Study Title: Tocotrienol-rich vitamin E (Tocovid Suprabio) and its effects on diabetic microvascular complications: nephropathy, retinopathy and neuropathy: A double-blinded placebo controlled, multicentre clinical trial with dose ranging study to study mechanisms of action of tocotrienol in comparison to tocopherol

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Abstract

Diabetes mellitus is a leading cause of morbidity and mortality worldwide. The prevalence of diabetes in Malaysia has soared to 16.8% in 2019. Diabetes can lead to serious microvascular complications affecting kidney, eye and nerve functions. In recent years, there is growing interest towards tocotrienols for they are found to be a more potent antioxidant as well as having greater antiglycemic, anticholesterolemic, anti-inflammatory, neuroprotective and cardioprotective properties as compared to tocopherols. Our recent research found that tocotrienol-rich vitamin E at a dose of 200mg twice daily significantly improves nerve conduction in patients with diabetic neuropathy, renal function in diabetics with stage 3 kidney disease and reduces bleeding in the diabetic retina. The research aims to investigate the effects of low dose tocotrienol-rich vitamin E and alpha-tocopherol on peripheral neuropathy in type 2 diabetes mellitus patients; determine if supplementation of tocotrienol-rich vitamin E at lower dose improves nerve conduction parameters, eye and renal function and if tocotrienol-rich vitamin E is superior to alpha-tocopherol (200IU). This is a prospective, multi-centered, randomized, double-blinded, placebo-controlled study involving patients with type 2 diabetes mellitus with reasonable glycaemic control (HbA1c between 6.0 - 9.0%) and diabetic neuropathy as assessed by nerve conduction study using 100mg and 200mg of Tocovid SupraBio® and 200IU of α-tocopherol once per day for 24 weeks. These findings could impart value-added products of the palm-oil industry into the medical sector particularly for treatment of diabetes and its microvascular complications.

Proposal

Section 1: Study Title

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Section 3: Introduction and Literature Review

3.1 Diabetes Mellitus

3.1.1 Global Burden of Diabetes

Diabetes mellitus (DM) is a chronic disease of impaired carbohydrate metabolism secondary to insulin insensitivity and/or insulin secretory dysfunction. There are multiple types of DM and each of it are as a result of a complex interplay between environmental factors and genetic predisposition. The most common type of DM is Type 2 Diabetes Mellitus (T2DM). Obesity increases insulin insensitivity and/or resistance in the body and as a compensatory mechanism to maintain normal glucose tolerance more insulin is secreted by the beta cell in the pancreas. Overtime, the function of the pancreas declines hence decreasing the amount of insulin secreted. Insufficient endogenous insulin production to normalized glucose results in hyperglycemia. (1, 2) According to World Health Organization (WHO), diabetes mellitus affects 422 million adults globally with an estimate annual cost beyond USD $1.3 trillion.(3) The soaring growth in numbers had nearly quadrupled from 108 million affected individuals

in 1980.(4) Furthermore, it is projected that by 2040, the prevalence of diabetes will snowball up to 642 million, with disproportionate growth in developing countries.(5) The main driving force behind the surge in figures is reflected by the parallel increase in prevalence of obesity.

According to the National Health and Morbidity Survey (NHMS) 2011, the prevalence of diabetes in Malaysia has escalated by 31% over a duration of 5 years.(6) This also indicates that there are currently around 2.6 million adults living with diabetes in Malaysia.(6) Alarmingly, up to 9.2% of the adult population in Malaysia have undiagnosed diabetes due to the mild and asymptomatic nature of T2DM.(6) The exponential increase in the prevalence of diabetes in Malaysia has made it a major public health emergency. DM imposed a substantial burden not only restricted to the healthcare system but also the national economy.(6) The substantial rise in incidence of diabetes has been attributed to factors such as being overweight, obesity, physical inactivity.(6) Chronic hyperglycemia can result in severe life-threatening complications and life-changing complications. Zanariah Hussein et al. (2015) reported that the prevalence of combined microvascular complications such as nephropathy, retinopathy and neuropathy among diabetics was 75% and macrovascular complications was 29%.(7) WHO reported four million cases of deaths annually from diabetes associated complications for example, end stage kidney disease (ESRD), cardiovascular disease and stroke.(7)

3.1.2 The Basis of Glycaemic Control

Analysis of glycated haemoglobin (HbA1c) provides valuable information that reflects the cumulative glycaemic profile over the past three months based on the lifespan duration of haemoglobin in red cells. Over the last few decades, HbA1c monitoring has become the standard of care in practice. It is a reliable biomarker for chronic hyperglycemia which correlates well with the risk of diabetes complications in the long run.

The Diabetes Control and Complications Trial (DCCT) was the first multicenter, randomized clinical trial conducted to determine if intensive therapy and strict glycaemic control could possibly ameliorate and prevent complications of diabetes in Type 1 Diabetes Mellitus

(T1DM). The DCCT showed that intensive therapy of T1DM patients with strict diet, regular exercise and intensive insulin therapy successfully delayed the onset and slowed the progression of diabetic vascular complications such as retinopathy, nephropathy and neuropathy. At the mean of 6.5 years, intensive insulin therapy resulted in reduction in HbA1c level of 2.0% as compared to the control group (7.2% vs 9.1%). Furthermore, an overall relative risk reduction of 54% less albuminuria, 39% less microalbuminuria, 76% less diabetic retinopathy, 60% less neuropathy was observed at the end of 6.5 years for every reduction in HbA1c by 2%. (8)

Another major trial that was published in 1999, the United Kingdom Prospective Diabetes Study (UKPDS), attested the theory that tight glucose control ameliorates the progression of diabetic microvascular disease in T2DM subjects. The cohort was made up of 5102 newly diagnosed T2DM patients who were either given usual glucose treatment or intensive glucose lowering therapy with diet exercise and medications. They were then closely followed over 10 years. The intensive glucose control approach resulted in a decrease of 0.9% in HbA1c which conferred a relative risk reduction of 21% in retinopathy and 34% in albuminuria. Additionally, an overall 25% risk reduction in microvascular endpoints was achieved with tight glucose control. On the other hand, the DCCT and UKPDS trials failed to demonstrate significant risk reduction of macrovascular complications for example cardiovascular events. (9)

The major question when comparing both studies (DCCT, UKPDS) was the magnitude of decrement in relative risk reduction for microvascular complications. The outcome of DCCT was far more superior than UKPDS but if considered per 1.0% reduction in HbA1c, they were not significantly different. The difference could also be justified by the pathogenesis of the disease. The complications of T1DM are often solely secondary to insulin deficiency as a result a single-point approach is sufficient to ameliorate complications associated with the disease. On the other hand, T2DM is attributed to a combination of factors including insulin resistance, hypertension, dyslipidaemia, lifestyle factors and socioeconomic factors. Intervening solely on glucose control may not be adequate to replicate the same outcome.

Interestingly, this finding had raised awareness that multi-centric approach may be more favourable, especially for T2DM patients.

The discrepancy between the results of two trials were further evaluated by the Steno-2 trial. Intensive intervention was targeted on multiple risk factors of diabetic complications. At the end of the trial, the intervention group exhibits a relative risk reduction of 50% on diabetic nephropathy and comparable reduction in other microvascular complications. The impressive outcome of the Steno-2 trial corroborates that multifaceted management of T2DM is necessary. These trials had indeed reassured health policy makers that vigorous gluco-centric treatment along with multifactorial management of diabetes can significantly reduce the morbidity and mortality of the disease. (10)

Table 1 summarizes the mean relative risk reduction of contrasting outcomes with intensive treatment as compared to conventional treatment in T1DM and T2DM patients during three different randomized controlled trials namely DCCT, UKPDS, STENO-2 and its observational follow-up studies. (11)

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 with CON group during randomized controlled trials and observational follow up. (11)

In contrary to good metabolic memory whereby a long term risk reduction of DM complications is observed after early and intensive glycaemic control is achieved, “bad” metabolic memory refers to sustained harm secondary to chronic hyperglycaemia due to poor glycaemic control in patients with T2DM. (12) This theory is supported by three landmark studies namely the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) study, Action to Control Cardiovascular Risk in Diabetes (ACCORD) and Veterans Affairs Diabetes Trial (VADT). Table 2 shows the comparison of HbA1c levels at the start and end of study. These trials have shown that aggressive glycaemic control is potentially detrimental on cardiovascular outcomes in T2DM patients.(12) The disparity between the ADVANCE, VADT, ACCORD and UKPDS trials might be secondary to factors such as age and duration of diabetes. The median age of UKPDS patients was 54 while in ADVANCE it was 62. Furthermore, the duration of diabetes of the participants in ACCORD, VADT and ADVANCE studies were about 10, 12 and 8 years respectively, while participants in UKPDS consist of newly diagnosed diabetes. Additionally, only 7.5% of UKPDS participants had a history of macrovascular disease unlike ACCORD, VADT and ADVANCE which had 30%. (13-14)

Outcomes	Clinical Trials (36 words)								
	VADT			ACCORD			ADVANCE		
	START	END		START	END		START	END	
Mean HbA1c (%)	Both groups	CON	INT	Both groups	CON	INT	Both groups	CON	INT
	9.4	8.4	6.9	8.3	7.5	6.5	7.5	7.3	6.5

Table 2: Comparison of HbA1c levels at the start and end of the study for both groups

In conclusion, HbA1c is not an accurate indicator for long-term diabetic complications. Clinical studies have proposed that this “bad” metabolic memory can begin developing very early in diabetes without correlation with HbA1c levels. It is postulated that the accumulation and irreversible damage was likely due to accumulation of a biomarker Advanced Glycation Endproduct (AGE). Thus, proposing that AGEs is a long-lived biomarker unlike HbA1c which reflects short-term glycaemic control.

3.1.3 Vitamin E

In nature, Vitamin E is present in plants, vegetables and plant oils. Common food oils such as corn, olive, soybean and peanut oil contain largely tocopherols with little or no tocotrienols. Tocotrienol is found relatively limited in plant kingdom and food sources unlike tocopherol which is widely available. (15) Tocotrienols are found most abundant in certain vegetable oils for example, annatto seeds, rice bran oil, palm oil.(16) The extraction of crude palm oil from oil palm could yield up to 800mg/kg of tocotrienol. The total vitamin E content derived from palm oil consists of 30% alpha-tocopherol and 70% tocotrienol.(17) Studies have shown that ω-hydroxylase and α-tocopherol transport protein (αTTP) are essential components for the absorption of vitamin E. (18, 19) In the liver, αTTP facilitates the process of packaging and transporting vitamin E to other tissues through the bloodstream. αTTP has a higher affinity to α-tocopherol and has up 8.5-fold higher affinity to α-tocopherol than α-tocotrienol.(19) As a result, the unbounded vitamin E isoforms are susceptible to catabolization by cytochrome P450 -initiated ω-hydroxylation and oxidation by ω-hydroxylase. This has led to a huge debate concerning the potency of tocotrienol.

The plasma half-life of tocotrienol in humans was estimated to be approximately 2-4 hours.(20) A double-blind placebo-controlled study revealed that 250mg/day of Tocotrienol supplements for eight weeks in human subjects resulted in an increase of 0.8 µM plasma α-tocotrienol. (21) Tocotrienol has a limited bioavailability as it is a fat-soluble compound. SupraBio™, Tocovid™ and Naturale3 are self-emulsifying drug formulations of tocotrienols to increase absorption of oil-based compounds. With this formulation a threefold increment of

plasma α-tocotrienol (3µM) is detected in humans as compared to a previous study which yielded 0.8µM increment in the plasma α-tocotrienol level.(23)

Pharmacological studies and toxicological studies showed that tocotrienol supplementation in human subjects is relatively safe with no significant adverse events. Human subjects that were fed with 250mg/day of tocotrienol supplementation over 8 weeks and reported no side effects.(23) Our pilot study revealed minimal adverse events that were directly related to 400mg Tocovid SupraBio™. (24,25)

3.1.4 Superiority of Tocotrienols

The latest studies have demonstrated tocotrienols triumph with its antioxidant, anticholesterolemic, anti-inflammatory, anti-cancer, neuroprotective and cardioprotective properties. (17, 26) Since then, research on tocotrienol has only started to gain popularity. Notably, tocotrienol is 50 times more potent as an antioxidant as compared to tocopherol. This is because of its unique ability to distribute uniformly in the cellular bilayer. The uniform distribution of tocotrienol allows it to efficiently maximize its scavenging potential.(18, 27) Additionally, tocotrienol has an unsaturated carbon side chain that readily penetrates into tissues with saturated fatty layers for instance, liver and brain. (28) Due to tocotrienol's distinctive molecular structure, it has anti-cholesterolemic properties by inhibiting HMG-CoA reductase which is absent in tocopherols. (15, 29) Indeed, tocotrienol has gained popularity over the recent years and was named as the Vitamin E of the 21st century because of its great potency.(30)

Table 3: Previous studies that were conducted by our team showing various beneficial effects of Vitamin E.

Citation	Study type	Conclusions

Wan Nazaimoon & Khalid (1996) (31)	Human study (n = 32)	- Vitamin E is a potent antioxidant which reduced serum malondialdehyde (MDA) - There was no significant effects on lipid profile or HbA1c
Wan Nazaimoon & Khalid (2002) (32)	Animal study (rats)	- Tocotrienol improved HbA1c and blood sugar profile of diabetic rats - Tocotrienol reduced AGE in healthy rats
Mohamed Norazlina & Khalid (2002) (33)	Animal study (rats)	- Supplementation of Vitamin E derived from palm oil improved bone calcium in Vitamin E deficient rates unlike pure a-tocopherol supplementation which showed no differences
N.S.Ahmad & Khalid (2005) (34)	Animal study (rats)	- Tocotrienol supplementation protects bone better than tocopherols
Narimah & Khalid (2009) (35)	*In vitro*	- Both alpha tocopherol and gamma-tocotrienol showed anti-proliferation effects on hepatoma cells and cervical carcinoma cells
Hong Sheng & Khalid (2017) (36)	Animal study (rats)	- Tocotrienol exhibited anti-hypertensive, anti-cholesterolemic and cardioprotective effects - Tocotrienol improved HbA1c level and supress expression of circulating RAGE and AGE in the liver
Suzanne Tan & Khalid (2017) (25)	Human study (n = 45)	- At 2 months of Tocotrienol supplementation, significantly improve serum creatinine of diabetic nephropathy patients - There were no significant changes in the biomarkers as follows: Cystatin C , N ε -CML, AGE and RAGE.

Gerald Tan & Khalid (2019) (24)	Human study (n =45)	- Tocotrienol significantly improves serum creatinine and serum eGFR of diabetic nephropathy patients - There were no significant changes in the biomarkers as follows TNFR1, MDA, VCAM-1, Thromboxane-B.
Yeek Tat Ng & Khalid (2020) (37)	Human study (n = 80)	- Tocovid supplementation elevated the levels of serum NGF, in which its increase is postulated to reflect enhanced neuronal functions - Tocovid significantly improved the nerve conduction velocities of median, tibial and sural nerves

3.1.5 Role of Vitamin E in Diabetes

Poor glycemic control is complex and multifactorial, it is the main contributor for long-term complications associated with T2DM for example, diabetic kidney disease, diabetic retinopathy and diabetic neuropathy. The association between T2DM and oxidative stress has been well established, this is on the basis of hyperinsulinaemia and hyperglycaemia enhancing generation of reactive oxygen species hence contributing to the rise in oxidative stress. (38) The effects as a result of raising oxidative stress in T2DM includes impaired insulin signalling, β-cell function and promote haemoglobin glycation. (39)

Fang et al. (2010) showed that α-, γ-, and δ of tocotrienol increases co-factor PGC-1α and peroxisome proliferator-activated receptor α (PPARα) which was discovered to regulate energy metabolism in diabetes mellitus. Thus, defective PPARα is associated with insulin resistance in humans. It is found that PPAR ligands and tocotrienol are similar in terms of their chemical structure, hence it is hypothesized that the beneficial effects exhibited by tocotrienol are via PPAR regulation. (40)

3.2 Diabetic Kidney Disease

3.2.1 Introduction to Diabetic Kidney Disease

Diabetic Kidney Disease (DKD) is a known chronic microvascular complication of type I and II diabetes mellitus. DKD often progresses silently because one can have no symptoms to indicate therapy until dialysis or renal transplant is warranted. Chronic kidney disease (CKD) is defined as the presence of decline in glomerular filtration rate (GFR) of 60 ml/min per 1.73 m^2 or evidence of kidney damage for a period of more than 3 months, regardless of the cause. (25)

In 2004, Kidney Disease: Improving Global Outcomes (KDIGO) endorsed the framework for classifying CKD into five stages in accordance to the level of GFR and/or evidence of renal structural defects.(26) Kidney function deteriorates with GFR and stage 5 in KDIGO framework is classified as end stage renal failure (ESRF). (27) Unfortunately, the only management available now is restricted to renal replacement therapies for example hemodialysis or renal transplant. Both therapies are invasive, associated with risks and a huge financial burden. Hence, early diagnosis and adequate management are crucial.(28)

The prevalence of CKD stage 3-5 was 10.6%, invariably most of these patients will progress to stage 4 or end stage renal disease.(29) CKD Stage 3a comprises the largest stage of CKD with an approximate figure of 84%. Normal physiologic age-related changes in the kidney usually begin after the age of 30-40 where a fall in GFR of 1ml/min/1.73m^2 is estimated. Studies have shown that a steep decline in eGFR > 3ml/min/1.73m^2 is also an indicator of progression to ESRD. High levels of albuminuria are associated with higher risk of developing ESRF even with mildly to moderately low eGFR as illustrated in Figure 1. (30)

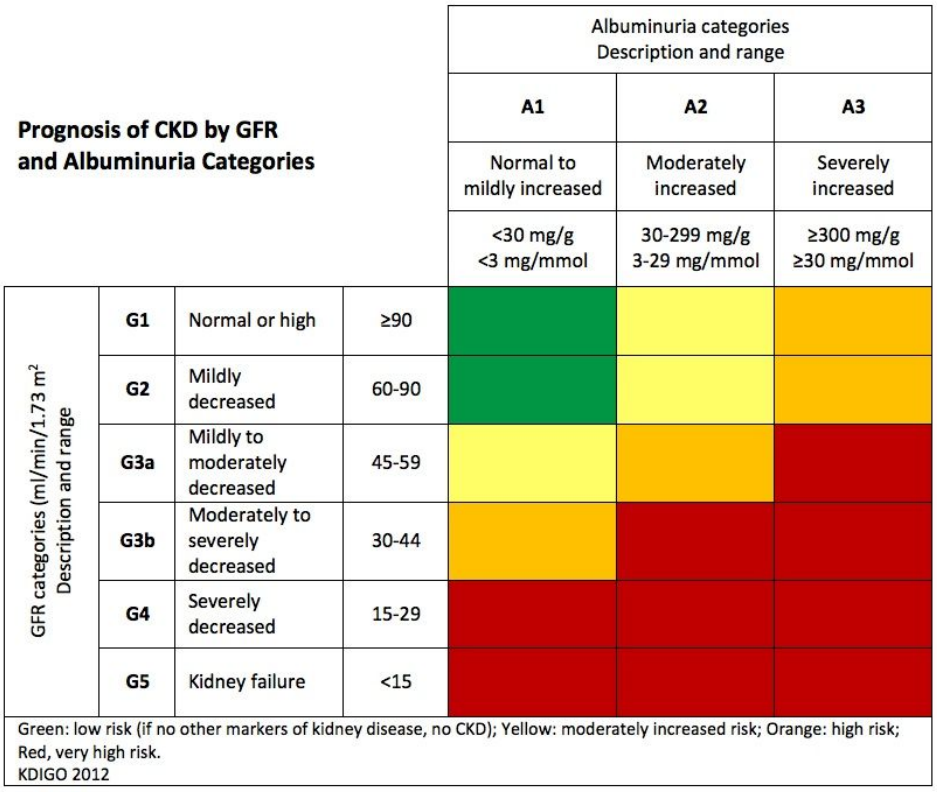

Figure 1: Classification of Chronic Kidney Disease (30)

The natural history of DKD has historically been taught as a stepwise course starting with hyperfiltration, presence of urinary albuminuria, progressive increment in microalbuminuria and subsequent decline in GFR and kidney function. However, many do not develop the disease in accordance with the classical trend. Interestingly, 60% developed the disease without progressing to albuminuria. (31) It is found that the mortality rate quadrupled in patients with albuminuria but preserved glomerular function.(1) In the early stages of DKD, there is typically a 25-50% increase in GFR function secondary to increase in sodium/glucose reabsorption and reduction in sodium delivery to the macula densa. (32) As the disease progresses, GFR begins to decline because of fewer functional nephrons consequently poor renal excretion.(33) Excessive synthesis and accumulation of extracellular matrix which leads to mesangial expansion leads to a further decline in GFR.(33)

The treatment goal for diabetic kidney disease is to delay or prevent further progression to end stage renal failure. Nevertheless, the main strategy remains to be optimal glycaemic

control of 7%, blood pressure control less than 130/80mmHg and perhaps dietary protein restriction. Studies have shown that angiotensin-converting enzyme inhibitors (ACE-i) or angiotensin receptor blockers (ARD) are effective in delaying progression of DKD in normotensive patients with albuminuria >30mg/g. (30)

STENO-2 study revealed that with aggressive management consisting of optimal glycemic control, cholesterol-lowering drugs, healthy lifestyle along with renin-angiotensin system (RAS) inhibitors at early stages, it is possible to impede the pathophysiological progress of diabetic nephropathy. (40, 41) The major barrier in the clinical scenario is because there are no biomarkers which can accurately and efficiently recognize patients who are at early risk of developing DKD. Conventional biomarkers for instance eGFR, creatinine, UACR are still being heavily relied upon to detect, monitor and predict the outcome of DKD. These conventional biomarkers are definitive in terms of disease monitoring, but when it comes to being a diagnostic tool, it remains questionable. (42, 43)

Our research team has attempted to utilize potentially novel biomarkers, such as Cystatin C, N ε -CML, AGE, Receptor for Advanced Glycation Endproducts (RAGE), Malondialdehyde (MDA), Tumour Necrosis Factor Receptor (TNFR1), Vascular Cell Adhesion Molecule-1 (VCAM-1) to discover its relationship with microvascular complications of diabetes including diabetic kidney disease. After 8 and 12 weeks of tocotrienol-rich vitamin E Tocovid supplementation, there was no significant change in these biomarkers. It could be potentially due to the short time frame of the study whereby it is impossible for AGEs to be broken down. Although there were no significant changes in the biomarkers as mentioned above, tocotrienol-rich vitamin E supplementation was found to improve kidney function. (44, 45) This signifies that there lies an underlying mechanism that is unrelated to neither anti-oxidant nor anti-inflammatory pathways that has not been discovered. Another possibility is that the biomarkers measured in the sera do not accurately reflect what is happening within the renal tissues.

3.2.2 Role of Tocotrienol in Diabetic Kidney Disease

The role of vitamin E in DKD remains unclear despite several investigations being performed to evaluate its effects on DKD.(41) Furthermore, after the setback from MICRO-HOPE study that revealed negative nephro-protective and cardio-protective results from tocopherol supplementation, many researchers became disinterested in this study area and as a result, research interest on vitamin E has dramatically plummeted.(42)

Bolignano et al. (2017) performed a systematic review investigating the role of vitamin A, C, E, zinc, selenium, ubiquinone or methionine supplementation as antioxidant on DKD. The study confirmed reno-protective effects as assessed by a reduction in urinary albumin excretion. However, the results were inconclusive due to heterogeneity of the outcomes and small sample size. Hence, this suggests that supplementation may be more appropriate if it is targeted to a selected population. (43) Another meta-analysis confirmed that prolonged supplementation of vitamin E or/and C is potentially beneficial in ameliorating endothelial dysfunction in T2DM with a body mass index (BMI) less than ≤ 30.(44)

Majority of studies in regards to vitamin E were performed using α-tocopherol which has different biological properties from tocotrienol. Hence, it is justifiable that even with the negative effects observed, it should not represent as inefficacy of tocotrienol but instead it is because of a different micronutrient supplementation.(41) The mechanisms which vitamin E influences the renal function in DKD has been largely postulated to be related to its antioxidant activity and anti-inflammatory. However, it may be more complex than it seems as our pilot study has tested otherwise. On the other hand, evidence with regard to the role of tocotrienol in DKD remains scant. Until now, limited animal studies and even fewer clinical trials were conducted to elucidate its possible benefits. In fact, our pilot phase study was one of the pioneer clinical trials that has been established. (44, 45)

Kuhad et al. (2009) reported that tocotrienol not only ameliorate diabetes, but also reverse the damage induced to the kidney via regulation of TNF-α and caspase-3 induced NF-κ β signalling cascade.(45) As expected, effects exerted by tocotrienol are far better as compared to tocopherol. Interestingly, the combination of insulin-tocotrienol yielded better results than individual treatment.(45) Perhaps this synergistic effect could be used as a

learning point for future human studies. Another animal study by Siddiqui et al. (2013) showed that tocotrienol improved serum lipid and glycaemic profile in T2DM rats which attenuated lipid-induced nephropathy. (46) Interestingly, tocotrienol supplementation also downregulated expression of TGF-β which is crucial in DKD progression. By modulation of TGF-β expression, its downstream signals such as fibronectin and type IV collagen in the kidney cells were downregulated. (46)

Khatami et al. (2016) was the first clinical trial study that has investigated the effects of tocotrienol on DKD.(47) It has involved a total of 60 participants who were randomly assigned into two groups. Those within the active group consumed up to 1200IU/day of vitamin E for 3 months. At the end of 3 months of vitamin E supplementation, the active group had significantly lower urine protein to creatinine ratio (UPCR), urinary protein, plasma MDA and serum AGE. This impressive result signifies that vitamin E supplementation indeed improves kidney function via alleviation of oxidative stress in diabetic nephropathy patients. One of the study limitations is that it was not clearly stated whether tocopherol-rich vitamin E or tocotrienol-rich vitamin E were used.(47)

The other two remaining clinical trials consist of our pilot phase study consisting of 2 separate phases. A total of 45 subjects were enrolled in this randomized controlled trial with equal subjects in each treatment arm. The primary aim of the study was to determine the effects of 400mg tocotrienol-rich Vitamin E supplementation daily in DKD patients. Notably, 8-12 weeks of tocotrienol-rich vitamin E has shown promising results with improvement seen on parameters of kidney function. However, these possible renoprotective effects seemed to be, irrespective to the anti-oxidative and anti-inflammatory properties of tocotrienol. (24,25)

All in all, even the mechanisms of action of Vitamin E in DKD remains unclear, its beneficial effects in DKD should not be refuted. This knowledge gap needs to be bridged with larger scale studies to further support the role of Vitamin E treatment in these patients.

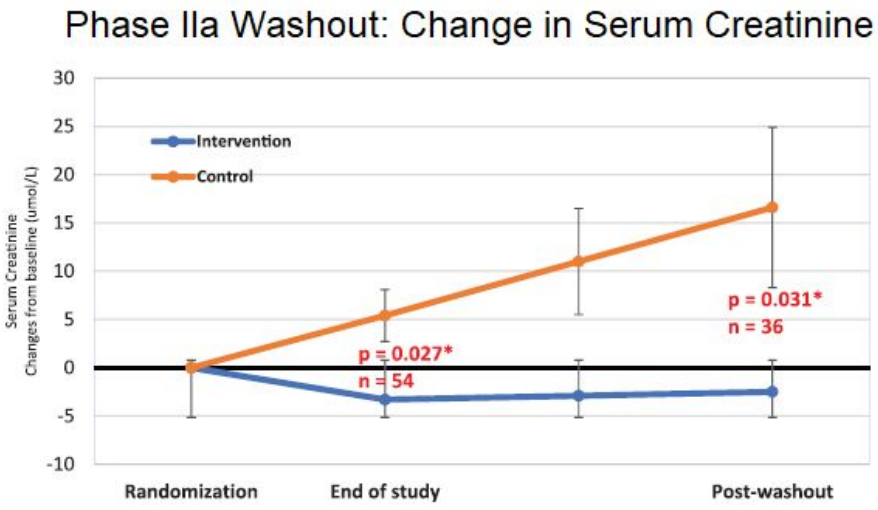

Figure 2: Change in serum creatinine over time between groups

Figure 2 illustrates the trend of serum creatinine in patients taking Tocotrienol-rich Vitamin E compared to the placebo group. There was a downwards trend of serum creatinine in the intervention group while there was a steady rise in serum creatinine in the control group. The increasing trend of serum creatinine in the control group is reflected by a steady decrease of renal function as shown in the figure below. In contrast, the eGFR of the intervention group remained stable around the baseline value.

Owing to the complexity of the physiopathological mechanism involved, our team did further clinical studies based on the pilot phase study to confirm the findings and explore other possible mechanisms. This ongoing study involves an increased number of patients with tighter inclusion and exclusion criteria for a duration of 12 months. We studied the role of growth factors such as TGF-β 1 and Nerve Growth Factor in the treatment of Diabetic complications involving the kidneys, eyes and retina. This study used tocotrienol-rich vitamin E Tocovid given as 200mg twice a day. The study ended in march/April and will have a 3 months wash out period now affected by the Covid imposed CMO.

The median age of the sample was 67 years old (IQR 14), with male as the dominant gender at 64.4%. The cohort is also made up of 57.6% Malay, 25.4% Chinese,13.6% Indian which is representative of the national demographics of Malaysia. Most participants have long

standing diabetes range from 12-18 years, with the mean duration of diabetes of 16.53 years (SD 8.29). Besides, the majority have a good HbA1c control of 7.57% (SD 1.02) and relatively well-controlled blood pressure control of 132/76mmHg. Majority of the patients have stage 3A chronic kidney disease. The mean of estimated glomerular filtration rate (eGFR) is 55.90ml/min/1.732 (SD 19.22) while the mean of serum creatinine was 120.60 µmol/L (SD 41.63). A large number of samples have moderate to severe microalbuminuria of >30mg/mmol.

The results analysed at 12 months are very positive and confirms the findings published earlier for 2-3 months

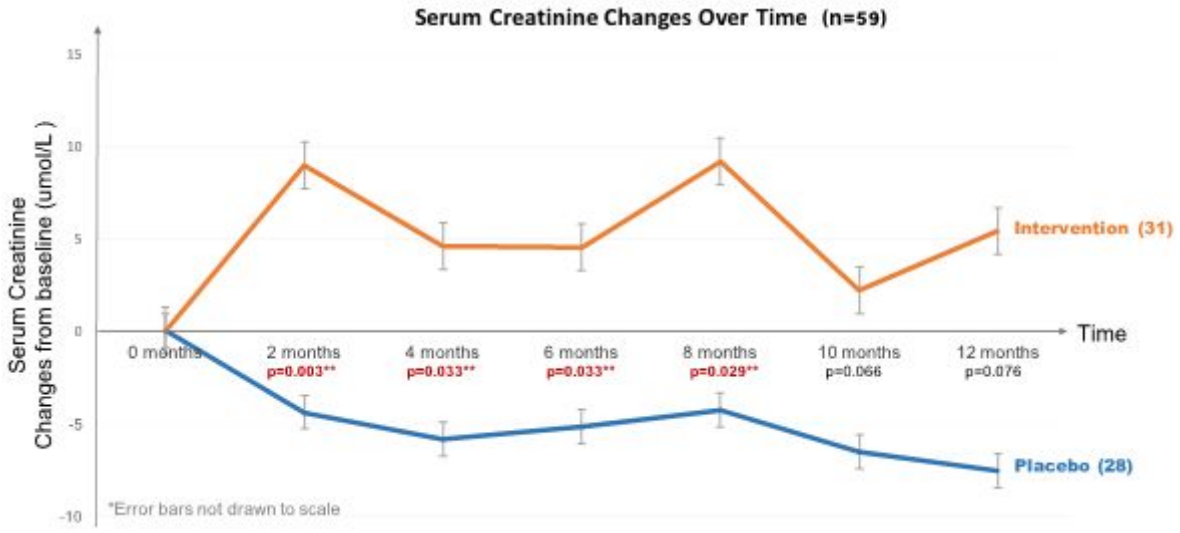

Figure 3: Changes in serum creatinine over time between placebo and intervention group

The graph in Figure 3 shows comparison of serum creatinine changes over time between the groups. As illustrated, the intervention group has a relatively steady reduction in serum creatinine levels unlike the control groups where there is a drastic increase in serum creatinine. The serum creatinine levels of the control group were constantly above the baseline value which indicates a decline in renal function.

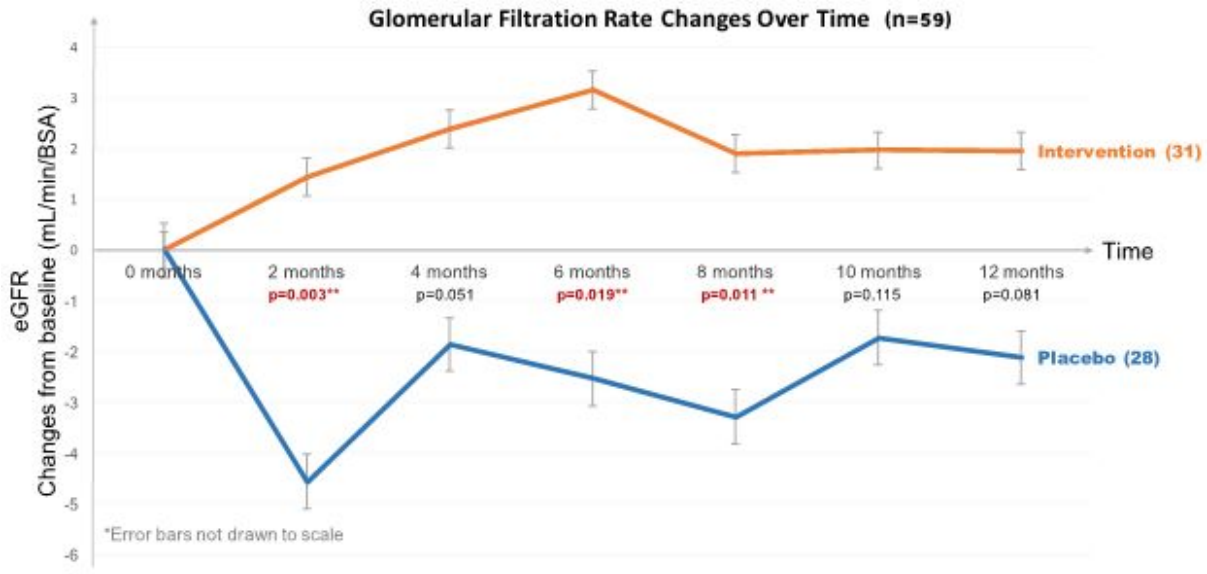

Figure 4: Changes in eGRF over time between intervention and control groups

The graph as illustrated in Figure 4 compares the eGFR changes between the groups over time. The eGFR value of the intervention group increases gradually until six months of supplementation before it stabilizes at eight months. Contrastingly, the eGFR of the control group demonstrated a downward trend over a year. The changes in glomerular filtration rate between both groups remains statistically significant up till eight months of the study. This reflects a declination in renal function in the control group while tocotrienol ameliorates renal function in the intervention group.

We then analysed as a subgroup those patients with Stage 3 Diabetic Kidney Disease i.e. with an EGFR between 30 – 60 mmol/min/bsa. As shown in Figure 5 and 6, tocotrienol-rich vitamin E given as Tocovid significantly improved renal function both in terms of lowering serum creatinine and improvement in estimated glomerular filtration rate (eGFR) respectively.

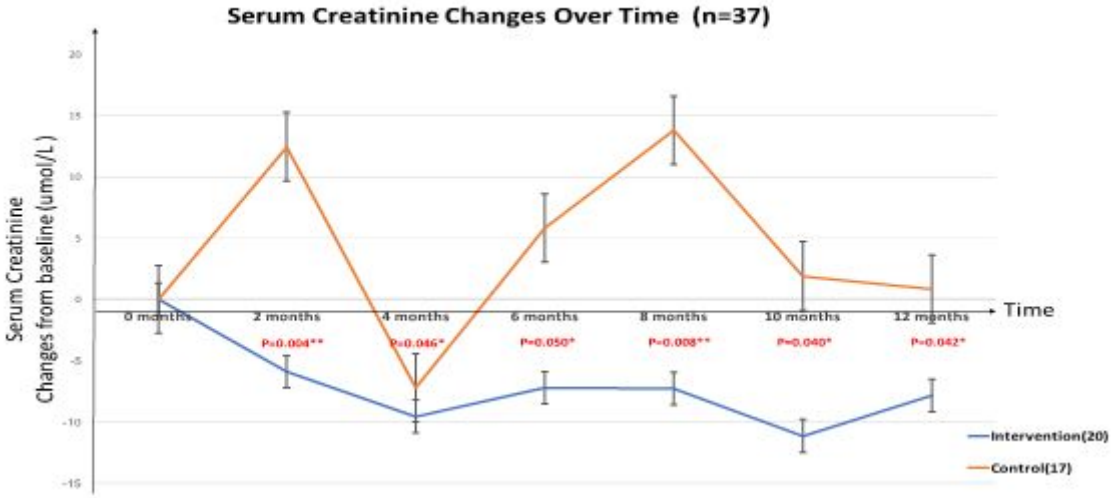

Figure 5: Changes in serum creatinine over time between intervention and control groups (eGFR between 30 – 60 mmol/min/bsa)

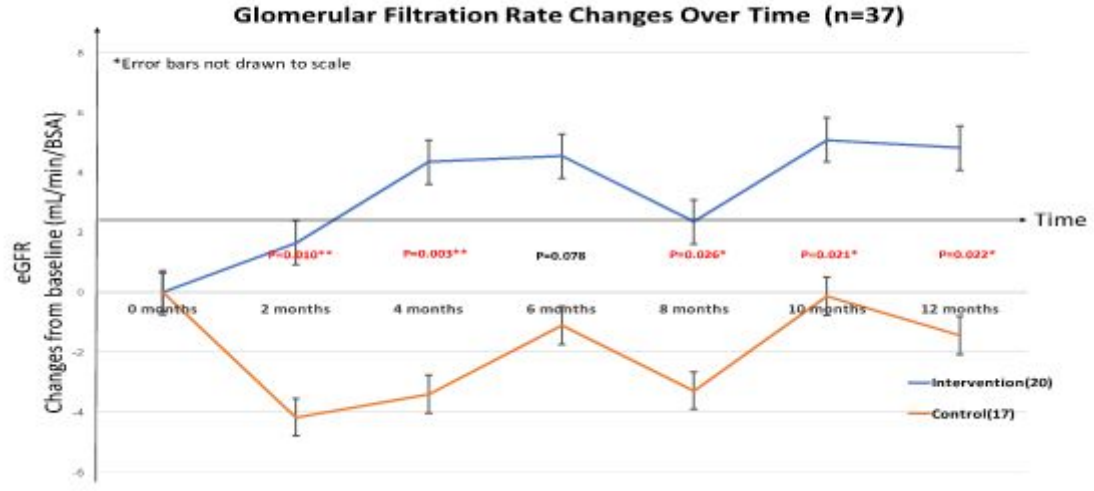

Figure 6: Changes in eGRF over time between intervention and control groups (eGFR between 30 – 60 mmol/min/bsa)

This study shows that tocotrienol-rich Vitamin E is very effective in protecting the kidneys in diabetics with significant renal deterioration i.e. stage 3 kidney disease .

3.3 Diabetic Peripheral Neuropathy

Diabetes mellitus leads to neuropathic syndromes which are often classified to acute or chronic subtype and focal or diffused neurological deficit.(48) Among all the subtype classifications, typical diabetic peripheral neuropathy (DPN), also known as distal symmetrical polyneuropathy (DSPN), accounts for approximately 75% of diabetic neuropathies,(49,50) and is the commonest neuropathic syndromes in diabetic patients.(48-51)

Globally, DPN is the commonest cause of neuropathy, and approximately 50% of the diabetic population are affected by this condition.(48,50) Out of these patients with DPN, half of them are asymptomatic,(50,54) while the other half will either present with foot ulceration due to diminished sensation or distressing neuropathic pain.(55) DPN accounts for the major proportion of hospitalization and it is the most common cause of non-traumatic amputation among all of the diabetic complications.(49,56) This suggests that DPN remains a serious complication of diabetes as it impairs the quality of life and causes significant morbidity as well as mortality.

3.3.1 Risk Factors of Diabetic Peripheral Neuropathy

The major risk factors of DPN are duration of diabetes, glycemic control and age.(58) There is a significant association between duration of diabetes and DPN, and this correlation is independent of diabetic individuals' age.(59) However, it is crucial to note that the strict glycemic control may decrease the risk of DPN, particularly in T1DM;(60,61) and some diabetic patients develop DPN even before the onset of diabetes.(62) Glycemic control, as assessed by levels of HbA1c and fasting plasma glucose, is another important risk factor for DPN. For instance, it was shown that for every 1% rise in HbA1c or 1mmol/L increment in fasting plasma glucose, the risk of DPN increases by approximately 15%.(59) Meanwhile, age was confirmed to be strongly associated with the risk of DPN and the risk approximately doubled with each decade of life.(58,59)

Other than that, high blood pressure, dyslipidemia and obesity has also been regarded as the major risk factors of DPN.(58) Additional risk factors of DPN include height, smoking history and reduced physical activities. Based on the above mentioned risk factors of DPN, it is important to classify them to either modifiable or non-modifiable risk factors. In a nutshell, it is important to control the modifiable risk factors of DPN as a preventive measure for the development as well as progression of the disease.

3.3.2 Vitamin E and Diabetic Peripheral Neuropathy

Generally, there are limited literatures regarding the beneficial effects of tocotrienol-rich vitamin E on DPN. This section will mainly focus on the studies carried out regarding vitamin E and its effects on DPN.

In 1998, Tutuncu NB et al conducted a study to investigate the improvement of diabetic neuropathy with vitamin E supplementation. 21 participants with T2DM were recruited and randomly assigned to receive either the intervention or placebo, namely 900mg and placebo respectively for six months. The result showed improvement in the vitamin E group in terms of nerve conduction.(63) Another study carried out by Rajanandh MG et al in 2014, showed similar results. In this randomized controlled trial, 92 patients with DPN were recruited and given vitamin E (intervention) or pregabalin (placebo). It was shown that the intervention group had reduction in terms of the total pain score as well as random blood sugar level.(64) Furthermore, vitamin E in combination with evening primrose oil with a dosing of 400mg daily and 500-1000mg daily respectively for a year were given to 80 patients with DPN.(65) It was concluded that vitamin E is beneficial in DPN as it reduces neuropathic pain among the diabetic patients.(65) Nevertheless, these studies did not specify the proportion of tocotrienol in the vitamin E and thus the effect cannot be evaluated accurately.

There was a recent publication which explored the effect of tocotrienol on DPN. In this research study, 300 patients were recruited and given 400mg/day (intervention) or placebo for a year. They inferred that tocotrienol did not improve DPN in terms of Total Symptom Score (TSS), Neuropathy Impairment Score (NIS) and NCS.(66) However, this study mainly focused on TSS and NIS, which are both subjective, as endpoints for the research. Moreover,

there are test-to-test variability in NCS conducted, and the results were not the main focus in the study.(67)

In addition to the studies above, Kuhad A et al conducted a study on diabetic rats, evaluating the effects between tocotrienol in combination with insulin and DPN. As a result, tocotrienol with insulin improves neuropathic pain in the diabetic rats, and reduces inflammatory cytokines as well as decreased oxidative stress.(68)

In conclusion, based on the above evidence, the effects of tocotrienol on DPN need to be further explored because it actually showed a promising role in the treatment of diabetic complications, especially DPN.

3.3.3 Effects of Tocotrienol-Rich Vitamin E on Diabetic Peripheral Neuropathy

We did a multicentre placebo controlled trial of tocotrienol-rich vitamin E Tocovid versus placebo in long term diabetics with neuropathy for 12 months. Preliminary data analysed at 8 weeks were very encouraging and have since been published this year: The mean/median of change between all the parameters were calculated and compared between Tocovid and placebo groups. As for the nerve conduction parameters, it is illustrated that all the velocity parameters in the every nerve significantly increased among the Tocovid group as compared to the placebo group. Nevertheless, the action potential for all nerves were not statistically different between Tocovid and placebo groups.

Table 4: Mean/Median Change of Nerve Conduction Parameters between Tocovid and Placebo Groups from Baseline to 8 Weeks

Mean/Median Change between Baseline and 8 Weeks Post-Intervention			
Median Sensory Nerve	**Tocovid (N=62)**	**Placebo (N=60)**	**p-Value**
Nerve Conduction Parameters			
Conduction Velocity (m/s)[b]	1.25 (3.35)	0.00 (2.90)	**<0.001***
Peak Velocity (m/s)[b]	0.95 (2.40)	-0.15 (2.33)	**<0.001***
NP Amplitude (µV)[a]	1.70 ± 5.95	1.15 ± 4.93	0.580
PP Amplitude (µV)[b]	3.15 (11.35)	1.40 (8.30)	0.213
Sural Sensory Nerve	**Tocovid (N=49)**	**Placebo (N=45)**	**p-Value**
Nerve Conduction Parameters			
Conduction Velocity (m/s)[b]	1.60 (1.80)	-0.60 (2.10)	**<0.001***
Peak Velocity (m/s)[a]	1.14 ± 1.64	-0.54 ± 1.62	**<0.001***
PP Amplitude (µV)[a]	1.11 ± 2.57	0.43 ± 2.99	0.243
NP Amplitude (µV)[b]	0.10 (4.95)	-0.40 (2.10)	0.470
Tibial Motor Nerve	**Tocovid (N=70)**	**Placebo (N=70)**	**p-Value**
Nerve Conduction Parameters			
Conduction Velocity (m/s)[b]	0.75 (2.25)	-0.90 (3.50)	**<0.001***
Distal Amplitude at Ankle (mV)[b]	0.80 (1.95)	0.60 (1.83)	0.291

N: Sample size; NP: Negative peak; PP: Positive peak.
**Data is significant if p<0.05.*

[a] *Data presented as mean ± standard deviation; p-value obtained using independent t-test, assumptions were fulfilled.*

[b] *Data presented as median (interquartile range); p-value obtained using Mann-Whitney U test.*

3.3.4 Vitamin E and Other Diabetic Complications

Besides improvement in DPN, Vitamin E has been shown to ameliorate other diabetic complications.(69) In terms of diabetic nephropathy, a randomized controlled trial conducted by Khatami PG et al which recruited 60 participants portrayed the beneficial effects of Vitamin E on diabetic nephropathy.(70) The improvement of diabetic nephropathy was assessed by reduction in urinary protein, protein-to-creatinine ratio as well as improvement in other biomarkers including serum TNF-α, AGEs, MDA, matrix metalloproteinases and insulin levels.(70) Nonetheless, the Vitamin E constituents were not specified in this study. A more recent publication by Tan SMQ et al verified that tocotrienol supplementation significantly reduced serum creatinine levels, indicating its beneficial effect in managing diabetic nephropathy.(71)

Vitamin E has also been proven to pose a beneficial effect on diabetic retinopathy. Animal models have also shown that angiogenesis in diabetic retinopathy can be inhibited by tocotrienol.(72) In human subjects, Bursell S et al demonstrated that vitamin E significantly improved diabetic retinopathy in terms of increasing retinal blood flow.(73) In addition, the study also concluded that vitamin E normalized creatinine clearance among diabetic patients.(73) Another prospective clinical trial conducted by Jain AB et al concluded that Vitamin E supplementation could potentially prevent diabetic complications, particularly in diabetic retinopathy and cardiovascular events.(74)

All in all, the benefits of Vitamin E on diabetes are apparent but we should note that most of the studies did not specify the constituents of Vitamin E in the papers. Based on the superiority of tocotrienol over tocopherol, we believe that our research study involving tocotrienol-rich Vitamin E will be able to ease diabetic complications, especially diabetic peripheral neuropathy.

3.4 Justification of the Study

Diabetes is a major public health emergency that should receive paramount attention. As a result of poor glycemic control, diabetics develop diabetes associated microvascular and macrovascular complications. With this exponential growth in numbers, diabetes mellitus carries a tremendous burden to both the healthcare system and patient. Moreover, approximately a third of T2DM patients suffer from diabetic nephropathy which is also the primary cause of end stage renal disease worldwide. To date, therapy for DKD has made very little advancement and treatment measures remain to be conventional such as optimal glycemic control and blood pressure monitoring.

Our pilot studies, which aim to evaluate the effects of tocotrienol-rich vitamin E (Tocovid Suprabio) in DKD showed significant reduction in serum creatinine and improvement in serum eGFR compared to controls at 2 and 3 months. (24, 25) Interestingly, at 6 months post washout, the beneficial effects of Tocovid Suprabio persisted compared to the controls. There was no significant reduction in HbA1c, and novel biomarkers measured for example Cystatin C, Nε-CML, AGE, Receptor for Advanced Glycation Endproducts (RAGE), Malondialdehyde (MDA), Tumour Necrosis Factor Receptor (TNFR-1), Vascular Cell Adhesion Molecule-1 (VCAM-1). This clearly indicates that Tocotrienol supplementation is beneficial for DKD although the mechanism of action remains elusive.

In conclusion, our group had shown the beneficial effects of tocotrienol-rich vitamin E in a variety of metabolic diseases. In the last 6 years we have shown the beneficial effects on diabetes in animal models and have then progressed onto clinical trials in patients with long standing diabetes. We showed that tocotrienol-rich vitamin E could improve diabetic kidney disease for at least one year, improve diabetic eye disease, and had highly significant improvement on nerves damaged by diabetes. These were most effective in patients with lower levels of Vitamin E. The studies were done with patients given Tocovid 200mg twice a day. Preliminary studies by our group showed that the effects were also significant with doses of only 200mg daily at as early as 2 months. We plan to extend this study to a greater number of patients given 200 mg daily and 100mg daily for up to 6 months, assessing the

effects on all 3 parameters diabetic kidney damage, diabetic nerve damage and diabetic retinopathy.

3.5 Conclusion

Our group had shown the beneficial effects of Tocotreinol rich vitamin E in a variety of metabolic diseases. In the last 6 years we have shown the beneficial effects on diabetes in animal models and have then progressed onto clinical trials in patients with long standing diabetes. We showed that tocotrienol-rich Vitamin E could improve diabetic kidney disease for at least one year, improve diabetic eye disease, and had highly significant improvement on nerves damaged by diabetes.

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Preliminary studies by our group showed that the effects were also significant with doses of only 200mg daily at as early as 2 months. We plan to extend this study to a greater number of patients given 200 mg daily and 100mg daily for up to 6 months, assessing the effects on all 3 parameters diabetic kidney damage, diabetic nerve damage and diabetic retinopathy.

Section 4: Hypotheses and Objectives

4.1 Research Questions

I. What is the effect of lower doses of tocotrienol-rich vitamin E, on the renal function of patients with diabetic kidney disease as assessed by serum creatinine, estimated glomerular filtration rate (eGFR) and urine albumin creatinine ratio (UACR)?
II. Similarly what is the effect of lower doses of tocotrienol-rich vitamin E on diabetic neuropathy and diabetic retinopathy in these patients?
III. What is the mechanism by which tocotrienol-rich vitamin E improves these functions in organs damaged by long standing diabetes since our earlier studies

showed that they do not correlate with HbA1c, AGE products, RAGE, inflammatory markers such as VCAM-1 and Cystacin, antioxidants MDA, or TNFR-1, TGF-β 1 etc.?

IV. Do the levels of tocopherol and/or tocotrienol correlate with serum TGF-β 1, serum creatinine and estimated glomerular filtration rate (eGFR)?

V. Are these beneficial effects only seen in tocotrienol-rich Vitamin E preparations, in contrast to pure tocopherols?

4.2 Hypotheses

- The beneficial effects of tocotrienol-rich vitamin E in diabetic complications can be seen at doses lower than 200mg per day within 2 months of treatment
- The beneficial effects are mainly due to tocotrienols in the tocotrienol-rich vitamin E preparations

4.3 Objectives

Primary objective:

- To investigate the effects of increasing doses tocotrienol-rich Vitamin E on kidney disease, peripheral neuropathy and retinal disease in patients with long standing type 2 diabetes mellitus

Secondary objectives:

- To investigate the mechanisms by which tocotrienol-rich vitamin E improves nerve function, protects the retina and gives renal protection in diabetics of 10 or more years duration suffering from these complications
- To investigate the superiority of tocotrienol-rich Vitamin E to natural alpha-tocopherol (200IU)

Section 5: Methodology

5.1 Study design

This is a prospective, multi-centered, randomized, double-blinded, placebo-controlled study involving patients with type 2 diabetes mellitus with reasonable glycaemic control (HbA1c between 6.0 - 9.0%) and diabetic neuropathy as assessed by nerve conduction study using 100mg and 200mg of Tocovid SupraBio® and 200IU of α-tocopherol once per day for 24 weeks. The study duration and timeline are shown in Diagram 1 below.

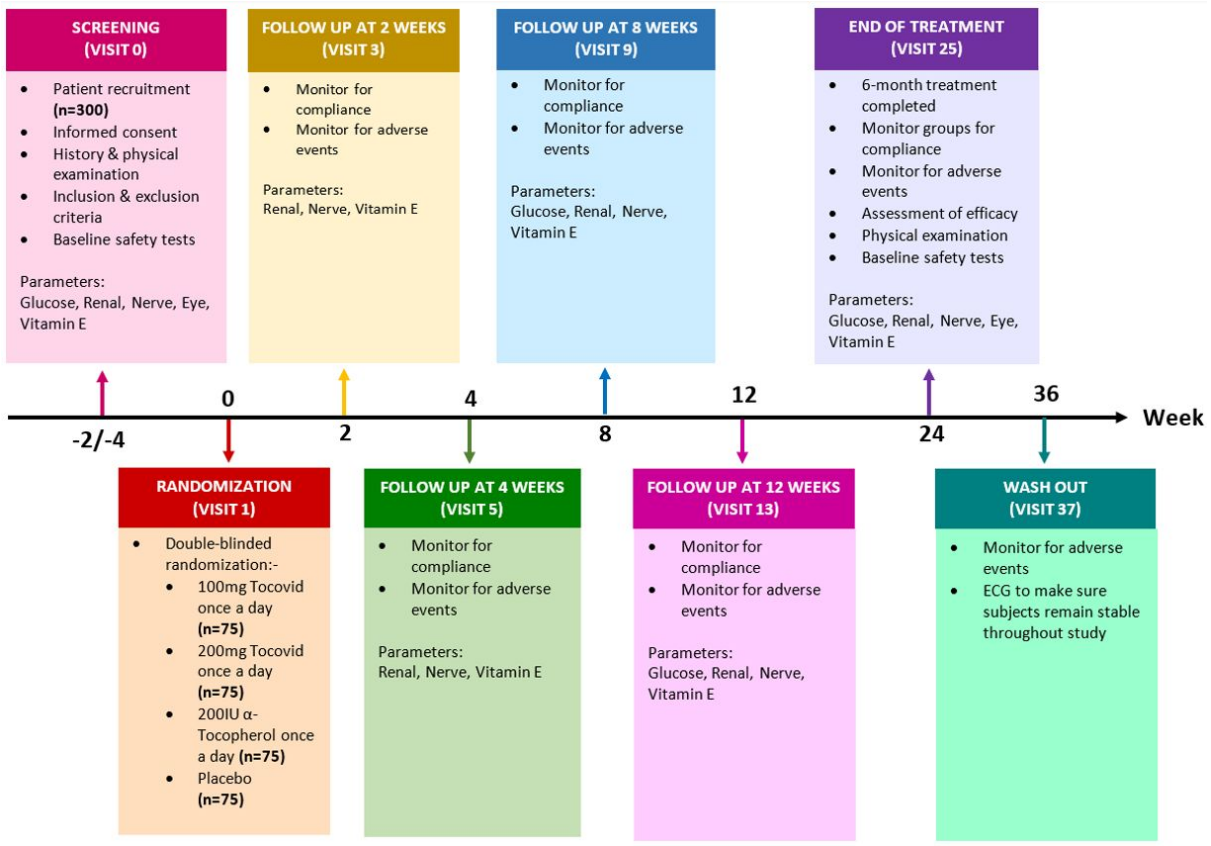

Diagram 1: Study timeline

Screening (Visit 0)

Potentially eligible participants from the existing pool of patients at the Clinical Research Centre (CRC), Monash University Malaysia are invited to participate in the clinical trial. The following information will be advised to the patients before the screening visit:

- ✔ Patient to fast for 8 hours (no food/drinks, only plain water allowed)
- ✔ No strenuous activity the night before
- ✔ Medication
    - DO NOT take hypoglycemic medication
    - Take usual dose antihypertensive and other medications
    - Bring all medications/supplements that are new
- ✔ Laboratory/Diagnostic Test Results
    - Bring all investigative test results for review
- ✔ If a patient is a premenopausal female participant, ensure that she is NOT menstruating during the appointment visit. This is important because it may affect

the accuracy of the test results if the instructions are not followed. Investigators will remind the participants via telephone call the day before all visits.

- ✔ Nerve conduction studies
    - DO NOT wear tight pants as a stimulating electrode will be placed on the skin at the back of their knees
    - DO NOT apply lotion or cream on the lower limbs as these may prevent electrodes from sticking properly
- ✔ Fundus camera
    - DO NOT wear contact lens as their pupils will be dilated with an eye drop

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

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

- ✔ In the screening visit, a thorough history-taking and physical examination will be done to ensure the participants meet the inclusion/exclusion criteria.

(Refer to the inclusion and exclusion criteria section below).

- ✔ Patient's blood pressure and anthropometric measurements such as weight, height and waist circumference will be routinely carried out in every visit.
- ✔ Further baseline and screening tests will only be done on subjects who meet the recruitment criteria thus far.
- ✔ The baseline tests include ECG, Renal Function Test (RFT), Urine FEME, Liver Function Test (LFT) and lipid profile.
- ✔ The screening tests include serum vitamin E, fasting blood glucose (FBG), HbA1c, urine dipstick and UACR.
- ✔ The complete list of the baseline and screening tests can be obtained in Table 2 below.
- ✔ Thus, a total of 25 ml of blood samples (5 tubes) and urine samples will be obtained and processed on the same working day at our CRC.

- ✔ Retinal photographs will also be taken for all subjects.
- ✔ Peripheral nerve conduction studies will be done on all subjects.
- ✔ Subjects will be asked to fill in a neuropathic pain questionnaire for investigators to assess and treat their nerve problems.
- ✔ Subjects who are premenopausal women will undergo a urine pregnancy test. If the urine pregnancy test is positive, a serum pregnancy test will be done.

Randomization Visit (Visit 1)

- ✔ Once the test results are out, the investigators will call the subjects to arrange an appointment to review their test results and inform them regarding their eligibility to participate in this clinical trial.
- ✔ Subjects who fulfilled the inclusion/exclusion criteria will be invited to return to the CRC for randomization (1-2 weeks after the screening visit).
- ✔ Subjects will be randomized 1:1:1:1 into the double-blind treatment period for 6 months. Randomization is stratified based on the patient's age, gender, HbA1c and duration of diabetes.
- ✔ The drug manufacturer will prepare the 100mg, 200mg Tocotrienol-rich Vitamin E (Tocovid SupraBio®), 200IU Vitamin E (natural α-Tocopherol) and placebo capsules. The capsules will be labelled as A, B, C or D.
- ✔ Therefore, both investigators and patients are unaware which one is the study drug and this prevents selection bias.
- ✔ The interventional group will receive either oral 100mg OD of Tocotrienol-rich Vitamin E, 200mg OD of Tocotrienol-rich Vitamin E, 200IU OD of α-Tocopherol and the control group will receive oral placebo OD.
- ✔ Each cohort will consist of 75 subjects, making a total of 300 subjects with an allowance of maximum 10% dropout per group.
- ✔ During this visit, blood samples will be collected to determine the baseline Vitamin E levels for the subjects.

Follow-Up Visit (Visit 3 and 5)

- ✔ During these follow-up visits, investigators will monitor for adverse events and may choose to withdraw the subject from the study based on his/her discretion in the case that any adverse event occurs.
- ✔ Investigators will monitor the subject's compliance by counting the remaining capsules.
- ✔ Anthropometric measurements, blood pressure, capillary FBG and urine FEME will be regularly performed during these visits.
- ✔ Blood samples will be taken to assess the patients' kidney function and determine Vitamin E levels.
- ✔ Nerve conduction study for both tibial motor nerve and sural sensory nerve will be repeated in every visit as well as neuropathic pain questionnaire to assess the change in nerve parameter.

Follow-Up Visit (Visit 9 and 13)

- ✔ These visits will be exactly the same as follow-up visits 3 and 5 with the addition of HbA1c and plasma FBG test.

End of Treatment Visit (Visit 25)

- ✔ This is the end of the 6-month double-blind treatment period.
- ✔ During this visit, investigators will continue to monitor for adverse events and compliance.
- ✔ Anthropometric measurements, blood pressure, finger-prick test, and urine dipstick will be routinely carried out in this final visit.
- ✔ Blood and urine samples will be taken as per screening visit for investigations: Renal Function, HbA1c, and UACR. Vitamin E levels will also be assessed. Nerve conduction study will be conducted for all subjects. Subjects will be asked to repeat the same neuropathic pain questionnaire that was done in previous visits for comparison. Retinal photography will be taken for eligible patients.

- ✔ Additionally, baseline safety tests including LFT, ECG, lipid profile and full blood count will be repeated to ensure subject‘s baselines are within the normal range and remain stable throughout the study.

Washout Visit (Visit 37)

- ✔ Investigators will monitor for any adverse event that occurs after the subjects stop taking the investigational products.
- ✔ As usual, anthropometric measurements, blood pressure, capillary FBG and urine FEME will be carried out in this visit.
- ✔ ECG will be done to ensure the subjects have remained stable throughout the study.

Test		V0	V1	V3	V5	V9	V13	V25	V37
Safety tests	Anthropometric measurements	■	■	■	■	■	■	■	■
	BP	■	■	■	■	■	■	■	■
	ECG	■						■	■
	Lipid profile	■						■	
	LFT (including AST and ALT)	■						■	
	UPT (Premenopausal female)	■	■					■	
Parameter 1: Renal	RFT (BUSE, Creatinine & eGFR)	■		■	■	■	■	■	
	UFEME	■	■	■	■	■	■	■	■
	UACR	■						■	
Parameter 2: Glucose	HbA1c	■				■	■	■	
	FBG (plasma)	■				■	■	■	
	FBG (finger-prick test)	■	■	■	■	■	■	■	■
Parameter 3: Nerve	Nerve conduction study (NCS)	■		■	■	■	■	■	
	Neuropathic Pain Questionnaire	■		■	■	■	■	■	
Parameter 4: Eye	Fundus camera	■						■	
Parameter 5: Vitamin E	Plasma Vitamin E		■	■	■	■	■	■	

Table 2: Investigation tests to be done at each visit

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 Sunway Campus, and Clinical School Johor Bahru, Klinik Kesihatan Tanglin Hospital and Thomson Hospital in Kota Damansara, Malaysia and Diabetes Clinic University Institute Technology Mara (UiTM) at Sungai Buloh. Those patients who have renal or nerve or retinal disease will be invited to be screened for the study at the Clinical Research Centre in Sunway Campus, the Clinical School in Johor Bahru or UiTM in Sungai Buloh.

5.3 Sample Size

- ✔ The total sample size of the study is 300 participants.
- ✔ Each cohort consists of 75 subjects with an allowance of maximum 10% dropout per cohort.
- ✔ The sample size was calculated based on the mean sural nerve conduction velocity at 8 weeks from our previous study published by Ng et al.(75). The mean sural nerve conduction velocity for the Tocovid SupraBio® group is 45.32 ± 5.30 m/s and 42.29 ± 4.51 m/s for placebo after 8 weeks of treatment.
- ✔ A minimum sample size of 256 subjects, 64 in each arm, is sufficient to detect a clinically important difference of 3.03 m/s between groups in improving sural sensory nerve conduction velocity assuming a standard deviation of 5.30 using an one-way ANOVA with pairwise comparisons to achieve 80% power and a 5% level of significance. Taking into account a maximum dropout of 10% and unforeseeable factors, a total sample size of 300 subjects for 4 treatment arms is required.

5.4 Inclusion and Exclusion Criteria

5.4.1 Inclusion Criteria

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

- ✔ Subject, or legal representative, has voluntarily signed and dated an Informed Consent Form
- ✔ Subject is 30-75 years of age at the initial Screening visit.
- ✔ 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% .
- ✔ If a subject has hypertension, he/she must have a stable blood pressure control for the past 2 months with not more than 10% change and the BP range should be < 145/90 mmHg.

Subject has at least one of the following symptoms of diabetic peripheral neuropathy:

- ✔ Reduction in nerve conduction velocity (baseline conduction velocity < 40 m/s)
- ✔ Reduction in amplitude
- ✔ Abnormality found in clinical assessment (Eg: pain, light touch, temperature, position sense, vibration and reflexes)

Subject has either one of the following or both:

- ✔ Inactive diabetic retinopathy as assessed by retinal photography
- ✔ Mild to moderate nephropathy which is defined by UACR of >20mg/g or eGFR between 30 - 60 ml/min/bsa (Stage 3 CKD)

5.4.2 Exclusion Criteria

Subjects will be excluded from entering the study if they meet ANY of the following criteria:

- ✔ Subject has unstable glucose control (more than 10% change in HbA1c levels over the last 2 months)
- ✔ Subject has poor blood pressure control, BP > 160/100
- ✔ Subject is pregnant during screening
- ✔ Subject has urine protein > 150g/dL during screening
- ✔ Subject has current urinary tract infection during screening (symptomatic or definitively on urine dipstick: presence of pyuria, nitrites and red blood cells)
- ✔ Subject has known non-diabetic kidney disease, such as kidney stones, etc
- ✔ Subject has a corrected visual acuity of less than 20/200
- ✔ Subject with eye diseases such as media opacity, amblyopic and glaucoma
- ✔ Subject on anti-epileptic or sedative medications
- ✔ 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.
- ✔ Subject is taking other water-soluble antioxidants for the past 2 weeks or fat-soluble antioxidants for the past 1 month
- ✔ Subject is a heavy smoker (≥20 sticks/day) or has stopped smoking for less than 1 month
- ✔ Subject has elevated liver enzymes (serum ALT and/or serum AST >3x the upper limit of normal)
- ✔ Subject with severely deranged renal profile. (Stage 5 CKD; eGFR ≤15 ml/min/1.73m^2)

5.5 Collection, Storage and Use of Biospecimens

- ✔ For the purpose of this study, blood samples will be collected from subjects and will be stored in serum-separating tube (SST) tubes.

- ✔ Refer to Table 2 for the list of blood tests.
- ✔ Urine samples will be taken for urine dipstick test and UACR test.
- ✔ Urine dipstick will be done at every visit while UACR test will be done only during Screening Visit and End of Treatment Visit.
- ✔ The blood and urine samples obtained will be processed on the same working day at our CRC, Monash University Malaysia.
- ✔ Validation of all test results will be done by Dr Badariah and Professor Khalid.
- ✔ Serum and plasma samples will be allocated into tubes of 1 ml each, snap-freezed and stored at -80°C for future testing of other biomarkers and measurement of Vitamin E levels respectively.
- ✔ Processing of serum and plasma 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 will be done at our study site.
- ✔ Subsequently, all biological specimens except serum and plasma samples will be discarded using local protocol at most two working days after validation of results.
- ✔ Therefore, specimens (blood and urine samples) will not be stored and kept for more than 3 (THREE) working days from collection until disposal.

There will be no genetic testing involved in this study.

5.6 Milestones

Results from our phase 2b study showed there was a statistically significant improvement ($p < 0.001$) in the nerve conduction studies parameters after 8 weeks of Tocovid SupraBio® supplementation and we have successfully published a paper on this finding in Nutrients 2020(75). In addition, our previous study in 2018 showed that 400mg of Tocovid SupraBio® once a day for 12 weeks significantly improved renal function as assessed by serum creatinine and eGFR, which remained significant 6-9 months after treatment ceased. This finding was published in Metabolism 2019 (37).

Section 6: Impact

6.1 Scientific Impact

- Address research gap of the effects of low dose tocotrienol-rich vitamin E on type 2 diabetes patients and determine the most effective dose
- Address research gap on the lack of validation and quantification methods for low levels of tocotrienol and tocopherol in human plasma
- Determine the mechanisms by which these beneficial effects by tocotrienol are mediated

6.2 Social Impact

- Potentially reduce the number of diabetic patients progressing into kidney, eye and nerve failure at lower costs using lower dose tocotrienol-rich vitamin E; consequently reducing the burden of diabetes disease and cost for our country.
- Impart value-added products of the palm-oil industry into the medical sector particularly for treatment of diabetes and its microvascular complications

Section 7: Background of Research Team

7.1 Scientific Background

Several publications on effects of tocotrienol-rich vitamin E in relation to diabetes have been published by our research team: -

Publications

Cheng, H., Ton, S., Tan, J., & Abdul Kadir, K. (2017). The Ameliorative Effects of a Tocotrienol-Rich Fraction on the AGE-RAGE Axis and Hypertension in High-Fat-Diet-Fed Rats with Metabolic Syndrome. *Nutrients*, *9*(9), 984. doi: 10.3390/nu9090984

Tan, S., Chiew, Y., Ahmad, B., & Kadir, K. (2018). Tocotrienol-Rich Vitamin E from Palm Oil (Tocovid) and Its Effects in Diabetes and Diabetic Nephropathy: A Pilot Phase II Clinical Trial. *Nutrients*, *10*(9), 1315. doi: 10.3390/nu10091315

Tan, G., Tan, S., Phang, S., Ng, Y., Ng, E., Ahmad, B., Palanisamy, U. & Kadir, K. (2019). Tocotrienol-rich vitamin E improves diabetic nephropathy and persists 6–9 months after washout: a phase IIa randomized controlled trial. *Therapeutic Advances In Endocrinology And Metabolism*, *10*, 204201881989546. doi: 10.1177/2042018819895462

Ng, Y., Phang, S., Tan, G., Ng, E., Botross Henien, N., & M. Palanisamy, U., Ahmad, B., Kadir, K. (2020). The Effects of Tocotrienol-Rich Vitamin E (Tocovid) on Diabetic Neuropathy: A Phase II Randomized Controlled Trial. *Nutrients*, *12*(5), 1522. doi: 10.3390/nu12051522

7.2 Technical Background

Our research team has experience in conducting human clinical trial since 1990s in UKM and since 2004 in Monash University. We routinely perform phlebotomy, laboratory based work such as biochemistry assays and liquid chromatography techniques and conduct statistical analysis using SPSS statistical package. As stated above, we have published numerous papers on vitamin E and tocotrienols.

7.3 Research Team Members

1. **Professor Emeritus Dato' Dr Khalid Abdul Kadir**

Professor of Medicine at Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, and the Endocrinology and Internal Medicine Specialist at the Thomson Hospital, Damansara, Malaysia.

2. **Dr Badariah Ahmad**

Senior lecturer in Physiology at Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia and Senior Lecturer/Clinician at Monash Diabetes and Hypertension Clinic at Tanglin Primary Care Clinic, Kuala Lumpur.

3. **Associate Professor Uma Devi M. Palanisamy**

Associate Professor in Biochemistry at Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia.

4. **Dr Nevein Bottross**

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5. **Dr Ho Loon Shin**

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6. **Noras'kin Binti Mohamad**

Senior technical officer at Monash Medical Precinct, Monash University Malaysia.

7. **Ungku Zulaikha Ungku Omar**

Technical officer at Monash Medical Precinct, Monash University Malaysia

8. **Pang Pei Ling**

Senior technical officer at Clinical School of Johor Bahru, Monash University Malaysia

9. **Savithiri A/P M K Gopal**

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10. **Chui Chor Sin**

Research assistant at Clinical School of Johor Bahru, Monash University Malaysia

11. **Chuar Pei Fen**

Research assistant at Monash Medical Precinct, Monash University Malaysia

12. **Sonia Phang Chew Wen**

PhD candidate at Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia

Section 8: Gantt Chart

Proposed start and end date: January 2021 - December 2022

Project Activities (months)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Protocol Ethical clearance & permission from relevant authorities																								
Screening and patient recruitment																								
Data collection																								
Statistical analysis																								
Final report preparation																								
Study closure report to NIH/MOH: study findings dissemination																								

Section 9: Budget

9.1 Budget Breakdown

Item and breakdown	Estimated cost
1. Remuneration for study subjects • Target: 300 • No of visits: 8 • RM 30 per subject per visit	300 x 8 x RM 30 = **RM 72,000** **<u>Subtotal: RM 72,000</u>**
2. Doctor's consultations • RM 120 for first visit • RM 60 x 7 subsequent visits = RM420	(RM 120 + RM 420) x 300 subjects = **RM 162,000** **<u>Subtotal: RM 162,000</u>**

3. Drugs
• Tocovid SupraBio® 100 mg
  • 1 capsule x 75 subjects x 168 days = 12,600 capsules
  • RM 2/capsule
• Tocovid SupraBio® 200 mg
  • 1 capsule x 75 subjects x 168 days = 12,600 capsules
  • RM 4/capsule
• Alpha-tocopherol
  • 1 capsule x 75 subjects x 168 days = 12,600 capsules
  • RM 1/capsule
• Placebo
  • 1 capsule x 75 subjects x 168 days = 12,600 capsules
  • RM 1/capsule
	12,600 x RM 2 = **RM 25,200**
	
	12,600 x RM 4 = **RM 50,400**
	
	12,600 x RM 1 = **RM 12,600**
	
	12,600 x RM 1 = **RM 12,600**
	
	<u>Subtotal: RM 100,800</u>

4. Laboratory test fees
• Certain blood and urine tests will be sent to BP labs for processing:
  • Renal Function Test (RFT)
  • RM 15/test
  • 2 visits (At 2 and 4 weeks)
  • RFT + HbA1c + Fasting Blood Glucose (FBG)
  • RM 60/test package
  • 2 visits (At 8 and 12 weeks)
	RM 15 x 2 x 300 subjects = **RM 9,000**
	
	RM 60 x 2 x 300 subjects = **RM 36,000**
	
	RM 160 x 2 x 300 subjects = **RM96,000**

• RFT + UACR + HbA1c + FBG + LFT + lipid profile
  • RM 160/test package
  • 2 visits (At screening and end of study)
• HPLC
  o Methanol: RM 53 x 20 units = RM1,060
  o n-hexane: RM 90 x 6 units = RM540
  o HPLC column = RM2,497
  o Guard cartridges = RM1,626
  o Vial inserts: RM 437 x 28 packs of 100 = RM12,236
  o Filter paper = RM320
  o Microcentrifuge tubes: RM 258 x 12 packs = RM3,096
	RM21,375
	
	<u>Subtotal: RM162,375</u>

5. Additional Tests
• ECG strips, labours, reports
  • RM 30/test
  • 2 visits (At screening and end of study)
• Fundus camera
  • RM 120/test
  • 2 visits (At screening and end of study)
• Nerve conduction study
  • RM 150/test
  • 6 visits (At screening, 2, 4, 8, 12 weeks and end of study)
• Consumables for nerve conduction study
  • Disposable surface electrodes
  • RM 500/box of 500 pcs
  • 300 subjects x 6 tests = 1800 tests
  • 1800 tests x 2 pcs/test = 3600 pcs (7.2 boxes ≈ 8 boxes)
  • Felt pad for bipolar stimulator (reusable)
  • RM 150/pack of 10
  • Alcohol swabs for cleaning site
  • RM 15/box of 100 pcs
  • 300 subjects x 6 tests = 1800 tests
  • 1800 tests x 1pcs/test = 1800 pcs (18 boxes)
  • Disposable non-sterile latex gloves
  • RM22/box of 100 pcs
  • 300 subjects x 6 tests = 1800 tests
  • 1800 tests x 2pcs/test = 3600 pcs (36 boxes)
	RM 30 x 2 x 300 subjects = **RM 18,000**
	
	RM 120 x 2 x 300 subjects = **RM 72,000**
	
	RM 150 x 6 x 300 subjects = **RM 270,000**
	
	RM 500 x 8 = **RM 4,000**
	
	RM 150 x 1 = **RM 150**
	
	RM 15 x 18 = **RM 270**
	
	RM 22 x 36 = **RM792**

	Subtotal: RM 365,212

6. Blood taking procedure
• Phlebotomy
  • RM 20/session
  • 6 visits
• Consumables
  • Disposable non-sterile latex gloves: RM 22/box of 100 pcs
  • Alcohol swabs for cleaning site: RM 15/box of 100 pcs
  • Tourniquet: RM 20 (reusable)
  • Butterfly vacutainer: RM 500/box of 200 pcs
  • Cotton swabs for site post blood taking: RM 10/bag of 100 pcs
  • Omnifix roll to secure swab at site post blood taking: RM 4/roll
  • EDTA Vacutainer tubes
  • EDTA-Fluoride Vacutainer tubes
  • SST Vacutainer tubes
  • Biohazard bag
	RM 20 x 6 x 300 subjects = **RM 36,000**
	
	RM 22 x 10 = **RM 220**
	
	RM 15 x 48 = **RM 720**
	
	RM 20
	RM 500 x 25 = **RM 12,500**
	
	RM 10 x 30 = **RM 300**
	
	RM 4 x 10 = **RM 40**
	
	Supplied by BP Lab
	
	Subtotal: RM 49,800

7. Proteomics • Bradford assay: RM 740/kits x 3 • Ig/G /albumin removal kit: RM 2,106/kit x 3 • Easy Mini MS prep kit: RM 2,250/kit x 3 • ELISA kits: RM2,500/kit x 6	RM 740 x 3 = **RM 2,220** RM 2,106 x 3 = **RM 6,318** RM 2,250 x 3 = **RM 6,750** RM 2,500 x 6 = **RM15,000** **Subtotal: RM 30,288**
8. Publications • 3 papers • RM 10,000/publication	RM 10,000 x 3 = **RM 30,000** **Subtotal: RM 30,000**
9. Protective Equipment • Disposable facemask, 3ply: RM45/box of 50 pcs • Disposable non-sterile latex gloves: RM 22/box of 100 pcs	RM45 x 150 = **RM6,750** RM22 x 200 = **RM4,400** **Subtotal: RM 11,150**
(A) Estimated total cost	**RM 983,625.00**
(B) SST (6%)	**RM 59,017.50**
(C) Contingency (10%)	**RM 98,362.50**
Grand total cost (Sum of A, B & C)	**RM 1,141,005.00**

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