RESEARCH PROTOCOL

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The effect of dietary interventions on endothelial glycocalyx dimensions and barrier function in South Asian patients with diabetic nephropathy

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)
AE	Adverse Event
AR	Adverse Reaction
ASAT	Aspartate Aminotransferases
ALAT	Alanine Aminotransferases
BMI	Body Mass Index
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CRF	Clinical Research File
CRP	C-reactive Protein
CV	Curriculum Vitae
DTSQ	Diabetes Treatment Satisfaction Questionnaire
EQ-5D-5L	EuroQol 5 Dimensions 5 Levels questionnaire
GFR	Glomerular Filtration Rate
ESRD	End Stage Renal Disease
EU	European Union
FMD	Fasting Mimicking Diet
GCP	Good Clinical Practice
GRAS	General regarded as safe
HbA1c	Hemoglobin A1c
Hb	Hemoglobin
HDL	High Density Lipoprotein
HS	Heparan Sulfate
Ht	Hematocrit
IB	Investigator's Brochure
IC	Informed Consent
IGF-1	insulin-like growth factor 1
IL	Interleukin
IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier
LDL	Low Density Lipoprotein

LED	Light-Emitting Diode
LUMC	Leiden University Medical Centre; in Dutch Leids Universitair Medisch Centrum
MCP-1	Monocyte Chemotactic Protein 1
METC	Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)
MHI	Microvascular Health Index
PBR	Perfused Boundary Region
RBC	Red Blood Cell
SAE	Serious Adverse Event
SDF	Sidestream Dark Field
SOD	Superoxide Dismutase
SPC	Summary of Product Characteristics (in Dutch: officiële productinformatie IB1-tekst)
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
TNF-α	Tumor necrosis factor-alpha
Wbp	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgegevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale: Micro- and macrovascular complications are the pathological hallmarks of diabetes mellitus, with diabetic nephropathy as one of the most serious microvascular complications. South Asians have a high incidence of type 2 diabetes and a higher change to progress to end-stage renal disease than West European patients, which may be due to a higher sustained systemic and glomerular inflammation. The endothelial glycocalyx, covering endothelial cells, is essential for maintaining vascular homeostasis. In the diabetic environment, impairment of the glycocalyx can be induced by invading macrophages which excrete the glycocalyx degrading enzyme heparanase and its activator cathepsin L. In the glomerulus, impairment of the glycocalyx results in increased permeability for albumin alongside renal and vascular inflammation. SDF-imaging is a non-invasive technique to visualize the endothelial glycocalyx and obtains parameters that reflect the microvascular endothelial status, combined in the Microvascular Health Index (MHI). Here we investigate if dietary interventions, which either provide the nutritional building blocks to support and maintain the endothelial glycocalyx or reduce the overall inflammatory burden, can aid in restoration of the glomerular endothelial glycocalyx and in turn result in a reduction of albuminuria and further delay, or prevent renal damage.

Objective: The primary aim of the study is to improve microvascular endothelial health between baseline and 3 months expressed by the Microvascular Health Index, using a non-invasive imaging technique, upon two different dietary interventions compared to the placebo. The secondary objective is to monitor the dietary effects after 3 months on systemic inflammation, metabolic parameters and albuminuria. Finally, we aim to determine new urinary heparan sulfate fragment biomarkers which can be related to specific glomerular extracellular heparanase activity, a possible central player in local glomerular damage and renal inflammation.

Study design: A randomized, placebo controlled, 3-arm intervention field study for 3 months with additional 3 month follow up measurements.

Study population: Patients from South Asian descent in primary care medical centres in the The Hague area between 18 and 75 years old with type 2 diabetes mellitus and proven microalbuminuria.

Intervention: One group receives a monthly 5-day fasting mimicking diet, one group receives daily 4 capsules of a food supplement and one group receives daily 4 placebo capsules, all for 3 consecutive months.

Main study parameters: The primary endpoint is an improvement of the Microvascular Health index in the diet group compared to the placebo group and in the food supplement group compared to the placebo group, after 3 months of the intervention.

Secondary endpoints: reduce albuminuria, decrease inflammation (urinary heparanase and MCP-1 expression, serum CRP and MCP-1 levels), reduce occurrence of specific urinary HS domains, improve metabolic parameters; fasting glucose, HbA1c, C-peptide, IGF-1, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides serum levels, waist circumference, waist-to-hip ratio, body weight and systolic blood pressure after 3 months of the fasting mimicking diet and after 3 months of the food supplement.
The legacy effect of the interventions is also determined, 3 months after stopping with the intervention.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: Eligible patients are randomly assigned to the fasting mimicking diet, food supplement or placebo arm. At baseline, after 3 months and at 6 months, the MHI is measured by non-invasive SDF imaging of the microcirculation under the tongue, and blood and urine samples are collected. In the FMD arm, dosages of the hypoglycemic drugs are adapted during the 5 days of the diet prevent hypoglycemia. Glucose monitoring is done during the diet and at the end of each month in all intervention arms through a finger prick blood sample.

1. INTRODUCTION AND RATIONALE

Micro- and macrovascular complications are the pathological hallmarks of diabetes mellitus. Diabetic nephropathy is one of the most serious microvascular complications associated with diabetes and the incidence of type 2 diabetes is rapidly increasing. Despite better regulation of hyperglycaemia and blood pressure, many patients still develop end-stage renal disease (ESRD).

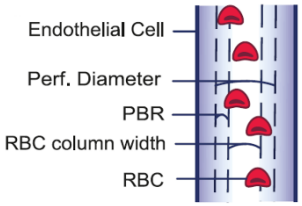
To date, albuminuria is one of the first signs of microvascular dysfunction in the kidney and is an independent risk factor for disease progression. Changes in the endothelial glycocalyx function have shown to be important in the pathophysiology of glomerular diseases and development of albuminuria [1]. The glycocalyx is a carbohydrate-rich layer that covers the luminal surface of vascular endothelial cells. It consists of proteoglycans containing glycosaminoglycans, and incorporated plasma- and endothelium-derived glycoproteins, which together form a dynamic and interactive network at the vascular endothelial surface. The main glycosaminoglycan chains expressed in the glycocalyx are heparan sulfate (HS) and hyaluronan, which constitute 90% of the total amount [2].
The endothelial glycocalyx is essential for vascular homeostasis, it plays an important role in many vascular physiological processes such as mechanotransduction in response to shear stress [3], regulation of inflammatory and thrombotic responses [4] and providing selective permeability for circulating cells and proteins [5]. In the glomerulus, the glycocalyx covers the fenestrated endothelium and contributes to the glomerular filtration barrier [6]. Impairment of the glomerular glycocalyx leads to increased vascular permeability, which allows proteins such as albumin to pass through the fenestrated endothelium and enter into the subendothelial space [7, 8].

The endothelial glycocalyx is a dynamic network with a continuous balance between renewal, modification and degradation. This balance can be affected by mechanical as well as biochemical influences. Heparanase appears to be a crucial mediator in this process, as it is the only known mammalian enzyme that is capable of breaking down heparan sulfate [9, 10]. Activated heparanase has the ability to cleave heparan sulfate side chains, which results in modification of the glycocalyx. Under physiological circumstances heparanase activity is tightly regulated [11]. However, in glomerular diseases such as diabetic nephropathy, heparanase has been found to be released in renal tissue and urine [12-14].
Enhanced heparanase activity contributes to an imbalance in vascular glycocalyx function, with the diabetic environment being one of the strongest known inducers of heparanase activity. Circulating glycated serum proteins and inflammatory cytokines in diabetic patients

contribute to persistent activation of endothelial cells and attraction of monocytes. Diabetic nephropathy is characterized by glomerular inflammation with renal influx and accumulation of macrophages [15-17]. During sustained activation, endothelial cells are stimulated to produce proheparanase. This proheparanase can be turned into active heparanase in the extracellular environment, by the enzyme cathepsin L, which is secreted by local activated macrophages [11, 18]. Activated heparanase in turn can also activate macrophages through its toll-like receptors, creating a positive feedback loop [19].

In the South Asian population, the incidence of type 2 diabetes is very high [20-22]. These patients have a 40-fold higher chance to progress to end stage renal disease compared to West European diabetic patients [23]. This may be due to a higher systemic inflammation in these patients, which causes endothelial activation and induces glomerular inflammation [24-26]. Therefore, these high risk South Asian patients would benefit the most from early therapies that reduce systemic and glomerular inflammation and restore glycocalyx dimensions.

Diabetes is a systemic multi organ disease causing systemic microvascular complications and endothelial glycocalyx impairment. One way to investigate the endothelial glycocalyx is through sidestream darkfield (SDF) imaging. SDF imaging is a non-invasive technique that visualizes the sublingual microvasculature with a hand-held camera. This technique showed that red blood cells (RBCs) maintain a certain distance from the endothelium due to the presence of the glycocalyx. Damage to the glycocalyx allows RBCs to penetrate deeper towards the endothelial surface, which is expressed by an increased perfused boundary region (PBR) [27]. In previous studies, the PBR measured with SDF imaging was able to discriminate between different patient groups such as dialysis patients [28], lacunar stroke patients [29] , critically ill patients [30], patients with ESRD [31], and patients with type 2 diabetes [32].



Lee et al 2014, Deeper Penetration of Erythrocytes into the Endothelial Glycocalyx Is Associated with Impaired Microvascular Perfusion

Other parameters however, give more insight into the quality of the microvascular perfusion, such as RBC filling percentage, capillary density and flow dependency [33].
Combining these parameters, Glycocheck BV developed the quantifiable Microvascular Health Index (MHI). The Microvascular Health Index ranges from 0.00 to 10.00 with a score between 0.00 – 1.00 reflecting a clinically perturbed glycocalyx and perfusion in the

microvasculature, a score between 1.00 – 2.00 a less than average health of the microvasculature, 2.00 – 3.00 an average microvascular health, between 3.00 and 5.00 an above average and a score above 5.00 a good functional microcirculation with adequate endothelial glycocalyx coverage and flow mediated adaptation.

In the last years, the endothelial glycocalyx has been targeted to ameliorate albuminuria in diabetic nephropathy. Therapeutics such as sulodexide and avosentan, intended to improve glycocalyx dimensions, failed to demonstrate albuminuria lowering effects or introduced limiting side effects in randomized controlled trials with diabetic patients [34-36].
However, new promising dietary interventions, that support endothelial glycocalyx function and reduce inflammation without introducing limiting side effects, are available.
Marine organism-derived fucosylated and sulfated polysaccharides that mimic glycosaminoglycans may have beneficial effects in diabetic patients by stabilizing the imbalanced glycocalyx. Fucoidan, extracted from brown seaweed, is one of those sulfated polysaccharides and is the main component of the food supplement Endocalyx™.
Administration of the supplement in a mouse model of aging revealed beneficial vascular effects by reducing mesenteric macrophage accumulation and increasing capillary density. Moreover, daily administration of Endocalyx™ in healthy volunteers improved the quality of the endothelial glycocalyx, measured with SDF-imaging, compared to no treatment (unpublished data, H. Vink).
Simultaneously, the Longo group showed that a monthly cyclic 5-day periodic fasting mimicking diet (FMD) was able to rejuvenate the immune system, decrease tissue inflammation and extend life- and health span. In experimental studies, a fasting mimicking diet improved insulin sensitivity and reduced circulating inflammatory cytokines, which are involved in sustained activation of endothelial cells [37, 38]. Furthermore, two clinical trials revealed long lasting beneficial effects after 3 monthly FMD cycles on metabolic parameters (fasting glucose, systolic blood pressure and blood IGF-1 levels) and systemic inflammation in subjects with a high cardiovascular risk at baseline [38, 39].
This fasting mimicking diet may lead to beneficial effects in the diabetic kidney by several mechanisms. First, amelioration of glucose levels will decrease the amount of glycated serum proteins, which are one of the most potent inducers of heparanase. In addition, fasting induces upregulation of intra-glomerular autophagy [40]. Autophagy is a highly regulated lysosomal protein degradation pathway that removes protein aggregates and damaged or excess organelles to maintain intracellular homeostasis and cell integrity. Impairment in autophagy is thought to be involved in the pathophysiology of diabetic nephropathy and contributes to endothelial dysfunction [41-43]. Hyperglycaemia upregulates autophagy in several renal cell types such as mesangial cells, endothelial cells, and podocytes. However,

prolonged hyperglycaemia deprives autophagy, which is associated with increased severity of diabetic renal damage. [43] As autophagy is upregulated by fasting, the FMD may lead to a more balanced glomerular endothelium and removal of aggregated proteins or cell debris, which leads to a less pro-inflammatory milieu. This, in turn, causes less attraction of inflammatory cells, less activation of heparanase and thus less impairment of the glycocalyx. These dietary interventions, the Endocalyx™ food supplement and FMD, may be promising alternative interventions to support the imbalanced microvascular glycocalyx in diabetic patients by supplying glycocalyx substitutes and reducing glomerular inflammation.

2. OBJECTIVES

Primary Objective:

1. To investigate whether a fasting mimicking diet improves the Microvascular Health Index between baseline and 3 months in type 2 diabetic South Asian patients with microalbuminuria in comparison to the placebo group.
2. To investigate whether intervention with the food supplement Endocalyx™ improves the Microvascular Health Index between baseline and 3 months in type 2 diabetic South Asian patients with microalbuminuria in comparison to the placebo group.

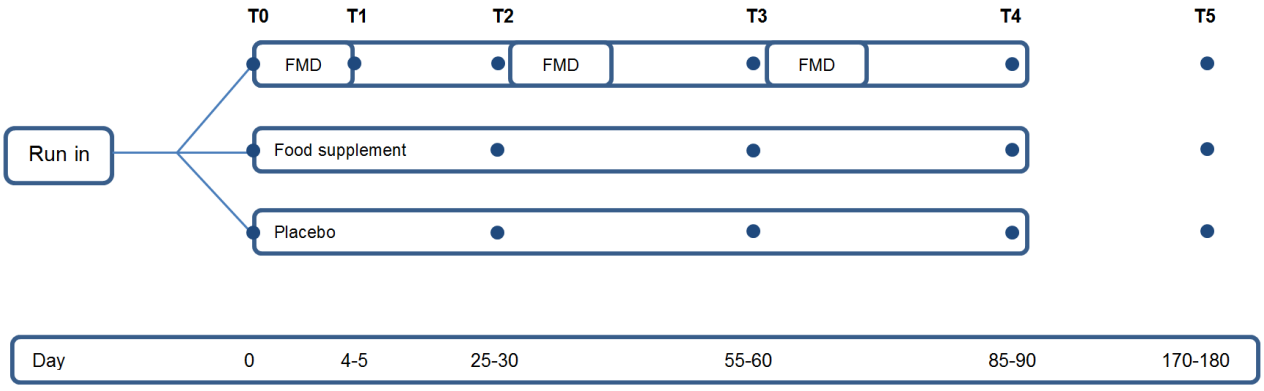
Secondary Objectives:

If the fasting mimicking diet and the food supplement after 3 months can:

- Decrease inflammation (urinary heparanase and MCP-1 expression, serum CRP and MCP-1 levels).
- Reduce occurrence of specific urinary HS domains.
- Reduce albuminuria
- Improve metabolic parameters; fasting glucose, HbA1c, C-peptide, IGF-1, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides serum levels, waist circumference, waist-to-hip ratio, body weight and systolic blood pressure.

3. STUDY DESIGN

A 6-month, single center randomized controlled trial to study the effects of dietary interventions on improving endothelial health in South Asian patients with diabetic nephropathy. In total 90 South Asian patients with type 2 diabetes and proven microalbuminuria between 18-75 years of age will be recruited at general practitioner centers in the The Hague region. Eligible patients will be included for a period of 6 months. Patients will be randomly assigned to receive the fasting mimicking diet, food supplement or placebo for a period of 3 months. The primary endpoint is an improvement of the Microvascular Health index after 3 months in the diet group compared to the placebo group and in the food supplement group compared to the placebo group, measured with SDF-imaging. After 6 months, which is 3 months after stopping with the interventions, patients will also return for a follow-up measurement to investigate possible legacy effects of each intervention.
The food supplement and placebo arm will be double blinded. The placebo arm will also be used as a control group for the FMD arm.
Study procedures will take place at the practice of the general practitioner and will be performed by LUMC research staff. The general practitioner will not perform any of the study procedures. Flow chart I gives an overview of the study design and time points for study visits.



Flow chart I

4. STUDY POPULATION

4.1 Population (base)

The study population consists of South Asian patients with type 2 diabetes and microalbuminuria between 18-75 years old. In total 90 patients will be included in the study.

4.2 Inclusion criteria

In order to be eligible to participate in this study, the following criteria must be met:

1. South Asian patient with diabetes mellitus type 2.
2. Female or male, aged between 18 and 75 years.
3. Body Mass Index ≥18.5.
4. CKD-EPI >45 ml/min/1.73m² .
5. Proven microalbuminuria defined as albumin/creatinine ratio ≥0.3 and <30 mg/mmol (single portion) or 3-300 mg albumin per day (24 hours urine collection) in the last twelve months.
6. Systolic blood pressure ≤ 180 mmHg.
7. Treatment with hypoglycemic drugs: metformin, sulphonylureas and/or insulin.
8. Subject is willing to participate in the study, must be able to give informed consent and the consent must be obtained prior to any study procedure.
9. Patients must be able to adhere to the study visit schedule and protocol requirements.
10. If female and of child-bearing age, patients must be non-pregnant, non-breastfeeding, and use adequate contraception.

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

1. Any concurrent illness, disability or clinically significant abnormality that may, as judged by the investigator, affect the interpretation of clinical efficacy or safety data or prevent the subject from safely completing the assessments required by the protocol.
2. Non-diabetic renal disease e.g. known polycystic kidney disease.
3. Use of LMW heparin and/or immunosuppressive drugs.
4. Significant food allergies for galactose, nuts, soy, tomato, corn, grape, melon and artichoke, which would make the subject unable to consume the food provided.
5. Signs of active infection or autoimmune disease, requiring systemic treatment.
6. A psychiatric, addictive or any disorder that compromises ability to give truly informed consent for participation in this study.

7. Malignancy (including lymphoproliferative disease) within the past 2-5 years (except for squamous or basal cell carcinoma of the skin that has been treated with no evidence of recurrence).
8. Use of any other investigational drug.
9. Patient has enrolled another clinical trial within last 4 weeks.

4.4 Sample size calculation

The primary outcome will be improvement of the Microvascular Health Index after 3 months. The sample size is chosen according to a pilot study with 13 healthy volunteers receiving the food supplement Endocalyx™ for 3 consecutive months. After 3 months, a 31% improvement in Microvascular Health Index was demonstrated, expressed in a significant increase of the MHI from 1.05 at baseline, to 1.38 at 3 months (improvement of 0.33).
Group sample sizes of 23 per group achieve 80% power to detect a difference of 0.35 between the null hypothesis that group improvement means are 0.0 and the alternative hypothesis that the improvement mean of group 2 or 3 is 0.35 with estimated group standard deviations of 0.41 with a significance level (alpha) of 0.05000 using a two-sided two-sample t-test. If we anticipate a 25% drop out rate per group, we will need 30 patients per group. Therefore, we decided to enrol a total number of 90 patients in this study.

5. TREATMENT OF SUBJECTS

5.1 Investigational product/treatment

Patients will be randomly assigned to receive either the fasting mimicking diet, the food supplement or the placebo.

The FMD arm will follow 3 cycles of the diet for 3 consecutive months. One cycle consists of 5 continuous days of the diet followed by 25 days of normal food intake.

Patients in the food supplement and placebo arm will receive 4 capsules per day, for 3 consecutive months. The food supplement and placebo arm will be double blinded.

Patients in the supplement group (placebo or food supplement) will be instructed to not change their diet during the study.

5.2 Use of co-intervention

Patients included in the study can be on standard diabetic care consisting of oral hypoglycemic drugs or insulin. During the study, the diabetes medication will/can be altered, which is explained in paragraph 5.3. Included patients can also be on standard antihypertensive care with antihypertensive medication. The antihypertensive drugs can be altered during the study and is left to the discretion of the investigator. However, preferably RAAS inhibitors will not be altered during the study as this can influence heparanase activity and albuminuria.

Other co-medication is allowed with the exception of immunosuppressive (such as prednisolone) and low molecular weight heparin. Use of co-medication should be documented.

5.3 Glucose monitoring

<u>Endocalyx and placebo group</u>

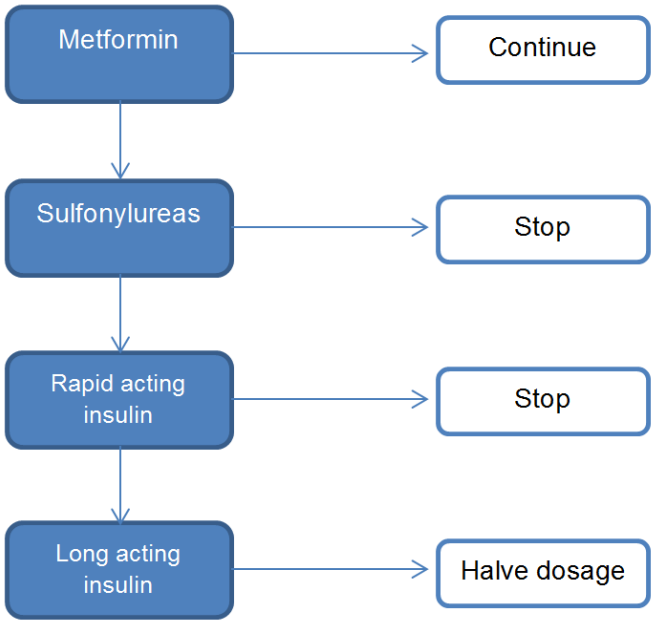
Patients are asked at baseline if they ever experienced hypoglycemia. The symptoms of hypoglycemia are explained at baseline and glucose monitoring will be done at the end of every month (T2, T3 and T4). Patients that use rapid acting insulin are instructed to track glucose levels during one day (before every meal and before going to bed in the evening) in the week prior to the study visit. At the study visit, fasting glucose will be measured with a finger prick blood sample. Patients are asked if they experienced hypoglycemia during the last month. If the fasting glucose at the study visit is between 4.0 and 15.0 mmol/L and the patient did not experience hypoglycemia during that month, the dosage (for that patient) of hypoglycemic drugs is not altered. However, if the patient did experience hypoglycemia during that month or the measured fasting glucose at the study visit is below 4.0 mmol/L, the dosage of hypoglycemic drugs will be altered according to the investigators opinion.

FMD group

Patients that use sulfonylureas or insulin will be provided with a glucose meter and strips at baseline. The device and strips will be provided for free. These patients will get an instruction from the investigator or research staff on how to self-measure glucose levels.
Patients are asked at baseline if they have ever experienced hypoglycemia. The symptoms of hypoglycemia are explained and provided in a patient letter to patients that use sulfonylureas or insulin. The patient letter contains a telephone number and instructions on what to do if the patient experiences symptoms of hypoglycemia or if the self-measured glucose drops below 4.0 mmol/L. Patients are instructed to call the investigator if they experienced hypoglycemia during weekdays and the internal resident on duty at the Haaglanden Medical Center in the evening/night of weekdays and on weekend days. Treatment of hypoglycemia will be performed according to the investigator's or physician's decision and the guidelines of hypoglycemia. The dosages of the hypoglycemic medication can be adjusted if needed.

To prevent hypoglycemia during the diet, the dosages of oral hypoglycemic drugs and insulin will be altered during the 5 days of the diet. These alterations are shown in flow chart II. Sulfonylurea's and rapid acting insulin will be stopped, and the dosage of long acting insulin will be halved during the 5 days of the diet. The sulfonylurea drugs will be stopped one day prior to the start of the diet. Metformin will be continued during the diet. Patients that still use long actin insulin during the diet are asked to measure fasting glucose levels during the 5 days and track glucose levels 4 times throughout the second day of the diet. These patients will be contacted by the investigator during the diet to monitor the glucose levels.
Patients will return to their normal dosage of oral hypoglycemic drugs or insulin for the next 25 days of the month. Patients that return to sulfonylureas or long- or rapid acting insulin are instructed to measure the fasting glucose for the first 3 days after the diet. The investigator or one of the research staff will contact these patients by phone in the first following days after returning to their normal diet.
At the end of every month, the previous FMD cycle is evaluated together with the glucose monitoring, by measuring fasting glucose levels with a finger prick blood sample. Patients that use rapid acting insulin are instructed to track glucose levels during one day (before every meal and before going to bed in the evening) in the week prior to the study visit. If the fasting glucose at the study visit is between 4.0 and 15.0 mmol/L and the patient did not experience hypoglycemia during that month, the dosage (for that patient) of hypoglycemic drugs is not altered. However, if the patient did experience hypoglycemia during that month or the measured fasting glucose at the study visit is below 4.0 mmol/L, the dosage of hypoglycemic drugs will be altered according to the investigators opinion.

Flow chart II: Hypoglycaemic drug dosage adjustment during the 5-day FMD



5.4 Escape medication

Treatment of hypoglycemia will be performed according to the investigator's or physician's decision and the guidelines of hypoglycemia. In case of an allergic reaction to one of the ingredients of the diet or food supplement, standard treatment with anti-histamines will be given. Severe cases of allergic reaction will be treated with epinephrine, prednisolone and evaluated in the hospital.

6. INVESTIGATIONAL PRODUCT

6.1 Name and description of investigational products

6.1.1 Fasting Mimicking diet: Prolon™

The fasting mimicking diet that will be used in this study is called Prolon™ (further termed Prolon), produced by L-Nutra. Prolon is a 5 day plant-based, low caloric, low protein, diet that is designed for humans to attain water fasting like effects while minimalizing the burden of fasting.

6.1.2 Food supplement: Endocalyx™

Endocalyx™ (further termed Endocalyx) is a food supplement distributed by Microvascular Health Solutions. The supplement combines three complementary and synergistic working mechanisms to support the endothelial glycocalyx function, by supplying sulfated polysaccharides, anti-oxidant enzymes, and additional substrates for glycocalyx synthesis.

6.1.3 Placebo

The placebo will be produced by the pharmacy of the LUMC and will mimic the Endocalyx capsules.

6.2 Summary of findings from non-clinical studies

6.2.1 Fasting mimicking diet

Dietary restrictions such as intermitting fasting in non-clinical studies established beneficial effects on oxidative damage, inflammation and life span by inducing metabolic and cellular changes [44]. Fasting improved glucose and insulin sensitivity, reduced circulating inflammatory markers and insulin-like growth factor 1 (IGF-1) serum levels, a hormone associated with aging and cancer, and induced autophagy, which is impaired in diabetic nephropathy and contributes to glomerular inflammation and endothelial dysfunction [37, 43, 45].

The metabolic effects of the FMD were observed in non-diabetic and type 2 diabetic mice. In non-diabetic mice, a 4-day fasting mimicking diet twice a month reduced glucose and IGF-1 levels majorly at the end of the fasting cycle compared to the control group, whereas ketone bodies increased by 9 fold. These markers, however, returned to baseline levels within the days of refeeding [38]. In diabetic mice, alternate day fasting reversed high insulin and glucose levels to normal levels, despite similar overall food intake compared to the control group [46]. This was also seen in db/db mice, which develop insulin resistance in early stages and beta cell failure in later stages. Weekly cycles of a 4-day FMD reduced fasting glucose levels to nearly normal levels and improved glucose tolerance and insulin levels, which led to an overall better survival [37].

The fasting mimicking diet also obtained anti-inflammatory effects. In the db/db mice, inflammatory cytokines such as TNF-alpha, IL-12, and MCP-1 were supressed after multiple FMD cycles [37]. In C57BL/6 mice, prone to develop malignant lymphomas, inflammation in lymph nodes and the liver was reduced after multiple 4-day FMD cycles, with reduced tumour incidence and delayed onset of the disease [38].
Another interesting effect of fasting mimicking diets was the induction of beta cell regeneration in healthy and T1D human pancreatic islets by reducing IGF-1 levels and downregulating mTOR and PKA signalling [37]. Inhibition of the mTOR pathway causes an upregulation of autophagy, which is impaired in diabetic nephropathy. Others also showed that intermitting fasting or calorie restriction in rats with diabetic nephropathy obtained anti-inflammatory effects and upregulated autophagy by increasing SIRT1 expression in the kidney [47, 48]. The mTOR and SIRT1 pathways are important nutrient-sensing pathways and regulators of autophagy [49].

6.2.2 Food supplement

Administration of the Endocalyx supplement in a mouse model of aging revealed anti-inflammatory and beneficial vascular effects. In old mice, the food supplement increased arterial nitric oxide production by 50%, decreased mesenteric macrophage accumulation by 60%, lowered blood pressure by 25%, increased capillary density by 50%. These effects led to an improvement in microvascular health index compared to baseline (unpublished data, H. Vink).

6.3 Summary of findings from clinical studies

6.3.1 Fasting mimicking diet

The Longo group developed Prolon, a plant based low-protein low-caloric 5-day fasting mimicking diet for humans. This intermitting short period of fasting obtains the beneficial effects on IGF-1, inflammation, glucose and insulin levels similar of water fasting, while providing micronutrients to minimize the burden of fasting.
In a pilot study, 19 healthy volunteers followed this diet for 5 days per month for 3 consecutive months. At the end of the first 5-day cycle, fasting glucose, IGF-1 levels and body weight were significantly reduced and these effects remained after resuming a normal diet, one week after finishing the third cycle. As a marker for systemic inflammation and risk factor for cardiovascular disease, C-reactive protein levels were measured. For subjects with CRP levels in the moderate to high risk range at baseline, levels returned within the normal range after 3 FMD cycles [38].
After that, a larger randomized cross over trial with the Prolon diet was conducted with 71

healthy individuals that completed 3 cycles of the diet in 3 months. A significant reduction in BMI, total body fat, systolic blood pressure, and serum IGF-1, total cholesterol, HDL and LDL levels was seen one week after finishing the third FMD cycle. A post hoc analysis that stratified the effects of the diet by risk factor showed that only for subjects with prediabetic fasting glucose levels (>99 mg/dl) and for subjects with high CRP levels (>1.0 mg/L) at baseline, levels returned to normal ranges 5 days after finishing the third FMD cycle. Moreover, a voluntary follow-up 3 months after finishing the third FMD cycle showed that the significant beneficial effects on body weight and inflammation remained in all follow-up subjects. For subjects with at-risk levels at baseline, fasting glucose, IGF-1 levels and the systolic blood pressure were still significantly reduced 3 months after finishing the last FMD cycle, indicating that the beneficial effects of the diet may last for several months [39].
In conclusion, the fasting mimicking diet has a long lasting positive effect on metabolic factors but also obtains anti-inflammatory effects in subjects with a high cardiovascular risk profile.

6.3.2 Food supplement

A pilot study was conducted among 13 healthy volunteers receiving the Endocalyx food supplement. After 3 months, the Microvascular Health Index measured by SDF imaging improved by 31%. After 4 months, the Microvascular Health Index in the volunteers improved by 50%. This showed the beneficial effects of the food supplement on the microvasculature as it significantly increased capillary density and red blood cell filling percentage, and reduced the perfused boundary region (unpublished data, H. Vink).

6.4 Summary of known and potential risks and benefits

6.4.1 Fasting mimicking diet

A low caloric, low energetic diet such as the Prolon diet can give discomfort and symptoms such as hunger, fatigue, dizziness, muscle aches, headaches and possible fainting.
In the study with 71 healthy subjects following diet, no severe or life threatening adverse effects were reported. The most reported adverse effects were mild fatigue (45%), weakness (35%), headache (30%), dry mouth (18%), memory impairment (18%) and muscle pains (10%) [39]. The pilot study with 19 healthy subjects also did not report any severe or life threatening adverse effects of the FMD [38]. Another possible side effect may be an unknown allergic reaction to one of the ingredients of the diet.
Because of the low caloric intake during the 5 days of the diet, adequate measurements are taken to prevent hypoglycemia during the diet as illustrated in 3.2 and flow chart II.
Benefits of the diet are improvements in metabolic factors such as body weight, blood pressure, triglycerides, insulin sensitivity and fasting glucose. For patients with diabetic

nephropathy, benefits remain to be established but are potentially substantial with a reduction in albuminuria by decreasing systemic and glomerular inflammation burden which may improve the glomerular microvascular health.

6.4.2 Food supplement

In the pilot study with Endocalyx, no serious adverse effects were reported. One side effect that was reported was dizziness, as the endocalyx supplement lowered the systolic blood pressure. The supplement is already used in general practitioners offices in the United States and to date; no one reported any major side effects. Studies conducted with the individual ingredients also did not report any serious adverse effects, this is further explained in the structured risk analysis (paragraph 12). A possible side effect may be an unknown allergic reaction to one of the ingredients of the supplement.
Benefits of the Endocalyx food supplement in diabetic patients remain to be established but are mainly improving the microvascular health by supporting endothelial glycocalyx function.

6.5 Description and justification of route of administration and dosage

6.5.1 Fasting mimicking diet

The Prolon diet will be provided in a box were each day is separately packaged. The box consists of plant-based soups, bars, drinks, snacks, herbal teas, vitamins, and supplements. A specific combination of food is provided on each day for breakfast, lunch, dinner and snacks. The diet consists of a 5 day regime with day 1 providing 1,090 kcal (10% protein, 56% fat, and 34% complex carbohydrate), whereas days 2 to 5 provide 725 kcal (9% protein, 44% fat, and 47% complex carbohydrate) per day. The Prolon diet is lactose and gluten free but contains nuts, soy and tomato as ingredients. Water and herbal tea can be consumed without a limit during the 5 days of the diet. The characteristics of the diet are the lack of refined carbohydrate, very low protein content and high levels of healthy fats from plant based sources**.**

6.5.2 Food supplement

Composition of one Endocalyx capsule

Ingredient	Amount (mg/capsule)
Fucoidan (85%)	106.25 mg
Oxxynea OMD-MVHS Blend SOD (Superoxide dismutase, catalase and polyphenols)	120.00 mg
Glucosamine Sulfate	375.00 mg
Hyaluronic Acid 1800-3000 kDa	17.50 mg
Microcrystalline Cellulose	130.00 mg
Silicon Dioxide	2.00 mg
Total mg per capsule	750.75 mg

Fucoidan is a fucosylated and sulfated polysaccharide extracted from the brown algae species *Laminaria japonica* that mimics heparan sulfate, the main glycosaminoglycan of the endothelial glycocalyx.
Hyaluronan or hyaluronic acid is also a glycosaminoglycan that is incorporated in the endothelial glycocalyx.
Oxxynea OMD-MVHS Blend SOD contains superoxide dismutase (SOD) and polyphenolic enzymes extracted from olive, grape and artichoke. These enzymes act as antioxidants, capable of helping to prevent cellular and molecular damage caused by free radicals, such as reactive oxygen species, through scavenging the oxidants and inhibiting further activity.
Glucosamine sulfate is extracted from corn, and is an amino sugar and precursor in the biochemical synthesis of glycosylated proteins and lipids. It can increase synthesis of two main constituents of the endothelial glycocalyx, namely heparan sulfate and hyaluronan.
Silicon dioxide and microcrystalline cellulose are natural anti-caking agents used to prevent the components from sticking together.

6.5.3 Placebo

The placebo capsule will mimic the Endocalyx capsules and will consist of microcrystalline cellulose and silicon dioxide.

6.6 Dosages, dosage modifications and method of administration

6.6.1 Fasting mimicking diet

One FDM cycle consist of 5 continuous days of the Prolon diet followed by 25 days of normal food intake. Patients will follow 3 cycles for 3 consecutive months.

6.6.2 Food supplement

Patients will receive 4 capsules Endocalyx per day, in total 3 grams per day, for 3 consecutive months. The capsules are orally administered and can be taken with water, on an empty stomach or with food.

6.6.3 Placebo

Patients will receive 4 capsules of the placebo per day for 3 consecutive months. The capsules are orally administered and can be taken with water, on an empty stomach or with food.

6.7 Preparation and labelling of Investigational Medicinal Product

6.7.1 Fasting mimicking diet

Prolon will be produced and distributed by L-Nutra, a company based in Los Angeles, California. Prolon contains only plant-based, natural food components generally regarded as safe (GRAS, US Food and Drug Administration label).

6.7.2 Food supplement

Endocalyx is a food supplement that is distributed by Microvascular Health Solutions LLC in Alpine, Utah. Preparation and labelling of the Endocalyx is done according to the relevant good manufacturing practice (GMP) guidelines by CSB Nutrition Corporation. The food supplement will be re-labelled, blinded and distributed by the LUMC pharmacy.

6.7.3 Placebo

Preparation and labelling of the placebo is done according to the relevant good manufacturing practice (GMP) guidelines by the pharmacy of the LUMC. The placebo will be blinded and distributed by the pharmacy of the LUMC.

6.8 Drug accountability

6.8.1 Fasting mimicking diet

The Prolon boxes will be stored in the LUMC. The principal investigator is responsible for providing the Prolon box to the patients before the start of every cycle and is responsible for checking the expiration date on the packages in the boxes.

6.8.2 Food supplement

The supplements will be stored in the pharmacy of the LUMC. CSB nutrition is responsible for verifying if the supplements meet the release criteria. The investigator is responsible for providing the supplements to the patients at baseline and at the end of the first and second month.

6.8.3 Placebo

The placebo will be stored in the pharmacy of the LUMC. The pharmacy is responsible for verifying if the placebo meets the release criteria. The investigator is responsible for providing the placebo to the patients at baseline and at the end of the first and second month.

7. METHODS

7.1 Study parameters/endpoints

7.1.1 Main study parameter/endpoints

The primary endpoint is to improve the endothelial microvascular health expressed by an improvement in Microvascular Health Index between baseline and 3 months of treatment in South Asian patients with type 2 diabetes mellitus through:

1. The fasting mimicking diet in comparison to the placebo.
2. The endocalyx food supplement in comparison to the placebo.

7.1.2 Secondary study parameters/endpoints

Secondary endpoints include;

- Albuminuria: urinary excretion of albumin, using albumin/creatinine ratio in mg/mmol.
- Inflammation: urinary heparanase and MCP-1 expression, serum C-reactive protein, and MCP-1 levels.
- Metabolic response: fasting glucose, HbA1c, C-peptide, IGF-1, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides levels, body weight, waist circumference, waist-to-hip ratio and systolic blood pressure.
- Identifying specific urinary heparan sulfate domains.

To evaluate the legacy effects of the interventions, the primary and secondary parameters will also be assed three months after stopping the intervention.

7.1.3 Other study parameters

Urinary ketones are measured after the first 5 days of the first FMD cycle to determine the compliance of the patients in the diet group.
ASAT and ALAT are measured for monitoring liver function, serum creatinine level for monitoring kidney function and the frequency of hypoglycemia during the study is documented as safety endpoints.

7.2 Randomisation, blinding and treatment allocation

Eligible patients will be randomized during the run in period into the FMD, food supplement or placebo arm after given informed consent. Randomisation will be performed by the LUMC pharmacy. The placebo and food supplement group will be double blinded. The pharmacy will store the randomization code. The investigator or pharmacy can deblind the subjects in the occurrence of any AE, SAE, or abnormality in a laboratory assessment which, in the opinion of the investigator, warrants the subject’s permanent withdrawal from the trial.

Patient specific data will be stored under a subject identification code and randomization number for each patient. There will be a subject identification code list that links the codes to a subject. This list will be stored for 15 years by the principal investigator. Data will be presented for the complete intention-to-treat population as well as the per protocol population.

7.3 Study procedures

Patients will be seen in accordance with the schedule listed in the table below.

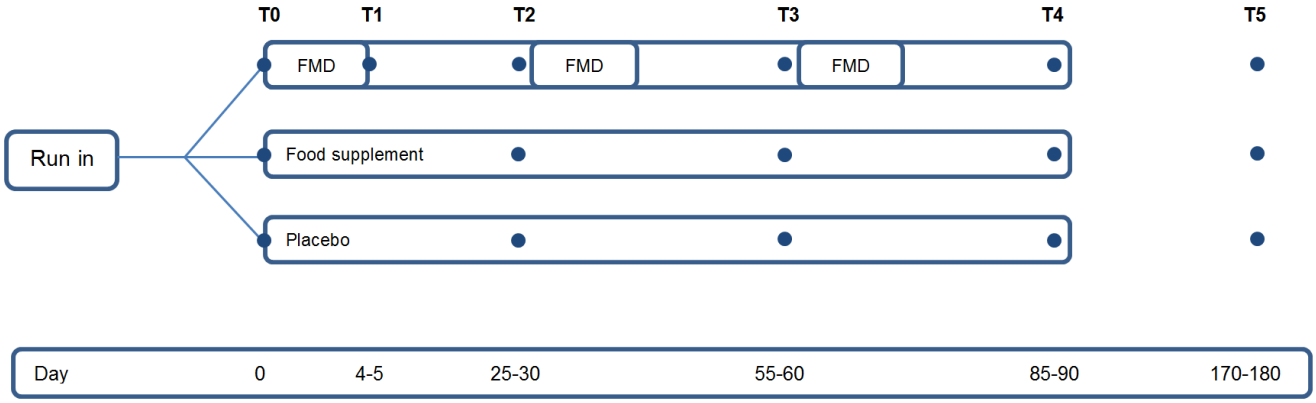


Table of all measurements and procedures performed in the study.

	Run in - 30 days	T0 Baseline	T1 Day 4-5 FMD only	T2 Day 25-30	T3 Day 55-60	T4 Day 85-90	T5 Day 170-180
In- & exclusion criteria	x						
Written informed consent	x						
Randomization	x						
Baseline characteristics		x					
SDF imaging		x				x	x
Survey		x				x	x
Urine collection [a]		x	x			x	x
Blood pressure		x		x	x	x	x
Blood sampling		x				x	x
Anthropometric measurements		x	x			x	x
Glucose monitoring			x	x	x	x	
Adverse events				x	x	x	x

[a] First morning urine of the morning of the study visit

Study visits

Patients are instructed to not eat or smoke the morning of the study visit.

Baseline characteristics

Baseline characteristics will be obtained at the beginning of the study which includes: date of birth, age, sex, ethnicity, relevant medical history, smoking, experience of hypoglycemia in the past and use of medication.

Surveys

The EuroQol 5 Dimensions 5 Levels questionnaire (EQ-5D-5L) will be used to measure the health-related quality of life of the patients. The EQ-5D-5L is an internationally validated questionnaire to measure the health status. It contains 5 health dimensions; mobility, self-care, usual activities, pain/discomfort, anxiety/depression and a scale to score the patients' health that current day. [50]

Patients' satisfaction about the interventions will be reviewed with the Diabetes Treatment Satisfaction Questionnaire (DTSQ). The DTSQ is developed to assess the diabetes treatment satisfaction and consists of eight items each rated on a seven-point scales. The items include satisfaction with current treatment, treatment convenience, flexibility of treatment, understanding of diabetes, continuity of treatment, recommending treatment to others with diabetes and perceived frequency of hyper- and hypoglycemia. [51]

Patients will fill in the DTSQ static version at T0 and the DTSQ change version at T4.

Urine collection

The first morning urine of the day will be collected on the day of the study visit. Levels of creatinine, albumin, heparanase, MCP-1 and HS-fragments will be measured in the urine samples. The presence of leukocytes or nitrite in the urine will be tested with a dipstick. In the FMD arm, urinary ketones will be measured after the first 5 days of the first cycle to assess the compliance of the patients. Analysis of the urine samples will be done in the LUMC and Radboud University Medical Center. The urine samples will be stored in the LUMC.

Laboratory tests

Blood sampling will be done at 3 study visits by vena puncture. At T0, T4 and T5, serum levels of Hb, Ht, Hb1ac, C-peptide, IGF-1, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, creatinine, C-reactive protein, MCP-1, ASAT and ALAT will be assed. Analysis and storage of the samples will be done in the LUMC.

In the FMD arm on day 5 of the first FMD cycle, blood ketone levels will be determined with a

capillary ketone meter.

Renal function

The CKD-EPI formula will be used for GFR estimation [52].

Blood pressure and anthropometric measurements

Systolic and diastolic blood pressure will be measured at the study visits by hand or automatic monitor. Height in centimeters and body weight in kilogram will be measured in indoor clothing but without shoes. Waist circumference and hip circumference will be measured in centimeters.

Glucose monitoring

Glucose monitoring is explained in paragraph 5.3.

SDF imaging

Sidestream dark field (SDF) imaging is a non-invasive technique that measures the endothelial glycocalyx with a handheld camera that visualizes the sublingual microvasculature. The CE-marked camera is put under the tongue of the patients and measures in total about 3000 vascular segments in the sublingual vessels. The investigator moves the camera to a different location in the mouth until the total amount of frames necessary is determined automatically. This takes about 2-4 minutes.
The camera uses green LED light to detect the hemoglobin in red blood cells and measures lateral red blood cell (RBC) movements, RBC filling percentage, vessel density, and local vascular perfusion efficiency. These parameters reflect the endothelial glycocalyx health status and are affected if the glycocalyx layer is impaired [32, 33]. The image acquisition is automatically mediated through the Glycocheck software (Glycocheck BV, Maastricht, the Netherlands), which combines these parameters and calculates the Microvascular Health Index (MHI). This index ranges from ranges from 0.00 to 10.00 with a score between 0.00 – 1.00 reflecting a clinically perturbed glycocalyx and perfusion in the microvasculature, a score between 1.00 – 2.00 a less than average health of the microvasculature, 2.00 – 3.00 an average microvascular health, between 3.00 and 5.00 an above average and a score above 5.00 a good functional microcirculation with adequate endothelial glycocalyx coverage and flow mediated adaptation.

In addition, capillary tortuosity will be determined by placing the SDF camera in four quadrants of the inner lip (duration about 1-2 minutes): the upper right quadrant, upper left quadrant, lower right quadrant and lower left quadrant. From these collected frames, the

capillary tortuosity will be assessed by scoring the number of twists per capillary in the capillaries in the field of view per image. The number of twists will be stratified as 0: no twists (or pinhead capillaries) to 4: four or more twists, described by Djaberi et al. [53]
The overall tortuosity score per subject will be determined by selecting the most frequent tortuosity score in the 12 studied microcirculatory imaging areas (3 microcirculatory imaging areas per quadrant).

7.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons or if continuing participation is deleterious for the subject's wellbeing. For example, ≥20% weight loss or an allergic reaction to the food supplement or diet ingredients will be a reason for withdrawal from further participation. All data generated up to the time of discontinuation from the study will be analysed and the reason for discontinuation will be documented.

7.4.1 Specific criteria for withdrawal

Subjects meeting the following criteria must be withdrawn from the trial:

- occurrence of any AE, or abnormality in a laboratory assessment which, in the opinion of the investigator, warrants the subject's permanent withdrawal from the trial.
- urgent medical reasons or if continuing participation is deleterious for the subject's wellbeing.
- patient noncompliance, defined as refusal or inability to adhere to the trial schedule or procedures.
- at the request of the subject, investigator or regulatory authority.
- subject becomes pregnant.
- subject dies or is lost to follow-up.

7.5 Replacement of individual subjects after withdrawal

The sample size calculation for this study is given in paragraph 4.4. A 25% protocol violator and/or drop-out rate is taken into account. Therefore an inclusion of 30 subjects per study group is aimed. However, drop-outs from this study that occur during the first 2,5 months because of schedule problems, pregnancy or non-diabetic medical reasons may be replaced in order to reach 23 subjects per study group to complete the study. If a subject discontinues from the study prematurely the reason must be fully evaluated and recorded appropriately in

source documentation and the eCRF. If the subject is being withdrawn because of an AE, that AE must be indicated as the reason for withdrawal.

7.6 Follow-up of subjects withdrawn from treatment

No follow up will be done for subjects who are withdrawn from the study except for regularly follow up at the general practitioner. In case of withdrawal because of an adverse event, subjects are followed as described in paragraph 9.4.

7.7 Premature termination of the study

The investigators can decide to terminate the study premature for urgent medical reasons. For example in the case of a high amount of unexpected serious side effects.

8. SAFETY REPORTING

8.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise the subject's health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

8.2 AEs, SAEs and SUSARs

8.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the fasting mimicking diet or food supplement. At every study visit the occurrence of an adverse event is evaluated. A telephone number is provided to all the subjects in the study in the informed consent form which they can contact in case of an (serious) adverse event during the study. All adverse events reported at the study visit by the subject or observed by the investigator or the research staff will be evaluated and recorded.

The occurrence of hypoglycemia is evaluated at every study visit in all intervention groups together with the glucose monitoring. Patients in the diet group that use sulfonylureas or insulin are instructed to call the investigator in case of hypoglycemia (see paragraph 5.3). In case of persistent or severe hypoglycemia, patients can contact the investigator on week days and the internal resident on duty at the Haaglanden Medical Center in the evening/night of weekdays and on weekend days. Treatment of hypoglycemia will be performed according to the investigator's or physician's decision and the guidelines of hypoglycemia.

Any (serious) adverse event recorded by the general practitioner or internal residents at the Haaglanden Medical Center will be reported to the investigator by email (a.i.m.van_der_velden@lumc.nl) or by telephone as soon as possible. The investigator is responsible for evaluating and recording all adverse events.

The laboratory results will be evaluated by the investigator. An abnormality in a laboratory assessment will be documented and is left to the discretion of the investigator. If, in the opinion of the investigator, the laboratory results jeopardise the safety of the subject, the subject will be withdrawn from the study. The following results or references ranges will require additional investigation and follow-up:

- ASAT and ALAT level ≥ than two times the baseline value.
- Creatinine level increase of more than 25% of the baseline value.
- CRP level ≥ 10 mg/dl.

8.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

All adverse events during the study reported or observed by the subject, investigator, general practitioner or internal residents at the Haaglanden Medical Center will be evaluated by the investigator for the criteria of a SAE. All serious adverse events will be recorded in SAE report forms by the investigator and send to the sponsor without undue delay after obtaining knowledge of the events. The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events. All SAE report forms will be stored in the trial master file.

8.3 Annual safety report

The sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC, competent authority, and competent authorities of the concerned Member States.

This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

8.4 Follow-up of adverse events

All adverse events will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol.

9. STATISTICAL ANALYSIS

9.1 Primary study parameter

SPSS will be used for all statistical analysis. Data analysis will be performed based on the intention to treat principle. Patients will be included in the analyses according to the treatment to which they were randomised.

The primary endpoint Microvascular Health index (MHI) will be summarized in mean with standard deviation or median with interquartile range. The effect within the group between baseline and 3 months is tested using a paired Student's t test (two-tailed). Differences in improvement of the MHI between baseline and 3 months between the FMD group and the placebo group and the food supplement and placebo group will be tested with an independent Student's t test.

9.2 Secondary study parameters

The categorical data will be summarized by the frequencies and percentages and tested within the group between baseline and 3 months using a Pearson's Chi-squared test. The continuous data will be summarized in mean with standard deviation in case of normal distribution or median with interquartile range in case of non-normal distribution. A paired two-tailed Student's t test or Wilcoxon signed rank test will be used to calculate the effect within the group between baseline and 3 months.

Differences in outcomes between the groups (diet vs placebo and supplement vs placebo) will be tested with a Student's *t* test for normally distributed data or Mann-Whitney *U* test for non-normally distributed data.

A paired two-tailed Student's t test or Wilcoxon signed rank test for differences of the primary and secondary parameters between baseline and 6 months within the group will be used to evaluate the legacy effect of the interventions 3 months after stopping with the intervention.

9.3 Other study parameters

Demographic data and other baseline characteristics will be summarised using descriptive statistics. The main analyses will also be performed in a priori defined subgroup analyses such as age at baseline, smokers vs non-smokers ect.

Additional explorative analyses involve the analysis of prognostic and predictive factors for achieving the primary or secondary endpoint, this will be investigated by using logistic regression. The level of significance for all tests will be set at $p<0.05$.

10. ETHICAL CONSIDERATIONS

10.1 Regulation statement

This study will be will be conducted according to the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) in accordance with the Medical Research Involving Human Subjects Act (WMO) and to the standards of Good Clinical Practice. The study protocol will be submitted for approval to the Medical Ethics Committee (METC) of the Leiden University Medical Center

10.2 Recruitment and consent

The study will not start before formal approval of the Medical Ethics Committee (METC) of the LUMC has been granted. Patients will be recruited at primary care centres in the The Hague region. The investigator or assigned research staff will provide oral and written information about the study and is responsible for the signed informed consent. Patients will be given as long as they require deciding if they want to participate in this study. Only after written informed consent is obtained will the patient be entered into the study.

10.3 Benefits and risks assessment, group relatedness

The prevalence of type 2 diabetes is high in South Asian patients [22].These patients have a 40 times higher risk of developing ESRD and a faster progression to renal failure compared to West European patients [54]. This is may be linked to increased systemic and glomerular inflammation in these patients [24, 55, 56]. Consequently, these patients will probably have an impaired endothelial glycocalyx with high heparanase activity and would benefit the most from treatments that support the glycocalyx homeostasis and reduce inflammation burden. To date, treatments for diabetic nephropathy are insufficient in preventing the development of ESRD. The interventions in this study can be used as add-on dietary interventions to reduce residual albuminuria and progression of renal disease.

The intervention burden in the FMD arm is for 5 days a month and in the other arms by taking daily capsules. Blood and urine sampling and non-invasive SDF imaging will be done at 3 time points in all intervention groups. In addition, patients are frequently monitored due to the possible effects of the interventions on glucose levels and insulin sensitivity. For the FMD group this means frequently measuring glucose values trough a finger prick.

Patients in the diet group have a risk of the occurrence of hypoglycemia during the study. In the days of the diet, this risk is reduced by stopping the hypoglycemic drugs that can cause hypoglycemia. After the diet, patients are asked to measure their glucose levels regularly. The dosages of the hypoglycemic drugs can be altered if needed.

Other risks of the interventions may be an allergic reaction to one of the components of the diet or the food supplement. However, if eligible patients already are known with specific food allergies, they are excluded from the study. Effects on kidney function and liver function will be monitored during the study, but are not expected as a result of the interventions. In our opinion, the risk of the interventions is reduced within our study design and therefore the limited side effects and possible beneficial effects of the interventions on metabolic parameters, microvascular health and albuminuria justify participation in this study.

10.4 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO. The sponsor also has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.
The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

10.5 Incentives

Participants will receive 200 euros to cover any expenses for participation in the study, which is composed of 40 euros per study visit. Patients that are withdrawn from the study will receive the incentive build up from baseline to the time point of the study visit they completed.

11. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

11.1 Handling and storage of data and documents

The blood samples, urine as well as data and documents will be coded by a subject identification code. The identification code will consist of the protocol ID number “88”; followed by a consecutive number for the subject, for example; 88-01, 88-02. The blood and urine samples will be coded by this identification code without randomization number but followed by the number for the sampling. Examples: 88-01-T0 (meaning patient one, sampling at T0). 88-032-T4 (meaning patient thirty two, sampling at T4).
There will be a subject identification code list that links the codes to a subject. Only the principle investigators have access to the subject identification code list. The subject identification code list and the data will be stored separately for 15 years by the principal investigator.

The data collected during the study visits will be entered directly into the eCRF (Castor) with exception for the questionnaires, laboratory results and SDF imaging parameters. The questionnaires will be filled out by the patient on paper during the study visit. The SDF imaging parameters will be collected in the Glycocheck software system. Patients are entered into the Glycocheck software under the subject identification code. Only the sex of the patient will be added in the Glycocheck software. The laboratory results will presented in HIX anonymously under patient name Glycotreat_trail_vrouw or Glycotreat_trail_man.. The investigator or research staff will enter this data into the eCRF. The questionnaire and laboratory source documents will be stored in a paper file in the LUMC. The Glycocheck software will be stored on a laptop in the LUMC.

Blood and urine samples will be send to the Radboud UMC, these samples will be labelled with the subject identification code and sampling number. The identification key will be kept at the LUMC.

11.2 Monitoring and Quality Assurance

Monitoring will be done according to the monitor plan by internal monitors of the LUMC.

11.3 Amendments

A ‘substantial amendment’ is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;

- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority. Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

11.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

11.5 Temporary halt and (prematurely) end of study report

The sponsor will notify the accredited METC and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the patient's last visit.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.
In case the study is ended prematurely, the sponsor will notify the accredited METC and the competent authority within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC and the Competent Authority.

11.6 Public disclosure and publication policy

The study results will be published in medical journals.

12. STRUCTURED RISK ANALYSIS

12.1 Potential issues of concern

a. Level of knowledge about mechanism of action.

Fasting mimicking diet

The fasting mimicking diet tricks the body into fasting which induces several beneficial effects by triggering (long lasting) adaptive cellular stress responses. The mechanisms that are typically induced by fasting due to metabolic shifts are a reduction of IGF-1 levels, insulin, fat storage, and endogenous glucose production, increased adipose lipolysis and fat oxidation, and use of glycerol and ketone bodies instead of glucose as preferred carbon sources. Moreover, it induces autophagy and reduces levels of pro-inflammatory cytokines and systemic inflammation [45].

Food supplement

The Endocalyx supplement combines three mechanisms to support the glycocalyx homeostasis; by supplying polysaccharides as glycocalyx constituents, stimulating glycocalyx synthesis by providing the substrate for glycosaminoglycan synthesis and by reducing reactive oxygen species-mediated endothelial dysfunction [57-59].

b. Previous exposure of human beings with the test products and/or products with a similar biological mechanism.

Fasting mimicking diet

Two trials were conducted with healthy volunteers receiving the Prolon diet. The results are provided in paragraph 6.3.1.

Food supplement

A pilot trial was conducted among healthy volunteers with the food supplement. The results are provided in paragraph 6.3.2.

Several trials have been conducted with the individual ingredients of Endocalyx. Fucoidan extracted from brown marine algae has been studied individually or in a combined supplement in randomized placebo controlled trials [60-62] and in cancer patients [63, 64] Brown algae seaweed supplements has also been studied in a small clinical trial with type 2 diabetic patients [65].

Hyaluronic acid and glucosamine sulfate are provided as supplements for patients with knee pain or osteoarthritis and have been studied in large randomized placebo controlled trials [66, 67]. Superoxide dismutase supplements have been studied in trials with middle-aged women and in adults with cardiovascular risk factors [68, 69]. Polyphenol supplements,

including grape polyphenols, have been studied in several human randomized controlled trials [70].

c. Can the primary or secondary mechanism be induced in animals and/or in *ex-vivo* human cell material?

Fasting mimicking diet

The systemic effects of the fasting mimicking diet on inflammation, insulin, IGF-1 and glucose levels were also seen in experimental studies [37, 38, 71]. The results of experimental studies are explained in paragraph 6.2.1.

Food supplement

In the experimental study, the food supplement Endocalyx improved the microvascular health index, increased capillary density, lowered blood pressure and decreased mesenteric macrophage accumulation in old mice.

The individual ingredients of the food supplement have been studied in experimental studies and human cells.

Administration of fucoidan in diabetic rats improved the endothelial function shown as an upregulation of nitric oxide synthesis, improved blood pressure, and shear stress induced vasodilatation [72]. The anti-inflammatory, anticoagulant and anticoagulant effects of fucoidan extracted from 9 different brown seaweed species were investigated in rats and human umbilical vein endothelial cells [73].

Glucosamine hydrochloride induced the production of hyaluronan in human synovial cells and chondrocytes as it its incorporated and used for glycosaminoglycan synthesis [74].

Treatment with high molecular weight hyaluronan in diabetic mice reduced diabetes induced renal injury by modulating CD44 signaling, reduced NF-κB activation and the expression of inflammatory cytokines, but had no effect on blood glucose levels [75].

d. Selectivity of the mechanism to target tissue in animals and/or human beings.

Fasting mimicking diet

The fasting mimicking diet has no specific target tissues, but obtains systemic effects triggered by cellular responses. Fasting mostly affects metabolic parameters and systemic inflammation.

Food supplement

The target site of the food supplement is the endothelial glycocalyx, which covers the endothelial cells of the systemic vasculature and therefore has systemic effects.

The ingredients of the Endocalyx went through a quality control for specification of composition and to detect contamination in the products (such as heavy metals or microbiology). These forms are provided in the supplementary.
Other toxicology studies and evaluation of the individual ingredients in animals or humans revealed no significant toxicological changes and confirmed the safety of the individual ingredients [67, 76, 77].

e. Analysis of potential effect.

Fasting mimicking diet

The Prolon diet is a pre-composed program that is provided in a box with separate boxes for each day. Therefore, the dosages of the ingredients remain the same throughout the study. In both clinical trials with the Prolon diet, no serious adverse effects were reported. The reported side effects were that of a low caloric diet such as mild fatigue, weakness, muscle aches, dry mouth and headache. The pilot study showed that changes in serum parameters for kidney and liver function remained within physiological ranges [38].

Food supplement

The pilot study with the Endocalyx food supplement reported no adverse effects of the supplement. One reported effect was dizziness, as endocalyx lowered the systolic blood pressure.
Studies with the individual ingredients:
Fucoidan: A study with rats to evaluate the anticoagulant effect of fucoidan extracted from Laminaria japonica showed no effects from daily administration of 300 mg/kg. Higher doses of 900mg/kg did however elevate clotting time [76].
In a study with volunteers taking 3 gram fucoidan per day, no clinical relevant changes was seen in coagulation markers [78]. In our study, patients receive about 400 mg fucoidan per day in the Endocalyx supplement, therefore, no effect on coagulation is expected.
Another randomized placebo-controlled trial with 300 mg fucoidan supplements showed no no clinically significant change in blood safety markers (haematology or biochemistry) and that it is was well tolerated without adverse effects [62]. Diarrhea was a reported side effect in patients taking 6 grams per day, which improved immediately after stopping fucoidan administration [79]. The last safety study with 20 healthy volunteers taking daily 4 gram fucoidan for two weeks showed no significant changes in haematological, kidney and liver parameters, and showed no significant changes in urine biochemical parameters or faecal and bowel states [80].

Hyaluronic acid: in several randomized controlled trials with oral administration of hyaluronic acid, with dosages ranging from 48 to 240 mg per day, no adverse events related to hyaluronan were reported [67]. A randomized controlled trial with patients taking daily 200 mg hyaluronan, no adverse changes in haematology, liver and kidney parameters were seen [81]. In our study, patients receive about 70 mg hyaluronic acid per day in the Endocalyx supplement.

Glucosamine sulfate: in large randomized placebo controlled trials with dosages of 1500 mg per day, no serious adverse effects linked to glucosamine sulfate occurred [66]. In our study, patients receive 1500 mg glucosamine sulfate per day in the endocalyx supplement.

Oxxynea OMD-MVHS Blend SOD : there are no adverse effects reported in the studies with vegetable superoxide dismutase supplementation [82].

f. Pharmacokinetic considerations.

Fasting mimicking diet

Not applicable.

Food supplement

Fucoidan: limited research is available on the pharmacokinetic of fucoidan. Orally administered fucoidan was detected in small amounts the serum of healthy volunteers [83]. Others determined the concentration fucoidan in the liver after administration in rats, demonstrating systemic distribution [84].

Hyaluronic acid: in rats and dogs, orally administrated HA was degraded to oligosaccharides by intestinal bacteria, absorbed in the large intestine and is subsequently distributed throughout the tissues [85, 86].

Glucosamine sulfate is absorbed for 80% and is metabolized by the liver and excreted via urine. The half-life after oral administration is 60 hours [87].

Oxxynea OMD-MVHS Blend SOD: a review on the bioavailability of polyphenols in humans showed that the mean time to reach maximum concentration of grape polyphenols (catechin) was 1.8 hours and for artichoke polyphenols (chologenic acid) 1.0 hours, with a half-live for catechin of 2.5 hours [88].

g. Study population.

For this study, south Asian patients, between 18 and 75 years, with diabetes mellitus type 2 and microalbuminuria will be included. If female and of child-bearing age, patient must be non-pregnant and non-breastfeeding.

h. Interaction with other products.

Fasting mimicking diet

Not applicable.

Food supplement

Several mouse studies indicated that intravenous glucosamine supplementation altered insulin sensitivity and glucose levels. However, randomized controlled trials with healthy individuals and type 2 diabetic patients demonstrated no significant increase in glycemic control, serum insulin or fasting glucose [89, 90]. During the study, glucose monitoring is done at the end of every month.

i. Predictability of effect.

Primary and secondary outcomes are the same for all the intervention arms.

Side dark field imaging is used to assess the effects of the interventions on the endothelial glycocalyx. A question can be whether mucosal glycocalyx thickness is representative for systemic changes. However, systemic activators in the diabetic environment will induce endothelial glycocalyx changes throughout the circulation. Changes in the mucosal glycocalyx were accompanied by changes in systemic glycocalyx thickness in diabetic patients [91]. In addition, glycocalyx changes in type 2 diabetic patients have shown to coincide in both the retinal and the sublingual microvasculature [92]. Heparan sulfate domains, heparanase, MCP-1 and albumin excretion are also used as parameters to evaluate the effect. Albuminuria is a known predictor of renal damage and a risk factor for renal failure. Furthermore, albuminuria is linked to dysregulation of the glycocalyx [1].

j. Can effects be managed?

Fasting mimicking diet

Patients receiving the fasting mimicking diet can return to their normal diet if there is a high incidence of adverse effect during the 5 days of the diet. Because of the low caloric intake during the diet, adequate measures are taken to prevent hypoglycemia induced by insulin or sulfonylureas. The dosages of these drugs are altered during the 5 days of the diet and patients are instructed to self-measure fasting glucose levels. At baseline, the instruction and

materials for self-measuring is provided to patients that use insulin or sulfonylurea derivatives. Moreover, a patient letter with the symptoms of hypoglycemia and instructions on what to do if the glucose level drops to 4.0 mmol/L, is provided to these patients. Patients that use long actin insulin during the diet are instructed to measure fasting glucose levels every day and track glucose levels during the second day of the diet. Patients that return to insulin or sulfonylurea derivatives are instructed to measure fasting glucose levels the 3 days after returning to their normal diet. These patients are contacted by the investigator or research staff by telephone. They are instructed to call the investigator if the glucose level drops to 4.0 mmol/L. At the end of the month, the last FMD cycle is evaluated for all patients together with additional glucose monitoring with a finger prick blood sample. If needed the dosages of hypoglycemic drugs can be altered. If the patients experience an allergic reaction to one of the ingredients of the Prolon diet, they are treated as mentioned in paragraph 5.4 and are withdrawn from the study. Effects on liver and kidney function are monitored during the study. The occurrence adverse effects is documented and evaluated at the end of every month.

Food supplement

At every study visit, the occurrence of adverse effects is documented and evaluated. The fasting glucose levels and blood pressure is measured at the study visits. If needed, the dosages of the hypoglycemic or anti-hypertensive drugs can be altered. Possible effects on liver and kidney function are monitored during the study. If patients experience an allergic reaction to the ingredients of the Endocalyx food supplement, they are treated as mentioned in paragraph 5.4 and are withdrawn from the study.

12.2 Synthesis

Fasting mimicking diet

The overall risk of the fasting mimicking diet is low to mediate. The diet does not contain any active substances and the safety and feasibility of the diet is established in two randomized controlled trials with healthy volunteers, which reported no serious adverse effects. However, the diet has not been used in diabetic patients. Patients that use sulfonylurea derivatives or insulin during the study are instructed and provided at baseline with a glucose meter for self-measuring of fasting glucose levels. Because of the low calorie and carbohydrate content of the diet, dosages of hypoglycemic drugs are altered or stopped during the 5 days to prevent hypoglycemia. Patients are instructed at baseline and a patient letter is provided with the symptoms of hypoglycemia and instructions what to do if the glucose level drops to 4.0 mmol/L.

Patients that still use long acting insulin during the diet are instructed to measure fasting glucose levels during the diet. Patients that return to insulin or sulfonylurea derivatives after the diet are also instructed to measure fasting glucose the consecutive three days after the diet. These patients are contacted by the investigator or research staff by telephone. Additional glucose monitoring will be done at the end of every month at the study visit with a finger prick. During the study, the dosages of the hypoglycemic drugs can be altered if needed. In case of hypoglycemia or serious adverse events, patients are instructed to call the investigator or the internal resident on duty at the Haaglanden Medical Center. Effects on liver and kidney function are also monitored during the study. In our opinion because of the study design with alterations in the hypoglycemic medication and frequent glucose monitoring the risk of hypoglycemia during the study is reduced to a low risk.

Food supplement

The overall risk of the Endocalyx food supplement is low. The individual ingredients of the supplement are already used as dietary supplements. Large randomized controlled trials with the individual ingredients, some with higher dosages of the ingredients than in the Endocalyx supplement, revealed no major adverse effects and showed the safety of the individual ingredients. In the pilot study with the supplement, no serious adverse effects were reported. One side effect that was reported was dizziness because of the effect on reducing blood pressure. The blood pressure is measured at every study visit and the dosages of the anti-hypertensive medicine can be altered if needed. Effects on glucose levels, insulin sensitivity, liver function and kidney function are also monitored during the study. Glucose monitoring is done at the end of the month with a finger prick blood sample, the dosages of the hypoglycemic drugs can be altered if needed. Moreover, the occurrence of adverse effects is documented and evaluated at the end of every month. Patients are provided with the telephone number of the investigator that they can call in the case of a serious adverse event.

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