

# The effects of vitamin E on non-proliferative diabetic retinopathy in type 2 diabetes mellitus: Are they sustainable with 12 months of therapy

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## Abstract

**Introduction:** Prolonged uncontrolled hyperglycaemia has shown to cause oxidative stress, inflammation, thrombosis and upregulation of angiogenesis in diabetics, which all contributes to diabetic retinopathy development and progression. Vitamin E is found to have anti-inflammatory, anti-oxidative, anti-thrombogenic and anti-angiogenesis which could play an important role in early treatment of diabetic retinopathy. This study aims to investigate the effect of Tocotrienol-rich vitamin E (Tocovid) on the progression of retinal microhaemorrhages and diabetic macular oedema in patients with diabetic retinopathy.

**Method:** This is a multi-centred, randomized, double-blinded, placebo-controlled trial which involved 55 eligible participants. The participants in the treatment group ( $n=22$ ) received Tocovid 200 mg twice daily while those in the placebo group ( $n=23$ ) would receive placebo twice daily. Both groups will be on the treatment for a total duration of 12 months. Both retinal signs will be assessed at baseline, 2 months, 6 months and 12 months of treatment to determine the progression of diabetic retinopathy. Serum vascular endothelial growth factor which reflects on the angiogenesis process in the eye was analysed as well at similar time points as the retinal findings.

**Results:** After 12 months of treatment, the placebo group had a significant increase of 23.42% in retinal microhaemorrhages ( $p < 0.05$ ), but the Tocovid group had no significant changes. Moreover, the Tocovid group showed a significant decrease of 48.38% in area of diabetic macular oedema over the 12 months period ( $p < 0.05$ ), but the placebo group had no significant changes. Meanwhile, there was no significant difference in serum vascular endothelial growth factor level when comparing between both groups.

**Conclusion:** These findings could indicate that Tocovid has an important role in preventing early diabetic retinopathy progression.

## Keywords

Type 2 diabetes mellitus, diabetic retinopathy, tocotrienol, vitamin E, anti-angiogenesis, anti-inflammatory, anti-oxidant, anti-thrombosis, retinal microhaemorrhages, diabetic macular oedema, serum vascular endothelial growth factor

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## Introduction

Diabetes mellitus is described as a chronic metabolic disorder as a result of hyperglycaemia due to insufficient insulin secretion or action or even both.<sup>1</sup> In 2017, type 2 diabetes mellitus (T2DM) has affected an estimated 415 million people and this number is expected to rise to approximately 600 million people in the year 2040.<sup>2</sup>

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes especially in T2DM

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individuals. DR is a massive burden towards diabetics as it could lead to visual impairment and eventually blindness.<sup>3</sup> Although multiple treatments have been introduced to treat DR, it still remains as one of the main contributors to adult blindness.<sup>4</sup> Classification of DR is based on the severity of retinal microhaemorrhages. Early Treatment Diabetic Retinopathy Study (ETDRS) severity scale is widely used to classify DR into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR).<sup>5</sup> NPDR can be further divided into mild, moderate and severe categories based on extent of microvascular lesions. Primarily, NPDR represents the early stage of DR and is usually asymptomatic while PDR represents the later stage. Diabetic macular oedema (DME) can occur at any stage of DR and is described as retinal thickening or exudate formation on the retina.<sup>6,7</sup> Currently, treatment strategies aimed at NPDR involve both blood pressure and strict glycaemic control.<sup>8</sup>

The exact pathophysiology of DR is not fully understood as it is highly complex and involves multiple pathways. Current pathways which have been highly researched on and postulated by researchers on how DR develops are polyol pathway, accumulation of advanced glycation end product (AGE), protein kinase C (PKC) pathway and hexosamine pathway.<sup>9</sup> Activation of these pathways would lead to upregulation of mediators, such as vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF) and insulin-like growth factor (IGF), which are responsible for DR development.<sup>9</sup>

Vitamin E is known to have various beneficial properties which include being an anti-inflammatory, anti-oxidative as well as anti-angiogenic agent.<sup>10</sup> Vitamin E occurs naturally as either tocopherol or tocotrienol based on its side chains which attached to the chroman ring.<sup>11</sup> Researchers have discovered that tocotrienol has far superior beneficial properties over tocopherol, which includes angiogenesis inhibition.<sup>10</sup> However, the exact role of vitamin E in DR has yet to be explored completely. Vitamin E has been researched thoroughly and found to have a significant impact on angiogenesis inhibition via regulating various mediators, especially reducing VEGF expression.<sup>12–15</sup> Therefore, vitamin E could be an important agent in attenuate early stage DR. To this date, the evidence of vitamin E effect on DR is insubstantial and requires further exploration.

VEGF is one of the hallmark factors which is linked to DR pathophysiology.<sup>16,17</sup> Retinal cells which include retinal epithelial, ganglion and endothelial cells are all able to produce VEGF.<sup>18</sup> The increase in this factor as a result of prolonged uncontrolled hyperglycaemia would result in neovascularization and blood retinal barrier disruption, thus leading to DR formation.<sup>16</sup> A meta-analysis conducted by Zhou et al.<sup>19</sup> successfully found that serum VEGF is a reliable angiogenesis biomarker to determine the severity and progression of DR.

The primary objective of this clinical trial is to investigate the effect of tocotrienol-rich vitamin E (Tocovid) on DR with 12 months of supplementation. The second objective is to investigate the effect of Tocovid on biomarker which reflects on angiogenesis, namely, serum VEGF.

## Materials and method

### Study design

Our research is a multicentre, prospective, double-blinded, placebo-controlled, randomized controlled trial to investigate the effect of Tocovid comparing with placebo on DR. This clinical trial was done in two separate clinical sites in the same country. The study involves treatment with Tocovid supplementation for NPDR in type 2 mellitus for a period of 12 months. This was conducted with the adherence to the standards of Declaration of Helsinki as well as Malaysian Good Clinical Practice (GCP) guidelines. This research was approved by the Monash University Human Research Ethics Committee (MUHREC) with project code 12090. Participants were recruited for a total duration of 2 months from November 2018 to January 2019.

### Participants

#### Inclusion criteria

Participants were aged between 25 and 75 years old with T2DM and stable haemoglobin A1c (HbA1c) levels (not more than 10% change over the last 2 months) were eligible for this study. Participants who have hypertension should have stable blood pressure control with than less than 150/90 mmHg.

Participants must be diagnosed with NPDR by an external qualified ophthalmologist. The diagnosis is done based on the International Clinical Disease Severity Scale (ICDSS) grading the NPDR as mild, moderate and severe through a coloured retinal photograph.

#### Exclusion criteria

Participants who were diagnosed with certain eye conditions previously, such as glaucoma and cataract, underwent laser photocoagulation therapy or had intravitreal anti-VEGF injection were excluded from this trial to prevent any interference with the retinal findings. Participants with other diseases such as active malignancy, liver conditions, inflammatory conditions and acute coronary syndrome were excluded from the trial. Besides that, participants were consuming other water-soluble anti-oxidants which includes vitamin C, glutathione or polyphenols in the past month, or if they were taking any fat-soluble anti-oxidants such as vitamin E in the past 2 months were not included in the trial. Chronic active smokers (>20 sticks/day) were excluded from the trial as well.

## Screening visits

Participants were mostly recruited from an existing pool who regularly attend diabetic reviews at Clinical Research Centre (CRC) in Monash University Sunway Campus and CRC Clinical School Johor Bahru. Other participants were referred by family doctors and endocrinologist. Patients were carefully selected to participate in this trial based on their past medical history to establish their eligibility.

Both informed and written consent were obtained with any prior to any screening procedures. All queries were answered accordingly. A thorough history and physical examination was done, followed by anthropometric measurement and blood pressure measurement. Blood tests such as fasting blood glucose and HbA1c were measured to determine the baseline diabetes parameters. Liver function tests, lipid profile and echocardiogram (ECG) were carried out as well as a safety precaution. Eligible participants were then invited back for the randomization process in 2–4 weeks.

## Randomization

The randomization process was done using a computer-generated sequence in a 1:1 ratio. A total of 55 participants were randomized and stratified according to gender, duration of DM (<15 years or  $\geq$ 15 years) and HbA1c level (either < or  $\geq$ 7.95%). The treatment group would receive Tocovid SupraBio™ 200 mg twice daily while the control group was given placebo. The exact constitution for Tocovid is as follows: d- $\alpha$ -Tocotrienol: 61.52 mg, d- $\gamma$ -Tocotrienol: 112.80 mg, d- $\delta$ -Tocotrienol: 25.68 mg, d- $\alpha$ -Tocopherol: 91.60 IU, Plant Squalene: 51.28 mg and Phytosterol Complex: 20.48 mg. Both Tocovid and placebo capsules were sponsored ExcelVite Pty Ltd. (Malaysia) and manufactured by Hovid Pharmaceuticals Berhad (Malaysia). The capsules are similar in terms of size, shape and colour and were labelled by ExcelVite as Drug A and Drug B. This allowed the identity of the investigational product to remain hidden from both participants and investigators till the end of the study to avoid selection and performance bias.

## Follow-up visits

Participants were followed up for every 2 months to ensure compliance and monitor for any adverse events from the investigational product. Compliance was calculated by pill count method. In each visit, the anthropometric measurements, blood pressure and fasting blood glucose were carried out. Retinal photographs and serum VEGF level were done at 2 months, 6 months and 12 months of treatment. Any modification to the participant's regular medication was done and recorded to ensure they are healthy throughout the trial.

**Table 1.** Specification of digital retinography system (DRS).

Component	Specifications
Field of view	45° × 40°
Fixation target	7 internal LEDs
Operating distance	37 mm
Exposure value	1.25
Sensor size	5 megapixels (2592 × 1944)
Sensor resolution	48 pixels/degree

## Sample size

The phase IIb trial recommended sample size was calculated using a sample size calculator available on <https://clincalc.com/stats/samplesize.aspx>. After adjusting the recommended formula for this trial, it is recommended that a total of 82 samples or eyes, 41 in each group, to demonstrate a 23.49% decrease in retinal microhaemorrhages when comparing the intervention and control group with a type 1 error rate ( $\alpha$ ) of 0.05 and type 2 error rate ( $\beta$ ) of 0.20 (power=80%). The 23.49% decrease value was obtained from the previous phase IIa trial result at the end of the study. After taking into account a dropout rate of 5%, the final recommended sample size was 44 in each group.

## Assessment of outcomes

The primary outcome variables of this study are percentage change of retinal bleed and DME. The secondary outcome in this study is the serum VEGF level which is measured using enzyme-linked immunosorbent assay (ELISA).

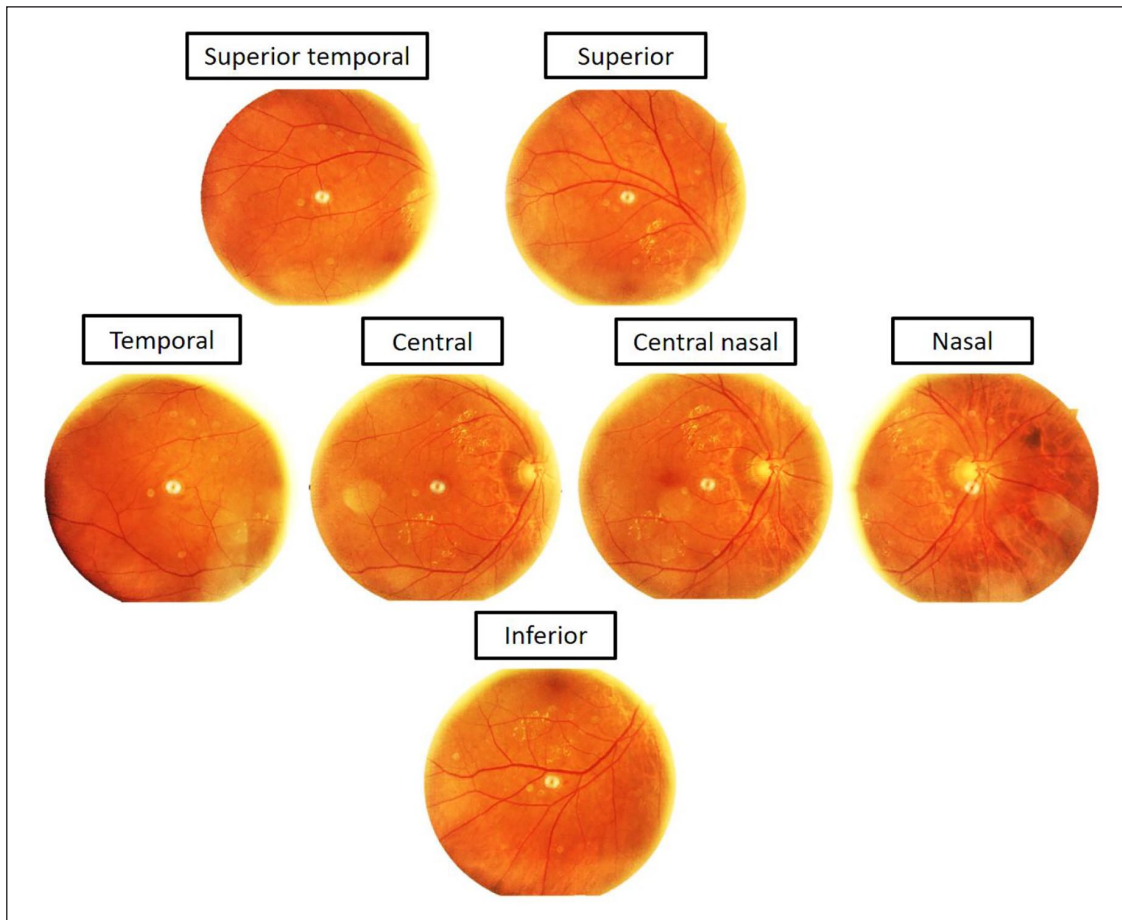
## Methodology of retinal photographs

### Preparation

Tropicamide 1% (Alcon®, Cointrin, Geneva, Switzerland) was used to dilate the pupil prior to the fundus examination. Two drops were applied to allow the pupil to dilate more than 4 cm. The fundal camera Digital Retinography System (DRS) (CenterVue, Fremont, CA, USA) was used to measure the pupil diameter. If the pupil size is less than 4 cm, Tropicamide 1% is reapplied at 20-min interval.

### Retinal photograph and process

After the pupil size is satisfactory, the participant's retinal photograph will be taken. Participants were told to remain still and focus on the light when the fundus photo is being taken. This is to ensure the best retinal image quality. Before commencing the procedure, light in the procedure room was switched off to prevent any light interference. The specification of the DRS fundus camera is provided in Table 1. A total of seven different retinal fields were captured for each eye as shown in Figure 1.



**Figure 1.** Seven different 45° × 40° retinal fields captured.

The seven different retinal fields were then transferred from the fundus camera to a computer to be merged using the Dual Align i2k Retina® Montage Software. An example of montage image is as shown in Figure 2.

### *Methodology of analysing retinal bleed and DME*

The primary outcomes of this trial, retinal bleed and DME were analysed using the following formulas:

1. Retinal microhaemorrhages =  $\frac{\text{Area of retinal bleed}}{\text{Net retina area}}$
2. Diabetic macular oedema =  $\frac{\text{Area of diabetic macular edema}}{\text{Net retina area}}$
3. Net retina area = Full retina area – area of artefact

The cause of artefacts in the retinal images could be from camera positioning to the lens, eyelids and eyelashes. The total area of retina will only be deducted by the area of artefacts to obtain the net retinal value if removal of artefacts is not possible. The primary outcome value is converted to percentage (%) during analysis as shown by the following formula

$$\frac{\text{Subsequent reading} - \text{initial reading}}{\text{Initial reading}} \times 100\%$$

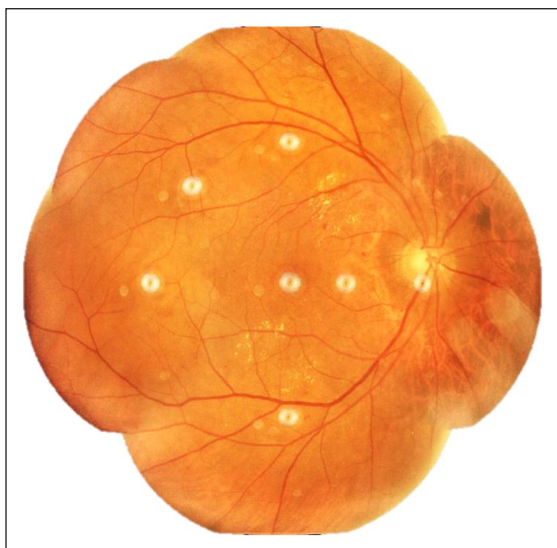
ImageJ software is used to measure both retinal microhaemorrhages and DME. This software allows 100× magnification to have better clarity in identifying retinal bleeds and DME. The area of interest will be outlined using the ‘area selection’ tool and subsequently measured using the ‘measure’ tool which is both preinstalled in the software as described in Figure 3. The measurement is then recorded to Microsoft Excel prior to analysis.

The area of retina and artefacts were measured similarly as the primary outcomes. The net retinal area was measured

in each retinal photography session as it is expected to have variation in values despite the same participant.

### Measurement reliability

To prevent inter-assessor variation, the area of both retinal microhaemorrhages and DME was done by the same

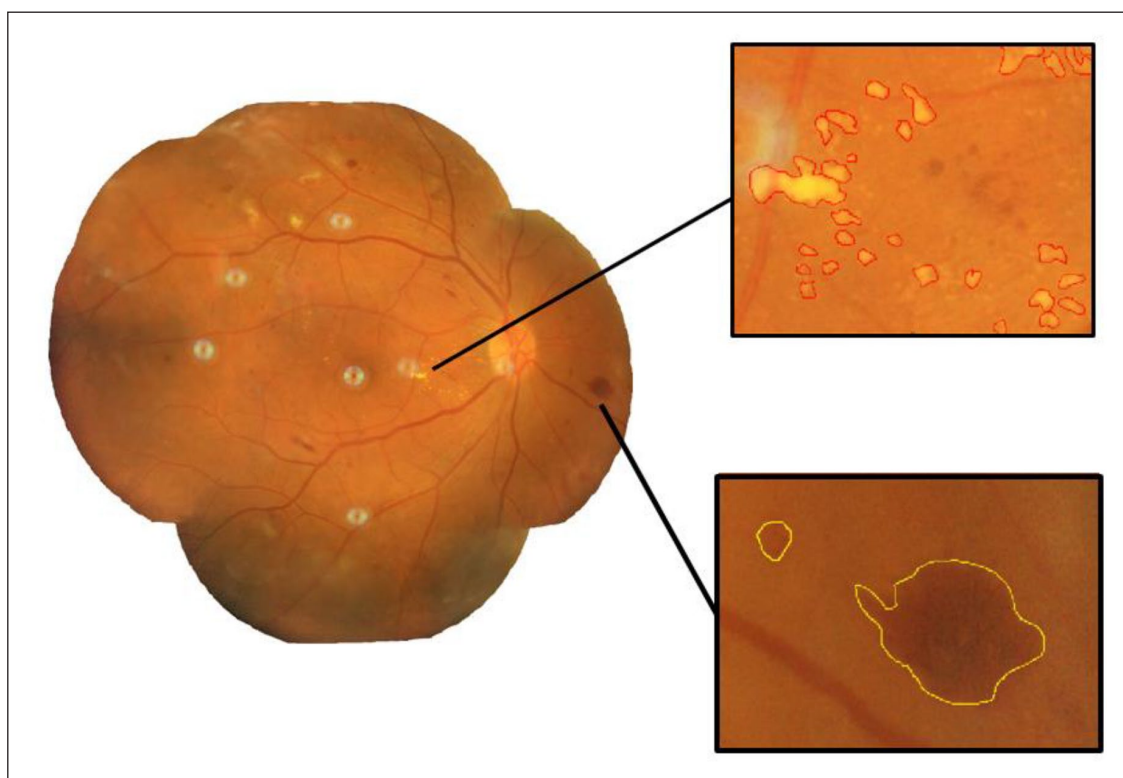


**Figure 2.** Merged retinal image using the i2k Retina® Montage Software.

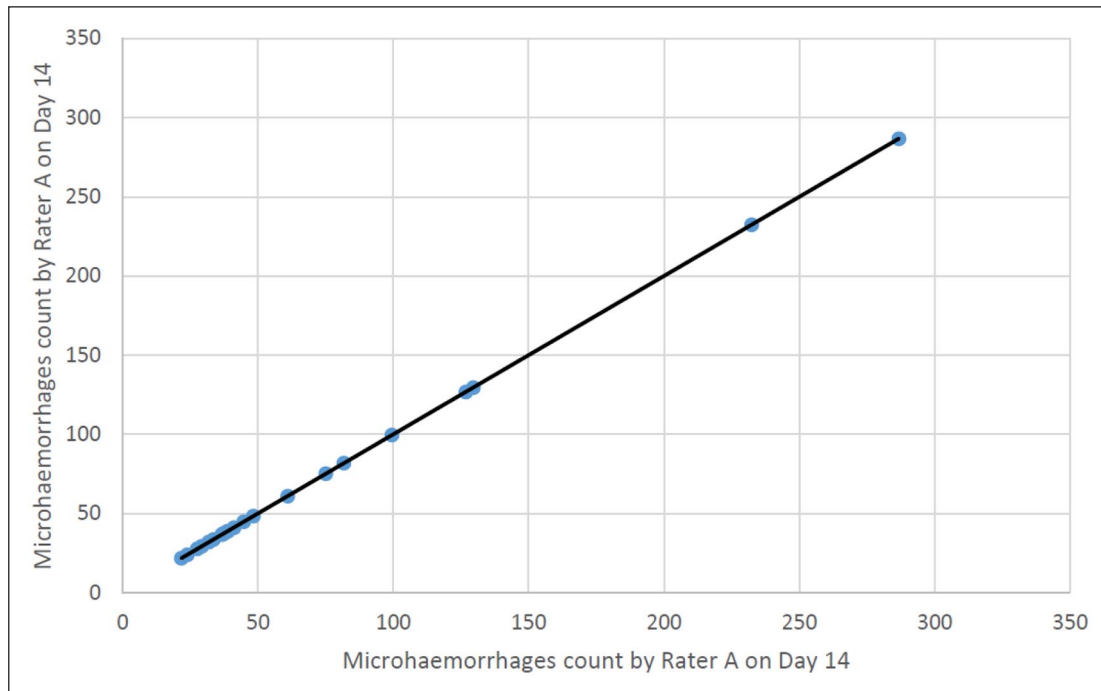
investigator. To ensure that the measurement is consistent and reliable, inter-rater and intra-rater analysis was done. 10% of retinal photographs were randomly selected to evaluate the reliability measurements, which consist of intra-rater and inter-rater reliability analysis. For intra-rater analysis, Rater A would analyse the same retinal photographs on Day 1 and later on Day 14. For inter-rater analysis, same retinal photographs were analysed by Rater B and the data are used to compared to Rater A's data on Day 1.

**Intra-rater reliability.** For the intra-rater reliability analysis, the interclass correlation coefficient (ICC) was significantly high with 0.95 ( $p < 0.001$ , 95% confidence interval (CI)=0.828 to 0.986) for intra-retinal microhaemorrhages and 0.92 ( $p = 0.002$ , 95% CI=0.542 to 0.988) for DME count which are presented in Figures 4 and 5, respectively. Based on Fleis (1988) interpretation of ICC, both of these values indicate excellent reliability with minimal variation for Rater A on Day 1 when compared on Day 14.<sup>20</sup>

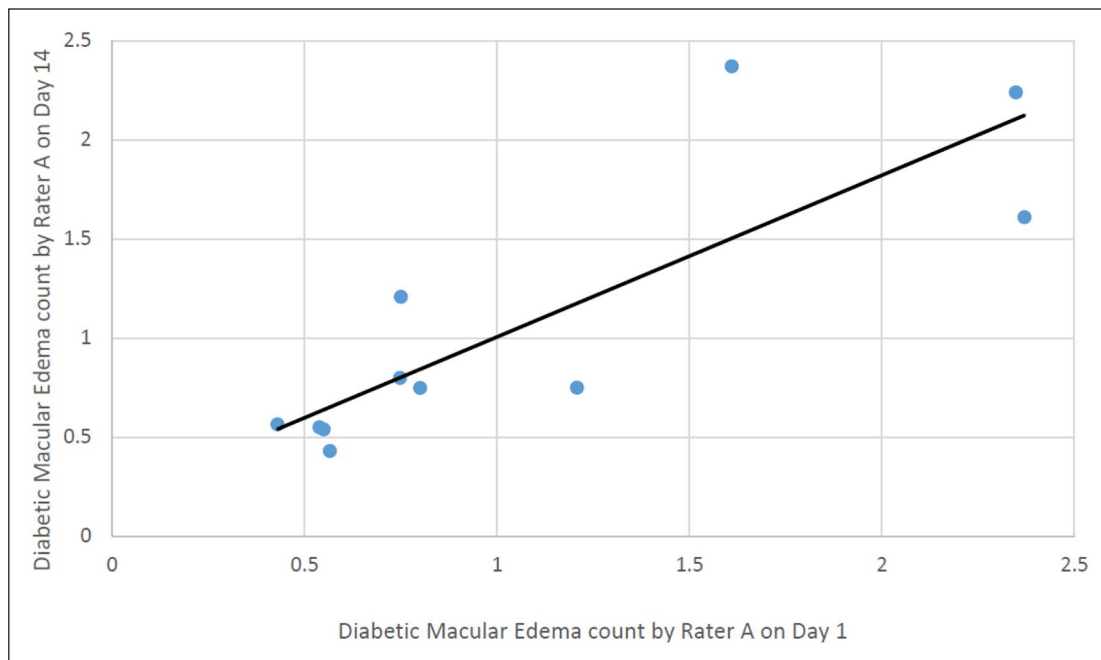
**Inter-rater reliability.** The ICC for the inter-rater reliability for retinal microhaemorrhages was significantly high with 0.832 ( $p < 0.001$ , 95% CI=0.492 to 0.952) while for DME was 0.524 ( $p = 0.114$ , 95% CI=-0.382 to 0.916) which can be seen in Figures 6 and 7, respectively. According to Fleis (1986) interpretation, the ICC values for retinal microhaemorrhages prove to be an excellent reliability.



**Figure 3.** Area of retinal bleed (yellow outline) and DME (red outline) with 100× magnification.



**Figure 4.** Intra-rater reliability scatterplot with best fit line on 22 samples of retinal microhaemorrhages. Each marker indicates a sample.

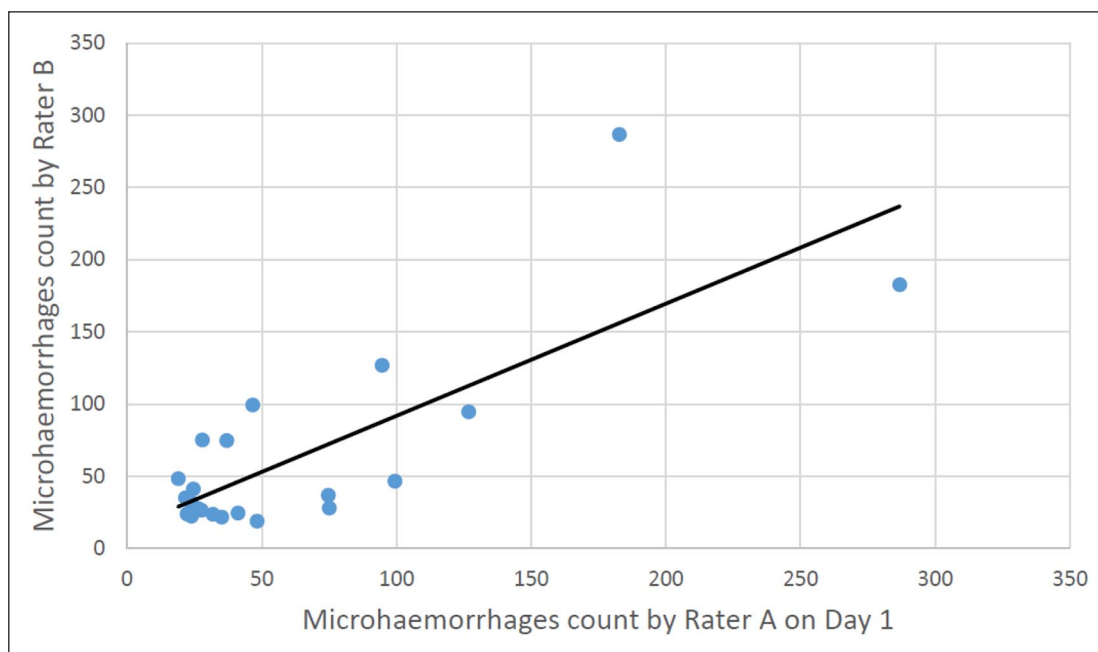


**Figure 5.** Intra-rater reliability scatterplot with best fit line on 12 samples of diabetic macular oedema. Each marker indicates a sample.

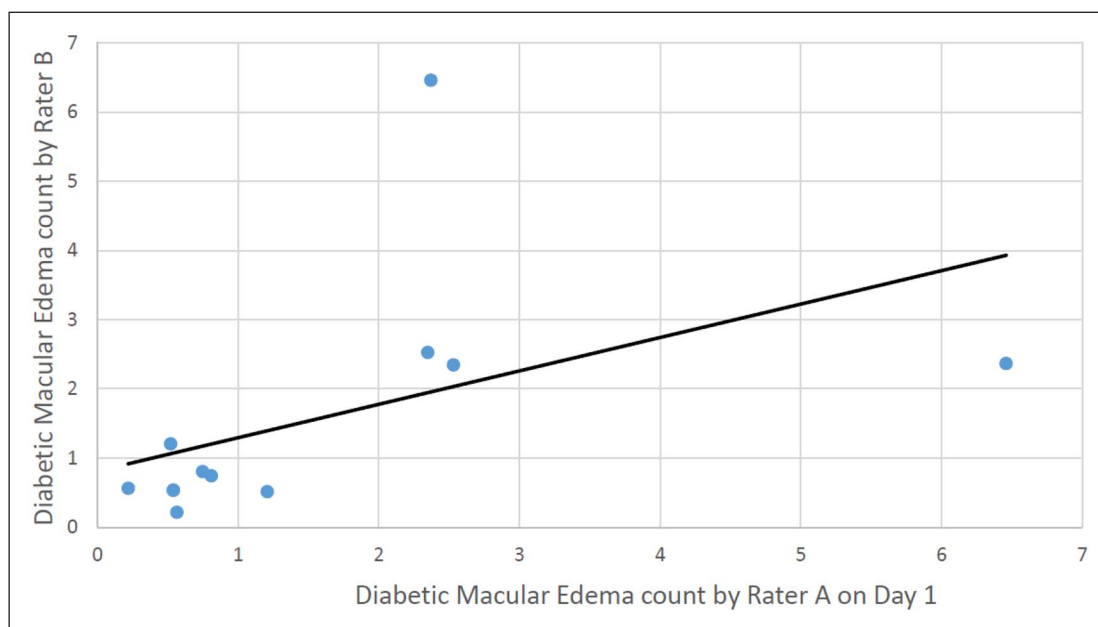
However, for DME, it falls under the moderate reliability category.<sup>20</sup> This could be attributed by the manual outline feature on the ImageJ software as well as the interpretation of DME which could be subjective and varies across raters.

### Serum VEGF

Blood samples were centrifuged using the Eppendorf Centrifuge 570R (Hamburg, Germany) the same day it was



**Figure 6.** Inter-rater reliability scatterplot with best fit line on 22 samples of retinal microhaemorrhages. Each marker indicates a sample.



**Figure 7.** Inter-rater reliability scatterplot with best fit line on 12 samples of diabetic macular oedema. Each marker indicates a sample.

collected. The serum was flash frozen and stored at  $-80^{\circ}\text{C}$  in Eppendorf tube and only processed at the end of the trial. The purpose of this is to reduce inter-assay variability. These tests were measured in duplicates and quantified by colorimetric method using ELISA with Tecan Infinite 200 PRO (Zürich, Switzerland) and the ELISA kits, Elabscience E-EL-H0111 (Houston, TX, USA). The ELISA kits had

intra-assay coefficient variances of 4% and inter-assay coefficient variances of 8%.

### Plasma tocotrienol and tocopherol levels

Blood samples were collected in EDTA tubes and centrifuged to obtain plasma. The plasma samples were flash

frozen and stored at  $-80^{\circ}\text{C}$  in Eppendorf tubes. Tocotrienol and tocopherol levels were measured for all patients using high-performance liquid chromatography (HPLC; HPLC 1200, Agilent, Santa Clara, CA, USA) with fluorescence detector based on a method by Che et al.<sup>21</sup> with modification. The plasma vitamin E levels were corrected for lipids.

## Statistical analysis

All statistical analysis was done using IBM SPSS Statistics v.25 (Armonk, NY, USA) database. Primary outcomes were analysed by comparing the difference in change between both groups and separately as well. Modified intention-to-treat (ITT) analysis was implemented in this trial to minimize attrition bias. To obtain the change seen in between visits, difference of data between the first and final visits was taken as a continuous variable. Normality was tested for all of the variables using the Shapiro–Wilk test. If the data were parametric, independent *t*-test and paired *t*-test were then performed on these variables. For non-parametric data, Mann–Whitney test, and either the Wilcoxon signed-rank test or Friedman were conducted. Friedman was conducted if it was noted the baseline variables were significantly different between both groups. For categorical variable, chi-square test or Fisher’s exact test was used to analyse depending on the suitability. The *p*-value of  $<0.05$  was set to deem statistically significant.

## Results

A total of 146 participants were assessed initially for their eligibility prior to being recruited in this trial. Out of these, 55 participants were successfully recruited and randomized. There were 27 participants in the Tocovid group which contribute 51 eyes and 28 participants which contribute 52 eyes. The ITT analysis was implemented on a total of five participants in this trial which four of them were lost to follow up and one had deceased. The summary of the flow diagram is as shown in Figure 8.

### Baseline characteristics

At baseline, the mean age of all patients was 62.45 years old with an average duration of diabetes of 17 years and HbA1c of 7.78%. Male participants contribute 58.18% of the cohort or 32 participants. It was noted there was significant difference in diastolic blood pressure, area of DME and serum VEGF level at baseline comparing between Tocovid and placebo groups which the Friedman test was used to cater to these differences. The other baseline characteristics have no significant difference comparing between both groups as shown in Table 2.

### Primary outcomes

The retinal parameters for retinal microhaemorrhage and DME were measured bilaterally for both eyes. Some

participants only had one eye measured as the other eye was excluded due to media opacity or was previously treated with DR using anti-VEGF injection or laser photocoagulation. A total of 103 eyes were analysed for retinal microhaemorrhage, 51 in the Tocovid and 52 in the placebo group. There was no significant difference in percentage area retinal microhaemorrhage at baseline comparing between both groups. At the end of the trial, the change of retinal microhaemorrhage was at 2 months, 6 months and 12 months of treatment was compared between the Tocovid and placebo groups. It was found there was no significant difference in change at 2 months, 6 months and 12 months of treatment between both groups. These findings are summarized in both Table 3 and Figure 9.

Separate analysis was done as well in this study to detect any significant changes within the group itself. When analysed individually using the Wilcoxon signed-rank test, the placebo showed a significant increase in median percentage area of retinal microhaemorrhage at 12 months of treatment, but there were no significant changes at other time points. However, in the Tocovid group, there were no significant changes in between any of the visits. These findings are summarized in Table 4 and Figure 10.

A total of 56 eyes were analysed for DME outcome, 28 each in Tocovid and placebo groups. There was no significant difference when comparing the median percentage change in area of DME between the Tocovid and placebo groups at 2 months, 6 months and 12 months of treatment. These findings are summarized in Table 5 and Figure 11.

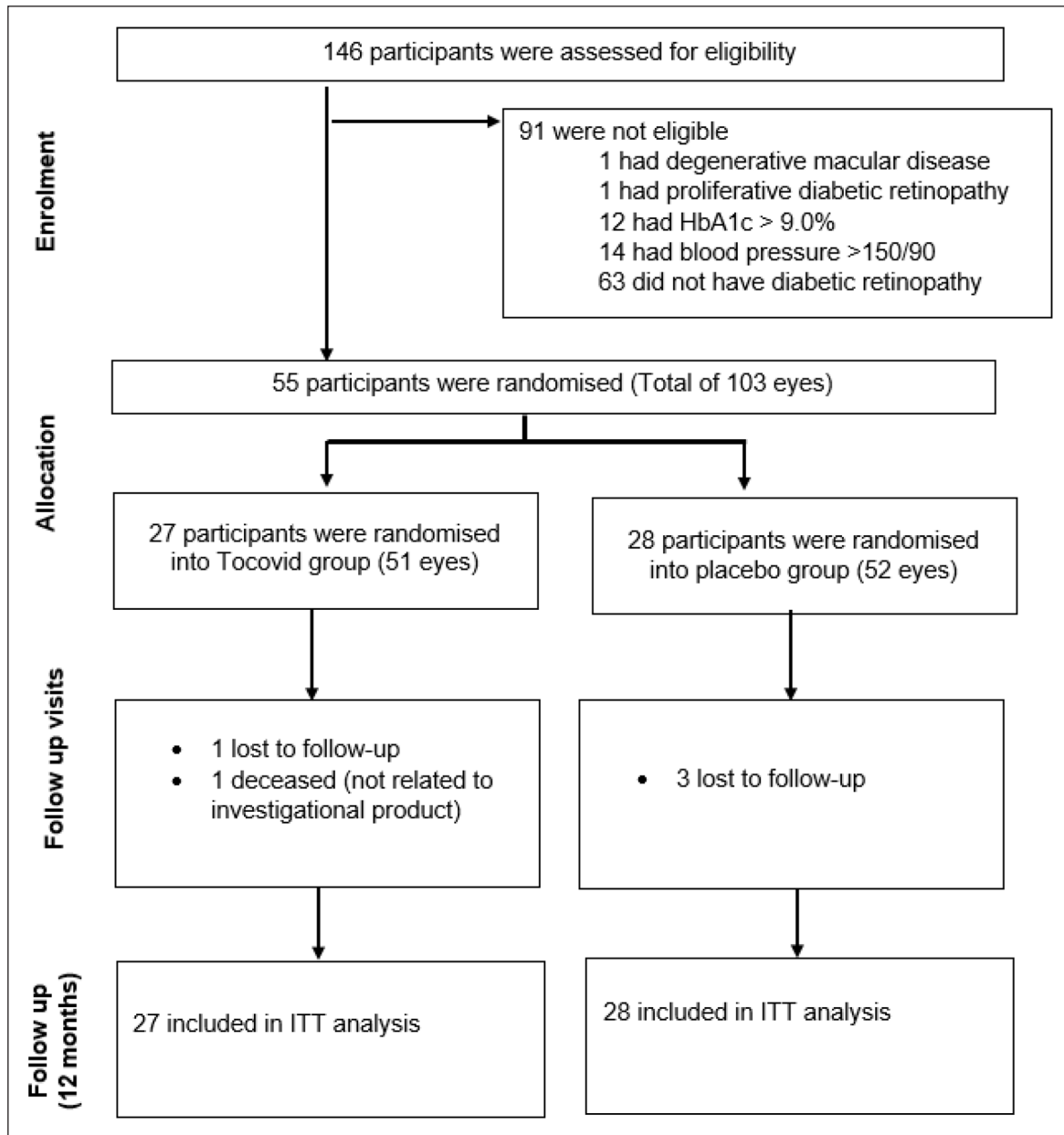
When separate analysis was done, it was noted that the baseline area percentage of DME was significantly different between the Tocovid and placebo groups. Therefore, the Friedman test was used to cater to this situation and analysed these both groups separately. The result from the Friedman test showed significant changes over the 12 months duration in the Tocovid group ( $p < 0.05$ ), but the placebo group did not show otherwise ( $p > 0.05$ ). Further analysis was done to determine these changes and was found there was a significant decrease in percentage area of DME at 2 months and 12 months of treatment. These results are summarized in Table 6 and Figure 12.

### Secondary outcome

The serum VEGF level was analysed at similar time points as the retinal findings which were 2 months, 6 months and 12 months of treatment. The change in serum VEGF level comparing between both groups across the 12 months duration was not significant. These results are outlined in both Table 7 and Figure 13.

Correlation between serum VEGF level and both retinal findings were explored as well in this trial. When comparing the baseline serum VEGF and area of retinal microhaemorrhage at baseline, there was a significant weak negative correlation between these two variables. However, there was no





**Figure 8.** Flow diagram of phase IIb trial.

significant correlation between serum VEGF level and area of DME at baseline as seen in Table 3.

The baseline area of serum VEGF level was found to be significantly different comparing between both the Tocovid and placebo groups. Therefore, separate analysis was done using the Friedman test to determine any significant changes within the group. In both Tocovid and placebo groups, there were no significant changes across the 12 months period of treatment. These changes are summarized in Table 9.

Parameters such as blood pressure and HbA1c level were measured at 2 months, 6 months and 12 months of treatment as both of these parameters could be a result of confounding factors to the retinal findings.<sup>22,23</sup> There were no significant

changes across the 12 months duration of systolic blood pressure and HbA1c level in both groups. However, the diastolic blood pressure in the Tocovid group had a significant decrease ( $p < 0.05$ ) in the 12 months duration which was not seen in the placebo group. Further analysis showed that the highest decrease was 5 mmHg which would not have a significant impact on the retinal findings. To add on, the impact of diastolic blood pressure alone on progression on DR has only been reported in cases of type 1 diabetes mellitus (T1DM) and not in T2DM.<sup>24</sup> All of these changes are shown in Table 10.

Levels of tocotrienols and tocopherol were measured in both Tocovid and placebo groups at baseline, 2 months,

**Table 2.** Baseline characteristics of study patients.

Baseline characteristics	Tocovid (n = 27)	Placebo (n = 28)	p-value	All patients (n = 55)
Gender			0.874 <sup>a</sup>	
Male	16 (59.3%)	16 (57.1%)		32 (58.2%)
Female	11 (40.7%)	12 (42.9%)		23 (41.8%)
Race			0.448 <sup>b</sup>	
Malay	10 (37.0%)	16 (57.1%)		26 (47.3%)
Chinese	10 (37.0%)	4 (14.3%)		14 (25.5%)
Indian	7 (25.9%)	7 (25.0%)		14 (25.5%)
Others		1 (3.6%)		1 (1.8%)
Age (years)	62.93 ± 9.64	62.00 ± 7.72	0.695 <sup>c</sup>	62.45 ± 8.65
Duration of T2DM (years)	18.30 ± 8.71	16.64 ± 7.71	0.459 <sup>c</sup>	17.45 ± 8.18
HbA1c (%)	7.53 ± 1.18	8.06 ± 1.34	0.112 <sup>c</sup>	7.80 ± 1.28
Systolic blood pressure (mm Hg)	139.77 ± 13.12	135.70 ± 18.15	0.289 <sup>c</sup>	137.70 ± 15.87
Diastolic blood pressure (mm Hg)	78.65 ± 7.88	81.11 ± 11.06	0.374 <sup>c</sup>	79.91 ± 9.62
Weight (kg)	73.93 ± 14.19	71.23 ± 16.52	0.567 <sup>c</sup>	72.56 ± 15.33
BMI (kg/m <sup>2</sup> )	27.70 ± 4.08	27.37 ± 5.30	0.800 <sup>c</sup>	27.53 ± 4.70
Ophthalmological parameters				
NPDR grades			0.724 <sup>b</sup>	
Mild	38 (74.51)	35 (67.31)		73 (72.81%)
Moderate	11 (21.56)	14 (26.92)		25 (24.27%)
Severe	2 (3.92)	3 (5.77)		5 (4.85%)
Percentage area of retinal bleed × 10 <sup>5</sup>	45.69 (71.99)	53.37 (146.31)	0.436 <sup>d</sup>	
Percentage area of DME × 10 <sup>3</sup>	1.052 (1.95)	2.982 (4.20)	0.049 <sup>*d</sup>	
Serum VEGF level	817.524 (374.25)	604.065 (627.96)	0.035 <sup>*d</sup>	730.14 (625.26)

n: number of participants; T2DM: type 2 diabetes mellitus; BMI: body mass index; NPDR: non-proliferative diabetic retinopathy; DME: diabetic macular oedema; VEGF: vascular endothelial growth factor.

Values with bracket are the number of patients (percentage, %). Plus and minus values are mean value ± SD.

<sup>a</sup>Chi-square test. Assumptions were fulfilled.

<sup>b</sup>Fisher's exact test. More than 20% of the cells have expected value of less than 5.

<sup>c</sup>Independent t-test. Assumptions were fulfilled.

<sup>d</sup>Mann-Whitney test. Assumptions were fulfilled.

**Table 3.** Comparison of change in percentage retinal microhaemorrhage between Tocovid and placebo groups.

Timeline	Median percentage change in retinal microhaemorrhage (%)				r	p-value
	Tocovid (n = 51)		Placebo (n = 52)			
	Median	IQR	Median	IQR		
Baseline to 2 months (%)	4.87	65.87	0.93	64.93	0.0002	0.885
Baseline to 6 months (%)	-0.67	79.21	0.02	119.60	0.003	0.584
Baseline to 12 months (%)	3.56	60.24	10.99	88.96	0.033	0.065

n: number of eyes; r: effect size from the Mann-Whitney test between groups (small effect:  $r \geq 0.1$ , medium effect:  $r \geq 0.3$ , large effect:  $r \geq 0.5$ ); IQR: interquartile range.

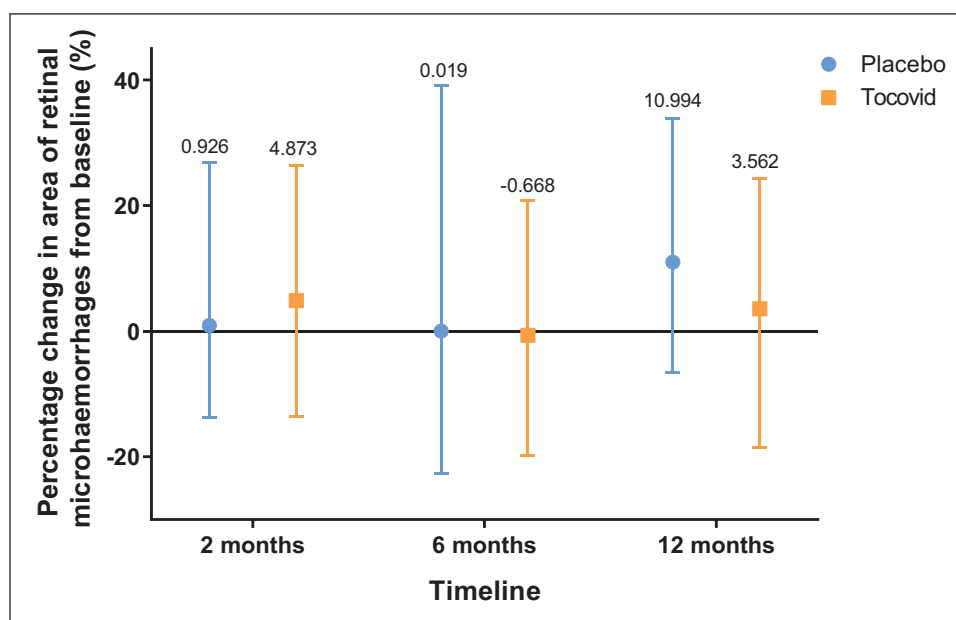
Data are not normally distributed (Mann-Whitney test).

6 months and 12 months of treatment (Table 8). There were no significant differences in the levels of tocotrienols and tocopherol at baseline between the groups as expected. At 2 months treatment, the levels of all isomers of tocotrienols in the Tocovid group increased significantly from 2 months until the end of the study at 12 months ( $p < 0.05$ ) as compared to the placebo group which remained similar to the

levels at baseline ( $p > 0.05$ ). These comparisons as mentioned are summarized in Table 11.

### Multiple linear regression

Multiple linear regression models were fitted to assess the effect of independent variables on change in retinal outcomes



**Figure 9.** Comparison of median percentage changes in area of retinal microhaemorrhages per area of retina between Tocovid and placebo groups. Error bars represent the interquartile range.

**Table 4.** Comparison of the area percentage retinal microhaemorrhage within the Tocovid and placebo groups.

Timeline	Tocovid (n=51)				Placebo (n=52)			
	Median	IQR	r	p-value	Median	IQR	r	p-value
Two months treatment								
Baseline	45.69	71.99	-0.144	0.146	53.37	146.31	-0.115	0.240
At 2 months	52.27	76.45			66.83	200.21		
Six months treatment								
Baseline	45.69	71.99	-0.010	0.918	53.37	146.31	-0.885	0.367
At 6 months	62.96	72.63			53.58	161.14		
Twelvemonths of treatment								
Baseline	45.69	71.99	-0.019	0.851	53.37	146.31	-0.245	0.013*
At 12 months	49.40	107.25			65.87	188.86		

n: number of eyes; r: effect size from the Wilcoxon signed-rank test within subjects (small effect:  $r \leq -0.1$ , medium effect:  $r \leq -0.3$ , large effect:  $r \leq -0.5$ ); IQR: interquartile range.

Data are not normally distributed (the Wilcoxon test).

\*Significant at  $p < 0.05$ .

(retinal microhaemorrhages and DME) from baseline to 2 months, 6 months and 12 months, respectively. Independent variables which may have impact on retinal outcomes were included in the base model: treatment (dummy variables 0 for placebo and 1 for Tocovid), age, duration of diabetes, HbA1c and blood pressure (systolic blood pressure (SBP) and diastolic blood pressure (DBP)) at baseline as well as at each visit (2 months, 6 months or 12 months). The final model was obtained using backward elimination to remove non-significant independent variables. Only significant independent

variables with p-value less than 0.05 were included in the final model. It was found that none of these independent variables are significant predictors for percentage change in retinal microhaemorrhages.

The same independent variables were used to fit a multiple linear regression models to predict percentage change in DME from baseline to 2 months, 6 months and 12 months. The significant factors on the percentage change in area of DME are displayed in Table 12. The equations for the final model at each time point are as follows

$$\text{Percentage change in DME}_{\text{baseline to 2 months}} = -2.167 + 0.026(\text{SBP}_{\text{baseline}}) - 0.253(\text{HbA1c}_{2 \text{ months}})$$

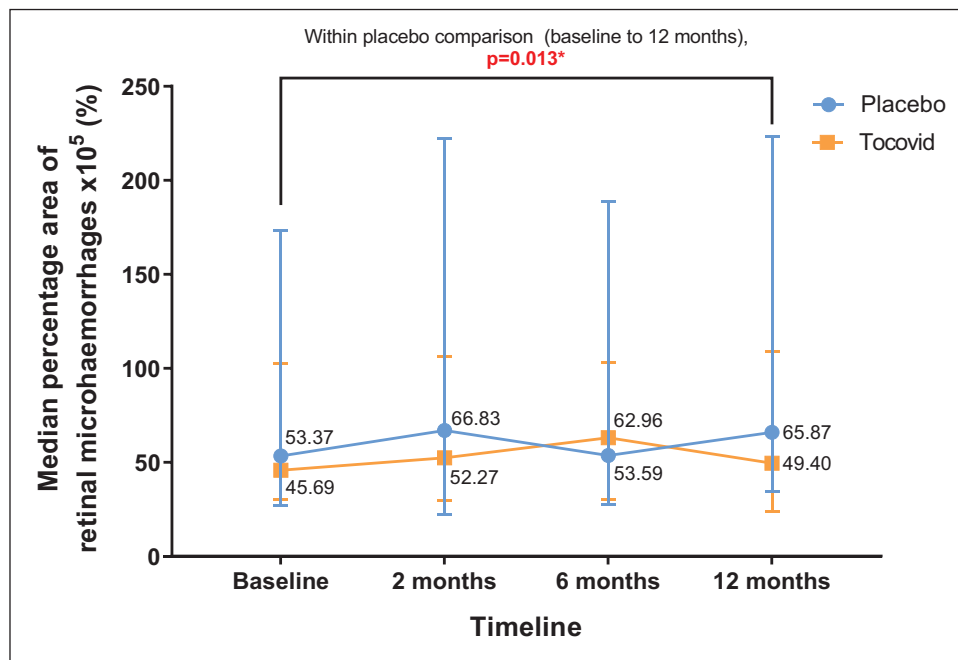
$$\text{Percentage change in DME}_{\text{baseline to 6 months}} = -7.196 + 0.941(\text{Tocovid}) + 0.034(\text{SBP}_{\text{baseline}}) - 0.821(\text{HbA1c}_{\text{baseline}}) + 1.047(\text{HbA1c}_{2 \text{ months}})$$

$$\text{Percentage change in DME}_{\text{baseline to 12 months}} = 0.400 - 0.877(\text{HbA1c}_{\text{baseline}}) + 0.647(\text{HbA1c}_{12 \text{ months}})$$

### Discussion

This study is a product of its successor which started and was completed whereby it found that with 8 weeks of Tocovid treatment, it was able to significantly improve diabetic nephropathy and diabetic neuropathy without any significant changes to blood pressure, HbA1c, inflammatory and

oxidative biomarkers.<sup>25,26</sup> This indicates that Tocovid was able to improve both diabetic nephropathy and neuropathy without the decreasing blood pressure and HbA1c, as well as not through suppressing oxidative stress and inflammation. On top of that, vitamin E has been highly researched and proved to have a significant role in suppressing angiogenesis in both human cells and animal studies by regulating various



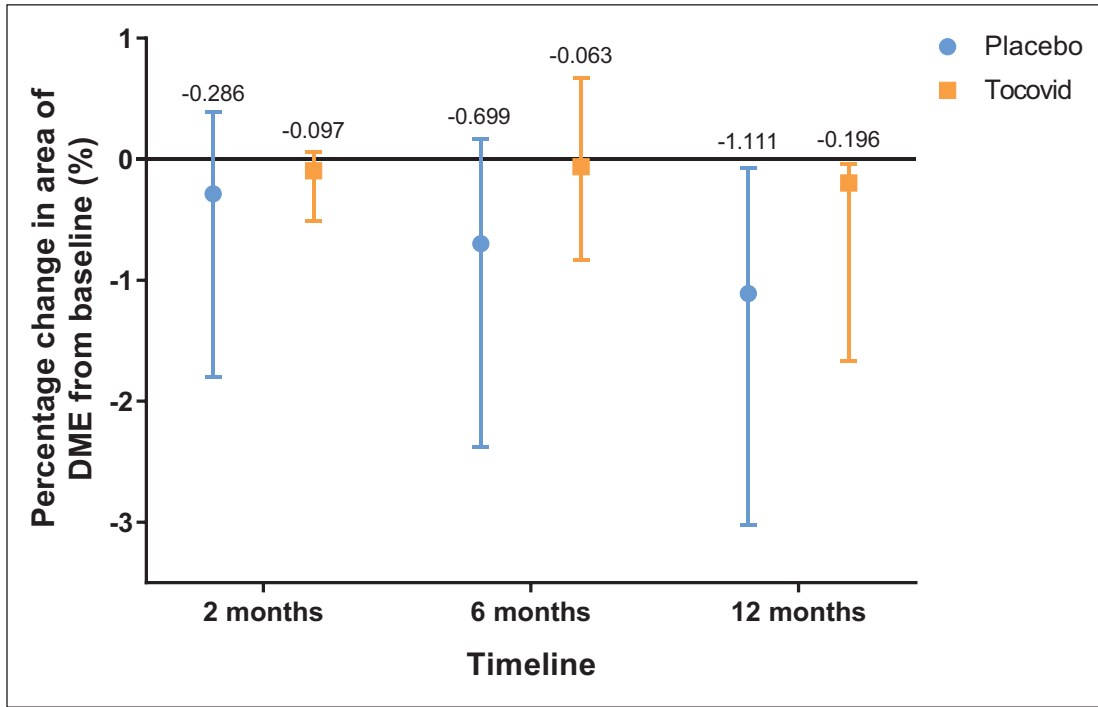
**Figure 10.** Comparison of median area of retinal microhaemorrhages per area of retina within groups from baseline to 12 months of treatment. Error bars represent the interquartile range.

**Table 5.** Comparison of change in percentage DME between Tocovid and placebo groups.

Timeline	Tocovid (n=28)		Placebo (n=28)		r	p-value
	Median	IQR	Median	IQR		
Baseline to 2 months (%)	-0.097	0.57	-0.286	2.19	0.032	0.376
Baseline to 6 months (%)	-0.063	1.63	-0.699	2.55	0.059	0.071
Baseline to 12 months (%)	-0.196	1.63	-1.110	2.94	0.039	0.142

DME: diabetic macular oedema; n: number of eyes; Visit 1: baseline; Visit 3: at 2 months treatment; Visit 7: at 6 months treatment; Visit 13: at 12 months of treatment; r: effect size from the Mann–Whitney test between groups (small effect:  $r \geq 0.1$ , medium effect:  $r \geq 0.3$ , large effect:  $r \geq 0.5$ ); IQR: interquartile range.

Data are not normally distributed (the Mann–Whitney test).



**Figure 11.** Comparison of median percentage changes in area of DME per area of retina between Tocovid and placebo groups. Error bars represent the interquartile range.

**Table 6.** Comparison of the area percentage of DME within the Tocovid group.

Timeline	Tocovid (n=28)			
	Median	IQR	r	p-value <sup>a</sup>
Two months treatment				
Baseline	1.052	1.95	-0.449	0.019*
At 2 months	0.692	1.90		
Six months treatment				
Baseline	1.052	1.95	-0.116	0.539
At 6 months	0.913	1.64		
Twelve months treatment				
Baseline	1.052	1.95	-0.615	0.001*
At 12 months	0.509	1.09		

n: number of eyes; r: effect size from the Wilcoxon signed-rank test within subjects (small effect:  $r \leq -0.1$ , medium effect:  $r \leq -0.3$ , large effect:  $r \leq -0.5$ ); IQR: interquartile range.

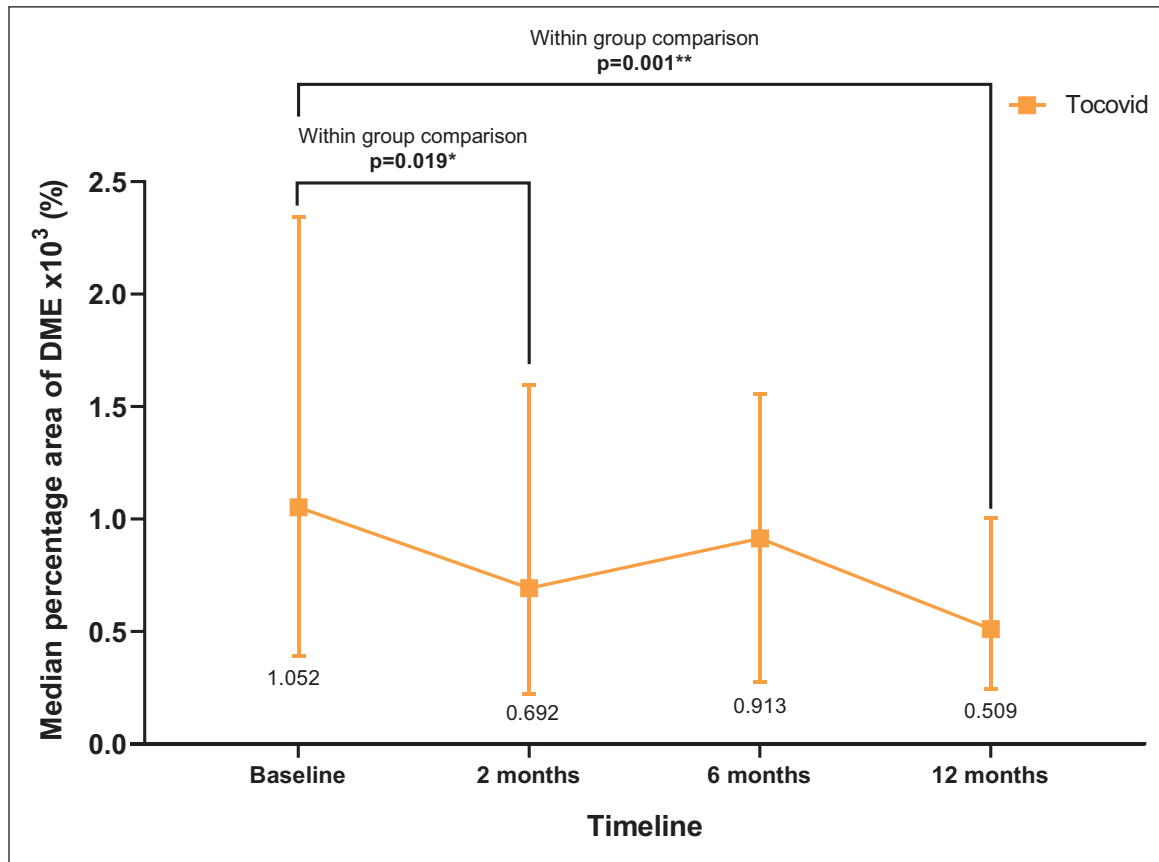
<sup>a</sup>Data are not normally distributed (the Wilcoxon test).

\*Effect size within group significant at  $p < 0.05$ .

mediators, especially VEGF.<sup>12,13,27</sup> However, clinical trial investigating the effect of vitamin E on DR is very limited and mainly focuses on tocopherol and not tocotrienol.<sup>28-30</sup> Therefore, this study aims to investigate if Tocovid could have a similar beneficial effect on the most common microvascular complication of diabetes which is DR.

In this randomized controlled trial, Tocovid 200 mg twice daily for 12 months had significant beneficial effect on DR by improving both retinal microhaemorrhages and DME. In the retinal microhaemorrhage analysis, the

placebo group had a significant increase of 23.42% of retinal microhaemorrhage area at 12 months whereas the Tocovid group had no significant changes across the 12 months of treatment. This could indicate that Tocovid treatment have a protective effect in preventing further retinal bleed formation. For the area of DME analysis, the Tocovid group had a significant decrease of 34.22% and 48.38% at 2 months and 12 months of treatment, respectively, whereas the placebo group had no significant changes across the 12 months duration.



**Figure 12.** Comparison of median area of DME per area of retina in the Tocovid group from baseline to 12 months. Error bars represent the interquartile range.

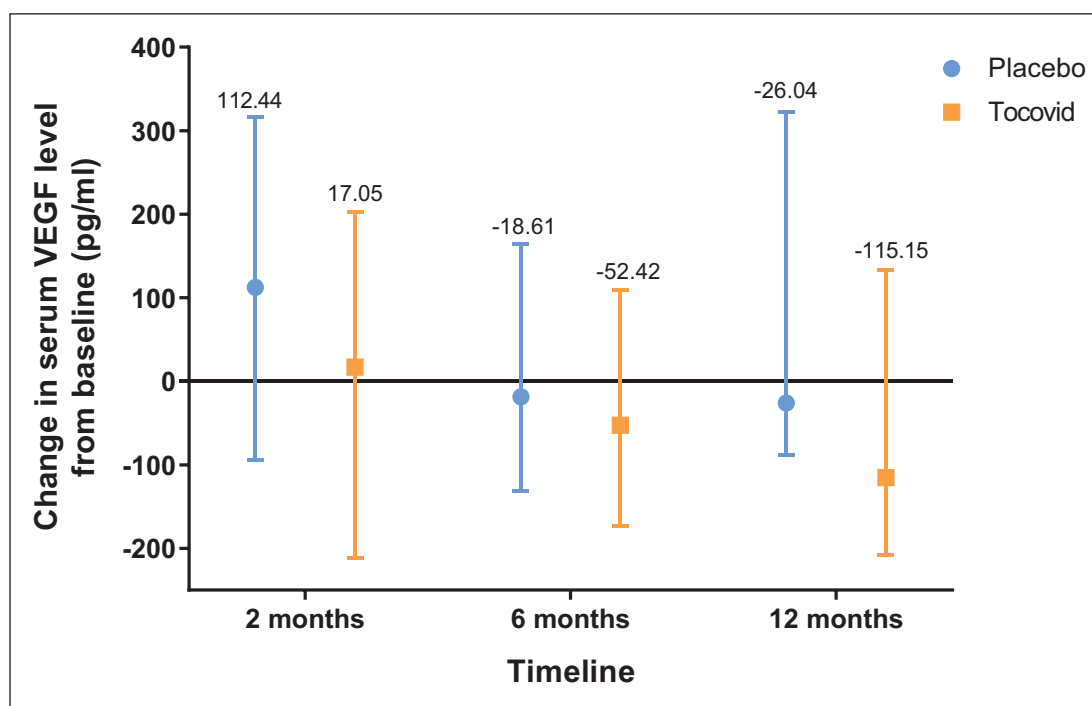
**Table 7.** Comparison of change in serum VEGF level between Tocovid and placebo groups.

Timeline	Tocovid (n=27)		Placebo (n=28)		r	p-value
	Median	IQR	Median	IQR		
Baseline to 2 months (pg/mL)	17.05	414.49	112.44	410.40	-0.224	0.114
Baseline to 6 months (pg/mL)	-52.42	282.39	-18.61	295.17	-0.926	0.354
Baseline to 12 months (pg/mL)	-115.15	342.08	-26.04	411.36	-0.224	0.114

VEGF: vascular endothelial growth factor; n: number of participants; r: effect size from the Mann–Whitney test between groups (small effect:  $r \leq -0.1$ , medium effect:  $r \leq -0.3$ , large effect:  $r \leq -0.5$ ); IQR: interquartile range. Data are not normally distributed (the Mann–Whitney test).

In our secondary outcome measurement, the serum VEGF level had no significant difference in change comparing between both groups and in the separate analysis had no significant change within both the Tocovid and placebo groups. These results are somewhat counterintuitive to our research hypothesis whereby we believed that Tocovid was able to improve DR by reducing VEGF level, and consequently decreasing angiogenesis process in the eye. In the correlation analysis, it was recognized that there was a significant negative correlation between serum VEGF level and retinal microhaemorrhages at baseline ( $p < 0.05$ ). This suggests that

increase of serum VEGF level would result in reduction of area of retinal microhaemorrhage. This significant weak correlation result could be explained by the serum VEGF biomarker being not sensitive enough to the changes in the eye. Serum VEGF being a systemic biomarker could be affected by various factors and conditions which includes inflammatory conditions and undiagnosed malignancies.<sup>31,32</sup> In addition, the correlation done in the meta-analysis conducted by Zhou et al.<sup>19</sup> showed significant correlation between serum VEGF level with the severity of DR using the ETDRS scale. The changes in area of both retinal parameters could be too



**Figure 13.** Comparison of median percentage changes in serum VEGF level between Tocovid and placebo groups. Error bars represent the interquartile range.

**Table 8.** Correlation between serum VEGF level and retinal findings at baseline.

Parameters	<i>n</i>	Serum VEGF level ( $r_s$ )	<i>p</i> -value <sup>a</sup>
Area of retinal microhaemorrhages	55	-0.247	0.012*
Area of diabetic macular oedema	55	-0.126	0.358

VEGF: vascular endothelial growth factor; *n*: number of eyes.

Weak correlation:  $r_s \leq -0.1$ , medium correlation:  $r_s \leq -0.3$ , strong correlation:  $r_s \leq -0.5$ .

<sup>a</sup>Spearman's correlation. Assumptions were fulfilled.

\*Correlation is significant at  $p < 0.05$ .

**Table 9.** Friedman's test comparing serum VEGF level within Tocovid and placebo groups.

Timeline	Tocovid ( <i>n</i> = 27)				Placebo ( <i>n</i> = 28)			
	Median	IQR	<i>W</i>	<i>p</i> -value <sup>a</sup>	Median	IQR	<i>W</i>	<i>p</i> -value <sup>a</sup>
Serum VEGF level								
Baseline	817.524	374.25	0.028	0.522	604.065	627.96	0.043	0.306
At 2 months	789.830	461.51			792.17	567.24		
At 6 months	729.83	461.51			724.74	696.71		
At 12 months	777.17	547.61			715.42	827.39		

VEGF: vascular endothelial growth factor; *n*: number of participants; IQR: interquartile range; *W*: Kendall's *W* uses Cohen's interpretation (small effect:  $r \geq 0.1$ , medium effect:  $r \geq 0.3$ , large effect:  $r \geq 0.5$ ).

<sup>a</sup>Data are not normally distributed (the Friedman test). Assumptions were fulfilled.

small-scale as well for serum VEGF level to be correlated and suggesting that serum VEGF is not a suitable biomarker to detect the effects of vitamin E on DR. Taken together,

these biomarker results suggest that the mechanism of action on how vitamin E was able to improve DR remains inconclusive.

**Table 10.** Comparison of HbA1c and blood pressures in Tocovid and placebo groups at baseline, 2 months, 6 months and 12 months of treatment.

Parameters	Tocovid (n=27)			Placebo (n=28)		
	Mean ± SD	F	p-value	Mean ± SD	F	p-value
HbA1c (%)						
Baseline	7.54 ± 1.24	2.223	0.116	8.08 ± 1.37	0.699	0.563
At 2 months	7.57 ± 1.24			8.10 ± 1.39		
At 6 months	7.10 ± 0.90			7.83 ± 2.03		
At 12 months	7.36 ± 1.15			8.09 ± 2.00		
Systolic blood pressure (mmHg)		2.593	0.086		1.453	0.261
Baseline	140.12 ± 13.67			135.70 ± 18.15		
At 2 months	132.68 ± 12.00			127.70 ± 13.32		
At 6 months	136.20 ± 23.50			129.29 ± 18.00		
At 12 months	137.68 ± 15.00			131.29 ± 14.99		
Diastolic blood pressure (mmHg)		4.905	0.013*		1.456	0.260
Baseline	80.58 ± 7.76			82.00 ± 12.03		
At 2 months	74.63 ± 8.20			77.33 ± 9.08		
At 6 months	79.63 ± 7.62			78.00 ± 9.91		
At 12 months	75.05 ± 8.35			76.24 ± 7.35		

n: number of participants; SD: standard deviation.

Repeated measures ANOVA. Assumptions were fulfilled.

\*Significant at  $p < 0.05$ .

**Table 11.** Comparison of plasma levels of tocotrienols and tocopherol in Tocovid and placebo groups at baseline, 2 months, 6 months and 12 months of treatment.

Levels of vitamin E (pg/mL)		Group		p-value
Isomer of vitamin E		Tocovid (n=27)	Placebo (n=28)	
α-Tocotrienol	Baseline	34.72 ± 23.4	33.52 ± 20.05	0.105
	2 months	210.21 ± 68.7	32.74 ± 7.12	0.015*
	6 months	203.99 ± 65.41	45.15 ± 19.69	0.026*
	12 months	223.89 ± 34.36	51.95 ± 12.62	0.045*
γ-Tocotrienol	Baseline	39.55 ± 9.87	37.55 ± 13.22	0.904
	2 months	114.17 ± 33.34	18.65 ± 7.79	0.008**
	6 months	131.54 ± 42.68	21.07 ± 7.55	0.015*
	12 months	113.89 ± 12.17	23.89 ± 12.17	0.049*
δ-Tocotrienol	Baseline	15.57 ± 12.36	14.64 ± 10.24	0.765
	2 months	52.91 ± 13.31	10.72 ± 3.80	0.004**
	6 months	45.56 ± 11.66	11.27 ± 3.10	0.007**
	12 months	44.80 ± 4.34	11.92 ± 7.41	<0.001***
α-Tocopherol	Baseline	1849.96 ± 273.62	1935.57 ± 289.74	0.831
	2 months	2366.64 ± 746.64	1565.70 ± 356.97	0.321
	6 months	2445.34 ± 779.01	1358.26 ± 409.07	0.208
	12 months	2323.41 ± 114.97	1192.38 ± 92.36	0.074

n: number of participants.

Data are presented as mean value ± SD. Analysed using repeated measures ANOVA. Assumptions were fulfilled. The plasma vitamin E levels have been corrected for lipid.

Significant at \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

The significant increase in levels of tocotrienols and tocopherol in the Tocovid group as compared to the placebo group at 2 months and throughout the study period until 12 months confirms the patients' compliance to

supplementation of Tocovid in this study. To the best of our current knowledge, this is the first research which investigated the effect of vitamin E on NPDR in T2DM patients.



**Table 12.** Predictors of percentage change in area of DME: backward elimination linear regression.

Percentage change in area of DME	Independent variable	Unstandardized coefficient, $\beta$	95% CI for $\beta$		$p$ -value	Adjusted $R^2$
			Lower	Upper		
Baseline to 2 months	Constant	-2.167	5.372	1.039	0.181	0.115
	Baseline SBP	0.026	0.005	0.047	0.018*	
	HbA1c at 2 months	-0.253	-0.489	-0.017	0.036*	
Baseline to 6 months	Constant	-7.196	-12.864	-1.529	0.014*	0.202
	Treatment	0.941	0.074	1.808	0.034*	
	Baseline SBP	0.034	0.001	0.066	0.043*	
	HbA1c at baseline	-0.821	-1.313	-0.330	0.001**	
	HbA1c at 6 months	1.047	0.428	1.665	0.001**	
Baseline to 12 months	Constant	0.400	-2.767	3.568	0.801	0.176
	HbA1c at baseline	-0.877	-1.352	-0.402	0.001**	
	HbA1c at 12 months	0.647	0.138	1.156	0.014*	

DME: diabetic macular oedema; CI: confidence interval; SBP: systolic blood pressure.

Assumptions were fulfilled.

Significant at \* $p < 0.05$ ; \*\* $p < 0.01$ .

One of the main limitations that were present in this trial was the consistency in retinal image analysis methodology for retinal microhaemorrhage and DME. Although intra-rater and inter-rater analysis was carried out during the analysis, the degree of variability in analysing the retinal findings remains a significant factor in the data's reliability. At this time, there is an automated analysis of fundus image being developed known as convolution neural network (CNN) which had excellent results in identifying signs of DR.<sup>32,33</sup> This method could be incorporated in future research works to allow a more consistent and reliable method in measuring retinal microhaemorrhage and DME.

Another source of weakness in this study which could be improved is the sample size. Although using the sample size calculator, the number of eyes (55 participants contributed 103 eyes total) was sufficient to detect a statistically significant change, the evidence is unable to convince clinician to integrate vitamin E as a treatment option for DR. However, the study could pave a way for future trials to include a larger number to determine that similar results in this study could be replicated in the bigger scale as well.

To sum it all up, DR is the most common diabetes microvascular complications as well as contributes to a main portion of vision loss among adults. Currently, treatment options aimed at early stage DR are restricted to only blood pressure and glycaemic control. Tocovid treatment for the 12-month duration has shown significant beneficial effect on DR by improving both retinal microhaemorrhage as well as DME. There were no changes in the serum VEGF level indicating that Tocovid did not reduce DR by inhibiting angiogenesis in the eye along with oxidative stress and inflammation. The challenge now is to identify the exact mechanism of action on how vitamin E is able to improve DR and replicating these results in future studies on a larger scale.

### Author contributions

K.A.K. contributed to the conceptualization. J.-I.H. contributed to the data curation and formal analysis. B.A. contributed to the funding acquisition. K.A.K., B.A., J.-I.H., E.Y.N., Y.C. and Y.Y.K. contributed to the investigation. K.A.K., B.A., J.-I.H., E.Y.N., Y.C. and Y.Y.K. contributed to the methodology. B.A. contributed to the project administration. K.A.K. and B.A. contributed to the resources. J.-I.H. and Y.Y.K. contributed to the software. K.A.K. and B.A. contributed to the supervision. K.A.K. and B.A. contributed to the validation. K.A.K. and B.A. contributed to the visualization. J.-I.H. contributed to the writing – original draft. K.A.K., B.A., J.-I.H., E.Y.N., Y.C. and Y.Y.K. contributed to the writing – review and editing.

### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

### Ethical approval

Ethical approval for this study was obtained from the Monash University Human Research Ethics Committee (MUHREC) with project code 12090.

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### Informed consent


Written informed consent was obtained from all subjects before the study.

## Trial registration

The trial is registered with the Australian New Zealand Clinical Trial Register ANZCTR code ACTRN12619001568101 titled – The Effects of Tocotrienol-Rich Vitamin E (Tocovid) on Diabetes and Diabetic Microvascular Complications: Kidney Disease (Nephropathy), Eye Disease (Retinopathy) and Nerve Impairment (Neuropathy).

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