same working day at our CRC. The baseline tests include ECG, renal profile, liver function test and lipid profile; these tests are important to ensure that the participants are fit and eligible to participate. The screening tests include serum Vitamin E, fasting blood glucose, HbA1c, urine FEME and UACR. A retinal photograph and nerve conduction study will be taken for all subjects. Subjects who are pre-menopausal women will undergo a urine pregnancy test. The tests are shown in **Table 1** below. Participant will be excluded if their test results meet any of the exclusion criteria.

Once the test results are ready, the investigators will call the participants to arrange an appointment to review their test results and inform them regarding their eligibility to participate. Patients who fulfilled the criteria will be invited to the CRC for randomization (2-4 weeks after the screening visit).

5.1.2 Randomisation (Visit 1)

Subjects will be randomized 1:1 into the double-blind treatment period for approximately 6 months. The randomization is done via a computerised randomisation software and the subjects will be stratified according to age, gender, duration of disease, glycaemic control and BMI.

The interventional group will receive oral 200mg BD of Tocotrienol and the control group will receive oral placebo BD. Each group will have 56 patients, with an allowance of maximum two dropouts per group.

Anthropometric measurements, blood pressure, FBG and urine FEME will be routinely carried out during this visit and every visit that follows.

5.1.3 Follow-up Visit (Visit 2, 4 and 6)

During Visit 2, 4 or 6, investigators will monitor for any adverse events. In the event it occurs, the investigator may choose to withdraw subject from the clinical trial based on his/her discretion. Subjects who are withdrawn will proceed to the discontinuation visit as described in Section 5.10) Investigators will also monitor subject's compliance to treatment via capsule count (refer Section 5.9).

5.1.4 Preliminary Analysis (Visit 3)

During Visit 3, investigators will monitor for adverse events and compliance. Anthropometric measurements, blood pressure, visual acuity, FBG and urine FEME will also be routinely carried out. Further investigations including HbA1c, UACR and biomolecular markers (AGE, MDA etc) will be conducted. Retinal photography and nerve conduction study will be done for all subjects.

Additionally, baseline safety tests including LFT, RFT, ECG and lipid profile will be conducted to ensure subject's baselines are within the normal range and has remained stable throughout the study.

Results from this visit will be analysed by an external independent assessor who will advise the correlation and significance of any improvement

5.1.5 Follow up visit (Visit 5)

During Visit 5, investigators will monitor for adverse events and compliance. Anthropometric measurements, blood pressure, visual acuity, FBG and urine FEME will also be routinely carried out. Further investigations including HbA1c, UACR and biomolecular markers (AGE, MDA etc) will be conducted.

Additionally, baseline safety tests including LFT, RFT, ECG and lipid profile will be conducted to ensure subject's baselines are within the normal range and has remained stable throughout the study.

5.1.6 Final Visit (Visit 7)

During the Final Visit, investigators will monitor for adverse events and compliance as in Visit 2 and 3. Anthropometric measurements, blood pressure, visual/colour acuity, FBG and urine FEME will also be routinely carried out during this visit. Further investigations including HbA1c, UACR, biomolecular markers (AGE, MDA etc) will be conducted. Retinal photography and nerve conduction study will also be conducted for all subjects.

Additionally, baseline safety tests including LFT, RFT, ECG and lipid profile will be conducted to ensure subject's baselines are within the normal range and has remained stable throughout the study.

5.1.7 Post-trial Follow-up Visit (Visit 8)

One month after conclusion of the trial, patients will be called back to review for any potential long-term side effects. Baseline safety tests will be repeated including LFT, RFT, ECG and lipid profile.

Patients will also be debriefed regarding the study.

The investigations and tests to be conducted at each visit in the study is summarised in Table 7 below.

Tests		**Screen (Visit -1)**	**Random (V1)**	**Visit 2**	**Visit 3**	**Visit 4**	**Visit 5**	**Visit 6**	**Visit 7**	**Visit 8**
Safety tests	Lipid profile	●			●		●		●	
	ECG	●			●		●		●	●
	LFT (including AST and ALT)	●			●		●		●	
	Anthropomorphic measurements	●	●	●	●	●	●	●	●	●
	Blood pressure	●	●	●	●	●	●	●	●	●
	UPT (female, premenopausal)	●	●		●		●		●	
Parameter 1: Renal	RFT (BUSE, Creat & eGFR)	●			●		●		●	
	Urine dipstick	●	●	●	●	●	●	●	●	●

	UACR	●			●		●		●	
Parameter 2: Glucose	HbA1c	●			●		●		●	
	FBG (plasma)	●			●		●		●	
	FBG (finger-prick test)	●	●	●	●	●	●	●	●	●
Parameter 3: Nerve	Nerve conduction study	●			●				●	
Parameter 4: Vit E	Serum Vit E	●			●		●		●	
	Serum AGE	●			●				●	
	Plasma MDA	●			●				●	
	Serum TNFR1	●			●				●	
	Serum Thromboxane B2	●			●				●	
	Serum NGF	●			●				●	
Parameter 5: Inflammatory markers	Serum Interleukin-6	●			●				●	
	Serum VCAM-1	●			●				●	
Parameter 6: Eye	Fundal camera	●			●				●	
	Visual acuity	●			●				●	

Table 7 Investigations and tests conducted at every visit in the study.

5.2 Study population

Data are collected from patients with type 2 diabetes who go for regular follow-up at the Clinical Research Centre of Monash University, Klinik Kesihatan Tanglin, Klinik Kesihatan Kelana Jaya, Thompson Medical Centre, Tun Hussein Onn Eye Hospital in Kuala Lumpur, Malaysia and Sultanah Aminah Hospital in Johor Bahru, Malaysia. Those patients who have renal or nerve or

retinal disease will be invited to be screened for the study at the Clinical Research Centers in Sunway Campus or the Clinical School in Johor Bahru.

5.3 Sample Size & Justification

The total sample size of the study is 116 participants. Each cohort (active vs placebo) comprises of 58 participants with an allowance of maximum 2 dropouts per cohort. This number is derived from an online sample size calculator available at: http://clincalc.com/stats/samplesize.aspx

The data keys into the calculator for our study group design was "two independent study groups" with "continuous primary endpoint". This implies that the two study groups will each receive different treatments and the primary endpoint is an average value of the data collected. For the statistical parameters, the anticipated average reduction in Intra retinal bleeds or serum creatinine or improvement in nerve conduction used for Group 1 (active) was 20 ± 5% and Group 2 (placebo) was 5%. The difference in % reduction of AGE products between both groups was about 20%. The enrolment ratio used was 1:1. The type I error rate (alpha cut-off) used was 5% (0.05) indicating a 5% chance of false positive. The type II error rate (beta cut-off) used was 20% (0.2) indicating a 20% chance of false negative. The results from the calculator are as shown below. The results from the calculator are shown below. Therefore, the minimum number of participants needed to be enrolled into the study in order to have sufficient statistical power to detect a treatment effect is 112. Table 8.1 and Table 8.2 illustrates the sample population and power of the study.

Sample Size	
Group 1 (placebo)	56
Group 2 (active)	56
Total	112

Table 8.1 Sample Size

Study parameters	
Mean, group 1	83.66
Mean, group 2	66.298 (20% dec)
Alpha	0.05
Beta	0.10
Power	0.9

Table 8.2 Power of study

5.4 Inclusion Criteria

To be eligible for initial entry into the study, subjects must meet all of the following criteria:

I.	Subject, or legal representative, has voluntarily signed and dated an Informed Consent Form approved by an Institutional Review Board (IRB)/Independent Ethics Committee (IEC), after the nature of the study has been explained and the subject has had an opportunity to ask questions. The informed consent must be signed before any study-specific procedures are performed.
II.	Subject is 35 - 75 years of age at the initial Screening visit.
III.	Subject has T2DM with stable glucose control (not more than 10% change in HbA1c levels over the last 2 months) and the HbA1c range should be within 6 - 9%.
IV.	If subject has hypertension, he/she must have stable blood pressure control for the past 2 months with not more than 10% change and the BP range should be <150/90 mmHg.
V.	Subject has eGFR of 30 – 60 mL/min/1.73m^2 OR UACR of 20 – 200 mg/g.
VI.	Subject has mild/moderate retinopathy as defined by:
VII.	Mild: At least one microaneurysm
VIII.	Moderate: Hemorrhage/microaneurysm, cotton wool spots, venous beading, and intraretinal microvascular abnormalities

Table 9 Inclusion Criteria

5.5 Exclusion Criteria

Subjects meeting any of the following criteria will be excluded from the study:

I.	Subject has poor diabetic control, HbA1c >9%.
II.	Subject has poor blood pressure control, BP>150/90
III.	Subject is pregnant during screening.
IV.	Subject has current urinary tract infection during screening (symptomatic or definitively on urine FEME: presence of pyuria, nitrites and red blood cells).
V.	Subject has known non-diabetic kidney disease, such as kidney stones, glomerulonephritis etc.
VI.	Subject on anti-epileptic or sedative medications
VII.	Subject has acute or severe chronic illness such as acute coronary syndrome, active tuberculosis, and previous or current history of cancer, liver or inflammatory disease, etc.
VIII.	Subject is taking other water-soluble antioxidants for the past 2 weeks or fat-soluble

	antioxidants for the past 1 month.
IX.	Subject is a heavy smoker (≥20 sticks/day) or has stopped smoking for less than 1 month.
X.	Patients with severely deranged renal profile. (Stage 4 - 5 CKD; eGFR <30ml/min/1.73m^2).

Table 10 Exclusion Criteria

5.6 Minimisation of Bias

5.6.1 Measures taken to Minimise Bias

Measures taken to minimize bias in our study include:

- Randomisation
    - Subjects will be stratified according to parameters such as age, gender, duration of disease, glycaemic control and BMI and will be randomised via computer-generated allocation schedule.
    - This is to ensure that each patient has an equal chance of receiving any of the treatments under study, generate comparable intervention groups, which are alike in all the important aspects except for the intervention each group receives.
    - It prevents both selection bias and insures against the accidental bias.
- Double-blinded and placebo-controlled
    - Both investigator and subject will be double-blinded against the intervention or control group.
    - Both intervention and control tablets will be indiscernible; of similar size, shape, colour and taste.
    - A moderator who is neutral and not involved in the trial will be selected to oversee the double-blinding process.
    - This is to minimise selection bias.

5.6.2 Maintenance of Randomisation Codes

Randomisation codes will be safeguarded by an independent operator at Monash University Malaysia. Consequently, any issues which arise will be advised accordingly by the independent assessor (Associate Professor Quek Kia Fatt)

5.6.3 Procedures for Breaking Codes

- Treatment blinding must only be broken in the event of emergency where knowledge of the treatment is necessary to provide acute medical care or where there is safety/clinical concern from the research team.

- In a non-emergency situation, the request may come from the Sponsor or the research.
- Where possible, all code break requests should be discussed with the Investigator, Sub-investigator or Sponsor before un-blinding. In all cases, the Investigator and Sponsor must be notified by the next working day of the code break request, although it is not necessary to inform the sponsor of the result of the request. All communication should be documented in the trial master file.
- All care should be taken to ensure that no unnecessary un-blinding of the study team occurs when they are not the requesting party of the code break.
- Requests for un-blinding should be accompanied with adequate subject details and reasons for requesting the code break. This information should be documented and should include the following:
    - Patient information and trial information
    - Identity of the person requesting the code break request
    - Reason for the code break request
    - Bottle or box number of the trial medication (if available to hand)

(Adapted from NHS UK Code Breaking/Un-blinding of Clinical Trials, training and procedure testing)

5.7 Route of Administration, Dosage and Treatment Periods

The interventional group will receive oral 200mg BD of Tocovid and the control group will receive oral placebo BD for a period of time of six months (Refer to **Section 1**).

5.8 Device/Process Specifications

Device		Model/Validation
Clinical	Sphygmomanometer	Omron 7130/7120
	Anthropometric tools • Weighing and height scale • Measuring tape	Charder Electronic MS 4900
	ECG machine	Edan SE1200
	Blood Glucose Testing Device	One Touch Ultra Easy
Laboratory	HbA1c processor	Afinion As100
	UFEME/UACE processor	Afinion As100
Ocular	Fundal camera	DRS
Neuropathy	Nerve conduction study machine	

5.9 Monitoring of Compliance

Adherence to treatment will be assessed by site performed capsule count during each visit. Every effort will be made to maintain adherence as close to 100% as possible. We will conduct monthly follow-up visits to monitor the patients.

5.10 Withdrawal Criteria

Subjects may withdraw at any time or be dropped from the trial at the discretion of the investigator should any untoward effects occur. In addition, a subject may be withdrawn by the investigator or SPONSOR if he/she violates the trial plan or for administrative and/or safety reasons. The investigator or trial coordinator must notify the SPONSOR immediately when a subject has been discontinued/withdrawn due to an adverse experience via telephone or fax.

Randomized subjects who discontinue blinded study medication will have a Discontinuation Visit as soon as possible. The Discontinuation Visit will mirror the final treatment visit (Refer to **Section 5.1.6**). Subjects who have withdrawn from the study will not be replaced.

5.11 Collection, Storage and Use of Biospecimens

For the purpose of this study, a total of 30ml of venous blood will be taken at screening visit (visit -1), follow-up visits on Visits 3, 5 and 7. The blood samples will be stored in EDTA tubes for HbA1c, floride-EDTA tubes for fasting plasma glucose and serum-separating tube (SST) tubes for renal profile, lipid profile, liver function tests and other biomolecular markers. Refer to **Table 1 in section 5.1** for the list of blood tests.

Urine samples will be taken for urine dipstick test, urine FEME and UACR test. Urinalysis will be done at every visit while UACR test will be done at alternate visits (namely Visits -1, 3, 5 and 7). The blood and urine samples obtained will be processed on the same working day at our Clinical Research Centre, Monash University Malaysia. Validation of all test results will be done by Dr Badariah and Professor Khalid.

Blood tests including lipid profile, renal profile, liver function test, fasting plasma glucose, HbA1c and urine test UACR will be sent to BP labs for processing. Two copies of the results will be delivered 2-3 working days later. One copy will be kept for recording purposes while the other will be given to the participant in the following visit.

Serum samples for AGE, MDA, TNFR1, IL-6, VCAM-1, Thromboxane B2 testing will be allocated into tubes of 1 ml each and frozen at -80 degrees Celsius. Processing of the serum samples may not be done on the same working day as it is done on a batch-to-batch basis to minimize inter-assay variation. Therefore, all screening, lab processing and interpretation of the investigation results will be done at our study site.

Subsequently, all biological specimens except the serum samples will be discarded using local protocol at most two working days after validation of results. Therefore, the specimens (blood and urine samples) will not be stored and kept for more than 3 (Three) working days from collection until disposal. The serum samples are kept for future testing of other biomarkers. There will be no genetic testing involved in this study.

5.12 Protection of Dignity and Privacy of Subjects in Future Research

Informed consent will be taken from participants before aliquoting their serum samples for future testing of other biomarkers. It will not be used for any genetic testing. The serum samples will be kept frozen until sufficient funds are available for testing.

5.13 Remuneration for Study Subjects

For all study subjects (both treatment and control arms), a specific appointment date for the screening and follow-up will be set by investigators, during which subjects will be required to travel to Clinical Research Center, Monash University Malaysia.

Investigators will provide remuneration of RM30.00 (Ringgit Malaysia thirty only) for each subject recruited to cover for transport expenses for their return trip to and from healthcare facility for the scheduled screening program. We are of opinion that this sum of remuneration is reasonable to cover for travel expenses alone and will not act as unjustified monetary incentive to influence study participation.

6.0 Treatment and Procedures

6.1.1 Permitted Medications/Treatments during Trial

Subjects will be allowed to continue all current medications and treatments provided it fulfills the inclusion/exclusion criteria and does not include the medications stated in Section 6.1.2.

6.1.2 Non-Permitted Medications/Treatments during Trial

Non-permitted medications or treatments during the trial include:

- Any supplementary antioxidants

(including but not limited to vitamin A, vitamin C, vitamin E, glutathione beta-carotene, selenium, zinc, manganese and alpha-lipoic acid)

- Any drugs that may cause sedation or drowsiness

(including but not limited to sedative/hypnotic drugs, narcotics, certain anti-anxiety, anti-depressants and anti-histamines)

6.1.3 Rescue Medication / Procedure

Given that the intervention (tocotrienol-rich Vitamin E from palm oil or Tocovid) is a form of supplement with no reported short-term adverse effects at the given dose, therefore no rescue medication/procedure is included in this protocol.

7.0 Assessment of Efficacy

The efficacy of the intervention (tocotrienol-rich vitamin E from palm oil or Tocovid) will be assessed by primary and secondary endpoints (refer to **Section 4.0** Study Endpoint). These endpoints will be recorded during the various visits as outlined in **Section 5.0** Methodology.

8.0 Assessment of Safety

The intervention (tocotrienol-rich Vitamin E from palm oil or Tocovid) is a form of supplement with no reported short-term adverse effects at the given dose. However, the investigators will monitor for adverse events during each monthly visit as described in **Section 5.0** Methodology. In between the monthly visits, patients have the liberty to reach out to the study team and site for consultation. In the event an adverse event occurs, the investigator may choose to withdraw subject from the clinical trial based on his/her discretion. Subjects who are withdrawn will proceed to the discontinuation visit as described in **Section 5.10**. The duration of follow-up of adverse events will also be based on the investigator and study team's discretion.

9.0 Statistics

Data analysis would be performed using IBM SPSS v.24. Logistic regression will be used to estimate the relative risks, its corresponding 95% confidence interval and p values based on the clinical trial data. Univariate and multivariate analysis of the data collected will also be calculated.

10.0 Risks and Benefits of Study

10.1 Potential risk(s) of Study

According to MIMS Malaysia and other previous trials, there is no reported adverse effects r eported with consumption of Tocovid Suprabio at the given dose of 200mg BD

10.2 Potential benefit(s) of Study

As discussed in the literature review, the potential benefits of tocotrienol in patients with T2DM are mainly its role as a potent antioxidant and optimisation of glycaemic and/or metabolic control. In addition, other benefits include its ameliorating diabetes-associated cognitive

decline, neuropathy, retinopathy, nephropathy, cardiovascular disease and improving endothelial function.

11.0 Statement on Ethical Issues

Study will be registered in National Medical Research Register (NMRR) and ethical approval will be sought from Medical Research and Ethics Committee (MREC). Study will be conducted along the guidelines outlined by the Malaysian Good Clinical Practice and all investigators strive to maintain strict conduct according to this protocol to maintain the privacy and confidentiality of the data gathered for the study.

12.0 Informed Consent and Voluntary Participation

The investigator must obtain documented consent from each potential subject prior to participating in a clinical trial. Consent must be documented by the subject's dated signature on a consent form along with the dated signature of the person conducting the consent discussion. A copy of the signed and dated consent form should be given to the subject before participating the trial.

The initial informed consent form and any subsequent revised written informed consent form and any written information provided to the subject must receive the MREC's approval/favourable opinion in advance use. The subject should be informed in a timely manner if new information becomes available that may be relevant to the subject's willingness to continue participation in the trial. The communication of this information will be provided and documented via a revised consent form or addendum to the original consent form that captures the subject's dated signature.

All participation in this clinical trial must be voluntary in nature with subject's and investigator's dated signature on informed consent sheet obtained.

13.0 Collection of Personal Information

The nature of this clinical trial requires personal information to be collected from the patients through history-taking. Only information pertaining to the eligibility of the subjects for recruitment into the clinical trial such as those specified in the inclusion and exclusion criteria will be obtained. In addition, subject's contact information and details will be recorded for the monitoring purposes. Once recruited, study subjects will only be identified by a study subject number (no relation to personal information or personal identifiers; not identifiable in any way) which is unique for this study and accessible only by investigators. The master list which

contains the patients' particulars will only be made accessible to the Principal Investigator. Data collection on data sheets and subsequent analysis will utilize only the study subjects' numbers. The master list of study subjects will be destroyed once findings for this study are published.

14.0 Confidentiality and security of source documents and study data

14.1 Protection of Privacy and Confidentiality of Personal Information

As previously mentioned, due to the nature of this study, personal information or personal identifiers essential to the study will be collected. Once recruited, study subjects will only be identified by a study subject number which is unique for this study and accessible only by investigators. The master list which contains the patients' particulars will only be made accessible to the Principal Investigator. Data collection on data sheets and subsequent analysis will utilize only the study subjects' numbers. The master list of study subjects will be destroyed once findings for this study are published.

14.2 Subject's Access to Personal Information and Study Data

Subject will be given access to their personal information and test results obtained during this clinical trial.

14.3 Medical Records and Study Data

Data gathered for this study will be stored in a password-protected computer and all data and findings generated are confidential, accessible only by investigators. Upon completion of the study and dissemination of the findings through publication, data in the computer will be copied to CDs and the data in the computer erased. CDs will be stored in a locked office of the investigators and maintained for a minimum of seven years after the completion of study. Data gathered for this study will not be used for any future study without prior approval from relevant regulatory bodies and/or ethical committee(s).

15.0 Publication Policy

No personal information or identifiers are collected as part of this study. Therefore, no personal information will be disclosed and subjects will not be identified in any way when the findings of the study are published.

16.0 Involvement of Vulnerable Subjects

16.1 Involvement of Minors

This clinical intervention trial **DOES NOT** involve minors. The study age population ranges from 35 to 75 years old.

16.2 Involvement of Other Vulnerable Subjects

This clinical intervention trial **DOES NOT** involve individuals who are vulnerable to coercion or undue influence. This includes the following populations:

- Adults unable to consent. This includes patients with: -
    - Dementia
    - Mental illness
    - Learning disability
    - Brain damage
    - Intoxication
    - Or any other condition which renders individual unable to give informed consent.
- Orang Asli
- Pregnant women
- Prisoners

17.0 Termination of Study

The study may be terminated prematurely for reasonable cause such as occurrence of serious adverse event provided that a written notice is submitted in advance of the intended termination.

The distributor company for tocotrienol-rich vitamin E from palm oil (Tocovid) will be informed about the cause and occurrence of the study termination.

Following the study termination for any reason, all subjects who have not withdrawn must be contacted. At this time, subjects who have not permanently discontinued the study drug will be scheduled for the final treatment visit. In subjects who permanently discontinued from study drug, a phone call can be completed to solicit information about any potential endpoint events and serious adverse events since the previous visit.

18.0 Finance and Insurance

18.1 Financial Budget

Item and breakdown	**Estimated cost**
1. Renumeration for study subjects • Target: 116 subjects • No of visits: 8 • MYR 30.00 per subject per visit	116 x 8 x MYR 30 = **MYR 27,840**
2. Drugs • Tocovid: 2 capsules X 58 X 180 days = 20,800 capsules • Placebo: 2 capsules X 58 X 90 days = 20,800 capsules	Sponsored by Hovid Pharmaceutical Berhad

3. Laboratory lab fees
• Certain blood and urine tests will be sent to BP labs for processing:
• HbA1c + RFT + LFT + FBG + lipid profile + UACR
  ○ MYR 84/test
  ○ 4 tests
  ○ 116 subjects
• Biomolecular markers will be process at local in-house chemical pathology laboratory in Biomedical Research Lab, Monash University Malaysia, costs incurred from purchasing of the following reagents are:
  ○ AGE – MYR 1700/plate
  ○ MDA – MYR 1200/plate
  ○ TNFR1 – MYR 1700/plate
  ○ Thromboxane B2 – MYR 1700/plate
  ○ IL-6 – MYR 1200/plate
  ○ VCAM-1 – MYR1700/plate
  ○ NGF – MYR 1700/plate
  ○ Each plate can be used for 48 samples
  ○ 3 tests will be performed
  ○ Total 8 plates will be required for 116 patients
  ▪ 116 X 3 / 48 = 7.25
	MYR 84 X 4 X 116 =
	MYR 38,976
	
	Sum = 1700 + 1200 + 1700 + 1700 + 1200 + 1700 + 1700
	= MYR 10,900
	
	MYR 10,900 X 8
	= **MYR 87,200**

4. Additional Tests • ECG machine and strips • Fundal camera • Nerve conduction study machine and electrodes • Snellen chart	Available in-house from Monash University Malaysia.
5. Consumables for blood taking procedure Disposable non-sterile latex gloves • Alcohol swabs for cleaning site • Tourniquets • Syringes • Needle for blood taking 21G (Green) units • Needle for blood taking 23G (Blue) units • EDTA Vacutainer tube • EDTA-Floride Vacutainer tube • SST Vacutainer tube • Biohazard bag • Cotton swabs for site post blood taking • Omnifix roll to secure swab at site post blood taking • Alcohol rub	Available in-house from Monash University Malaysia.
Estimated total cost	**MYR 154,016**

18.2 Insurance/Indemnity

This clinical trial is conducted under the sponsorship of Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia and Hovid Pharmaceuticals.

19.0 Gantt Chart

Project Activities	2018		2019					
	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Literature Review	**X**	**X**	**X**					
Study Protocol	**X**	**X**	**X**					
Ethical clearance & permission from relevant authorities		**X**	**X**					
Screening and recruitment of participants			**X**	**X**	**X**			
Data collection			**X**	**X**	**X**	**X**	**X**	
Data cleaning and finalize database							**X**	**X**
Statistical analysis							**X**	**X**
Full/final report submission							**X**	**X**
Study closure report to NIH/MOH: Study findings dissemination								**X**

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